Restoring Bee Diversity and Pollination Services through Revegetation

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ABSTRACT

Habitat loss is causing declines in native bees and reducing associated pollination services. Revegetation can be used to reverse these declines, and is a restoration technique attracting growing effort and resources. However, a lack of understanding around the quality of revegetation needed to support native bees and their ecological roles remains, limiting opportunities to improve revegetation outcomes.

This thesis aims to address this gap and compares floral and bee diversity, pollination services and pollination networks in revegetated landscapes ranging in habitat quality. In addition, novel molecular tools were explored to improve the ease of undertaking pollen identification and quantification, and applied these methods to describe pollination networks.

Field experiments were used to compare floral and bee diversity in revegetation sites varying in quality, together with remnant habitat and cleared land in South Australia. Pollination services within the same sites were measured using two native phytometer species, one pollinated by native bees only, and the other by both native bees and introduced honey bees. Bee diversity and richness were found to be higher within sites that were higher in floral diversity. In addition, while pollination services provided by honey bees were uniform across treatments, pollination by native bees was higher in higher diversity revegetation compared with lower diversity revegetation. Pollination networks were then generated using the bee collected pollen from field surveys.

Pollen identification is an important objective for many scientific fields, including pollination ecology and agricultural sciences, where the quantification of mixture proportions is sought after but remains challenging. Novel molecular hybridisation capture approaches can potentially improve upon current methods for identifying and quantifying taxa, and were applied to artificial pollen mixtures. This method uses complementary RNA baits to capture DNA barcodes of interest, and produces random length DNA fragments, which allow for the removal of PCR duplicates, reducing bias in downstream quantification. This metabarcoding approach was applied using two reference libraries for angiosperms (*matK* and RefSeq chloroplast) constructed from publicly available sequences. Taxon ID provided by the single barcode did not always have resolution to species or genus level. The RefSeq chloroplast database yielded better qualitative results at these taxonomic levels, but the database was limited in taxon coverage. This method was then applied to the native bee pollen. Pollination networks from these data revealed that high diversity revegetation sites had similar complexity and robustness to remnant revegetation, although the latter sites had much larger networks. Networks in low diversity revegetation were simple and potentially un-robust.

The main results of this thesis indicate that higher quality revegetation characterised by the establishment of a more diverse set of plant species has the potential to restore native bees and associated pollination services and networks. However, there is still a gap between pollination levels and networks observed in high diversity revegetation compared to remnant vegetation, as well as a substantial difference in bee composition, suggesting that preserving remnant vegetation should be the highest priority conservation action in any landscape.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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This thesis was written on Kaurna land.

CHAPTER 1: Literature review - Relationship between bee diversity and vegetation

Wild bees are vital for healthy ecosystems and provide important pollination services in natural and agricultural systems. As much as 80% of the world's flowering plants and up to a third of food production depends on animal pollinators (McGregor 1976). Australia has a very diverse bee community, and is home to over 1,650 (Batley and Hogendoorn 2009) of the world's 21,000 bee species. This number continues to grow as more species are described. Concerningly, evidence is growing that bees are in decline globally (Biesmeijer *et al.* 2006; Potts *et al.* 2010; Burkle *et al.* 2013; Goulson *et al.* 2015; Hogendoorn *et al.* 2020).

Bee declines

Pollinators, like most animals, are impacted by a host of parasites, diseases, and pathogens. These are natural, intricate parts of ecosystems, and help to regulate population sizes. However, the spread of non-native parasites and diseases, and habitat loss (discussed in detail below) has contributed to some species declining (Goulson *et al.* 2015). Much of the evidence for bee declines, especially those reported in the media, are based on losses of managed honey bee (*Apis mellifera*) hives that were observed in America and Europe. The widespread collapse of honey bee colonies in the United States and Europe from 2006 – 2009 was significant enough to earn the name 'Colony Collapse Disorder', or CCD (Oldroyd 2007). The cause was determined to be multi-faceted, with multiple and compounding stressors, including parasites, diseases, pesticides, and extreme climate events (Vanengelsdorp *et al.* 2009). However, a significant driver of CCD was due to viruses passed to honey bees by the parasitic mite *Varroa destructor* (Goulson *et al.* 2015).

Honey bees and the Varroa destructor mite

The *Varroa* mite is believed to have caused the collapse of most feral bee colonies (wild colonies where honey bees are introduced) in America since its arrival in the 80s (Oldroyd 2007). Since its introduction and establishment in New Zealand in 2000, it has caused severe declines in feral honey bee colonies (Iwasaki *et al.* 2015), and is predicted to cost the economy between $365-661 million by 2028 (Howlett and Donovan 2010). Feral colonies are more severely affected by the *Varroa* mite, as apiarists can control *Varroa* with pesticides. The mite has spread worldwide, save only a few islands (Iwasaki *et al.* 2015). Its arrival in Australia is thought to be imminent and is expected to exterminate feral honey bee populations (Cunningham *et al.* 2002; Cook *et al.* 2007; Batley and Hogendoorn 2009). As of 2022, Australia had remained free of the mite, but a recent introduction in New South Wales was confirmed on the 22nd of

June (Australian Government 2022b), and this has led to the biggest outbreak on record in Australia. By the end of September 2022, the mite had been detected in over 100 locations, and emergency biosecurity zones were established throughout NSW to limit the spread. Economic losses if the mite becomes established throughout Australia are predicted to be $70 million per annum (Australian Government 2022a).

Despite the large attention that the plight of the honey bee has attracted, honey bees are in fact not declining, and their use as an umbrella species for bee conservation has been argued against (Iwasaki and Hogendoorn 2021). Even through the spread of the *Varroa* mite and subsequent colony losses, which poses challenges for apiarists, the number of managed honey bee hives has increased globally by approximately 45% in the last 50 years (Aizen and Harder 2009).

Wild bee decline

Wild bee decline is of greater concern compared with the loss of managed honey bee hives. Wild bees also provide vast amounts of free pollination services, and have severely declined across the Americas and Europe (Potts *et al.* 2010). Through the loss of bee diversity, lower levels of pollination services could lower crop yields, and demand for agricultural land could increase, further exacerbating biodiversity loss (Aizen *et al.* 2009). Although honey bees are considered the most important crop pollinators, they are also an introduced species in many parts of the world. They have become invasive, and colonised large parts of Australia (Paton 1996). While the issue has not been fully resolved (Paini 2004), honey bees are thought to compete with native pollinators (Paton 1993, 1996), which contribute important pollination services to crops (Garibaldi *et al.* 2013; Garibaldi *et al.* 2014; Rader *et al.* 2016). Feral honey bees visit at least 200 native plants and were observed to consume most of the resources of some plants (Paton 1996). They were the most abundant species recorded in a recent survey of bees across Australia, and were found to have a large overlap in resource use with native bees (Elliott *et al.* 2021). Honey bee competition with native bees is species specific, and largely negative, although current research is limited (Prendergast *et al.* 2022). Introduced bees help spread introduced weeds, and impede the pollination of native plants (Paton 1993; Brown *et al.* 2002).

The likely spread of the *Varroa* mite in Australia, and the potential resulting suppression of feral honey bee colonies, lends opportunities to develop pollination strategies that are not reliant on feral honey bees. This will be important to avoid a pollination deficit, given that a large amount of current crop pollination is provided for free by feral honey bees (Cunningham *et al.* 2002). Native bees are not susceptible to the *Varroa* mite, so increasing the pollination services they provide could be a strong action to safeguard pollination services in the future, and decrease the reliance on non-native wild bees that are ecologically damaging. The possible reduction of feral honey bees also provides opportunities for native bee conservation and restoration,

since native bees may benefit from the decreased competition with honey bees (Iwasaki *et al.* 2015). However, native bees and the pollination services they provide have not been studied adequately in Australia to date.

Habitat loss

Wild bee decline (in Australia wild bees are referred to as native bees, to distinguish them from invasive honey bees which are wild but native in other parts of the world) has been very difficult to quantify, because of the lack of robust baseline data, and difficulty in measuring population change. However, habitat loss is known to be a main driver of species decline (Biesmeijer *et al.* 2006; Potts *et al.* 2010; Burkle *et al.* 2013; Goulson *et al.* 2015; Hogendoorn *et al.* 2020), with a recent WWF (World Wildlife Fund for Nature) report finding a decline of 68% of 21, 000 monitored populations since the 1970s (WWF 2020). Habitat loss through agricultural intensification (Klein *et al.* 2007) and urbanisation creates one of the largest problems for pollinators, reducing foraging and nesting opportunities (Batley and Hogendoorn 2009; Goulson *et al.* 2015). In a meta-analysis on human disturbance and pollination, habitat loss and fragmentation were significantly associated with declines in wild pollinators (Winfree *et al.* 2009). Hanula *et al.* (2015) also predicted that future losses of pollinators are probable, given that land use change is likely to be the biggest driver of future biodiversity loss.

Australia is not free of the habitat degradation experienced overseas. Approximately 63% of the continental land area has been modified for human use (Australian Bureau of Statistics 2010), with many land types being disproportionately cleared. There has been a 40% loss of forest cover, and 80% of eucalypt forests have been modified, with as little as 3% of some woodland types remaining (Yates and Hobbs 1997). The remaining forest cover is highly fragmented (Gathmann and Tscharntke 2002; Bradshaw 2012). It is likely that bees reliant on these habitats have been adversely affected by the reduction in natural vegetation and vegetation quality. Habitat loss results in the physical loss of bees, and additionally in the loss of relationships bees have with plants. These relationships can be measured as pollination services, or described by pollination networks. Pollination networks are useful tools for understanding community structure, and shed more general understanding about ecological systems beyond a single species focus (Cusser and Goodell 2013). However, plant-pollinator mutualisms are unlikely to restore themselves (Cusser and Goodell 2013), without the return of the food and nesting resources required by the pollinators (Exeler *et al.* 2009; Roulston and Goodell 2011). This requires habitat restoration, which outside of agricultural systems lacks research.

Some revegetation efforts to date

The impact of habitat loss is now being recognised and some large-scale restoration programs are underway across the globe. The Partnership on Forest & Landscape Restoration recommended the restoration of 350 million ha of cleared and degraded

land worldwide by 2030 (Woerden 2014). More than four million ha of new forest has been planted in China annually since the 1990s, under China's 40 year, billion tree program (Xu 2011). In Australia, $2.55 billion has been allocated for emission reduction plantings (Australian Government 2014). More recently, $50 million was invested by the Australian Government for the 20 Million Trees project from 2014 - 2020, which, along with reforestation, aimed to improve the environment, sustainability and productivity of agricultural systems (Australian Government 2021). In South Australia, the Million Trees program resulted in almost 3 million local native plants being planted around the state capital of Adelaide, covering 1,500 ha of the metropolitan area, to reduce the carbon footprint of the city and improve biodiversity (Urban Biodiversity Unit *et al.* 2013).

It is difficult to assess the success of such restoration projects, since post-planting monitoring is often lacking. When measured, restoration success is often quantified as the number of trees planted, and survivorship of plantings (Ruiz-Jaén and Aide 2005b). But other metrics such as vegetation structure and ecosystem processes, including comparisons to reference sites, would give a clearer indication of true success (Ruiz-Jaén and Aide 2005a). The benefit of revegetation to bird life has been well documented in some cases (Munro *et al.* 2007; Paton and O'Connor 2009), but the benefit to bees, and whether this in turn improves pollination services, has not. Pollinators, and particularly bees, are a key element in successful restoration, since they maintain species diversity and ecosystem productivity (Fiedler *et al.* 2012), and some 87.5% of flowering plants rely to some extent on animal pollinators for seed set (Ollerton *et al.* 2011). Furthermore, pollinator diversity is considered an important factor in determining the quality of pollination services available. For example, positive associations have been found between pollinator diversity and crop fruit set (Garibaldi *et al.* 2015). Revegetation needs to be carefully thought out, and needs to include ecosystem specific planning (Bradshaw 2012), including pollinator consideration to ensure adequate pollination services.

Restoring bee diversity and pollination services

Pollination services are a key ecosystem function provided by bees. They are an essential element in successful restoration, since they maintain species diversity and ecosystem productivity (Fiedler *et al.* 2012). Hence, investing in restoration without regard for pollinators and the long-term viability of the system potentially wastes resources. To improve pollination services, it is necessary to focus on pollinator diversity rather than the abundance of select species (e.g., honey bees). Pollination services improve when a diverse bee assemblage is present (Garibaldi *et al.* 2015), which improves overall ecosystem biodiversity.

To improve pollination services through increased bee diversity, flower plantings in agricultural settings are a common approach and well explored overseas, and have

had positive results. Increases in bee diversity and abundance were observed along cropping edges (Venturini *et al.* 2016; Lowe *et al.* 2021). Many studies in Europe and the US focused on seed mixture composition for flower plantings (e.g. Harmon-Threatt and Hendrix 2015; Williams *et al.* 2015; Havens and Vitt 2016) (discussed further below), and ecological restoration could benefit if such considerations were implemented more widely. A US study found that the size of flowers in wildflower seed mixes, and staggered flowering times of flowers were important for attracting and maintaining a diversity of wild pollinators (Williams *et al.* 2015). Wildflower plantings adjacent to pollinator dependent blueberry crops increased the percentage of fruit set, and after three years the planting cost was compensated by the increased crop yield (Blaauw *et al.* 2014). Interestingly, honey bee abundance following restoration remained the same, but there was an increase in wild bee and syrphid abundance in crops adjacent to treatments (Blaauw *et al.* 2014). In a degraded agricultural landscape in California, small scale restoration in the form of hedgerow plantings significantly increased the occurrence of wild pollinators over eight years of monitoring, including more specialised and less mobile species which are often thought not to benefit from restoration in agricultural landscapes (Kremen *et al.* 2015). Furthermore, hedgerows particularly supported uncommon native bee species, creating a spill-over effect into nearby fields (Morandin and Kremen 2013). In a review by Venturini *et al.* (2016), the authors found that pollination reservoirs were effective at increasing the abundance of wild bees, with yield and profit increases. In some pollinator dependent crops the inclusion of pollinator reservoirs increased the crop production value from $198-$3,060/ha, and other ecosystem services were produced such as insect control which further improved yield and profit (Venturini *et al.* 2016).

Interventions other than flower plantings have also been successful at improving pollination diversity. As demonstrated in a prairie fen in Michigan, the removal of invasive weeds changed the plant and pollinator assemblage (Fiedler *et al.* 2012). The authors noted, however, that although generalist pollinators seemed to respond quickly to the restoration treatments, plant communities would take longer to properly recover, and specialist species may need more targeted restoration measures. A proposal to create complex habitats with open spaces suggests increases in pollinator diversity, since many pollinators need a combination of habitats to complete their life cycle (Winfree 2010; Hanula *et al.* 2015). Furthermore, there is a growing push to conserve bees and other pollinators in cities and urban environments, and this is done through a variety of methods including flower plantings and nesting sites provisions (Threlfall *et al.* 2015).

However, few projects currently target pollinator or bee conservation outside of agriculture (Menz *et al.* 2011). Heathland and grassland restoration in Europe effectively restored bee species abundance and richness to levels comparable with undisturbed sites (Forup and Memmott 2005; Forup *et al.* 2007; Exeler *et al.* 2009;

reviewed by Dicks *et al.* 2010), however, there is no evidence for the effects of reforestation (Dicks *et al.* 2010). In addition, the effect of restoration quality on pollination services has not been assessed. Indeed, a meta-analysis of the effect of restoration on wild bees found that nearly all of 28 restoration projects studied had plant community targets, without consideration of pollinators (Tonietto *et al.* 2018). Despite this, the authors found strong evidence of restoration benefits for bees in agricultural areas. Therefore, it remains of interest to explore the effect of reforestation and restoration on bees and pollination networks in natural settings.

Current limitations in pollination research

To address and understand the drivers of pollinator decline, combat biodiversity loss, and progress pollination research, insight is needed in the specific plants that support specific bees. This is best done by studying pollen collection, as pollen is often the limiting factor, and bees are more eclectic in their pollen than in their nectar choices (Minckley and Roulston 2006). To identify pollen, high-throughput methodologies have benefits over microscopic approaches (Bell *et al.* 2022). Furthermore, comprehensive understanding of invertebrate communities is an important element of successful restoration practice, but is often overlooked, and in the future, will increasingly be achieved with high throughput technologies (Heyde *et al.* 2022). Pollen identification (ID) is key to answering questions in many scientific fields, including within pollination ecology and agricultural sciences. Accurate pollen ID also supports the study of ancient plant communities (Clarke *et al.* 2020), human health (e.g. allergy research (Weber 1998)), and forensics (Alotaibi *et al.* 2020).

Traditional methods for pollen ID rely on microscopic observation of diagnostic characteristics of the pollen exine. This method is limited in accuracy and throughput, while being time consuming potentially constraining many projects. It also requires a high level of expertise, which can be hard to come by as it has been undervalued in many instances. The microscopy-based pollen ID limitations are well established. In most cases, taxa can only be identified to family or genus (Kraaijeveld *et al.* 2015; Richardson *et al.* 2015b; Smart *et al.* 2017). The time-consuming nature of microscopy-based ID limits throughput, and usually allows for only a subsample of each sample to be examined, meaning that rare taxa are often missed (Bell *et al.* 2016; Smart *et al.* 2017). Particularly limiting Australian research is that microscopy it cannot distinguish different species of Myrtaceae, a dominant plant family (Thornhill *et al.* 2012). Improved methods for pollen ID could improve opportunities for answering a variety of questions, and specifically in better understanding pollination networks.

Pollen metabarcoding

Due to current pollen ID limitations, and the need for IDs in many fields, alternative ID methods have been sought. Molecular approaches are being developed to progress

from traditional, microscopy led pollen ID. DNA barcoding, or metabarcoding when dealing with mixed samples, has advanced taxon ID in many research fields, has been explored extensively for pollen ID, and has been shown to provide accurate ID at high taxonomic resolution and with high sample throughput (Wilson *et al.* 2010; Keller *et al.* 2015; Kraaijeveld *et al.* 2015; Richardson *et al.* 2015a; Richardson *et al.* 2015b; Bell *et al.* 2017; de Vere *et al.* 2017; Bell *et al.* 2019; Suchan *et al.* 2019). In particular, metabarcoding is able to recover a taxonomic ID from as few as five pollen grains (Pornon *et al.* 2016), and the method has been shown to be superior to microscopy-based methods, with far more genera identified (Keller *et al.* 2015; Richardson *et al.* 2015b).

Pollen metabarcoding has also been used as a tool for constructing pollination networks, with 2.5 times as many interactions recovered in networks constructed using metabarcoding (Pornon *et al.* 2016). Pollen DNA metabarcoding was used to describe Australian alpine pollination networks. Findings included less specialisation and higher diversity in networks derived from pollen metabarcoding versus microscopic identification (Encinas-Viso *et al.* 2022). Similarly, pollen metabarcoding of moth pollination networks found more individuals carrying pollen, and more species per individual, compared with microscopic assessment alone (Macgregor *et al.* 2019).

Database limitations

The accuracy of metabarcoding is limited, however, by barcode choice and comprehensiveness of reference databases, since only taxa with reference sequences can be detected. Database repositories have been established where references can be stored and accessed, and these are growing. The animal CO1 barcode database is growing, with the single barcode being able to differentiate most animal taxa (Ratnasingham and Hebert 2007). However, the selection of effective barcodes for plant ID has presented a much greater challenge, since CO1 is not variable enough in plants to provide taxonomic resolution (CBOL Plant Working Group 2009). The success of standard barcodes relies on sequence variability to allow good taxon resolution, and conserved primer binding sites to allow for sequence analysis across a broad range of taxa. The most common barcoding approach uses PCR to amplify the barcode using primer sites, followed by sequencing and comparison to a reference database. When reference sequences for target species are absent, the similarity to the closest sequence(s) in the database can be used to generate a genus or family ID (Liu *et al.* 2019).

Quantification

Despite the demonstrated strengths of metabarcoding, the inability to answer quantitative questions regarding sample composition remains problematic. In pollination research, it is often desirable to know the relative proportions of taxa in a pollen sample. This information can shed light on the preference of pollinators or

abundance of resources, and can improve understanding of pollination networks and ecosystem robustness, which in turn can help guide pollination services restoration (Dormontt *et al.* 2018). Currently, there is mixed success in comparisons of relative proportions of DNA sequencing reads to starting pollen proportions for mixed samples (Bell *et al.* 2017). Positive correlations have been found between proportions of sequences and DNA mixes using *trnL* and ITS1 barcodes (Pornon *et al.* 2016), sequence proportions and starting pollen proportions using ITS2 (Keller *et al.* 2015), and between averaged *rbcL* and *matK* sequence abundance (Richardson *et al.* 2015a). A meta-analysis of 22 ecological studies of plants and animals that used metabarcoding with 7 markers, found only a weak positive association between starting biomass and sequences recovered, with large uncertainty (Lamb *et al.* 2019).

The weak or negative results arise from bias at several steps in the sample to sequence pipeline. Biases occur which can affect both the qualitative (whether the correct taxa are identified), and quantitative (proportion within mixture) aspect of metabarcoding. Any bias affecting qualitative accuracy can affect quantitative accuracy, by potentially lowering some taxa below the detection limit. Inaccurate quantitative estimates can occur due to a range of factors, but unequal PCR replication (mostly affecting related taxa) and variable barcode copy number (particularly affecting chloroplast loci (Golczyk *et al.* 2014) which contain the standard plant barcodes) likely play the greatest roles in introducing bias (Krehenwinkel *et al.* 2017). In fact, Pawluczyk et al. (2015) found up to a 2000 fold difference in DNA quantity between taxa and loci after PCR. PCR-free methods are being explored as a means to overcome these quantitative challenges, and they show improvement in quantification over PCR-based metabarcoding, for example, genome skimming and chloroplast assembly (Lang *et al.* 2019), Whole Genome Shotgun sequencing (Bell *et al.* 2021), and MinION Reverse Metagenomics (Peel *et al.* 2019). However, these methods have other drawbacks. Genome skimming and Whole Genome Sequencing (WGS) require a larger amount of DNA, which can be difficult to obtain from small solitary pollinators (Lang *et al.* 2019; Bell *et al.* 2021), and MinION Reverse Metagenomics requires the user to curate their own reference databases (Peel *et al.* 2019).

Hybrid capture

One method that could overcome the shortcomings of pollen metabarcoding and improve accuracy and quantification compared to existing methods of metabarcoding is hybridisation (hybrid) capture. Hybrid capture is a target enrichment technique that has recently been applied to environmental/ecological studies. In traditional PCR amplification methods, primers are bound to conserved barcode primer sites to amplify the barcodes. This creates exact copies of the barcodes that cannot easily be distinguished from the PCR duplicates. The hybrid capture approach uses sonication to randomly fragment the DNA after DNA extraction, creating a random DNA fragment soup. Chloroplast loci (genes) for which baits were designed are then ‘fished out’ of

the soup using the complementary baits (Waycott *et al.* 2021). Given that each DNA fragment has in theory a unique length, PCR duplicates (amplicons having same sequence and length) can be eliminated bioinformatically and only one copy of every captured sequenced read or read pair is retained. This could enable more accurate downstream quantification of relative taxon abundances based on the number of reads mapping to references. It therefore has the potential to remove PCR bias from the quantification analyses, which generates large quantitative bias in amplification-based metabarcoding approaches, and can cause taxon-specific amplification bias (Pawluczyk *et al.* 2015; Krehenwinkel *et al.* 2017). This could result in more accurate pollen quantification of mixed samples, and could reveal more detailed pollination networks.

Pollination networks

Observing pollination networks is a useful and common approach for examining ecosystem structure and function. Pollination networks are a very useful tool for assessing restoration success, since sustainable and long-term restoration can only exist with healthy pollinator networks (Kaiser-Bunbury *et al.* 2009; Cusser and Goodell 2013; Kaiser-Bunbury *et al.* 2017; Bell *et al.* 2022). Traditional approaches for reconstructing pollination networks consist of monitoring pollinators visiting flowers, which is limited by the large amount of time needed for pollinators to visit the observed flowers. In addition, such visitation networks only partially predict pollen transport networks (Popic et al 2013), and are biased towards specialists, which is contrary to most views that pollinator communities are generalist dominated (Bosch *et al.* 2009; Cusser and Goodell 2013; Encinas-Viso *et al.* 2022). Studies have begun exploring pollination networks constructed from the pollen carried by pollinators, and compared these with visitation records alone. Studies that used microscopy to identify pollen yielded networks with more interactions and fewer specialist species (Bosch *et al.* 2009; Burkle *et al.* 2013).

Project summary and expectations

The majority of existing pollination research has been related to agriculture and crop pollination services in Europe and North America, and the majority of pollination restoration, including floral restoration, has been done for agricultural purposes (Winfree 2010). There has been comparatively little research done in Australia, and little research outside of agricultural contexts. It is widely believed that the *Varroa* mite will establish in Australia at some point and eliminate the majority of feral honey bee colonies. In mid 2022 there was a *Varroa* outbreak and as of late 2022 containment measures are still underway (Australian Government 2022a). Since 65% of Australian crops rely to some extent on pollination services provided by honey bees (Gibbs and Muirhead 1998, cited in Keogh *et al.* 2010), and pollination deficits have been found in natural habitats, the loss of feral honey bees will possibly impact food

production. The consequence for farmers is likely to be very expensive, for a loss of productivity and higher demand for pollination are sure to drive up the cost of hiring honey bee hives for pollination (Cook *et al.* 2007). It could also lead to more agricultural expansion, if productivity is lowered, which would be ecologically damaging. In Australia, research is needed to find the most effective ways of improving pollination networks through improvements to bee diversity, and safeguarding against future pollinator declines.

In this thesis, I evaluate the benefit of two broadly classified, common revegetation approaches on the restoration of bee diversity, pollination services, and pollination networks, by also using a novel high throughput DNA metabarcoding method for pollen ID. Floral and bee diversity were measured within established revegetation sites across four areas of the Adelaide hills. For each chapters 2 and 4 , I compare large scale, 'low diversity' tree plantings with smaller scale 'high diversity' biodiversity plantings. These treatments were coupled with positive (native vegetation) and negative (cleared land) controls. For chapter 2, within these treatments, flower and bee diversity were measured, as well as bee-associated pollination services. Phytometer seed set was used as a proxy for pollination services. Phytometers are plants that are used to measure an environmental response, and have been used to measure pollination services in a number of studies (e.g. Orford *et al.* 2016; Castle *et al.* 2019; Olynyk *et al.* 2021). Two species of Australian native plants were used as phytometers, *Arthropodium strictum* which is pollinated by native bees only and *Cullen astralasicum* which is pollinated by both native bees and non-native honey bees. In chapter 3, I aim to demonstrate the effectiveness of hybrid capture DNA metabarcoding for identifying taxa in a pollen mix, and determining the accuracy of estimations of relative taxonomic abundances. To achieve this, I used two different reference databases, a *matK* database which is commonly used in amplicon metabarcoding, and a RefSeq whole chloroplast database. I explored whether, and how closely, the sequence composition of mixed pollen samples reflected starting proportions, to test the potential for broader application of hybrid capture metabarcoding as a useful tool in pollination research. Lastly, in chapter 4, I apply DNA metabarcoding to reconstruct pollination networks using pollen collected from the bees surveyed within the revegetation sites from chapter 2. The goal was to identify the pollen species in addition to the plants visited by bees, and gain deeper insight into pollination dynamics restored in revegetated landscapes.

The expectations from this study were:

- To find a gradient in floral diversity increasing from the cleared negative control through to remnant vegetation, and increasing bee diversity in response, since floral diversity has been linked to improved bee richness.
- That the higher diversity sites would have more pollination services, since pollination services have been linked to bee diversity.

- Given that feral honey bees are ubiquitous in the landscape the study took place in, pollination services provided by honey bees may be equal across sites with varying floral diversity.
- For metabarcoding of mock pollen mixtures, the RefSeq database would produce more accurate qualitative and quantitative results, since many more potentially informative gene regions were recovered using the chloroplast bait set used for hybrid capture, and PCR bias could be controlled.
- Pollination networks will be smaller and less complex in simple revegetation, and more complex networks would be restored in complex revegetation sites, in accordance with higher species diversity and pollination services.

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CHAPTER 2: Bee diversity and pollination services improve with revegetation effort

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Name of Principal Author (Candidate)	Dona Kireta		
Contribution to the Paper	Designed the study, collected and analysed the data, and wrote the manuscript.		
Overall percentage (%)	85%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
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Bee diversity and pollination services improve with revegetation effort

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DK, AJL, KH designed the experiments; DK, RL, KH undertook the field work and taxon identifications; DK, GRG analysed the data; AJL, KH supervised the project, and acquired funding; DK wrote the first draft of the manuscript; all authors contributed substantially to revisions.

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Abstract

Habitat loss is causing declines in native bees and reducing pollination services. Revegetation can be used to reverse these declines, and is a restoration technique attracting growing efforts and resources. However, a lack of understanding around the quality of revegetation needed to support native bees and their pollination services remains, limiting opportunities to improve revegetation outcomes. To assess this gap, we compared floral and bee diversity, and pollination services in revegetated landscapes ranging in habitat quality. We surveyed floral and bee diversity at established revegetation sites together with remnant habitat and cleared areas in South Australia. We also measured pollination services using two native phytometer species, which can be pollinated by native only, or both native bees and introduced honey bees (*Apis mellifera)*. We found that bee diversity and richness were higher within treatments that were higher in floral diversity. In addition, while pollination services provided by honey bees were uniform across treatments, pollination by native bees was higher in higher diversity revegetation compared with lower diversity revegetation. These results indicate that higher quality revegetation characterised by the establishment of a more diverse set of plant species, has the potential to restore native bee diversity and associated pollination services. However, there is still a significant gap between pollination services recorded in high diversity revegetation compared to remnant vegetation. These results suggest preserving remnant vegetation should still be the highest priority conservation action in any landscape, and provide important implications for restoration practitioners and landowners wishing to support landscape-level bee diversity and pollination services.

Key words:

bee diversity, phytometers, pollination services, revegetation, South Australia

Implications for practice

- Current revegetation approaches do not restore the levels of bee diversity and pollination services found in remnant vegetation. Therefore, conservation of remnant vegetation should be highly prioritised whenever possible.
- Restoration efforts should focus on planting high quality revegetation with diverse plant species for restoring bee diversity and pollination services.
- Simple tree plantings should be avoided for bee-oriented restoration goals, as they do not provide benefits over and above cleared/de-forested areas.

Introduction

Wild bees are vital for healthy ecosystems and provide important pollination services in natural and agricultural systems. Up to 80% of the world's flowering plants and a third of food production is dependent on animal pollinators (McGregor 1976). Australia is home to over 1,650 (Batley and Hogendoorn 2009) of the world's 21,000 bee species, and this number continues to grow as more species are described. Worryingly, there is mounting evidence that bees are in decline globally (Biesmeijer *et al.* 2006; Potts *et al.* 2010; Hogendoorn *et al.* 2020).

Whilst this decline has been largely unquantified, due to the difficulty in measuring change and the lack of robust baseline data, habitat loss is a main driver of species decline (Biesmeijer *et al.* 2006; Potts *et al.* 2010; Hogendoorn *et al.* 2020), with a recent report finding a decline of 68% of 21, 000 monitored populations since the 1970s (WWF 2020). Habitat loss through agricultural intensification (Klein *et al.* 2007) and urbanisation creates one of the largest problems for pollinators, reducing foraging and nesting opportunities (Batley and Hogendoorn 2009; Goulson *et al.* 2015). In a meta-analysis on human disturbance and pollination, habitat loss and fragmentation were significantly associated with declines in wild pollinators (Winfree *et al.* 2009), and continuing future losses are likely (Hanula *et al.* 2015).

Australia is no exception to this trend. Approximately 63% of the continental land area has been modified for human use (Australian Bureau of Statistics 2010), with many land types being disproportionately cleared. There has been a 40% loss of forest cover, and 80% of eucalypt forests have been modified, with as little as 3% of some woodland types remaining (Yates and Hobbs 1997). The remaining forest cover is highly fragmented (Gathmann and Tscharntke 2002; Bradshaw 2012). It is likely that bees reliant on these habitats have been adversely affected by the reduction in vegetation area and quality, and that the potential downstream loss of pollination services through the loss of bees is likely to have economic ramifications.

The impact of habitat loss is now being recognised and some large-scale restoration programs are underway across the globe. The Partnership on Forest & Landscape Restoration recommended the restoration of 350 million ha of cleared and degraded land worldwide by 2030 (Woerden 2014). More than four million ha of new forest has been planted in China annually since the 1990s, under China's 40 year, billion tree program (Xu 2011). In Australia, $2.55 billion has been allocated for emission reduction plantings (Australian Government 2014). More recently, $50 million was invested by the Australian Government for the 20 Million Trees project from 2014 - 2020, which, along with reforestation, aimed to improve the environment, sustainability and productivity of agricultural systems (Australian Government 2021). In the state of South Australia, the Million Trees program resulted in almost 3 million local native plants being planted around the state capital of Adelaide, covering 1,500

ha of the metropolitan area, to reduce the carbon footprint of the city and improve biodiversity (Urban Biodiversity Unit *et al.* 2013). Much of this restoration can be broadly grouped into two categories. The first is large-scale, low species diversity, simply structured revegetation, which is often done by direct seeding. Examples are large-scale tree plantings for carbon sequestration. The second type is smaller scale, ecological plantings with higher species diversity and more complex habitat structure, such as land offset plantings.

It is difficult to assess the success of such restoration projects, since post-planting monitoring is often lacking. When measured, restoration success is often quantified as the number of trees planted, and survivorship of plantings (Ruiz-Jaén and Aide 2005b). But other metrics such as vegetation structure and ecosystem processes, including comparisons to reference sites, would give a clearer indication of true success (Ruiz-Jaén and Aide 2005a). The benefit of revegetation projects to bird life has been well documented (Munro *et al.* 2007; Paton and O'Connor 2009), but the benefit to bees and whether this in turn improves pollination services, has not. Pollinators, and particularly bees, are a key element in successful restoration, since they maintain species diversity and ecosystem productivity (Fiedler *et al.* 2012), and some 87.5% of flowering plants rely to some extent on animal pollinators for seed set (Ollerton *et al.* 2011). Furthermore, pollinator diversity is considered an important factor in the quality of pollination services available. For example, positive associations have been found between pollinator diversity and crop fruit set (Garibaldi *et al.* 2015), while some restoration methods aimed at increasing pollinator abundance in farms reported increased fruit set and weight (Blaauw *et al.* 2014).

Investing in restoration with regard to the long-term viability of the system potentially better exploits limited resources. Revegetation should be carefully thought out, and should include ecosystem specific planning (Bradshaw 2012), including consideration of pollinators. Flower plantings for bees in agricultural settings are increasingly common and improve pollinator abundance and richness in field edges (Lowe *et al.* 2021). Many studies in Europe and the US focused on seed mixture composition for flower plantings in agriculture (e.g. Harmon-Threatt and Hendrix 2015; Williams *et al.* 2015; Havens and Vitt 2016) and ecological restoration could benefit if such considerations were implemented more widely. Indeed, a meta-analysis of the effect of restoration on wild bees found that nearly all of 28 restoration projects studied had plant community targets, without consideration of pollinators (Tonietto *et al.* 2018). Despite this, the authors found strong evidence of restoration benefits for bees. Heathland and grassland restoration in Europe effectively restored bee species abundance and richness to levels comparable with undisturbed sites (Forup *et al.* 2007; Exeler *et al.* 2009; reviewed by Dicks *et al.* 2010), but there is no evidence for the effects of reforestation (Dicks et al 2010). In addition, the effect of restoration quality on pollination services has, as far as we know, not been assessed.

Here, we explore the effect that the quality of various established revegetation areas has had on bee-associated pollination services, using bee surveys and phytometer seed set. Phytometers are plants that are used to measure an environmental response, and have been used in other studies to measure pollination services (e.g. Hardman *et al.* 2016; Castle *et al.* 2019; Olynyk *et al.* 2021). We used two species of Australian native plants as phytometers to measure pollination services, one that is pollinated by native bees only and another that is pollinated by both native bees and non-native honey bees (*Apis mellifera)*. We compared large scale, 'low diversity' tree plantings with smaller scale 'high diversity' biodiversity plantings. We coupled observations of seed set with surveys of flower and bee diversity in these revegetation sites as well as in positive (native remnant vegetation) and negative (cleared land) controls. We expected to find increased bee diversity in the more florally diverse revegetation sites, compared with low diversity sites, since floral diversity has been linked to improved bee richness (Cusser and Goodell 2013). We further expected that the pollination services to the phytometers would vary and improve with improving bee diversity. In addition, because feral honey bees are ubiquitous in the landscape we were working in, we predicted that high pollination services provided by honey bees would be found in the less florally diverse sites.

Methods

Bee diversity and pollination services were measured in revegetated sites across the Adelaide Hills in South Australia, using phytometer (i.e., a plant used to measure an environmental response) fruit set as a proxy to estimate pollination services provided by bees. We accompanied this with concurrent surveys of floral resources for bees, and bee surveys within the same sites.

Field sites

The study area incorporated four sites in the Mount Lofty Ranges, South Australia. The area underwent extensive land clearance prior to the 1990s, with only 10% highly fragmented pre-European vegetation remaining across the ranges, and 4% original vegetation remaining in the adjacent Adelaide plains (Bradshaw 2012). The region has a Mediterranean climate, with generally hot, dry summers, and cool, wet winters. Average rainfall ranges between 600 mm and 1000 mm annually.

Field work was conducted in spring and summer (September – February) of 2019-20. However, sampling during this period was hampered by unprecedented weather conditions, with intense heatwaves in early summer, culminating in catastrophic fire danger which precluded sampling at times (detailed below)(Australian Bureau of Meteorology 2020, 2021).

The field sites selected were pre-established restoration projects that utilised two broadly classified revegetation methods: low and high diversity plantings – see below. Additionally, revegetation sites were paired with negative ('Cleared' vegetation) and positive ('Remnant' vegetation) controls. The Cleared sites were used for different purposes (one used for pasture, one was an unused sports oval, two were simply deforested), but all were regularly mowed. Revegetation projects (in the area) were generally not large enough to allow multiple, independent experimental replicates within one site, since the home range of bees is relatively large, so three replicates of each revegetation 'treatment' were used in four spatially distinct sites across the Mt Lofty Ranges (Fig. 1). All sites had both controls, two of the sites had both types of revegetation treatment present, and two sites had either one or the other revegetation treatment (plus the controls), resulting in 14 plots (Table 1). The furthest sites were 48.3 km apart (Onkaparinga to Millbrook), and the nearest were 5.8 km apart (Clarendon to Craigburn), which is beyond the normal foraging distance of honey bees (Visscher and Seeley 1982) and far beyond the foraging range of wild bees (Gathmann and Tscharntke 2002; Greenleaf *et al.* 2007; Zurbuchen *et al.* 2010).

Low diversity sites (Low Div) were simple plantings usually containing fewer than five plant species of trees and large shrubs, such as *Acacia*, *Dodonaea, and Eucalyptus* (tough species resilient to grazing) and were planted in rows, sometimes with direct seeding. High diversity (High Div) revegetation sites were more complex plantings, aimed toward replicating remnant vegetation, and planted using tube stock of higher species diversity (10 + species), such as *Acacia, Chrysocephalum, Eucalyptus, Goodenia, Grevillea, Hakea, Hardenbergia, Leptospermum, Myporum, Scaevola.* These plantings had more complex structure, with multiple strata including understorey, mid and upper canopies. Additionally, the High Div revegetation often had ongoing maintenance which included weed control and infill planting.

The sites used were established by separately funded and managed restoration projects. Millbrook and Clarendon were SA Water sites, and revegetation was mainly done for biodiversity offsetting and water quality improvement, and received ongoing monitoring and maintenance. Craigburn and Onkaparinga revegetation projects were part of the Million Trees program that ran from 2003 to 2014, and included community led and targeted restoration efforts (Urban Biodiversity Unit *et al.* 2013), with little or no ongoing maintenance.

Uncontrolled variables between the sites included different planting years, different species used and different levels of post-planting maintenance (Table 1). However, planted vegetation at all the sites was considered mature given that all species flowered, except some *Eucalyptus* species.

Table 1. Revegetation with various treatments present at each site

Treatment Site	Cleared	Low Div	High Div	Remnant	Year restoration began	Other treatments
Craigburn	✓	✓	✓	✓	2009	none
Clarendon	✓	✓	✓	✓	2014	Weed control/infill planting
Millbrook	✓		✓	✓	2017	Weed control/infill planting
Onkaparinga	✓	✓		✓	2011	none

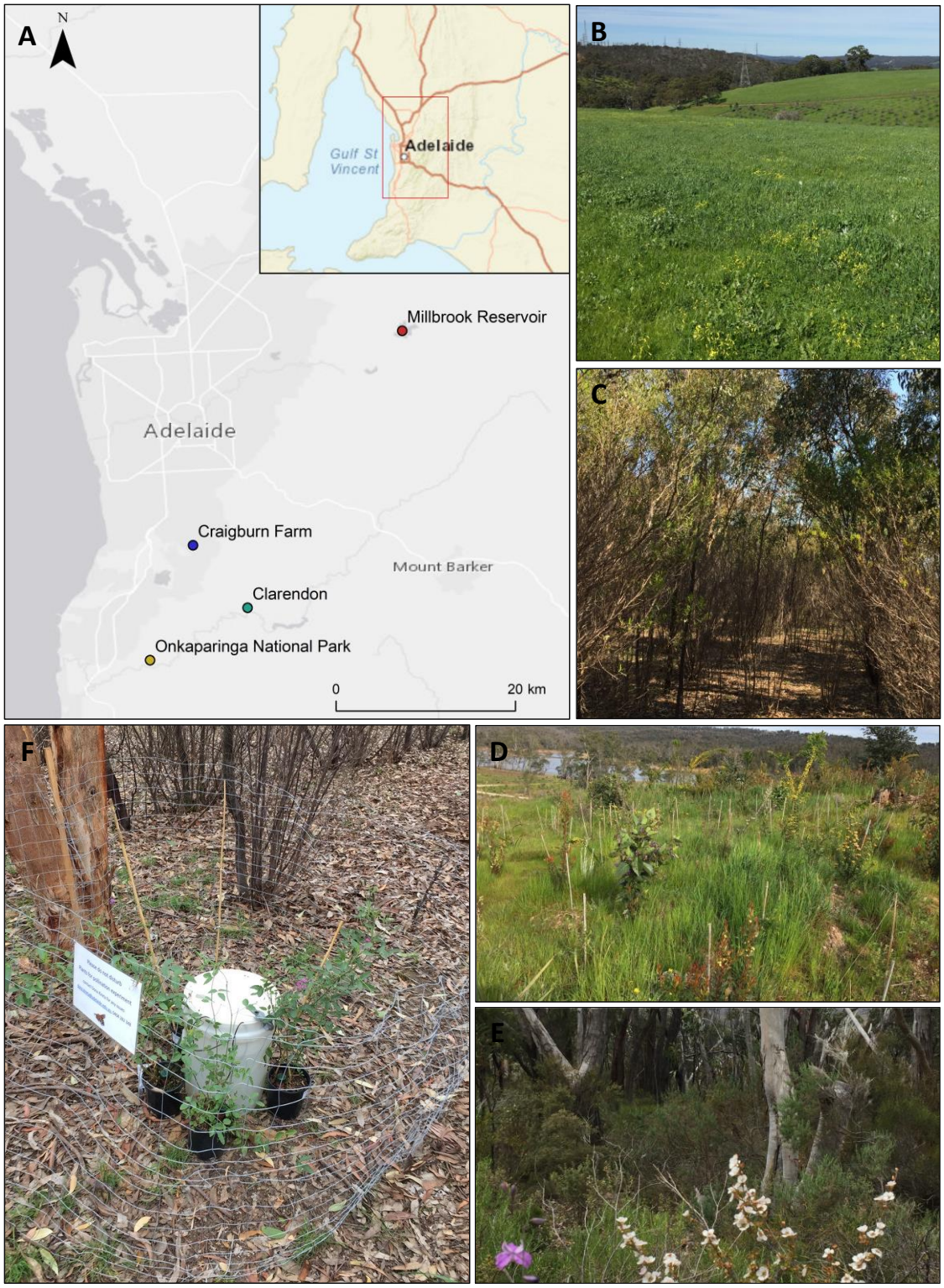

Figure 1. A: Locations of the revegetation sites used for bee surveys and pollination experiment. **B-D:** example photographs of the vegetation treatments; **B** negative control (cleared vegetation); **C** low diversity revegetation; **D** high diversity revegetation; **E** positive control (remnant vegetation). Millbrook reservoir had high diversity revegetation, remnant, and cleared land types; Craigburn Farm and Clarendon had high diversity and low diversity revegetation, remnant and cleared land types; and Onkaparinga National Park had low diversity revegetation, remnant and cleared land types. **F** Phytometer set up for measuring

pollination services (in a low diversity site): plants were fenced and connected to a Blumat® watering spike.

Quadrat design

A permanent 25 by 25 m quadrats was established within each treatment site. This size was chosen based on the High Div revegetation strategy employed by SA Water, where restoration effort was concentrated in 'diversity nodes', which were fenced to protect them from kangaroo grazing, which can be intense.

Flower surveys

Once per month during the flowering season (spring and summer 2019-20) flower surveys were conducted simultaneously with bee surveys. An exeption was in December 2018, when access to Clarendon and Millbrook was impeded by extreme fire danger, resulting in 7 of 84 data points missing. Within the 25 m quadrat, every flowering plant was recorded along three, meter-wide, parallel transects, that were spaced 10 m apart. The number of flowers per plant was recorded on a categorical metric scale (Brosi *et al.* 2007), for the total area of 75 m^2 surveyed. Unknown species were vouchered and identified using local floristic guides (Dashorst and Jessop 2006; Prescott 2012), and when deemed necessary, verified by staff from the State Herbarium of South Australia. The flower records from the six survey times (one survey each month to capture changing floral assemblages) were consolidated into one list, in which each species was kept only for the 'primary flowering' period, which was recognised by the number of flowers. For example, an *Acacia sp.* in transect 1 that flowered in September and October, but had more flowers in September, had only September records retained, and the records in other months were excluded. However, if another plant of the same species flowered in transect 2 in October, this was kept if it was the 'primary' instance of flowering. In effect, each plant was recorded once, to ensure individual flowers were included only once in the abundance measure. For the analysis, the midpoint of the flower number range was used for each plant recorded. Species that were known not to provide resources for bees were not recorded, such as grasses.

Bee surveys

Bee surveys were conducted at the same time as flower surveys. Bees visiting flowers were caught using an insect net over a period of 10 minutes inside and 10 minutes outside of each quadrat, to standardise sampling effort. Since native bees are only active when it is sunny and warm, sampling was restricted to sunny days with temperatures above 18°C and little wind. Captured bees were placed immediately on ice for pinning and subsequent identification. Bees were identified using a combination of morphological and DNA barcode methods. Morphological identifications were done where possible using keys to the genera in Michener (2007) and keys, when available, to the species (referred to in Michener 2007). For the

remainder of the samples, bee legs were plated and sent to the International Barcode of Life project for CO1 barcoding as part of a project to generate a barcode reference library of Australian native bee species (Hogendoorn *et al.* 2015).

Honey bees were not included in the bee surveys, although they were present and abundant at every sampling time point. Honey bees reach high densities locally with many feral hives occupying remnant paddock trees (Williamson *et al.* 2018), and their abundances in the field sites were likely related to proximity to feral colonies or managed hives. As these bees forage at large distances (Beekman and Ratnieks 2000), it is unlikely that their presence was affected by smaller scale habitat quality.

Pollination services

Two species of plants were used as phytometers to assess pollination services at each site: *Cullen australasicum* (Schltdl.) J.W.Grimes (native scurf-pea), and *Arthropodium strictum* R.Br. (chocolate lily). *C. australasicum* self-pollinates when its flowers are tripped (i.e. the lip pushed open), and can be pollinated by honey bees (Wang *et al.* 2010), whereas *A. strictum* is buzz pollinated and therefore not pollinated by honey bees (Gunn *et al.* 2020). Many Australian native bee species buzz pollinate (Smith and Saunders 2019), and therefore, the use of these two species could provide some insight into the pollination services provided by native and non-native bees in the landscape. On a practical level, these species were chosen because they can grow and flower in pots. Flowers are produced sequentially along a flower stem, and each flower produces small fruit when pollinated, which is easily counted. *C. australasicum* fruit contains one seed, and *A. strictum* fruit can contain many.

The phytometers were acquired in 2018 as tube stock, transferred to 140 mm pots, and established in the greenhouse. The plants were placed in the field in early September 2019 as each plant developed a flower stem, and were collected once flowering ended by late November 2019. Five plants of each species were placed within a chicken wire enclosure (to protect from grazing) within the 14 treatment plots (Fig. 1F). Ceramic watering spikes from Blumat® (Eurolux, Australia) were used to keep the plants watered for the duration of the field experiment (Fig. 1F). Following flowering, plants were collected and placed back into the greenhouse to allow the fruit to ripen. The fruit was collected once dry and counted, and flower petioles were counted to determine total numbers of flowers, since unpollinated flowers dropped off.

Analyses

To test whether treatments were different with respect to floral and bee diversity, Shannon diversity and species richness were calculated for each of the 14 sample plots in RStudio version 1.3.1 (RStudio Team 2020) using the 'vegan' R package (Oksanen *et al.* 2020). Shannon diversity was used since it accounts for species richness and relative abundance, contrary to Simpson which does not account for abundance. We

compared ANOVA and linear models with site as a random effect (as used to compare pollination services), to assess whether Shannon diversity differed between treatments. ANOVA had a lower AIC value than other models so this was ultimately used. Assumptions of normality were tested and met using Shapiro-Wilk tests and by plotting residuals. Species richness was used alongside diversity because we later extrapolated bee richness. For floral species richness (count data) a generalised linear model (GLM) with a Poisson error distribution and log link function was used to test differences between treatments. As the bee richness matrix contained many zeros, especially within the cleared treatment, we transformed the data by taking the log of response with 1 added as a constant to improve linearity and normality (e.g. Corcos *et al.* 2017). ANOVA was used to test for differences between the treatments. Finally, we ran Tukey's post-hoc tests using the 'agricolae' package for diversity, and 'multcomp' package for richness, to determine pairwise differences among treatments (de Mendiburu 2020).

To compare the completeness of bee sampling between sites, we built individual-based species rarefaction curves for each treatment at each site using the rarecurve function from 'vegan'. Following this, species richness was extrapolated to account for differences in sample size using the estimateR function from 'vegan'.

The sites were compared lastly in terms of flower and bee composition with a principal coordinates analysis (PCoA) performed with the dbrda function from the 'vegan' R package. Bray-Curtis and Jaccard distances were compared with very little difference between the two. Ultimately, Bray-Curtis distance was used to account for differences in relative abundance as well as species presence/absence. The flower data was square root transformed prior to Bray-Curtis dissimilarity since it appeared that some dominant plant species were driving the ordination.

Fruit for *C. australasicum* was counted and weighed (each fruit only contained one seed), and *A. strictum* seeds were extracted from fruit, counted and weighed. ANOVA tests showed no statistical difference in seed weight, or number of seeds per fruit (for *A. strictum*), between treatments, so only fruit number was further considered.

To determine whether revegetation quality had an effect on pollination services, generalised linear mixed effect models (GLMMs) were used. We used the glmer function from the 'lme4' package (Bates *et al.* 2015) to perform logistic regression after converting flower and fruit counts to a binary variable representing success or failure per flower. The data were overdispersed, likely due to large variation between treatment replicates (at different sites). This was expected since the sites varied geographically and in their ecological structure. An observation level random effect was added to the model (plant ID), to deal with overdispersion and non-independence of samples originating from the same plant. We also included site and site*plant interaction as random effects in the model. There was no significant difference between the models with different random effects. The model with plant ID as a

random effect had the lowest AIC value and was therefore chosen: $glmer\big(Fruit\ set \sim Treatment + (1|plant\ ID) + (1|Site)\big)$. We used the contrast function from the 'emmeans' R package, with method set to pairwise, and default adjustment for multiple comparisons (Tukey), for post-hoc testing to find statistical difference between the predicted mean fruit set of the vegetation treatments.

Results

Flower surveys assessed 26,599 flowers from 72 plant species within the surveyed sites. Bee surveys resulted in 242 bees collected from 34 flowering species within the same sites. The *Cullen australasicum* phytometers produced 8,274 flowers, of which 1,887 produced fruit. The *Arthropodium strictum* phytometers produced 2,210 flowers, of which 358 produced fruit. Plant and bee site species lists are found in the supplementary materials (Table S1; Table S2), as is a phylogenetic tree representing bee diversity across the study (Figure S1).

Diversity and richness – flowers and bees

The treatments differed significantly in flower diversity (one-way ANOVA, DF = 3, $p < 0.001$; Fig. 2). Tukey's HSD post-hoc comparison test showed that Cleared sites and Low Div revegetation did not differ in diversity. Cleared and Low Div both had lower diversity than High Div and Remnant sites, while the latter two did not differ significantly in flower diversity (Table S3).

The species accumulation curves suggest that our sampling effort underestimated bee species richness within all sites, since no curves reached an asymptote (Fig. 3). Clarendon Remnant had the highest number of individuals caught, more than double any other site.

The treatments also differed significantly in bee diversity (one way ANOVA, DF = 3, $p < 0.005$; Fig. 2A). Tukey's HSD post-hoc comparison test showed that Cleared and Low Div did not differ significantly and had the lowest bee diversity. Low Div and High Div did not differ significantly in bee diversity (one way ANOVA, DF = 3, $p = 0.07$). High Div and Remnant were also not significantly different, but Remnant had higher bee diversity than Cleared and Low Div sites (Table S3; Fig. 2A).

The treatments differed significantly in floral species richness, observed bee richness, and extrapolated bee richness (floral richness: $\chi^2 = 94.0$, DF = 3, $p < 5e\text{-}16$; bee richness, one way ANOVA: DF = 3, $p < 0.005$; extrapolated bee richness, one way ANOVA: DF = 3, $p < 0.0005$; Table S4; Fig. 2B). The pairwise differences between treatments in floral richness, observed bee richness and extrapolated bee richness all mirrored those of floral diversity (Fig. 2B).

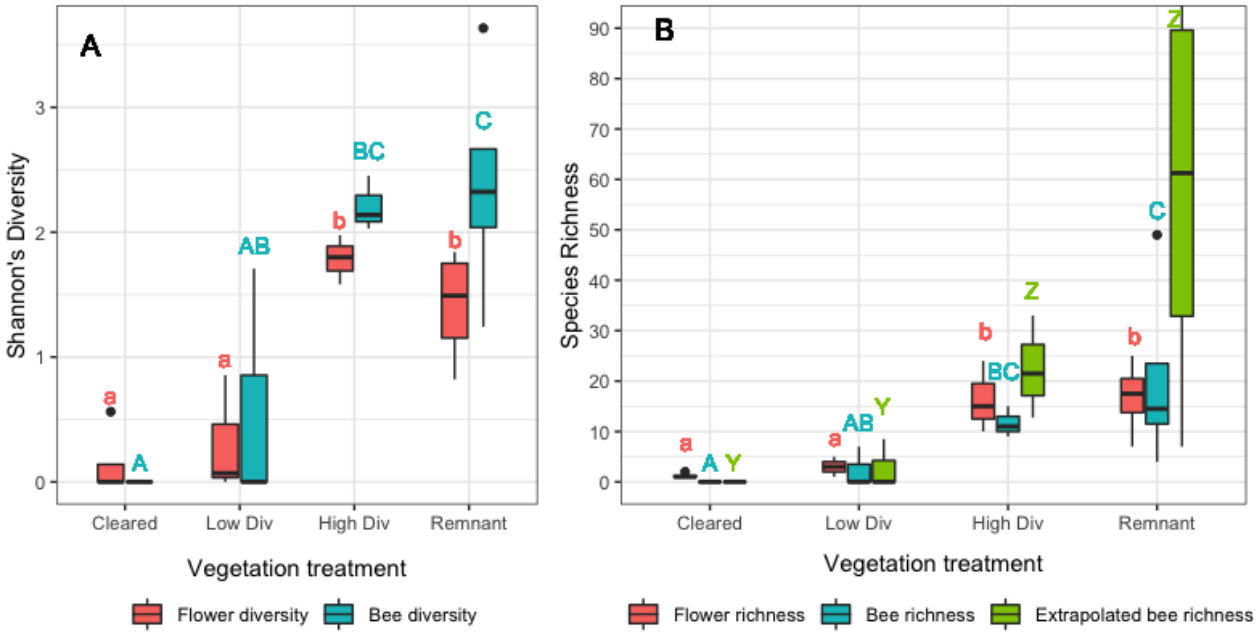

Figure 2: Box plot of Shannon's species diversity (**A**), and species richness (**B**) in four vegetation treatments: low diversity (Low Div) and high diversity (High Div) revegetation, with negative (Cleared) and positive (Remnant) controls. Bars depict survey results for flower diversity/richness (pink, left), observed bee diversity/richness (aqua, right/middle), and extrapolated bee richness (green, right). Top whisker for remnant extrapolated bee richness extended to 115.4, but was cropped for better resolution. Letters indicate significance between treatments according to post hoc tests within each diversity index, e.g. tests compared differences between flower diversity across treatments, not between flower and bee diversity within a treatment.

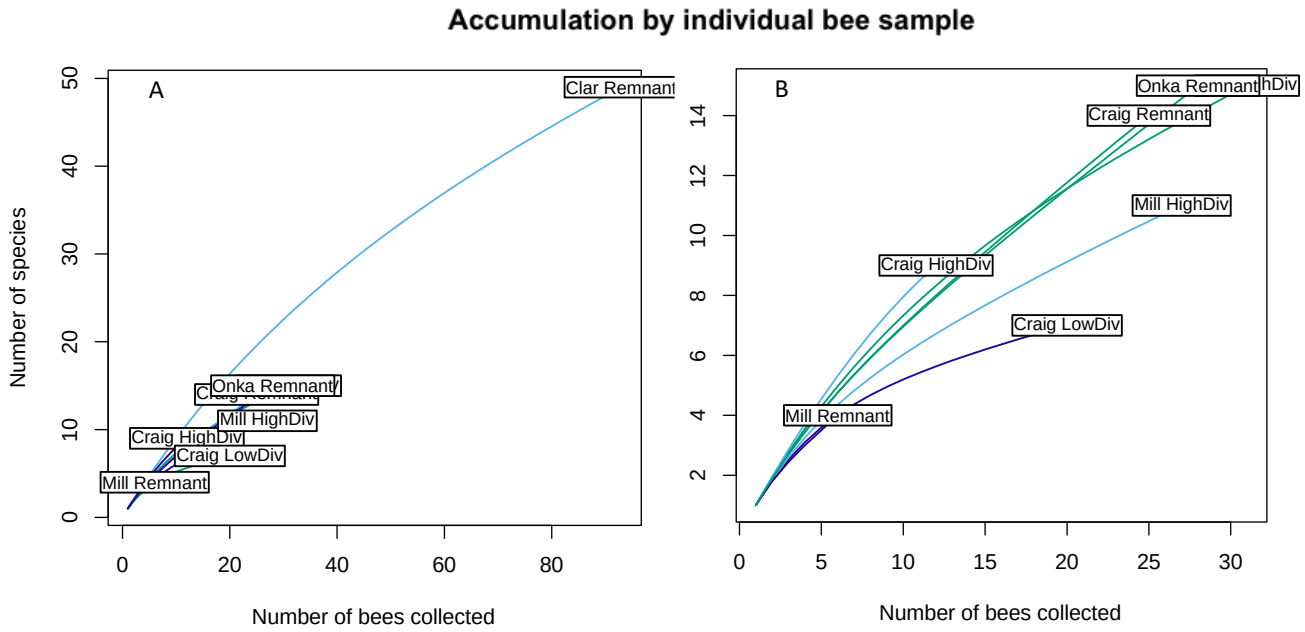

Figure 3: Species accumulation curves of bees surveyed in 4 sites and 4 vegetation treatments. Curves for negative controls (4 sites), Onkaparinga Low Div and Clarendon Low Div were absent, as no bees were captured. (**A**) All sites with bees captured; (**B**) the same plot with Clarendon Remnant excluded for better resolution. The top two labels of plot B overlap and read Onkaparinga Remnant and Onkaparinga High Div.

Community composition - floral and bee

The sites varied in their floristic composition as well as their diversity (Fig. 4; A). Three of the four Cleared controls clustered together, the fourth Cleared site overlapped with Clarendon Low Div. Two of the three High Div revegetation sites also clustered together. The Remnant treatments were similar along MDS1, but scattered across MDS2, as were the Low Div sites plus Craigburn High Div.

The bee community composition differed between the sites, with only Clarendon and Millbrook High Div sites having close proximity on the plot, indicating that these sites had similar bee communities (Fig. 4; B).

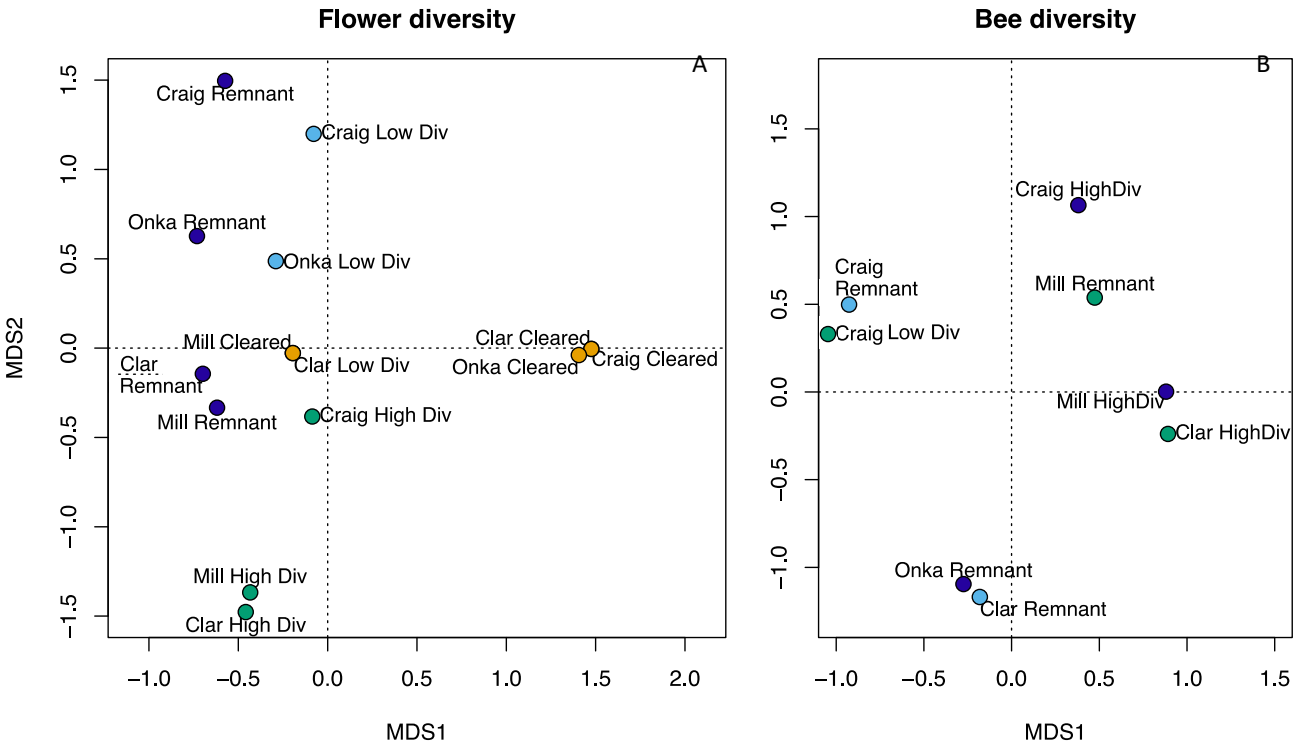

Figure 4: Ordination plot depicting site similarity based on (**A**) floral diversity and (**B**) bee diversity. Site abbreviations used: Clar = Clarendon, Craig = Craigburn, Mill = Millbrook and Onka = Onkaparinga. Colours indicate treatment (orange = cleared, light blue = low diversity, green = high diversity, dark blue = remnant).

Pollination services

The proportion of fruit set was not significantly different between vegetation treatments for *C. australasicum* phytometers ($\chi^2 = 3.75$, DF = 3, p = 0.29, Table S5, Fig. 5). However, there was a significant difference in fruit set between treatments for *A. strictum* ($\chi^2 = 14.59$, DF = 3, $p < 0.01$). Post-hoc tests revealed that Remnant had significantly higher fruit set than Cleared, Low Div and High Div, and that the latter two did not differ significantly due to high variance within the treatments (Table S6; Fig. 6 B). Fruit set was observed in the cleared controls for both species (*C. australasicum* = 0.20, *A. strictum* = 0.09), despite no bees being observed during surveys. The proportion of fruit set was overall higher for *C. australasicum* (max 1) than for *A. strictum* (max 0.5).

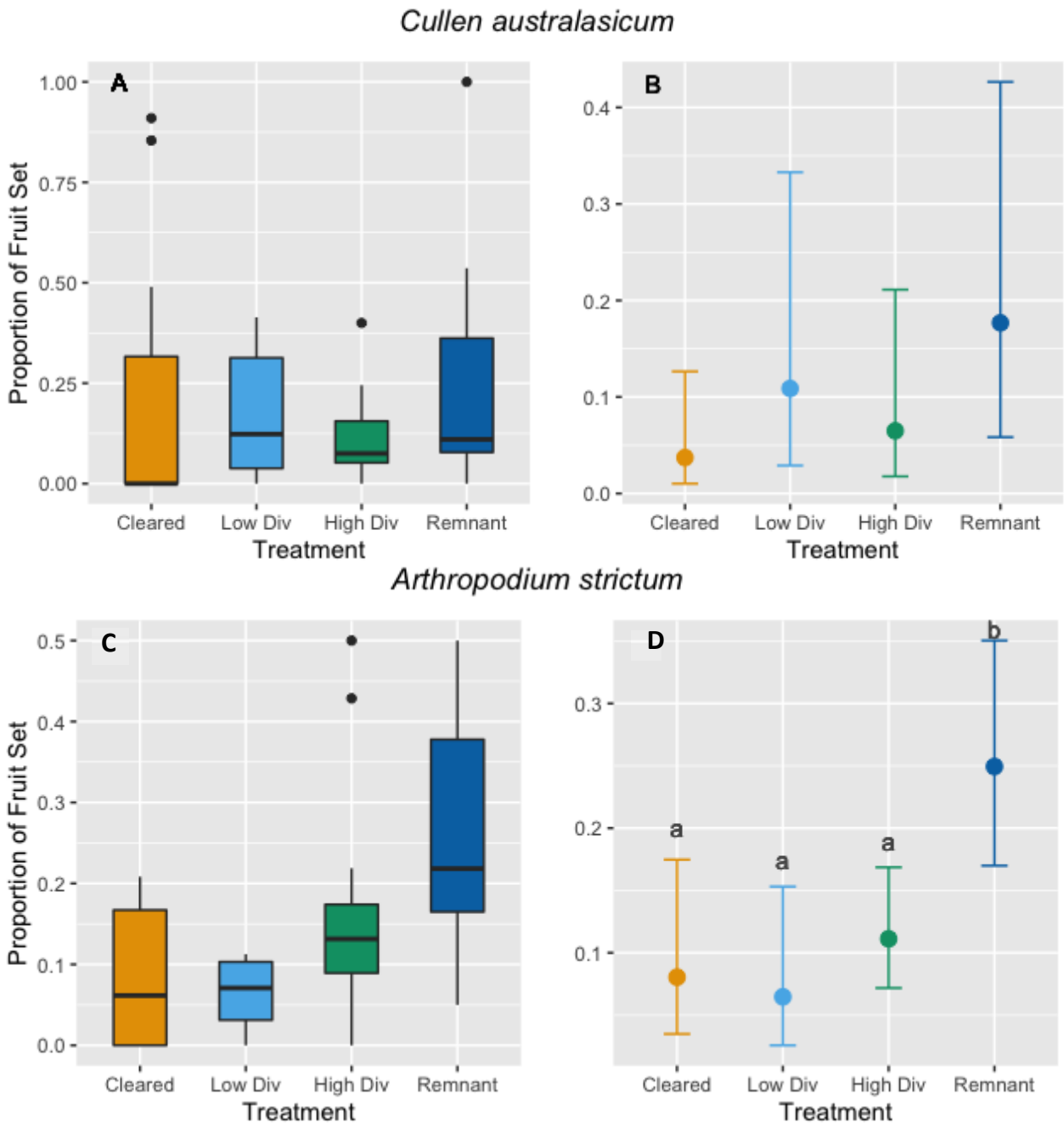

Figure 5: Plots of the proportion of fruit set for *Cullen australasicum* (top: **A**, **B**), and *Arthropodium strictum* (bottom: **C**, **D**) phytometers. **A**, **C**: Box plots of fruit set per phytometer. **B**, **D**: post hoc test showing predicted mean fruit set of phytometers with error bars showing 95% confidence limits, and letters in **D** indicating significant differences. Colours indicate treatment (orange = cleared, light blue = low diversity, green = high diversity, dark blue = remnant).

Discussion

Main findings

This study provides an Australian assessment of pollination services provided in habitats of different quality and restoration status. We found that increases in floral diversity in revegetation corresponded with increases in bee diversity, up to levels on par with bee diversity in remnant vegetation. Pollination services were different depending on the phytometer species. Fruiting success was not predicted by the observed bee diversity for the species *Cullen australasicum,* which is pollinated by generalist bees including honey bees, whereas fruiting success was higher in remnant vegetation for the phytometer species *Arthropodium strictum*, which is pollinated only by buzz pollinating native bees. We discuss these findings below and suggest limitations that may have led to underestimates of bee diversity.

Diversity and richness – flowers and bees

Bee diversities found in remnant patches were unmatched in either type of revegetation, even where similar levels of floral diversity were achieved. In revegetated sites, bee diversity may increase when diverse floral resources are provided. Floral diversity was lower and similar between cleared land and low diversity revegetation, and was primarily composed of invasive weeds, plus early spring flowering *Acacia* in the revegetation sites. High diversity revegetation and remnant vegetation had similar and high floral diversity, indicating that revegetation projects focusing on high planting diversity had managed to restore baseline levels floral diversity.

As was expected, bee diversity and richness increased with increasing floral diversity and floral richness, suggesting that bee diversity can recover toward remnant levels provided that high diversity plantings are established, and maintenance is continued long-term. The slope of the species accumulation curves, plus the large difference between observed and extrapolated bee richness in higher quality vegetation, indicate that despite the sampling effort, many species were missed, particularly in the remnant vegetation.

Although the data indicate that low diversity revegetation performed well in terms of recovering bee diversity and species richness, this result is caused by an outlier as, except for three individuals, all bees captured in low diversity sites were caught from a single flowering *Eucalyptus* tree at Craigburn. This tree contributed 20 bee samples, while across all sample sites 120 bees were caught from flowering *Eucalyptus*, comprising 49.6% of all bees captured. This indicates that *Eucalyptus* is a very important bee resource in the Mt Lofty Ranges irrespective of understorey floral diversity in the associated habitat.

Pollination services

Pollination services measured in remnant patches were also unmatched in either type of revegetation, although the result differed between the phytometers. There was no effect of revegetation quality on pollination services, as measured by fruit set, for *C. australasicum* which is pollinated by honey bees and native bees. However, pollination services to *A. strictum*, the buzz pollinated phytometer, increased with vegetation quality, and remnant vegetation had significantly higher levels of pollination than other vegetation treatments.

The uniform pollination of *C. australasicum* can be explained by the foraging behaviour of honey bees. Although *C. australasicum* is pollinated by both native and non-native bees, honey bees were extremely abundant in the landscape, as they are in many parts of Australia (Paton 1993; Oldroyd *et al.* 1997). They forage over large distances (Beekman and Ratnieks 2000), and were observed to forage on the phytometers in the lower diversity revegetation sites. This result is consistent with similar studies: there was no change in honey bee abundance with revegetation adjacent to agricultural plots (Morandin and Kremen 2013), and blueberry crops (Blaauw *et al.* 2014), but wild bees increased in both studies, meaning that honey bees were abundant in the poorer pre-revegetation landscape.

Honey bees do not pollinate *A. strictum* as it is a buzz pollinated plant. The native buzz pollinating bees are less likely to survive in poorer habitats that have insufficient floral resources, and this probably explains the lower levels of pollination services seen in cleared and low diversity revegetation, compared with higher diversity revegetation and remnant sites. Although there is some variation in the estimated distance travelled by solitary bees (Gathmann and Tscharntke 2002; Greenleaf *et al.* 2007; Zurbuchen *et al.* 2010), it is clear that their foraging range is much smaller than that of honey bees. Given short foraging distances, solitary bees are highly dependent on the habitat in the immediate surroundings of their nests, and it is suggested that a close and dense network of nesting and foraging sites is needed for their conservation (Gathmann and Tscharntke 2002; Zurbuchen *et al.* 2010). In addition, buzz pollinated plants such as *A. strictum* do not produce nectar, and therefore solitary bees would require nectar sources in the immediate surroundings to be able to visit these plants.

The high extrapolated bee richness explains the higher level of pollination services to *A. strictum* in remnant vegetation. Although pollination services in high diversity revegetation were higher than in low diversity revegetation, the lack of statistically significant differences between them was likely contributed to by modest sample sizes and high variance within treatments. There was further less opportunity to see treatment effects for the *A. strictum* phytometer because fruit set was naturally low (evidenced in the remnant treatments), and therefore somewhat stochastic in the small sample. Low proportional fruiting success has been documented in other buzz pollinated natives, e.g. *Dianella revoluta* (Duncan 2003; Duncan *et al.* 2004).

Furthermore, since *A. strictum* flowers sequentially along the stem (Gunn *et al.* 2020), some of the earlier set fruit matured before plant collection and may have been predated on by birds, which we have observed previously. Assuming that extrapolated values of bee richness are the less biased estimate, we conclude that the levels of bee richness/diversity required to restore pollination services to remnant levels are much higher than those found in revegetation sites.

Project limitations

The small samples sizes of bees was likely due to a combination of the difficulty of sampling on flowering *Eucalyptus*, difficult site access, and adverse weather conditions. *Eucalyptus* trees were present at all sites, but flowering between the sites was inconsistent, likely because of drought conditions and extremely low spring rainfall in preceding years, and the extreme heat wave in summer (Law *et al.* 2000; Australian Bureau of Meteorology 2020, 2021). *Eucalyptus* flowering is influenced by fire and seasonal rain (Law *et al.* 2000). *Eucalyptus* trees were only sampled in our surveys within low diversity revegetation, as those trees were mature enough to flower but had not grown too tall to access. By contrast, in remnant and high diversity sites, eucalypts were not sampled, because the trees were either too young to flower (see Table 1), failed to flower, or were too tall to access, the latter in particular in remnant sites.

Our experimental design also had some limitations. It would have been ideal to have multiple replicates within the revegetation sites, but unfortunately the revegetation sites were too small for independent sampling as the home range of bees is relatively large from 50 - 200 m, and these would have been pseudo-replicates. Hence, we used multiple sites with similar revegetation treatments, and had positive and negative controls at each site. This meant there were uncontrolled differences in addition to the revegetation design. Other studies use additional trapping methods such as pan traps, to supplement small catch numbers. However, we opted not to do this because it is becoming increasingly clear that traps are more attractive to bees when there are fewer floral resources in the landscape (Baum and Wallen 2011; Westerberg *et al.* 2021). Furthermore, the data from this study was required in subsequent studies that utilised the pollen collected by the bees, so other trapping methods were not appropriate as they would have introduced contamination.

It would also have been ideal to space the phytometer plants throughout the treatment rather than having them grouped, this was not practical in our study. To allow the phytometers to receive adequate pollination, we kept them in the field for the duration of their flowering, which was approximately 2 months. This required a watering system to keep them hydrated, and therefore they needed to be close together. This may have increased the attractiveness of the treatments, particularly

the negative control and low diversity revegetation, where other flower resources were poor, and increased the pollination services measured.

Lastly, we are aware that bee restoration success could be influenced by a number of other factors not tested here. The Island Biogeography Theory states that the number of species colonising an island (revegetation site) is determined by the immigration and extinction of species, which in turn depend on the distance to the source population (remnant land), and the size of the island (revegetated land) (MacArthur and Wilson 2009). This means the closer that revegetation is to remnant land, and the larger the planting is, the more species will colonise it through targeted dispersal, and by random chance. Hence, with time and larger plantings, species diversity could continue to increase. Revegetation size and landscape context may be interesting and important avenues for future study.

Conclusion

Bee diversity and pollination services found in remnant sites were unmatched in any type of revegetation, even where similar levels of floral diversity were restored. To protect native bees, the retention and management of remnant vegetation is therefore of the utmost importance, and should be a priority. In revegetated sites, bee diversity and pollination services may increase with time and with area revegetated, but only when diverse floral resources are provided.

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Supplementary material: Bee diversity and pollination services improve with revegetation effort

Table S1: Floral plant species list for each survey site

Site	Treatment	Species
Clarendon	Cleared	*Arctotheca calendula*
	Low Div	*Acacia pycnantha*
	High Div	*Arctotheca calendula*
		Billardiera cymosa
		Chrysocephalum apiculatum
		Chrysocephalum semipapposum
		Dianella revoluta
		Divisia leptofyla
		Eucalyptus leucoxylon
		Goodenia amplexans
		Hakea carinata
		Hypochaeris sp.
		Myoporum viscosum
		Pultenaea largiflorens
		Vittadinia blackii
		Vittadinia cuneata
		Acacia acinacea
	Remnant	*Acacia myrtifolia*
		Arthropodium strictum
		Asteraceae sp.
		Burchardia umbellata
		Calytrix tetragona
		Chamaescilla corymbosa
		Dianella revoluta
		Dillwinia hispida
		Drosera sp.
		Eucalyptus sp.
		Freesia sp.
		Glossodia major
		Goodenia blackiana
		Grevillia lavandacea
		Hakea rostrata
		Hibbertia sp. _3
		Hibbertia sp._4
		Hibbertia sp._1
		Ixodia achillaeoides
		Leptospermum continentale
		Lucopogon sp.
		Olearia ramulosa
		Platylobium obtusangulum
		Spyridium parvifolium
		Thysanotus patersonia

Craigburn	Cleared	*Arctotheca calendula*
	High Div	*Acacia myrtifolia*
		Arctotheca calendula
		Dodonea viscosa
		Echium plantagineum
		Eucalyptus leucoxylon
		Goodenia amplexans
		Grevillia lavandacea
		Grevillia sp._1
		Grevillia sp._2
		Olearia ramulosa
	Low Div	*Acacia acinacea*
		Acacia paradoxa
		Arctotheca calendula
		Dodonea viscosa
		Echium plantagineum
	Remnant	*Acacia paradoxa*
		Arthropodium strictum
		Burchardia umbellata
		Caesia calliantha
		Fumaria capreolata
		Hibbertia sp. _3
		Hibbertia sp._1
Millbrook	Cleared	*Hypochairis sp.*
	High Div	*Acacia acinacea*
		Acacia myrtifolia
		Arctotheca calendula
		Arthropodium strictum
		Billardiera cymosa
		Bulbine bulbosa
		Chrysocephalum apiculatum
		Chrysocephalum semipapposum
		Dianella revoluta
		Grevillia lavandacea
		Hakea carinata
		Hakea rostrata
		Hardenbergia violacea
		Hibbertia sp._2
		Hypochaeris sp.
		Kennidia prostrata
		Leptorhynchos squamatus
		Leptospermum myrsinoides
		Linum marginale
		Scaevola albida
		Tricoryne elatior
		Vittadinia cuneata
		Wahlenbergia sp
		Xerochrysum bractiatum
	Remnant	*Arthropodium strictum*

		Billardiera cymosa
		Brunonia australis
		Dillwinia hispida
		Diuris sp.
		Drosera sp.
		Goodenia blackiana
		Hibbertia sp. _3
		Hibbertia sp._1
		Hibbertia sp._2
		Hypochaeris sp.
		Kennidia prostrata
		Leptospermum sp.
		Pultenaea largiflorens
		Calytrix sp.
		Thelymitra antennifera
Onkaparinga	Cleared	*Arctotheca calendula*
		Trifolium sp.
	Low Div	*Acacia paradoxa*
		Acacia verticilata
		Sinapis sp.
	Remnant	*Acacia myrtifolia*
		Acacia verticilata
		Anagallis arvensis
		Arthropodium strictum
		Boronia coerulescens s sp.coerulescens
		Burchardia umbellata
		Caesia calliantha
		Calytrix tetragona
		Chamaescilla corymbosa
		Daviesia ulicifolia
		Dillwinia hispida
		Diuris sp.
		Dodonea viscosa
		Eucalyptus sp.
		Glossodia major
		Helichrysum scorpioides
		Hibbertia sp._1
		Thysanotus patersonia
		Wahlenbergia sp.

Table S2: Bee species list at each survey site

Site	Treatment	Bee species
Clarendon	High Div	*Amegilla (Zonamegilla) murrayensis*
		Homalictus sp. BIN:AAM1019
		Lasioglossum (Chilalictus) clelandi
		Lasioglossum (Chilalictus) expansifrons

	Lasioglossum (Chilalictus) lanarium
	Lasioglossum (Chilalictus) repraesentans
	Lasioglossum (Chilalictus) sp.
	Lasioglossum (Chilalictus) willsi
	Lasioglossum (Parasphecodes) hiltacus
	Lasioglossum sp.
	Leioproctus (Euryglossidia) sp. BIN:AEC5195
	Leioproctus (Leioproctus) platycephalus
	Leioproctus (Leioproctus) sp._b
	Lipotriches (Austronomia) australica
	Trichocolletes venustus
Remnant	*Callohesma calliopsella*
	Euhesma (Euhesma) bronsis
	Euriglossina sp. BIN:AEC4857
	Euryglossa capitata
	Euryglossina (Euryglossina) cf. hypochroma
	Euryglossina (Euryglossina) hypochroma
	Euryglossina (Euryglossina) stygica
	Exoneura (Brevineura) sp._b
	Exoneura (Exoneura) sp._a
	Exoneura sp. BIN:AAY4584
	Exoneura sp. BIN:ABX9558
	Exoneura sp. BIN:AEC5987
	Homalictus (Homalictus) punctatus
	Homalictus (Homalictus) sp. BIN:AAM1019
	Homalictus sp. BOLD:AEC2529
	Homalictus sp. BIN:AAM1019
	Hylaeus (Euprosopis) elegans
	Hylaeus (Euprosopis) honestus
	Hylaeus (Prosopisteron) sp. BIN:AAX2611
	Hylaeus (Prosopisteron) sp. BIN:AEC3080
	Hylaeus (Prosopisteron) sp. BIN:AEC3194
	Hylaeus (Prosopisteron) sp. BIN:AEC4499
	Hylaeus (Rhodohylaeus) proxima
	Hylaeus sp. BOLD:AEC1767
	Hylaeus sp. BOLD:AEC2227
	Lasioglossum (Chilalictus) clelandi
	Lasioglossum (Chilalictus) erythrurum
	Lasioglossum (Chilalictus) erythrurum BOLD:AEC6142
	Lasioglossum (Chilalictus) imitans
	Lasioglossum (Chilalictus) littleri
	Lasioglossum (Chilalictus) mundulum
	Lasioglossum (Chilalictus) sp.

		Lasioglossum (Chilalictus) sp._b
		Lasioglossum (Chilalictus) speculatum
		Lasioglossum (Chilalictus) vitripenne
		Lasioglossum (Chilalictus) willsi
		Lasioglossum (Ctenonomia) sp._c
		Lasioglossum (Parasphecodes) sulthica
		Lasioglossum(Chilalictus) vitripenne
		Leioproctus (Euryglossidia) sp. BIN:AEC5195
		Leioproctus (Leioproctus) cupreus
		Leioproctus (Leioproctus) maculatus
		Leioproctus (Leioproctus) plumosus
		Leioproctus (platycephalus group)
		Leioproctus cupreus
		Leioproctus sp. BIN:AEC3994
		Lipotriches (Austronomia) australica
		Lipotriches (Austronomia) flavoviridis
		Lipotriches (Austronomia) moerens
Craigburn	Low Div	*Callohesma calliopsella*
		Euryglossa adelaidae
		Euryglossa sp.
		Homalictus (Homalictus) punctatus
		Hylaeus (Prosopisteron) sp. BIN:AEC4499
		Hyphesma atromicans
		Megachile (Hackeriapis) tosticauda
	High Div	*Lasioglossum (Chilalictus) lanarium*
		Lipotriches (Austronomia) flavoviridis
		Megachile (Eutricharaea) obtusa
		Megachile (Eutricharaea) sp.
		Megachile (Hackeriapis) oblonga
		Megachile (Torridapis) apicata
		Megachile (Unplaced) ordinaria
		Megachile sp.
		Megachilidae sp.
	Remnant	*Callohesma calliopsella*
		Euryglossa adelaidae
		Homalictus (Homalictus) punctatus
		Hylaeus (Rhodohylaeus) proxima
		Lasioglossum (Chilalictus) bicingulatum
		Lasioglossum (Chilalictus) brunnesetum
		Lasioglossum (Chilalictus) clelandi
		Lasioglossum (Chilalictus) speculatum
		Leioproctus (Leioproctus) maculatus
		Leioproctus (Leioproctus) sp._b

		Leioproctus (unplaced) sp. BIN:AEC1806
		Lipotriches (Austronomia) flavoviridis
		Megachile sp.
		Pachyprosopis (Pachyprosopis) haematosoma
Millbrook	High Div	*Lasioglossum (Chilalictus) clelandi*
		Lasioglossum (Chilalictus) lanarium
		Lasioglossum (Chilalictus) plorator
		Lasioglossum (Chilalictus) repraesentans
		Lasioglossum (Chilalictus) sp. BOLD:AEJ4129
		Leioproctus (Leioproctus) maculatus
		Leioproctus (Leioproctus) platycephalus
		Leioproctus (unplaced or irroratus group) *sp.* BIN:AEC399
		Lipotriches (Austronomia) australica
		Lipotriches (Austronomia) flavoviridis
		Trichocolletes venustus
	Remnant	*Lasioglossum (Chilalictus) lanarium*
		Leioproctus (Leioproctus) maculatus
		Leioproctus (Leioproctus) sp._b
		Trichocolletes venustus
Onkaparinga	Remnant	*Euryglossinae sp.*
		Exoneura sp.
		Homalictus (Homalictus) punctatus
		Hylaeus (Euprosopis) honestus
		Hylaeus (Prosopisteron) sp. BIN:AEC4499
		Hylaeus (Prosopisteron) sp. near BIN:AEC2886
		Hylaeus (Prosopsteron) sp. BIN:AEC4499
		Hylaeus (Rhodohylaeus) proxima
		Hylaeus sp.
		Lasioglossum (Chilalictus) brunnesetum
		Lasioglossum (Chilalictus) sp. BIN:AEC5169
		Lasioglossum (Chilalictus) sp._a
		Lipotriches (Austronomia) australica
		Trichocolletes venustus
		Xanthesma (Xanthesma) argosomata

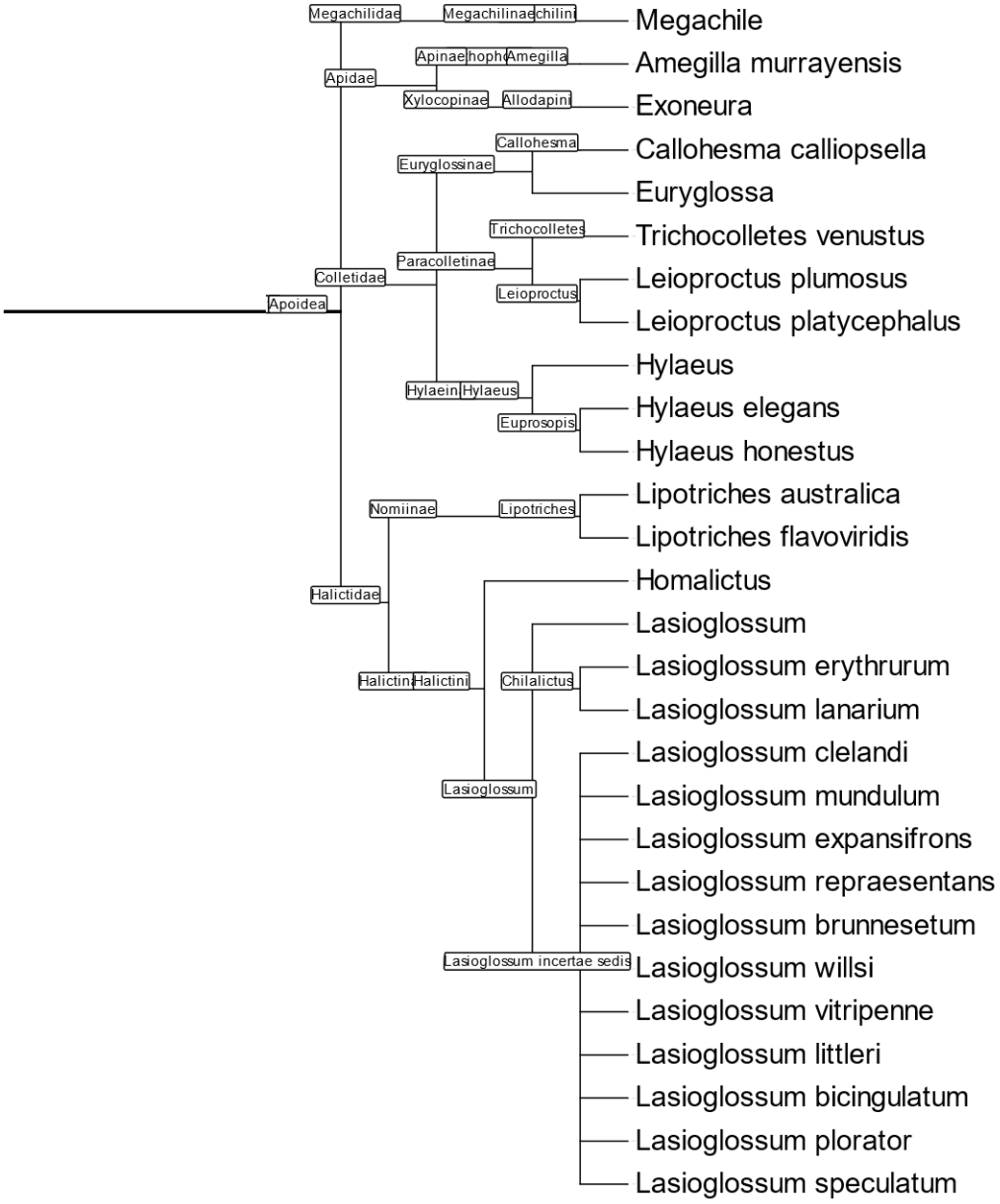

Figure S1. Phylogenetic tree providing an overview of bee diversity across the study. Built using a list of unique species from the study with phyloT v2, based on NCBI taxonomy. Note: this figure represents a very abbreviated list of species, since many species were identified to morphotypes, and many species were not available in NCBI at the time of building (June 2023).

Table S3: Tukey's pairwise comparison statistics for means of Shannon's diversity indices for flower and bee surveys in four vegetation treatments. LCL and UCL are lower and upper 95% confidence limits.

	Flower diversity				Bee diversity			
contrast	Est.	LCL	UCL	P val.	Est.	LCL	UCL	P val.
Low Div - Cleared	0.168	-0.712	1.05	0.94	0.570	-1.069	2.208	0.72
High Div - Cleared	1.65	0.766	2.53	<0.001	2.207	0.568	3.845	<0.01
Remnant - Cleared	1.27	0.456	2.09	<0.005	2.381	0.864	3.898	<0.005
High Div - Low Div	1.48	0.537	2.42	<0.005	1.64	-0.115	3.389	0.069
Remnant - Low Div	1.1	0.223	1.98	<0.05	1.811	0.173	3.450	<0.05
Remnant - High Div	-0.375	-1.25	0.505	0.58	0.174	-1.464	1.813	0.99

Table S4: Tukey's pairwise comparison statistics for means of species richness for flower and bee surveys, plus extrapolated bee species richness in four vegetation treatments.

	Flower richness				Bee richness				Extrapolated bee richness			
contrast	Est.	S.E.	Stat.	adj. P val.	Est.	LCL	UCL	P val.	Est.	LCL	UCL	P val.
Low Div - Cleared	0.88	0.56	1.57	0.37	0.69	-1.06	2.45	0.64	0.75	-1.34	2.84	0.70
High Div - Cleared	2.57	0.47	5.47	<5e-07	2.52	0.76	4.28	<0.01	3.09	0.99	5.18	<0.01
Remnant - Cleared	2.60	0.46	5.60	<1e-07	2.75	1.12	4.38	<0.005	3.75	1.81	5.69	<0.001
High Div - Low Div	1.69	0.36	4.67	<5e-05	1.83	-0.05	3.7	0.057	2.34	0.10	4.57	<0.05
Remnant - Low Div	1.72	0.36	4.84	<1e-05	2.06	0.30	3.81	<0.05	3.0	0.90	5.09	<0.01
Remnant - High Div	0.03	0.19	0.13	1.0	0.23	-1.53	1.99	0.977	0.66	-1.43	2.75	0.771

Table S5. The estimated regression parameters, standard errors, z-values and significance of the difference in fruit set of *Cullen australasicum* and *Arthropodium strictum* with the cleared site (GLMM), assuming a binomial model. Bold indicates significance > 0.05.

Fixed effect (Treatment)	***Cullen australasicum***				***Arthropodium strictum***			
	Est.	S.E.	z	P val.	Est.	S.E.	z	P val.
(Intercept)	-3.25	0.674	-4.83	1.38E-6	-2.44	0.451	-5.4	6.58E-08
Low Div	1.15	0.984	1.17	0.243	-0.235	0.662	-0.356	0.722
High Div	0.586	0.954	0.615	0.539	0.359	0.515	0.696	0.486
Remnant	1.72	0.924	1.86	0.0635	1.33	0.51	2.62	<0.01

Table S6. Comparison between treatments of fruit set in *Arthropodium strictum*. Columns are estimate of the difference between treatment means (Est.) using Tukey's correction a family of 4 estimates, with standard errors (S.E.). Values with a significance **< 0.05** are indicated in bold. Results are on the log odds ratio scale. P value adjustment: calculated using the emmeans package in RStudio.

Pairwise comparison bet. treatments	Est.	S.E.	z	P val.
Cleared - Low Div	0.235	0.662	0.356	0.985
Cleared - High Div	-0.359	0.515	-0.696	0.90
Cleared - Remnant	-1.335	0.51	-2.618	<0.05
Low Div - High Div	-0.594	0.549	-1.081	0.701
Low Div - Remnant	-1.57	0.548	-2.866	<0.05
High Div - Remnant	-0.976	0.349	-2.794	<0.05

CHAPTER 3: A novel approach for pollen identification and quantification using hybrid capture-based DNA metabarcoding

Statement of Authorship

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Overall percentage (%)	70%		
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A novel approach for pollen identification and quantification using hybrid capture-based DNA metabarcoding

Running head

Pollen ID via hybrid capture metabarcoding

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Key words

Pollen metabarcoding, Pollen quantification, Hybridization capture, Target enrichment

Abstract

Increasing effort is being invested into exploring optimal molecular methods for identifying mixtures of plants, and in particular pollen. Pollen identification (ID) is an important objective for many scientific fields, including pollination ecology and agricultural sciences, where the quantification of mixture proportions is sought after but remains challenging. Traditional pollen ID relies on microscopy, but this is time consuming and requires expertise, while being limited in accuracy and throughput. Molecular barcoding approaches have been explored and offer increased accuracy and throughput. The most common approach (amplicon sequencing) uses PCR amplification to isolate DNA barcodes, but this introduces significant bias, impairing downstream quantification. We explore here a novel molecular hybridisation capture approach to improve upon current methods for identifying and quantifying taxa, applied to artificial pollen mixtures. The method uses complementary RNA baits to capture DNA barcodes of interest, and produces random length DNA fragments, which allow for removal of PCR duplicates, reducing bias in downstream quantification. We tested the metabarcoding approach using two reference libraries constructed from publicly available sequences, using the *matK* plastid barcode, and RefSeq complete chloroplast references for angiosperms. We found that taxon ID provided by the single barcode did not always have resolution to species or genus level. The RefSeq chloroplast database yielded better qualitative results at these taxonomic levels, but the database was limited in taxon coverage (relative to the species used here) and introduced identification issues. At family level, both databases yielded comparable qualitative results, but the RefSeq database performed better quantitatively. A restricted *matK* database containing only the species included in the artificial mixtures yielded sequence proportions were highly correlated with input pollen proportions, demonstrating that hybridization capture could be a useful tool for metabarcoding and quantifying pollen mixtures. The choice of reference database remains one of the most important factors affecting qualitative and quantitative accuracy.

Introduction

Pollen identification (ID) is important for many scientific fields. Key areas are pollination ecology and agricultural sciences, but accurate pollen ID also supports the study of ancient plant communities (Clarke *et al.* 2020), human health (e.g. allergy research (Weber 1998)), and forensics (Alotaibi *et al.* 2020). Traditional methods of pollen ID rely on microscopy to observe diagnostic characters on the pollen exine. This method is time consuming and requires a high level of expertise, while being limited in accuracy and throughput, and potentially constrains many projects. The limitations of microscopy-based pollen ID are well established. In most cases, taxa can only be identified to family, or in some cases genus (Kraaijeveld *et al.* 2015; Richardson *et al.* 2015b; Smart *et al.* 2017). The time-consuming nature of microscopy-based ID limits the throughput, and usually only a subsample of each sample can be examined, meaning that rare taxa are often missed (Bell *et al.* 2016; Smart *et al.* 2017).

Due to these limitations, alternative methods for pollen ID have been sought. DNA barcoding, or metabarcoding (mixed samples) has advanced taxon ID in many research fields, has been explored extensively for pollen ID, and has been shown to provide accurate identifications at high taxonomic resolution and with high sample throughput (Wilson *et al.* 2010; Keller *et al.* 2015; Kraaijeveld *et al.* 2015; Richardson *et al.* 2015a; Richardson *et al.* 2015b; Bell *et al.* 2017; de Vere *et al.* 2017; Bell *et al.* 2019; Suchan *et al.* 2019). In particular, metabarcoding is able to recover a taxonomic ID from as few as five pollen grains (Pornon *et al.* 2016), and the method has the ability to ID many more genera than microscopy-based methods (Keller *et al.* 2015; Richardson *et al.* 2015b).

The accuracy of metabarcoding is limited, however, by the choice of barcode and comprehensiveness of reference databases, since only taxa with reference sequences can be detected. Database collections have been established where references can be stored and accessed, and these are growing. The cytochrome *c* oxidase subunit 1 (CO1) barcode is able to differentiate most animal taxa, and can be accessed through the Barcode Of Life Data system (Ratnasingham and Hebert 2007). However, the selection of effective plant ID barcodes has presented a much greater challenge, since CO1 is not variable enough in plants to provide taxonomic resolution (CBOL Plant Working Group 2009). The Consortium for the Barcode of Life (CBOL) Plant Working group recommends the chloroplast genome encoded maturase K (*matK*) and ribulose 1,5-biphosphate carboxylase (*rbcL*) as standard barcodes which can ID approximately 70% of all plant taxa, provided they are present in the reference database (CBOL Plant Working Group 2009). Other barcodes have also been recommended for specific groups of plants, or as supplementary barcodes, such as the *psbA-trnH* spacer (Kress and Erickson 2012). The success of standard barcodes relies on sequence variability to allow resolution of taxa, and conserved primer binding sites to allow for sequence analysis across a broad range of taxa. The common barcoding approach uses PCR to

amplify the barcode using primer sites, followed by sequencing and comparison to a reference database. When reference sequences for target species are absent, the similarity to the closest sequence(s) in the database can be used to generate a genus or family ID (Liu *et al.* 2019).

Despite the demonstrated strengths of metabarcoding, the inability to answer quantitative questions regarding sample composition remains problematic. In pollination research, it is often desirable to know the relative proportions of taxa in a pollen sample. This information can shed light on the preference of pollinators or abundance of resources, and can improve understanding of pollination networks and ecosystem robustness, which in turn can help restore pollination services in natural and agricultural settings (Dormontt *et al.* 2018). Currently, there is mixed success in comparisons of relative proportions of DNA sequencing reads to starting pollen proportions for mixed samples (Bell *et al.* 2017). Positive correlations have been found between proportions of sequence reads and DNA mixes using *trnL* and ITS1 barcodes (Pornon *et al.* 2016), sequence proportions and starting pollen proportions using ITS2 (Keller *et al.* 2015), and between averaged *rbcL* and *matK* sequence abundance (Richardson *et al.* 2015a). However, the latter study also found poor quantification with ITS2, and others found similarly less conclusive results, with weak correlations between sequence and starting sample proportions using ITS2 (Bell *et al.* 2019), and no conclusive results using ITS (Smart *et al.* 2017). A meta-analysis on metabarcoding used in 22 ecological studies found only a weak positive association between starting biomass and sequences recovered, with large uncertainty (Lamb *et al.* 2019). The weak or poor results arise from bias at several steps in the sample to sequence pipeline. Biases occur which can affect both the qualitative (whether the correct taxa are identified), and quantitative (proportion within mixture) aspect of metabarcoding. Any bias affecting qualitative accuracy can affect quantitative accuracy, by potentially lowering some taxa below the detection limit.

Factors including poor resolution of barcodes and biased representation within reference databases affect ID leading to inaccurate quantitative estimates. Additional factors include: differences in DNA isolation method (Pornon *et al.* 2016); amplification differences between taxa due to differences in primer binding affinity (Krehenwinkel *et al.* 2017) - which can lead to false negatives (when a present taxon is not identified) (Pawluczyk *et al.* 2015; Zinger *et al.* 2019) and downstream quantification biases; different barcode copy numbers (Krehenwinkel *et al.* 2017); DNA degradation bias (Krehenwinkel *et al.* 2018); and database quality issues (Richardson *et al.* 2017). Sequencing bias can also occur between both barcodes and taxa (Pawluczyk *et al.* 2015). Unequal PCR replication (mostly affecting related taxa) and variable barcode copy number (particularly affecting chloroplast loci (Golczyk *et al.* 2014) which contain the standard plant barcodes) likely play the greatest roles in introducing bias (Krehenwinkel *et al.* 2017). In fact, Pawluczyk et al. (2015) found up to

a 2000 fold difference in DNA quantity between taxa and loci after PCR. PCR-free methods are being explored as a means to overcome these quantitative challenges, and they show improvement in quantification over PCR-based metabarcoding, for example genome skimming and chloroplast assembly (Lang *et al.* 2019), Whole Genome Shotgun sequencing (Bell *et al.* 2021), and MinION Reverse Metagenomics (Peel *et al.* 2019). However, these methods have other drawbacks. Genome skimming and Whole Genome Sequencing (WGS) for example require a larger amount of DNA, which can be difficult to obtain from small solitary pollinators (Lang *et al.* 2019; Bell *et al.* 2021), and MinION Reverse Metagenomics requires the user to curate their own reference databases (Peel *et al.* 2019).

One method that could overcome these shortcomings and improve accuracy and quantification compared to existing methods of pollen metabarcoding ID is hybridisation (hereafter hybrid) capture. Hybrid capture is a target enrichment technique that has recently been applied to environmental/ecological studies. It can be used for degraded DNA, and has been used to create a reference database from herbarium specimens (Dormontt *et al.* 2018), explore historic ecological communities through sediment cores (Foster *et al.* 2021; Schulte *et al.* 2021), and phylogenetic studies (Nge *et al.* 2021). The method uses a probe, or bait, which is an RNA molecule complementary to the gene region of interest. Since the method does not rely on PCR to isolate the genomic regions of interest, it has the potential to remove PCR bias from the quantification analyses, which has been found to generate large quantitative bias in amplification-based metabarcoding approaches, and can cause taxon-specific amplification bias (Pawluczyk *et al.* 2015; Krehenwinkel *et al.* 2017).

For taxonomic ID, the bait is complementary to the barcode of interest (Waycott *et al.* 2021). The baits used in this study were designed to target 19 chloroplast genes (see Waycott *et al.* 2021), applicable to all angiosperm linages. To make them useful for such broad ranges of taxa, the baits do not need to match 100% to the barcode, 80-90% similarity will retrieve the target, and affinity can be controlled with the hybridisation temperature. The sequence overhang generated with hybrid capture baits can often recover complete or near complete chloroplast genomes. In traditional PCR amplification methods, primers are bound to conserved barcode primer sites to amplify the barcodes. This creates exact copies of the barcodes that cannot easily be distinguished from the PCR duplicates. Our approach uses sonication to randomly fragment the DNA after DNA extraction, creating a random DNA fragment soup. Chloroplast loci (genes) for which baits were designed are then ‘fished out’ of the soup using the complementary baits (Waycott *et al.* 2021). Given that each DNA fragment has in theory a unique length, PCR duplicates (amplicons having same sequence and length) can be eliminated bioinformatically and only one copy of every captured sequenced read or read pair is retained. This enables downstream quantification of relative taxon abundances based on the number of reads mapping to references.

The aim of this study was to demonstrate the effectiveness of hybrid capture DNA metabarcoding for identifying taxa in a pollen mix, and determining the accuracy of estimations of relative taxonomic abundances. We used two different reference databases, a *matK* database which is commonly used in amplicon metabarcoding, and a RefSeq whole chloroplast database. We expected that the RefSeq database would produce more accurate qualitative and quantitative results, since many more potentially informative gene regions were recovered using the chloroplast bait set used for hybrid capture, and PCR bias was controlled for. We explored whether, and how closely, the sequence composition of mixed pollen samples reflected starting proportions, to test the potential for broader application of hybrid capture metabarcoding as a useful tool in pollination research.

Materials and methods

Sample collection

A comprehensive experimental setup was made using pollen of three species from different families. The pollen from these taxa was visually distinct for easy morphological identification by non-experts (Fig. 1). This ensured that the taxa comprising each pollen pellet could be verified through morphology. Pollen was obtained from honey bee hives fitted with pollen traps. Honey bees forage on one species per foraging trip, so pollen pellets are usually comprised of a single species (Synge 1947; Visscher and Seeley 1982; Grüter and Ratnieks 2011). The hives had been placed in almond orchards (*Prunus dulcis*), brown stringybark plantations (*Eucalyptus baxteri*), and a field with flowering capeweed (*Arctotheca calendula*). *A. calendula* pollen is a distinctive orange colour which was easily separated from pollen pellets of other species that were present at the time of collection.

Pollen mixtures

We constructed 14 different pollen mixtures, with three replicates of each mixture. We used four negative controls (blanks), one for each extraction batch, totalling 48 samples/libraries.

The pollen mixture proportions were weight based. Each taxon varied in quantity from high to low abundance (Table 1, Fig. 2). The mixtures were suspended in ethanol and divided into three replicates for DNA extraction. Ethanol was used for suspension because it evaporated without leaving any residuals that may have affected subsequent DNA extraction and library preparation. Care was taken to strongly agitate the mixture before aliquoting.

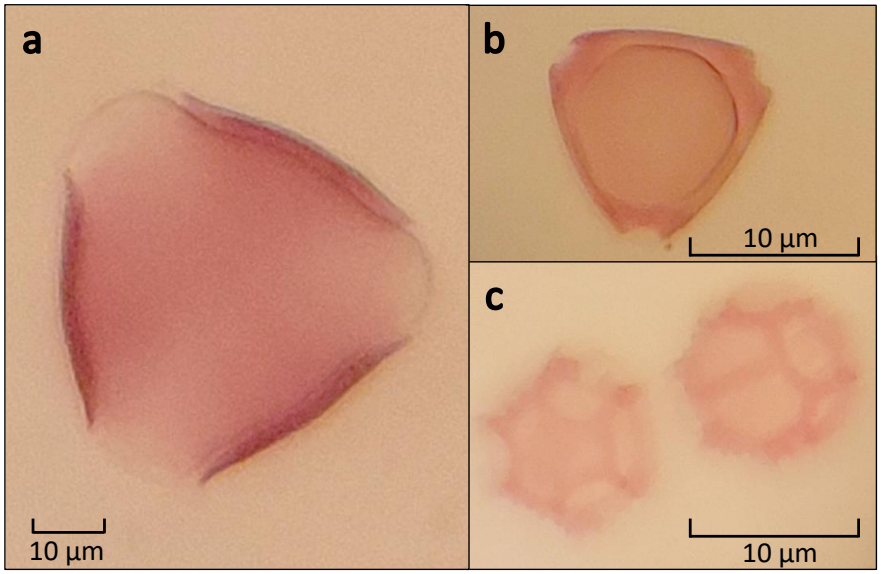

Figure 1. Images of the taxa of pollen used in artificial mixtures. **a)** *Prunus dulcis*, **b)** *Eucalyptus baxteri*, **c)** *Arctotheca calendula.* Photographs were taken from slides under a compound microscope by Leif Currie.

DNA extraction and library preparation

DNA was extracted from the pollen mixtures (9 mg) using the NucleoSpin® Food kit (Macherey-Nagel, Düren, Germany), with the "isolation of genomic DNA from honey or pollen" supplementary protocol. We modified the homogenisation and elution steps. We homogenised the dry pollen mixture aliquots using ceramic beads in 2 mL screw cap tubes on a Bead Ruptor 24 (OMNI International Inc.) at 6 m/s for 20 s cycles (3-4 minutes total) until a powder was formed. Sample tubes were submerged in liquid nitrogen between mill cycles to prevent DNA degradation caused by heat during bead beating, and to allow easier homogenisation by making the pollen brittle. The final elution step was done by passing the 60 µL of elution buffer through the spin column membrane twice instead of once, followed by spinning, to maximise DNA yield. Following extraction, DNA was quantified using a Quantus™ Fluorometer and QuantiFluor® dsDNA System (Promega, Madison, WI, USA), normalised to 2 ng/µL (samples with concentration lower than 2 ng/µL were used neat), and sonicated using a Bioruptor® Pico (Diagenode, USA) to create random length fragments (eight cycles of 15 s on, 90 s off).

Library preparation was done using an Eppendorf epMotion® 5075t - Liquid Handling Workstation. The DNA libraries were prepared using the NEBNext® Ultra™ II DNA Library Prep kit as described in the protocol by Waycott et al. (2021). In brief, custom made y-stubby adaptors were ligated to the DNA fragments. Each adaptor contained one of 48 unique 8 nucleotide in-line barcodes, which were combined in unique combinations (i.e. each sample received a unique combination of two barcodes ligated at each end) allowing downstream sample pooling. The libraries were amplified using

PCR (30 s at 94°C, followed by 17 cycles of 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s, a final extension at 72°C for 2 mins and held at 4°C). To reduce cost, libraries were pooled into groups of 16 according to estimates of library concentration. Pools were purified using a 1:1 volume concentration of MagNA Beads (Rohland and Reich 2012).

Hybridization capture

This study used the OZBaits_CP V1.0 universal plastid bait set for hybrid capture developed for targeted capture of angiosperm sequences (Waycott *et al.* 2021), following the myBaits® Targeted NGS Manual Version 4.01 hybridization protocol.

The baits were added to the pooled libraries and hybridized at 65°C for 48 hours. To avoid evaporation, chill-out™ red liquid wax (Bio-Rad Laboratories, Inc.) was added. Hybridised libraries were then amplified (2 min at 98°C, followed by 20 cycles of 98°C for 20 s, 60°C for 30 s, and 72°C for 45 s, a final extension at 72°C for 5 min and held at 8°C) with custom P7 and P5 Illumina adaptors. Following bait hybridization, target regions were bound to magnetic beads, samples were placed on a magnet and non-target regions were washed out of the product. Resulting libraries were visualised using the high sensitivity DNA assay of a 2100 Bioanalyzer (Agilent), and pooled in equimolar concentrations. Final purification used 1:1 MagNA, and final size selection at 350-600 bp was done using a 2 % agarose Pippin Prep gel cassette (Sage Science).

The unique combination of dual in-line molecular identifiers (adapter barcodes), and unique combination of dual-index primers were only used once for any library preparation in our lab to reduce contamination. The final library was sequenced at the Garvan Institute of Medical Research (Sydney, Australia) on one lane of an Illumina HiSeq X Ten with 2 × 150 cycle chemistry.

Bioinformatics pipeline: Sequence data processing and cleaning

Analyses were done using the Phoenix high performance computing cluster at the University of Adelaide, Australia. Samples were first demultiplexed via the indexes using Bcl2fastq, then demultiplexed via their internal barcodes using Sabre (Sabre-barcode-demultiplexing.). The barcodes had at least 2 degrees of separation, so one base pair mismatch was allowed.

We explored several analysis methods, including the pipelines developed by Sickel *et al.* (2015) and Bell *et al.* (2021) which were developed for metabarcoding and WGS respectively. However, we were unsuccessful in implementing methods using qiime2, which appeared incompatible with our non-amplicon data (we also attempted to use the q2-shogun and q2-metaphlan2 plugins for shotgun data, but were unable to overcome the errors encountered). We ultimately used a custom pipeline, which was similar to that of Bell *et al.* (2021), but used modified pre-processing steps, and additionally used Bracken (Lu *et al.* 2017)(see below) for improved quantification. We removed PCR duplicates using clumpify from BBtools (Bushnell 2021). Removing PCR

duplicates also made subsequent analyses faster and less memory intensive, since the dataset had been reduced by more than half. Sequence filtering and trimming was done using AdapterRemoval (Schubert *et al.* 2016). The 9th base following the 8 nt barcode, reads shorter than 30 nt, reads with a phred quality score < 20, and N tails were removed. Following this, Kraken2 was used to assign taxonomy to reads. Kraken2 is a k-mer based method, so it does not require pre-assembly of the sequences (Wood *et al.* 2019). It was used to classify reads at both species and genus classification levels. Bracken, which is a sister program to Kraken, was then used to estimate read abundance using the Kraken classifications (Lu *et al.* 2017). A minimum hit group threshold of 5 was set in Kraken (which is useful for custom databases), and a threshold of 5 set in Bracken. Bracken output was analysed using R (RStudio Team 2020).

We explored different reference database approaches for taxonomic identification, the first using a *matK* single barcode database, and the second using a complete chloroplast RefSeq database. The databases were downloaded (January 2022) and built using Kraken and Bracken. A list of all angiosperm species occurring in South Australia was obtained from the Atlas of Living Australia (https://www.ala.org.au/). The publicly available sequences for *matK* references were downloaded using this list. The RefSeq database consisted of all angiosperm chloroplast records available from the NCBI RefSeq database. *A. calendula* did not have a RefSeq chloroplast reference, so the chloroplast sequences available on NCBI were manually added to the database to ensure all taxa used in the mixtures were represented. At the time, 15 chloroplast sequences from 8 gene regions were available (Supplementary Table 1), and of the 8 regions, 6 matched barcodes targeted by the chloroplast bait set used (Waycott *et al.* 2021). For both databases, a modified version was created each including only the three taxa present in the pollen mixtures, to test the quantification independently of taxonomic ID. Then, to simulate a more realistic scenario where pollen identity is unknown, we repeated the analysis with the comprehensive database. The databases are referred to as wide (many taxa) and restricted (mixture taxa only).

Analysis

Linear regression was used to assess the correlation between the proportions of input pollen weight and resulting sequences. To determine if taxon rarity in the sample had an effect on taxon detection, we used binomial mixed effect models at each taxonomic level, with starting pollen weight proportion as the predictor variable, and a binomial response for detection success or failure. Mix ID was set as a random effect. All modelling was done in RStudio (RStudio Team 2020) using the lme4 package (Bates *et al.* 2015).

Results

After sequencing, we retrieved a total of 38,165,440 raw sequencing reads, with an average of 397,557 reads per sample. After filtering, 11,155,855 sequences were retained, an average of 116,207 reads per sample, and 27,009,585 reads were discarded of which an average of 234,035 sequences per sample were PCR duplicates.

Sample M2a had less than 600 reads sequenced after filtering, and was excluded from interpretation as this was likely the result of a technical error and thus unreliable. Of the four blanks, only one retained any reads after the quality filtering steps were carried out.

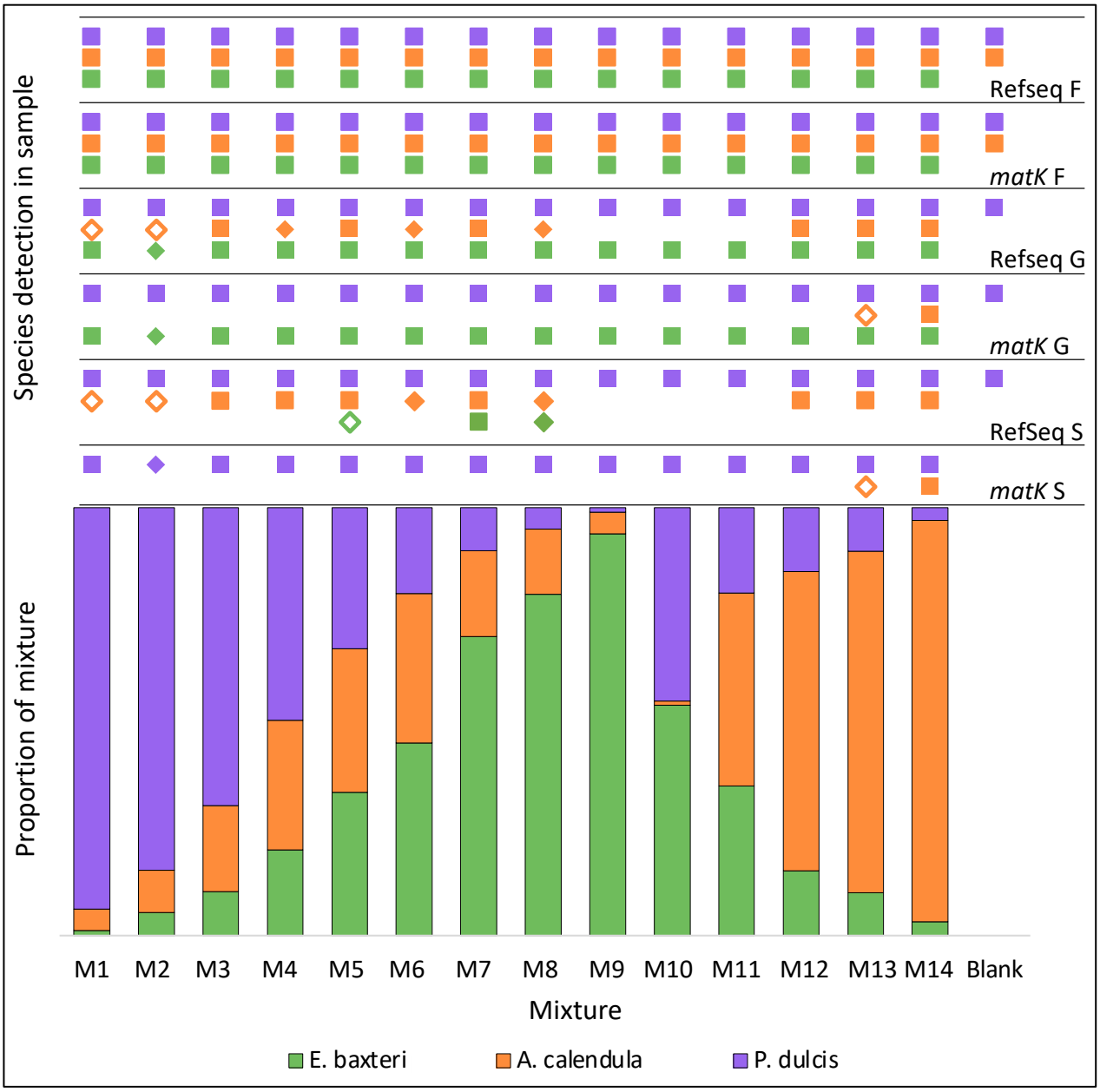

Figure 2. Stacked bar plot of the relative input proportions by weight of three pollen taxa (*Prunus dulcis*, *Arctotheca calendula* and *Eucalyptus baxteri*) in artificially constructed mixtures

(M1 - M14), and negative control (Blank). Symbols above each bar indicate whether taxa were detected in the mixture using metabarcoding with either *matK* or RefSeq databases, identified to family (F), genus (G) and species (S) levels. Solid squares indicate the taxon was detected in three mixture replicates, solid diamonds indicate detection in two of the three replicates, and hollow diamonds indicate detection in only one replicate.

MatK database

At the species level and using the wide *matK* database, *E. baxteri* was not detected in any sample. *Eucalyptus* was detected in all samples at genus level, apart from within the blank. *A. calendula* was detected in the same 5 samples at species and genus level. The five samples (plus a sixth with failed detection) were from mixes M13 and M14, which had starting proportions of pollen > 0.799, and no samples from mixes with lower starting proportions had positive IDs. *P. dulcis* had the best detection success, and was detected in all samples except the blank. At genus level, *Prunus* was detected in every sample, including the blank. At family level, all three taxa (*Myrtaceae*, *Asteraceae* and *Rosaceae*) were detected in every sample, except for the blank. In the blank no *Myrtaceae* was detected (Fig. 2; Supplementary Table 2).

False positives occurred when taxa which were not present in the sample were detected, or the opposite for false negatives, when taxa present in a sample were not detected. The percentage of false positive sequencing reads was 64.1% using the wide *matK* database at species level (Fig. 3C), 52.3% at genus level (Fig. 3E), and at family level there was a 10.7% false positive rate (Fig. 3G).

The relationship between input pollen proportion and proportion of reads was generally highly correlated (R^2 = 0.62 - 0.99). *E. baxteri* was undetected at species level, so a correlation could not be calculated. At genus level, R^2 = 0.96, but the proportion of reads fell far below the desired 1:1 input to output ratio. At family level, R^2 = 0.99, and the proportion of reads detected trended closer to the 1:1 ratio, although they remained below the desired level (Fig. 3H). *A. calendula* had the same relationship between input pollen and output reads at species and genus level, which was below the plot threshold, and had the lowest R^2 value (0.62) for both taxonomic levels. At family level, *A. calendula* was similarly correlated as *E. baxteri,* with R^2 = 0.97, and a trend along but consistently below the 1:1 ratio of input pollen to output sequences (Fig. 3H). *P. dulcis* had a very similar relationship between input pollen to output sequences at each taxonomic level (Fig. 3D, F, H), with high R^2 values (species R^2 = 0.75, genus and family R^2 = 0.98). However, the ratio of sequences to starting pollen proportions was positively biased in comparison to the desired 1:1 ratio in each scenario, and the deviation increased with decreasing taxonomic resolution (Fig. 3D, F, H).

The restricted *matK* database (containing only the three taxa used to make mixtures) naturally did not result in any false positives (Fig. 3A). The proportion of sequences

versus input pollen was linear and highly correlated for all taxa (R^2 = 0.97 – 0.99; Fig. 3B). The same higher than expected proportion of sequences for *P. dulcis* was seen, but *E. baxteri* and particularly *A. calendula* sequence proportions were much closer to the expected 1:1 ratio (Fig. 3B).

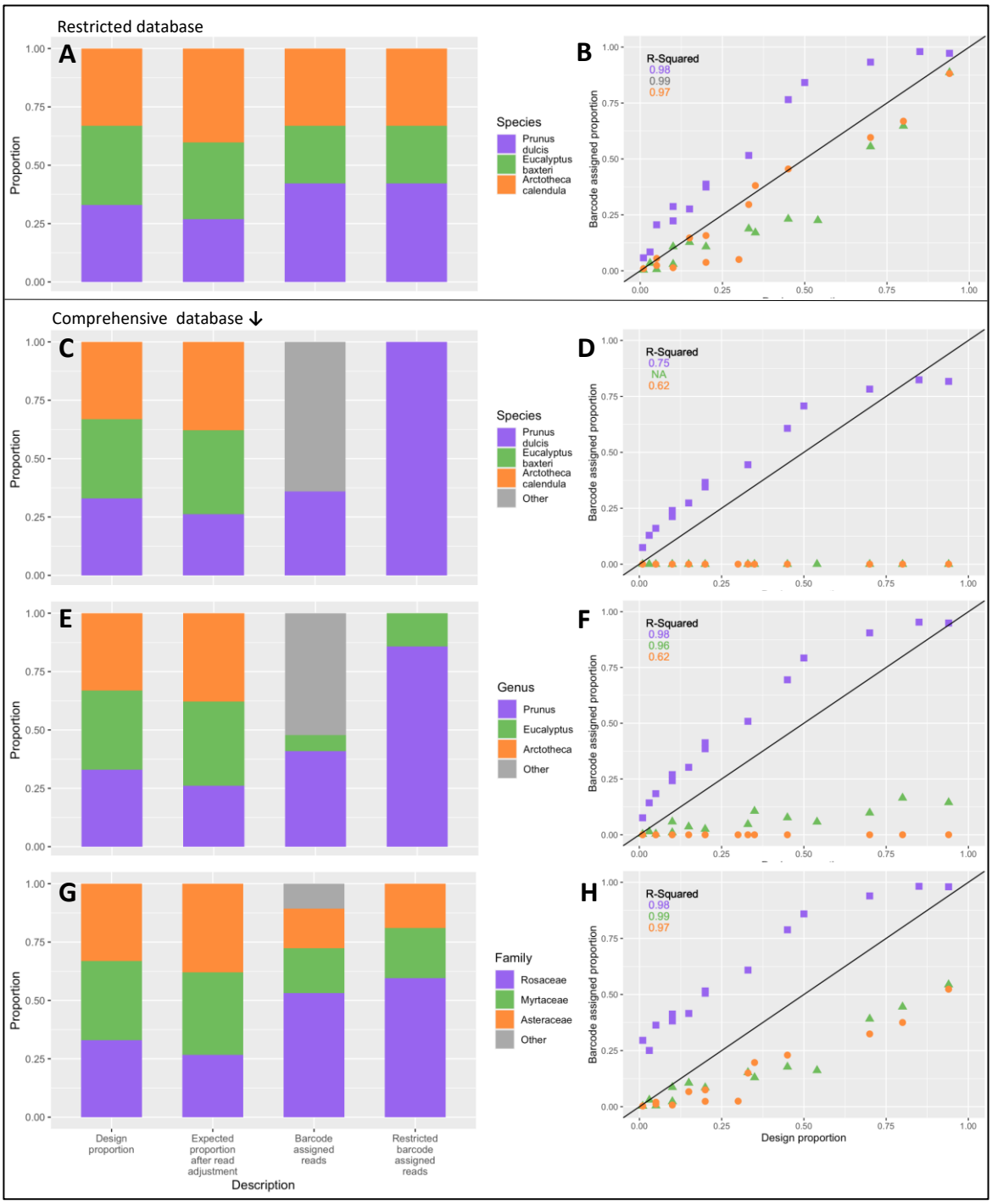

Figure 3. ***Left side:*** Summary of taxon proportions averaged across samples, with taxonomic assignments made using a ***matK*** reference database. Proportions were averaged across samples. Columns from left to right are: 1) original design proportion according to weights of pollen, 2) expected proportion after read correction (given the 14 mixtures had different numbers of reads per taxon), 3) total barcode assigned reads, 4) barcode assigned reads with

other taxa excluded. ***Right side*****:** Sequence proportions versus input (design) proportions of pollen; **A-B:** taxonomic assignment to **species** level made using a restricted *matK* database (only containing three taxa used in mixtures); **C-D:** taxonomic assignment to **species** level using a comprehensive *matK* database; **E-F:** taxonomic assignment to **genus** level using a comprehensive *matK* database; **G-H:** taxonomic assignment to **family** level using a comprehensive *matK* database.

RefSeq database

Using the comprehensive RefSeq database, and at species level, *E. baxteri* and *A. calendula* (although detected in some samples) were found in such low quantities that they were not plottable (Fig. 4C - D). *P. dulcis* sequence proportions were strongly correlated with input pollen proportions (R^2 = 0.97), and closely tracked the 1:1 ratio until the input pollen proportions reached 0.5, beyond which sequences occurred below the expected level (Fig. 4D). At genus level, *Arctotheca* was found at equally low abundances as *A. calendula* at species level. *Eucalyptus* was found at approximately half the expected proportion (Fig. 4E), but was strongly correlated with input pollen proportion (R^2 = 0.98). *Prunus* had slightly higher sequence proportions than expected (Fig. 4F), and was less linear (R^2 = 0.9) with a similar flattening of the curve above 0.5 starting pollen proportion, similar to *P. dulcis* at species level. At family level, all three taxa showed strong correlations between input pollen and sequence proportions (R^2 = 0.81 – 1) and plotted along the 1:1 ratio, although *Rosaceae* (*P. dulcis*) had the least linearity, as previous (R^2 = 0.81; Fig. 4H). *Myrtaceae* (*E. baxteri*) sequence proportions were at expected levels overall, and *Asteraceae* (*A. calendula)* and *Rosaceae* were below and above expected levels respectively (Fig. 4G). Only *P. dulcis* was detected in the blank at all three taxonomic levels, *A. calendula* was detected only at family level, and *E. baxteri* was not detected at all. This was the same as for *matK* except for *P. dulcis* detection at species level.

The percentage of false positive sequencing reads was 72.5% using the wide RefSeq database at species level (Fig. 4C), 47.4% at genus level (Fig. 4E), and a 9.6 % false positive ID rate at family level (Fig. 4G).

The restricted RefSeq database (containing only the three taxa used in the mixtures) also naturally did not result in any false positives. The proportion of output sequences versus input pollen was strongly linear for all taxa (R^2 = 0.96 and 0.97). *E. baxteri* and *P. dulcis* points showed more scatter on the plot than for *matK* for samples with less than 0.25 starting pollen proportion. *A. calendula* was close to zero and the other two taxa had higher than expected proportions (Fig. 4B). *E. baxteri* overall had approximately expected read quantities, but *A. calendula* had much lower, and *P. dulcis* much higher than expected read proportions (Fig. 4A).

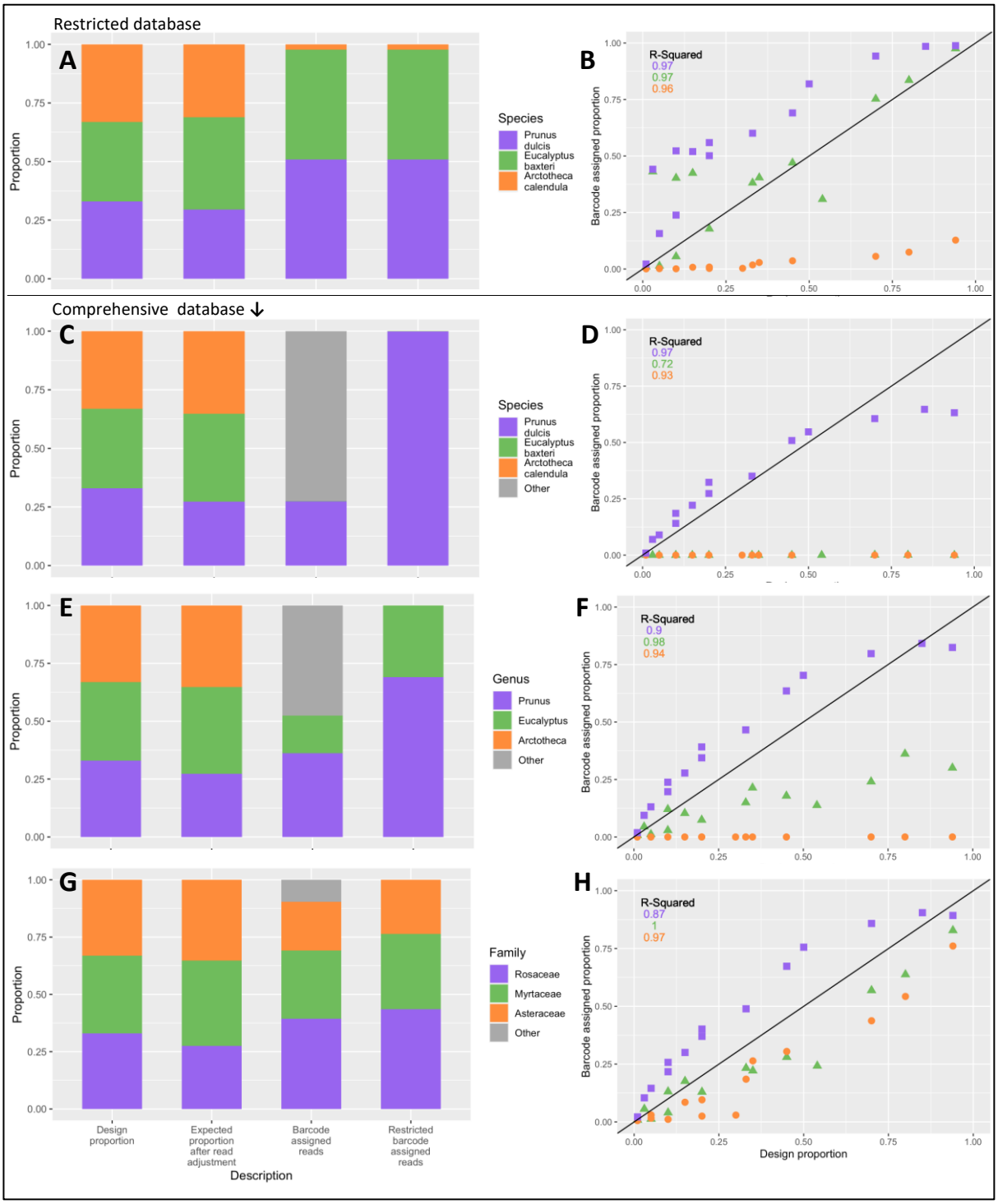

Figure 4. ***Left side:*** Summary of taxa proportions averaged across samples, with taxonomic assignments made using a **RefSeq** reference database. Proportions were averaged across samples. Columns from left to right are: 1) original design proportion according to weights of pollen, 2) expected proportion after read correction (given the 14 mixtures had different numbers of reads per taxon), 3) total barcode assigned reads, 4) barcode assigned reads with other taxa excluded. ***Right side*****:** Sequence proportions versus input (design) proportions of pollen; **A-B:** taxonomic assignment to **taxa** level made using a restricted database (only containing three taxa used in mixtures); **C-D:** taxonomic assignment to **taxa** level using a comprehensive RefSeq database; **E-F:** taxonomic assignment to **genus** level using a

comprehensive RefSeq database; **G-H:** taxonomic assignment to **family** level using a comprehensive RefSeq database.

Sample rarity

The detection of taxa was successful regardless of the amount of starting pollen in the mix. Starting pollen quantities did not have a significant effect on the detection, using either barcode database for assignment, at any taxonomic level (species, genus or family). Taxon detection versus input pollen proportion was tested in 24 combinations using the four reference databases. In nine cases, the taxon was detected at every pollen input level (every sample), so it was not possible to model (Table 1).

Table 1. Mixed model with binomial distribution to determine if pollen proportion in pollen mixtures affected the success or failure of taxonomic identification to three taxonomic levels.

Barcode db	Taxonomic level	Mix taxa	Est.	S.E.	Z-val	P-val
matK restricted	Species	*E. baxteri*	14.42	19.72	0.73	0.46
		A. calendula	-7.20	6.81	-1.06	0.29
		P. dulcis	Response is constant			
matK wide	Species	*E. baxteri*	14.43	19.72	0.73	0.46
		A. calendula	-11.20	35.72	-0.31	0.75
		P. dulcis	-7.13	6.83	-1.04	0.30
	Genus	*E. baxteri*	14.30	19.91	0.79	0.47
		A. calendula	-11.20	35.72	-0.31	0.75
		P. dulcis	-7.13	6.83	-1.04	0.30
	Family	*E. baxteri*	14.43	19.72	0.73	0.46
		A. calendula	-7.20	6.82	-1.06	0.29
		P. dulcis	-7.13	6.83	-1.04	0.30
RefSeq restricted	Species	*E. baxteri*	Response is constant			
		A. calendula				
		P. dulcis				
RefSeq wide	Species	*E. baxteri*	9.03	6.85	1.32	0.19
		A. calendula	-1.98	2.51	-0.79	0.43
		P. dulcis	Response is constant			

Genus	*E. baxteri*	14.43	19.72	0.73	0.46
	A. calendula	-2.03	2.58	-0.78	0.43
	P. dulcis	Response is constant			
Family	*E. baxteri*	Response is constant			
	A. calendula				
	P. dulcis				

Discussion

We used hybrid capture to metabarcode artificial pollen mixtures and evaluated the efficacy of taxon ID, and quantification of sequence proportions relative to the original pollen mixture. We constructed reference databases using Kraken2 and publicly available references from NCBI. We found that the ID of taxa within the pollen mixture provided by a single barcode did not always have resolution to species or genus level. The RefSeq chloroplast database yielded better qualitative results at these taxonomic levels, but the database was limited in taxon coverage (relative to the species used here) and read assignment issues likely occurred due to this. At family level, both databases yielded equally good qualitative results, but the RefSeq database performed better quantitatively. This result was not mirrored with restricted databases that only contained the mixture species, probably because *A. calendula* did not have a RefSeq chloroplast genome, and hence it performed better in the wide database which had other *Asteraceae* at Family level. We found overall that this hybrid capture method and bioinformatic pipeline performed well in identifying taxa at higher taxonomic levels, and found close to a 1:1 ratio of input pollen to output sequences depending on the database used. Database quality and choice had a large effect on result accuracy, since our molecular approach seemed to account for potential PCR bias. We discuss these results and limitations to this method as it stands.

Taxon identification

MatK database

At species level the *matK* database resulted in high levels of false negatives. This was unsurprising as the two standard plant barcodes recommended by CBOL for plant ID can discriminate only approximately 70% of plant species, plus there could have been additional reductions in the resolution since this figure relates to longer barcode sequences, rather than the short fragments generated here. Additionally, species within the *Myrtaceae* and *Asteraceae* families (two of the three taxa used here) can be difficult to ID (Gao *et al.* 2010; Arstingstall *et al.* 2021). One of the reasons can be high chloroplast similarity in not so closely related *Eucalyptus* species (Bayly *et al.* 2013),

which can make barcoding difficult. In this study, *Eucalyptus* may have been difficult to identify at species level because it had the most related taxa present in the database.

Prunus dulcis was readily identified at every taxonomic level, while *Eucalyptus baxteri* was more readily detected at genus level *(Eucalyptus)*, and *Arctotheca calendula* was only readily detected at family level *(Asteraceae)*. In the last case, however, there were no other species of *Arctotheca* in the database (there are only 4-5 accepted species in total), which meant that when the reads did not match the *matK* barcode, the closest matches were more distantly related species, contributing to the high false positive rate at genus level. Since there were many other *Prunus* and *Eucalyptus* species present in the database, *P. dulcis* and *E. baxteri* reads had many more closely related options to match to if the sequence did not match correctly, resulting in more accurate genus level IDs. In early analysis exploration with a database containing only one species per genus, the results yielded were poorer, with more false negatives at genus and family levels. This could occur because the hybrid capture method does not extract the entire barcode, so potentially important parts are missing, and the read matches to a different reference. This indicates that it could be important to have closely related species and some 'redundancy' in databases to achieve more accurate genus (if not species) level ID.

Refseq database

Except for *P. dulcis*, which was identified in every sample using the RefSeq database, we had less difficulty identifying the other taxa in the samples compared with the *matK* results. Unlike with *matK, E. baxteri* was identified in some samples at species level, and *Eucalyptus* was readily identified at genus level. At species level, the RefSeq database resulted in more false positives than the *matK* database results, but there were fewer false negatives as well. For results from both databases, the high false positive rate could be attributed to the Illumina sequencing, which is very sensitive and can easily pick up contamination. Although, most are likely explained by misidentification of sequences that came from the true positive species, since the false positive rate drops off at the higher taxonomic levels (although still not zero at family level).

A. calendula had a poorer representation in the RefSeq database. It did not have a publicly available chloroplast reference at the time of database curation, and the database also did not contain other *Arctotheca* species. Instead, the 15 chloroplast sequences available at the time of this study were added to the database (see methods). This most likely led to the much lower than expected abundance of *A. calendula* using the restricted database. With only the 15 gene regions *A. calendula* reads could possibly hit, versus the entire chloroplast genome for the other two taxa, many of the *A. calendula* sequences which did not match the 15 reference regions well, could have matched to regions of the complete chloroplast references for the other taxa, and increased the quantity of reads to those. However, at family level, and

with the wide *RefSeq* database, the proportion of *A. calendula* was closer to expected levels, since with other *Asteraceae* in the database there was more redundancy, and *A. calendula* could match to other more closely related taxa. Again, this suggests that in cases where databases are missing necessary taxa, it is useful to have references of closely related taxa which can provide genus level IDs.

Sample rarity

There was no relationship between pollen input proportion and detection rate. This result was also found by Bell *et al.* (2019), who additionally tested the influence of other taxa on identification. In both this study and ours, there appears to be a greater influence of taxon identity than rarity on detection.

Comparison of single barcode vs whole chloroplast database

The nature of the hybrid capture baits made the RefSeq database more appropriate for qualitative assessment for a couple of reasons. The first is that more sequences/reads were utilised (*matK* is only one of 19 loci targeted by baits). The *matK* database assigned approximately 1.5% to 3% of reads per sample to a reference, which was unsurprising given the other loci sequenced, but between 85% and 96% of reads assigned to the RefSeq database, resulting in more data being utilised. The second benefit is that the overhang that can occur as a result of randomly sized fragments matching to baits can be used. Unlike a single barcode database such as the *matK* database used here, where if the overhang falls outside of the barcode limits, it may prevent sequences from being assigned if the number of nucleotide mismatches exceeds the threshold set.

Quantification

A restricted database only containing the mixture taxa led to linear and highly correlated quantifications of taxon proportions for the *matK* database results, although there appeared to be taxon specific biases (these were present in all instances for both databases used). The RefSeq results, which closely followed the expected 1:1 ratio at family level, were less accurate using the restricted database. The factors discussed above affecting qualitative success also affected the quantification of relative proportions of the taxa. The greatest deviation from the expected ratio was *A. calendula* using the RefSeq database, likely because a whole chloroplast reference was not available for *A. calendula*, thus the sequences were less readily identified and were underestimated. It is evident from this that it is important wherever possible to have equivalent reference sequences for quantitative accuracy, even though the taxon was identified in many of the samples. The most readily identified species (*P. dulcis)* was overabundant in sequence reads. We expected that there would be a systematic bias arising from the different weights of the pollen taxa. *P. dulcis* was at least twice as large as the other two species meaning that fewer pollen grains would be present in the same weight, and since angiosperm pollen grains have the same number of cells, if

each taxon also had the same number of plastids per cell, then we would have expected it to have a lower proportion of sequences than the other two taxa. However, this assumption was not met, and *P. dulcis* was overabundant in all samples, rather than the reverse. This most likely occurred due to two reasons: the assumption about relatively equal numbers of plastids was not met, or the readiness of identification lead it to be overestimated. The number of plastids, and genome copy number of chloroplasts can vary greatly, from few to hundreds, between different species and tissue types, and tissue age (Morley and Nielsen 2016). While the tissue types were the same in this study, it is likely the species had different numbers of chloroplasts and chloroplast copy number accounting for some quantitative biases. There may also have been biases stemming from the laboratory, in the DNA extraction or sequencing steps, which favoured this taxon over the others.

Comparison with other studies

Compared to other studies, the hybrid capture method of our study, provides weaker qualitative results, whereas our quantitative results are equal or better. All studies considered had highly accurate qualitative results, although the reference databases used, and their breadth, varied.

Our study had accurate identifications at family level, but at species level, we only identified all species correctly in some samples using the RefSeq database. We had high levels of false positives for all species. This is similar to the study by Bell *et al.* (2021), who used a whole nuclear genome RefSeq database containing publicly available angiosperm species, and found their WGS method to be almost 100% accurate in identifying the species within their pollen mixtures, but they found high levels of false positives. In contrast to this study, we had more highly correlated DNA sequencing and pollen input proportions (R^2 = 0.72 – 1 for all taxa at all taxonomic levels), while they found an increasing correlation of R^2 = 0.60 and R^2 = 0.62 for species and genus levels. The amplicon metabarcoding used by Bell *et al.* (2019) found largely accurate taxonomic identifications, but only weakly correlated read proportions with *rbcL* and ITS2 barcodes. The study also found that some taxa were more readily detected, as we found with *P. dulcis*. Similar to our comparison between a *matK* and RefSeq database and the results, Bell *et al.* (2021) found more accurately identified taxa at both species and genus level using a RefSeq database compared to *rbcL* and ITS2 amplicon sequencing (from Bell *et al.* (2019)).

The study using RevMet by Peel *et al.* (2019) reliably identified plants in mixed-species samples using their custom database containing 54 species at proportions of ≥ 1%, with ‘few’ false positives and negatives. However, the method was only able to quantify high and low abundance levels of taxa. Lang *et al.* (2019) also found accurate qualitative results, with a 100% accurate identification rate in all samples, at levels as low as 0.2% of the total mixture. However, their database contained only the species

used in their mixtures. Comparatively, our study (although using far fewer species) also had a 100% accurate identification rate of taxa in the samples using the database only containing those samples. The study found significantly and highly correlated sequencing reads with pollen count proportions (R^2 = 86.7%), on par with our quantitative results.

Database selection and limitations

A comprehensive discussion detailing the current limitations of database availability exists in Bell *et al.* (2021) under the section "4.3 Present feasibility of WGS and future research direction". The main points are that the availability of whole genome or plastid references required for the WGS method used in their paper (and for the RefSeq database used here) are far below that of the number of ITS2 and *rbcL* sequences available. Further, without many upgrades to currently available sequences, this method will remain limited, and researchers may be forced to create their own references which is time consuming and costly. A workaround may be a bioinformatical method for combining data from multiple barcodes into a single analysis, which could utilize the vast quantity of single barcode references already available.

Applications and Conclusion

We have demonstrated that a hybrid capture approach with high throughput sequencing is an appropriate method for metabarcoding pollen mixes. The strength of using hybrid capture lies in the ability to target multiple genomic regions, potentially utilising more informative loci without prior knowledge about the target taxa. Yet, it remains that there is still no applicable method to combine multiple barcodes in a single analysis, so using a RefSeq chloroplast library generated better results than a single *matK* barcode library. However, there are far fewer plastid sequences available compared with barcode sequences, and missing taxa in the database could lead to issues with downstream quantification. Conversely, when the taxa present were known and the database restricted to just those present, the *matK* barcode library resulted in relatively accurate and highly correlated sequence proportions compared with input pollen proportions. The current limitations of this molecular method pertain to its application in the Australian context, where the flora is dominated by *Eucalyptus* species that were not well identified. However, in lower diversity environments like crop settings, this method could prove highly valuable, as the potential for misidentification can be limited by known site details. This method could be applied to pollinator-collected pollen samples, but care should be taken with reference choice and database curation, particularly when extracting quantitative information.

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Supplementary Information: A novel approach for pollen identification and quantification using hybrid capture-based DNA metabarcoding

Table 1: Initial proportions of three species of pollen in artificial mixes with three replicate samples (Samp.) per mix (M). Species used were *Eucalyptus baxteri* (E, green), *Arctotheca calendula* (A, orange), and *Prunus dulcis* (P, purple).

	Proportion based on weight (mg)			MatK wide Sp			MatK wide Gen			MatK wide Fam			RefSeq wide Sp			RefSeq wide Gen			RefSeq wide Fam		
Samp.	E prop	A prop	P prop	E	A	P	E	A	P	E	A	P	E	A	P	E	A	P	E	A	P
M1a	0.012	0.050	0.937																		
M1b	0.012	0.050	0.937																		
M1c	0.012	0.050	0.937																		
M2a	0.054	0.099	0.847																		
M2b	0.054	0.099	0.847																		
M2c	0.054	0.099	0.847																		
M3a	0.103	0.201	0.696																		
M3b	0.103	0.201	0.696																		
M3c	0.103	0.201	0.696																		
M4a	0.200	0.302	0.497																		
M4b	0.200	0.302	0.497																		
M4c	0.200	0.302	0.497																		
M5a	0.335	0.336	0.329																		
M5b	0.335	0.336	0.329																		
M5c	0.335	0.336	0.329																		
M6a	0.451	0.349	0.200																		
M6b	0.451	0.349	0.200																		
M6c	0.451	0.349	0.200																		
M7a	0.699	0.200	0.101																		
M7b	0.699	0.200	0.101																		
M7c	0.699	0.200	0.101																		
M8a	0.798	0.152	0.050																		
M8b	0.798	0.152	0.050																		
M8c	0.798	0.152	0.050																		
M9a	0.939	0.050	0.011																		
M9b	0.939	0.050	0.011																		
M9c	0.939	0.050	0.011																		
M10a	0.539	0.009	0.452																		
M10b	0.539	0.009	0.452																		
M10c	0.539	0.009	0.452																		
M11a	0.350	0.450	0.200																		
M11b	0.350	0.450	0.200																		
M11c	0.350	0.450	0.200																		
M12a	0.151	0.700	0.149																		
M12b	0.151	0.700	0.149																		
M12c	0.151	0.700	0.149																		
M13a	0.100	0.799	0.101																		

M13b	0.100	0.799	0.101																	
M13c	0.100	0.799	0.101																	
M24a	0.032	0.939	0.030																	
M24b	0.032	0.939	0.030																	
M24c	0.032	0.939	0.030																	
Blank																				

Table 2: Chloroplast barcodes and sequence details for references for *Arctotheca calendula* downloaded from NCBI in 2022

Barcode	Details
psbZ	trnS-psbZ intergenic spacer, partial sequence; PsbZ (psbZ) gene, complete cds; psbZ-trnG intergenic spacer and tRNA-Gly (trnG) gene, complete sequence; and trnG-trnfM intergenic spacer, partial sequence
psbA	PsbA (psbA) gene, partial cds; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence
ndhF	NADH dehydrogenase (ndhF) gene, partial cds
ndhF	voucher Trinder-Smith 143 (US) NADH dehydrogenase subunit F (ndhF) gene, partial cds
matK	maturase K (matK) gene, partial cds
rbcL	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds
matK	voucher BS0137 maturase K (matK) gene, partial cds
rbcL	voucher BS0137 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds
trnK	voucher Trinder-Smith 143 (US) tRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene, complete cds
ndhF	NADH dehydrogenase subunit F (ndhF) gene, partial cds; and ndhF-rpl32 intergenic spacer, partial sequence
rps16	ribosomal protein S16 (rps16) gene, partial sequence
trnL	trnT-trnL intergenic spacer, partial sequence; tRNA-Leu (trnL) gene, complete sequence; and trnL-trnF intergenic spacer, partial sequence
trnL	tRNA-Leu (trnL) gene, partial sequence; trnL-trnF intergenic spacer, complete sequence; and tRNA-Phe (trnF) gene, partial sequence
trnL	voucher Trinder-Smith 143 (US) tRNA-Leu (trnL) gene, partial sequence; trnL-trnF intergenic spacer, complete sequence; and tRNA-Phe (trnF) gene, partial sequence
rbcL	chloroplast partial rbcL gene for ribulose bisphosphate carboxylase large subunit, specimen voucher Savolainen V. & Powell M.P. 1801C (NBG)

CHAPTER 4: Restoration of pollination networks in revegetated sites using pollen metabarcoding

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Dona Kireta		
Contribution to the Paper	I designed the study, undertook the field and laboratory work, analysed the data, and wrote the manuscript.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	16/12/2022

Restoration of pollination networks in revegetated sites using pollen metabarcoding

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Abstract

Habitat loss is causing bee declines, which in turn is impacting interactions between plants and bees and potentially disrupting pollination networks. Revegetation can be used to reverse declining bee populations, and potentially restore networks. However, there is a lack of understanding around the quality of revegetation needed to support native bees and their ecological roles, limiting opportunities to improve revegetation outcomes. Here we aim to address this gap, and compare pollination networks in revegetated landscapes differing in habitat quality. Floral and bee surveys were conducted in established revegetation sites at four locations in the Adelaide Hills in South Australia. The revegetation sites varied in quality, with simple tree plantings, to more complex and biodiverse plantings, and were paired with remnant habitat and cleared land controls. To generate pollination networks, rather than using a traditional observation-based approach, we analysed the pollen collected from the bees using molecular DNA metabarcoding approaches, which have been found to reveal more interactions than observation-based networks. Rather than using PCR amplification of DNA barcodes, we used a newly developed hybrid capture method to isolate barcodes of interest for taxon identification. Pollination networks from these data revealed that complex revegetation sites had similar complexity and robustness to remnant revegetation, although the latter sites had much larger networks. Networks in simple revegetation were simple and un-robust - unlikely to withstand many future stressors. These results are supported by the flower and bee diversity found within the treatments, which increased with improving habitat quality. The results indicate that robust pollination networks can only be restored in sites with high floral diversity, but that remnant vegetation supports the best networks, and should be conserved wherever possible.

Introduction

Wild bees, the largest and most important group of pollinators, are critical elements in natural and agricultural systems. Animal pollinators provide pollination services to over 80% of flowering plants, and over 30% of the world's crops (McGregor 1976). Despite their importance, bees are severely affected by habitat loss, and are in decline globally (Biesmeijer *et al.* 2006; Potts *et al.* 2010; Burkle *et al.* 2013; Goulson *et al.* 2015; Hogendoorn *et al.* 2020), which is threatening their mutualistic relationships with plants.

Habitat loss has been identified as a main contributor of decline (Biesmeijer *et al.* 2006; Potts *et al.* 2010; Burkle *et al.* 2013; Goulson *et al.* 2015; Hogendoorn *et al.* 2020). In particular, agricultural intensification (Klein *et al.* 2007) and urbanisation create problems for pollinators by reducing food sources and nesting opportunities (Batley and Hogendoorn 2009; Goulson *et al.* 2015). In a meta-analysis on human disturbance and pollination, wild pollinator declines were found to be significantly associated with habitat loss and fragmentation (Winfree *et al.* 2009), and are likely to be futher impacted by future loss (Hanula *et al.* 2015). In addition to the physical loss of habitat, the relationship pollinators have with plants is also lost. These relationships are unlikely to restore themselves (Cusser and Goodell 2013), without the return of the food and nesting resources required by the pollinators (Exeler *et al.* 2009; Roulston and Goodell 2011). Pollinator-plant interactions are often described by pollination networks, which shed more general understanding on ecological systems beyond single species focuses (Cusser and Goodell 2013).

To reverse the negative impacts of habitat loss, large-scale restoration and reforestation projects have been undertaken in many countries. Some examples are China's 40 year, billion tree program (Xu 2011), the Great Green Wall program in Sub-Saharan Africa (Turner *et al.* 2021), and the Grain for Green program, also in China, which is the largest reforestation scheme up to 2016 (Hua *et al.* 2016). The Australian Government has also invested significant funds for restoration projects supporting a range of goals: $2.55 billion for plantings to combat greenhouse gas emissions (Australian Government 2014); $50 million toward the 20 Million Trees program (2014 – 2020), for reforestation, and improvements in environment, sustainability and agricultural productivity (Australian Government 2021); and, in South Australia, funds for the Million Trees project to reduce carbon footprint and increase biodiversity, by re-planting nearly 3 million native plants across 1,500 ha (Urban Biodiversity Unit *et al.* 2013). Much of this restoration activity can be broadly grouped into two categories. The first is large-scale, low species diversity, simply structured revegetation which is often done by direct seeding. Examples are large-scale tree plantings for carbon sequestration. The second type is smaller scale, ecological plantings with higher species diversity and more complex habitat designs, such as land offset plantings.

However, the success of such projects for the restoration of pollination networks is largely unknown, given that collection of baseline data and long term monitoring are often limited, and when evaluations are done, success is measured by the number of plants planted and the land area restored (Ruiz-Jaén and Aide 2005). Pollination networks are a useful ecological function metric for assessing restoration success, since sustainable and long-term restoration can only exist with healthy pollinator networks (Kaiser-Bunbury *et al.* 2009; Cusser and Goodell 2013; Kaiser-Bunbury *et al.* 2017; Bell *et al.* 2022).

Traditional approaches for reconstructing pollination networks consist of monitoring pollinators visiting flowers, which is limited by the amount of time needed for observations. In addition, such visitation networks only partially predict pollen transport networks (Popic *et al.* 2013), and are biased towards specialists, which is contrary to most views that pollinator communities are generalist-dominated (Bosch *et al.* 2009; Cusser and Goodell 2013; Encinas-Viso *et al.* 2022). Studies have begun exploring pollination networks constructed from the pollen carried by pollinators, and compared these with visitation records. Studies that used microscopy to identify pollen yielded networks with more interactions and fewer specialist species (Bosch *et al.* 2009; Burkle *et al.* 2013).

Molecular methods are being developed to progress both pollen and invertebrate identification. Traditional, microscopy-based pollen is identified by observing individual characteristics on the pollen exine, requires significant expertise and is limited in throughput and taxonomic resolution, as in many cases taxa can only be identified to genus or family level (Kraaijeveld *et al.* 2015; Richardson *et al.* 2015b; Smart *et al.* 2017). In addition, understanding the invertebrate community is an important but often overlooked element of successful restoration practice, which can only be achieved through high throughput technologies (Heyde *et al.* 2022). It has therefore been proposed that high throughput DNA-based methods be used to categorise plant-pollinator interactions (Bell *et al.* 2022).

Molecular barcoding using high throughput sequencing has successfully identified pollen in several studies to high taxonomic resolution (Wilson *et al.* 2010; Keller *et al.* 2015; Kraaijeveld *et al.* 2015; Richardson *et al.* 2015a; Richardson *et al.* 2015b; Bell *et al.* 2017; de Vere *et al.* 2017; Bell *et al.* 2019; Suchan *et al.* 2019). It has also been used as a tool for constructing pollination networks, with 2.5 times as many interactions uncovered in networks constructed with pollen metabarcoding, compared to networks constructed with visually identified pollen (Pornon *et al.* 2016). Pollen DNA metabarcoding using single or dual barcodes have been used to describe Australian alpine pollination networks, finding less specialisation and higher diversity in networks derived from pollen metabarcoding versus microscopic identification (Encinas-Viso *et al.* 2022). Similarly, pollen metabarcoding of moth pollination networks found more

moth individuals carrying pollen, and more species per individual, compared with microscopic assessment alone (Macgregor *et al.* 2019).

This study aims to evaluate the benefit of common revegetation approaches on the restoration of pollination networks, given their importance for healthy ecological systems. We have previously explored the restoration of bee diversity and pollination services in revegetated landscapes of varying floral diversity (Kireta *et al.*, unpublished; Chapter 2). We found that floral and bee diversity in low complexity revegetation were similar to sites with cleared vegetation, and diversity in complex revegetation sites was more similar to remnant vegetation. The pollination services measured within the same sites reflected this result. An unexplored question is whether pollination networks are also restored in revegetation sites, and to what extent they resemble those in remnant sites. We expect that pollination networks would be more limited and less complex in simple revegetation, and more complex networks would be restored in complex revegetation sites, in accordance with higher species diversity and pollination services. We used a newly developed high throughput DNA metabarcoding approach to reconstruct pollination networks in two broadly classified types of revegetation. The molecular method we used was a hybridization (hybrid) capture target enrichment-based approach, using a 20 nucleotide RNA bait, which is a molecular region complementary to multiple informative regions of the chloroplast plant genome, and proven to be useful for plant identification (Kireta *et al.*, unpublished, Chapter 3; Waycott *et al.* 2021). The goal was to identify species pollen in addition to the plants visited by bees, and gain deeper insight into pollination dynamics restored in revegetated landscapes.

Methods

This study compared pollination networks within revegetation sites differing in the diversity of plants and complexity of structure. The data was collected over two seasons, and the data from the second season had previously been used to assess floral and bee diversity and pollination services (Kireta *et al.*, unpublished; Chapter 2).

Field sites

Four study locations were used, situated in the Mount Lofty Ranges, South Australia (Figure 1). Up until the 1990s, this area suffered extreme land clearance. Today, only 4% native vegetation remains across the Adelaide plains, and 11% remains across the ranges, which remains highly fragmented (Bradshaw 2012). The climate in this region is Mediterranean, with hot dry summers, and cool wet winters which receive most of the annual rainfall, on average between 600 mm to 1000 mm.

Sampling occurred in spring and summer of 2018-19 and 2019-20. Unfortunately, climate conditions in Australia have become extreme in recent years, which affected

the study period in South Australia. Rainfall in the preceding years was well below average across the Mt Lofty ranges; September 2018 was the third driest September on record (Australian Bureau of Meteorology 2019), and January to April 2019 was the driest period ever recorded in the region. September 2018 was also the coolest on record, with unseasonal frosts, which were followed by heatwaves in mid January (2019), making it the warmest January on record (Australian Bureau of Meteorology 2020). The end of 2019 saw extreme heatwaves in December around Adelaide causing "catastrophic fire danger" in the Mount Lofty Ranges, followed by "extremely hot temperatures" in early January 2020 (Australian Bureau of Meteorology 2021). These conditions precluded December sampling in two of the four locations, leading to 7 of the 154 sampling time points missing from the final results.

Restoration sites were pre-established revegetation projects which fell into two broad categories. 'Simple' plantings consisted of less than five plant species, mainly combinations of *Eucalyptus*, *Dodonaea* and *Acacia* (hardy species resistant to grazing). These plantings were often direct seeded forming dense rows of large shrubs and trees. 'Complex' plantings attempted to replicate remnant vegetation, using a larger mix of flowering species (10 +) planted manually and creating more complex vegetation structure. The species used here varied, but often included *Goodenia, Arthropodium, Chrysocephalum, Eucalyptus, Grevillea, Hakea, Hardenbergia, Leptospermum, Scaevola,* and more. Often this complex revegetation also received ongoing maintenance. Three spatially distinct sites of each revegetation 'treatment' were used as experimental replicates to avoid pseudo-replication, since revegetation projects in this area were generally too small for independent sampling across replicates. A positive (remnant vegetation) and negative (cleared land) control were paired with each revegetation treatment. The sites were located far greater than the foraging distance of wild bees (Gathmann and Tscharntke 2002; Greenleaf *et al.* 2007; Zurbuchen *et al.* 2010). The closest sites were almost 6 km apart (Clarendon to Craigburn), and the furthest were 48 km apart (Onkaparinga to Millbrook). There were ultimately 14 treatment plots across 4 sites (Figure 1).

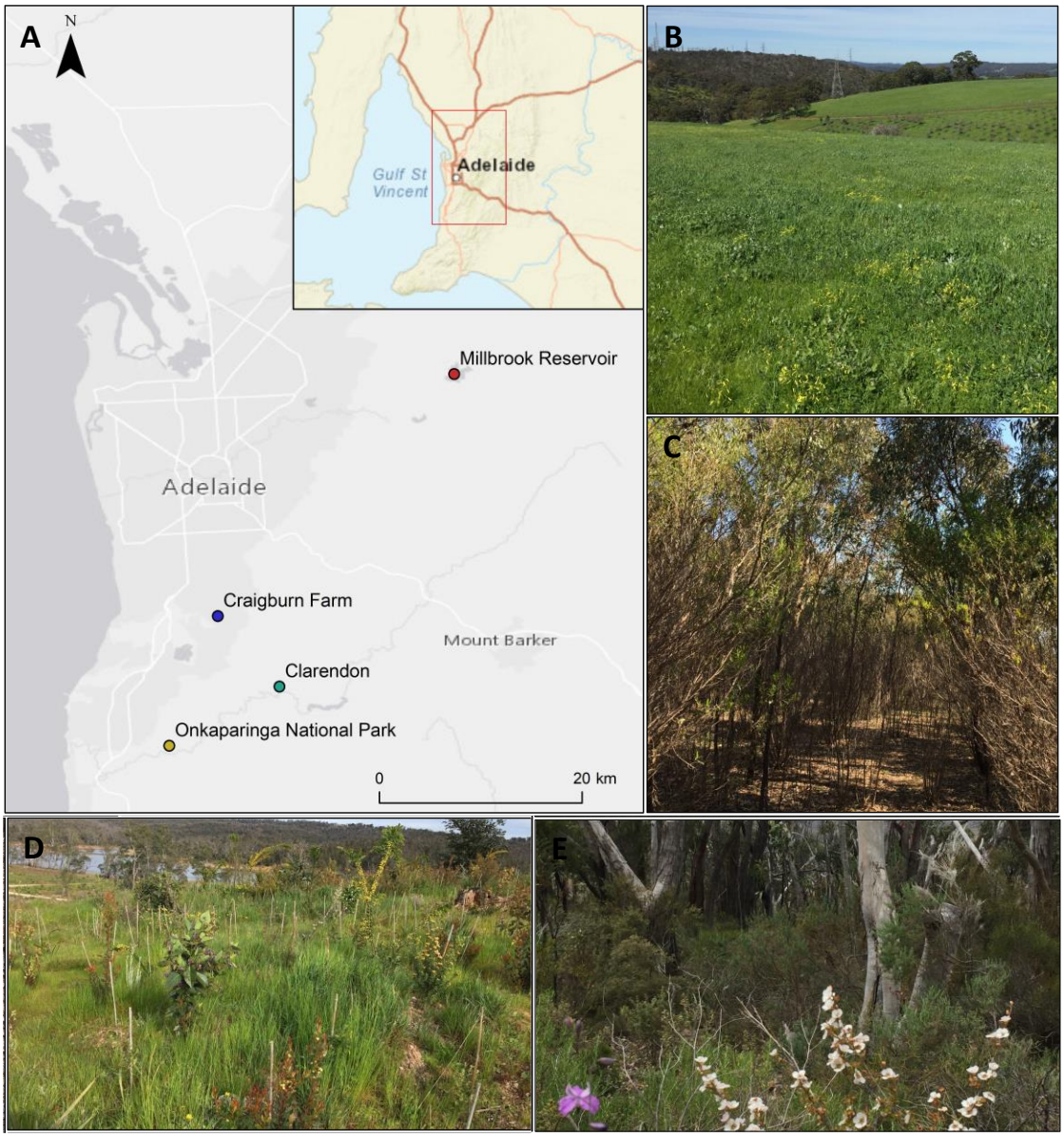

Fig. 1. A: Locations of the revegetation sites used for flower and bee surveys. **B-D:** example photographs of the vegetation treatments; **B** negative control (cleared vegetation); **C** simple revegetation; **D** complex revegetation; **E** positive control (remnant vegetation). Millbrook reservoir had complex revegetation, remnant, and cleared land types; Craigburn Farm and Clarendon had complex and simple revegetation, remnant and cleared land types; and Onkaparinga National Park had simple revegetation, remnant and cleared land types.

The restoration sites were developed by diverse projects with independent funding. At Millbrook and Clarendon, revegetation was motivated by biodiversity enhancement, offsetting, and water quality maintenance. These projects were monitored and maintained post planting. Craigburn and Onkaparinga sites were planted as part of the Million Trees program between 2003 to 2014 (Urban Biodiversity Unit *et al.* 2013). This project was community led, and had little to no post planting maintenance. Due to

these differences, uncontrolled variables existed between the sites (Table 1). Despite some *Eucalypt* individuals, all species at all sites were mature enough to flower.

Table 1. Revegetation sites detailing various treatments and variables

Treatment Site	Cleared	Simple	Complex	Remnant	Year of restoration	Other actions
Craigburn Farm	×	×	×	×	2009	-
Clarendon	×	×	×	×	2014	Extra planting/weed removal
Millbrook Res	×		×	×	2017	Extra planting/weed removal
Onkaparinga NP	×	×		×	2011	-

Surveys

Surveys were done within 25 by 25m permanent quadrats in each treatment site. Bees were surveyed once every month across spring and summer, from October to February 2018-2019, and September to February 2020. Bee sampling was done on warm, clear days above 18°C with little wind, when bees were most active. Bees were captured visiting flowers with a hand net, and put directly on ice for subsequent barcoding and identification (ID). Catching occurred for 10 minutes within the permanent quadrats, and a further 10 minutes outside of the quadrat in a zigzag fashion. The flower species the bees were visiting were recorded. Flower species were identified using Prescott (2012), and confirmed where necessary by botanists at the State Herbarium of South Australia. In addition, flower surveys were conducted at each sampling time point. Within the quadrat, three one-meter transects (75 m^2 total) were established, and every flowering plant attractive to bees was recorded along with the number of flowers on a categorical metric scale (Brosi *et al.* 2007). The flower surveys were merged into one list, and only one record of each plant was kept based on its peak flowering period, to avoid duplicating individuals. This resulted in a list of all flowering plants and flower abundance spanning the whole flowering season.

Honey bees were not included in the study, since they are an invasive species in native areas of Australia, and are not the target of restoration projects.

Metabarcoding and species ID

Sample preparation – bees without pollen

Bee samples were identified using both morphology and DNA barcoding. Where possible, morphological ID was done to genus level using keys in Michener (2007), and when possible to species level, using keys available referred to in Michener (2007). The remaining samples were identified through DNA barcoding. If the bees contained no pollen (e.g. most males/*Hylaeus*), bee legs were plated and barcoded using CO1 under an iBOL (International Barcode of Life) project aiming to create a reference library for all native Australian bees (Hogendoorn *et al.* 2015).

Sample preparation – bees with pollen

Bees with pollen were metabarcoded using the hybridisation (hybrid) capture approach, based on the method used by Kireta et al. (unpublished; Chapter 3), and described in Waycott *et al.* (2021), which uses the universal chloroplast bait set OZBaits_CP V1.0 which was developed for targeted hybrid capture of angiosperm sequences. We followed the myBaits® for Targeted NGS Manual Version 4.01.

A leg was taken from the bee and placed into 2 mL screw cap tubes. If the position of the scopa was not on the leg, it was scraped from the scopa, or if the bee had a small pollen load, it was washed with ethanol and spun to dislodge the pollen, then allowed to dry. A negative control was added in each extraction batch, and also later on in each hybrid capture run. Ceramic beads were then added and samples were homogenised using a Bead Ruptor 24 (OMNI International Inc.) for 20 second cycles at 6 m/s, interspersed with submersion in liquid nitrogen until a powder was formed. Liquid nitrogen helped with homogenisation and reduce DNA damage through friction heat.

DNA extraction and library preparation

DNA was extracted with the “isolation of genomic DNA from honey or pollen” protocol from the Macherey-Nagel NucleoSpin® Food kit (Düren, Germany). The homogenisation step was modified to the method described above, and the final product was eluted in 60 µL of elution buffer twice rather than once to increase DNA yield. DNA was quantified using the QuantiFluor® dsDNA System with the Quantus™ Fluorometer (Promega, Madison, WI, USA), then normalised to 2ng/uL (if the concentration was < 2ng/uL, DNA was used neat), and finally sonicated with the Diagenode Bioruptor® Pico (Diagenode, USA) to fragment DNA strands to random lengths (eight cycles 15 s on, 90 s off).

An Eppendorf epMotion® 5075t - Liquid Handling Workstation was used for DNA library preparation, using the using the NEBNext® Ultra™ II DNA Library Prep kit and protocol described in Waycott et al. (2021). The method uses custom-made y-stubby adaptors which ligate to the randomly sized DNA fragments. Forty-eight unique 8 nt in-line barcodes were incorporated into the adaptors, in unique combinations (one

adaptor ligated to each end of the DNA strand). This allowed downstream pooling. Libraries were amplified using PCR (30 s at 94°C, then 17 cycles of 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s, final extension at 72°C for 2 mins then 4°C hold). Gel electrophoresis was used to visualise the libraries (1.5% agarose gel, 1 x TBE buffer, 2uL library, 35 mins at 90 volts). Finally, groups of 16 libraries were pooled based on visual approximation of concentration, which reduced costs, then pooled samples were cleaned with MagNA Beads (Rohland and Reich 2012) at a 1:1 volume concentration.

Hybridization capture

The OZBaits_CP V1.0 universal plastid bait set was used for hybrid capture, which was developed to target 19 chloroplast genes for all angiosperm lineages (Waycott *et al.* 2021), and the myBaits® Targeted NGS Manual Version 4.01 hybridization protocol was used. After library pooling, the baits and 10uL of chill-out™ red liquid wax were added (reducing evaporation). Hybridization occurred at 65°C for 48h, followed by PCR amplification (98°C for 2 min, then 20 cycles of 98°C for 20 s, 60°C for 30 s, and 72°C for 45 s, and final extension at 72°C for 5 min and 8°C hold) using customised P7 and P5 Illumina adaptors. Magnetic beads were added to bind target regions, and non-target sequences were removed by placing samples on a magnet to separate the beads. Libraries were quantified with a 2100 Agilent Bioanalyzer with the high sensitivity DNA assay, and pooled to achieve equimolar concentrations. The final library was purified with MagNA beads at a 1:1 volume concentration, and was size selected at 350-600 bp with a Pippin Prep 2% agarose gel cassette (Sage Science). Sequencing was done on a single lane of an Illumina HiSeq X Ten with 2×150 chemistry at the Garvan Institute of Medical Research (Sydney, Australia).

Bioinformatics pipeline

The pipeline used here is based on that used by Kireta et al. (unpublished; Chapter 3) and Bell *et al.* (2021). It is a custom pipeline which runs on the Phoenix high performance computing cluster at the University of Adelaide, Australia. It involves demultiplexing firstly by the indexes with Bcl2fastq, and secondly by internal barcodes with Sabre. Since the barcodes contained two degrees of separation, we allowed one bp mismatch. Next, PCR duplicates were removed with clumpify from BBtools (Bushnell 2021), which makes analyses faster and reduces memory requirements. Then sequences were trimmed with AdapterRemoval, where the 9th base after the 8 nt in-line barcode was removed, plus all reads with < 30 nt and phred score < 20 were removed, and N tails trimmed.

Reference library

We constructed our own custom reference library for sequence ID. Previous work that compared hybrid capture metabarcoding with *matK* and RefSeq reference libraries found that the whole chloroplast RefSeq references yielded better results than using

the single barcode *matK* reference library with the bait set of 25 chloroplast regions (Kireta *et al.*, unpublished; Chapter 2). However, whole chloroplast references were much more limited than *matK* references. Publicly available RefSeq sequences missed 71 of the plant taxa observed in the field sites, while for *matK* only 23 species were not represented, but each species had at least a genus level representation. Accordingly, *matK* references were used for library construction.

To build the reference library, a comprehensive list of plants was used to download *matK* references from the NCBI nucleotide search portal. The plant list was based on all angiosperm species recorded in South Australia (obtained from the Atlas of Living Australia https://www.ala.org.au/), plus all plants species that bees were recorded visiting, any additional flowering species occurring in the field sites that were observed during sampling, and plant lists of species used for the revegetation projects (Table S2). In addition to public references downloaded from NCBI, we included references for 200 local plant species generated in house using the same hybrid capture method (unpublished).

Species identification and analysis

Following reference database creation, we used Kraken2 to assign taxonomy (at species level) to sequencing reads. Kraken2 does not require sequences to be pre-assembled, because it used a k-mer based assignment method (Wood *et al.* 2019). A threshold of 5 was set for minimum hit groups in Kraken, below which taxa were discarded.

To deal with pollen contamination which likely occurred via net transfer in the field, and possible lab contamination during sample processing, taxa found in the negative controls (blanks) were filtered out of the results unless they were the species that the bee was recorded visiting.

We used flowering species lists gathered during field sampling, and local information about the sites, to help inform and narrow down the species recovered through metabarcoding. For example, in the case that three *Goodenia* species were recovered on a single bee: it was improbable that a single bee captured visiting *Goodenia amplexans* had visited two additional *Goodenia* species*,* and knowing that the site only contained *Goodenia amplexans*, we replaced any additional *Goodenia* IDs with *Goodenia amplexans*. Similarly, barcoding results of two *Xanthorrhoea* species where only one species was recorded were reduced to a single species record. Where multiple plausible species of the same genus were recovered from a single bee, and where we were unable to distinguish which species was the true taxon, we reduced the ID to genus level only. We did this in particular with *Eucalyptus* and weedy *Asteraceae* species which were not identified to species level in the field. Although usually only a single individual flowered per sampling time point, we were unable to ID individual species, especially since up to four species occurred in many sites (Table S1).

There were approximately 10 possible species of *Eucaluptus* found across the whole study area (Table S1). Since the objective of this study was to compare the differences between networks in different vegetation treatments, and not absolute properties of the sites, we were comfortable knowing that some errors likely remain in the dataset. We assumed these were constant across the treatments, and arose from the large number false positives.

Shannon diversity was used to estimate diversity to compare floral and bee communities, since it accounts for species richness and abundance, contrary to Simpson diversity which does not account for abundance. One-way ANOVA was used to test for differences in diversity between treatments. Assumptions of normality were tested and met using Shapiro-Wilk tests and by plotting residuals. We then ran Tukey's post-hoc tests using the agricolae package to determine pairwise differences among treatments (de Mendiburu 2020).

The majority of ecological network analyses pool sampling data collected across space or time, and calculate overall networks. This is done because it is very difficult to collect enough data in a single sampling session that can be statistically analysed to high degrees of power, but it introduces forbidden links (Guimaraes *et al.* 2017). Due to phenological and spatial mismatches, some links between bees and plants cannot exist. An unrealised link that cannot exist (forbidden link, e.g. bee present but plant not flowering) is fundamentally different from an unrealised link that can exist (i.e. bee and flowering plant present, but bee does not visit plant). Therefore, the analyses of combined networks can lead to conclusions about the network structure (lots of unrealised links) that are unwarranted. A possible solution was to calculate pollination network and metrics individually for each sample point (50 in total), then compare these statistically to evaluate differences between treatments. We thus calculated separate networks for each of the sampling points. However, as discussed, the sample size for individual networks and metrics calculated per sampling unit was not large enough to reliably calculate sampling metrics, and impossible for 33 networks. In 15 of the 50 networks, nestedness could not be calculated, in 27 of the 50, specialisation could not be calculated, and in 23 of the 50, H2 could not be calculated (Table S3). This made it impossible to compare the network metrics between treatments using this approach.

An alternative approach to control for forbidden links is to use a null model approach (e.g. Fortuna and Bascompte 2006). We used the econullnetr package (Vaughan *et al.* 2017) in RStudio (RStudio Team 2020), which manages forbidden links using a table of resource availability input by the user. The package is a wrapper for the commonly used bipartite package (Dormann *et al.* 2008). A null network is calculated based on the assumption that interactions reflect the proportion of resources available, i.e., random resource use, using a table of flower survey data for resource availability. Then, network metrics are calculated for both the null and observed network, and an

iterative approach is used to estimate the probability that observed metrics are different from the null model. The package then outputs overall networks and metrics.

We assessed weighted nestedness, linkage density, weighted connectance, interaction evenness and network level specialisation (H2) across the networks. We used the 'generate_null_net' function and ran 999 iterations to compare the null model with observed results.

Results

A total of 349 bees were sampled, which were captured visiting flowers. Of the 349 bee samples, 150 were barcoded under the BOLD barcode for life project, and 121 bee samples containing pollen were metabarcoded using hybrid capture. The remaining 78 bees were species duplicates not containing pollen.

After sequencing, between 32,786 and 28,017,458 reads were generated per sample, and after filtering, between 4,360 and 8,175,644 reads remained per sample. A minimum of 11 and maximum of 277,454 reads were classified to species level per sample, assigning to between 2 and 53 species per sample above the minimum set threshold.

Species identification

A total of 114 species of bees were identified, that visited 42 species of flowering plant. Through metabarcoding of the pollen collected by these bees, 65 species of flowering plant were identified, of which 25 species were new (not observed during plant surveys). For the 121 bees that were also pollen metabarcoded, 79 samples returned positive results (after filtering and minimum thresholds), and 28 of those samples returned a species that matched the plant species which the bee was visiting during capture. A further 26 samples returned a species within the same genus, and 14 samples returned taxa within the same family of the plant species that the bee was visiting during capture. In total, 86 % of the successful samples had a taxon identified that matched the species, genus or family of the plant visited by the bee, in addition to other taxa identified.

Contamination

At the end of the bioinformatics pipeline, of the four negative controls included, two contained too few sequences to analyse, and two contained the following species: BC1; *Leptospermum continentale*, *Calytrix tetragona*, *Baeckea crassifolia*, *Spyridium parvifolium*, *Prunus persica*, *Atriplex cinerea*; BC5: *Spyridium parvifolium*, *Atriplex cinerea*. These species were removed from the metabarcoding results, unless they were present in the field site at the time of sampling.

Diversity results

The treatments differed significantly in flower diversity (one-way ANOVA, DF = 3, $p < 0.001$, Figure 2) and in bee diversity (one-way ANOVA, DF = 3, $p < 0.001$; Figure 2). Tukey's HSD post-hoc comparison test showed that Cleared sites and Simple revegetation did not differ in either floral or bee diversity. Cleared and Simple treatments both had lower floral diversity than Complex and Remnant sites, while the latter two did not differ significantly in bee diversity (Table S4; Figure 2).

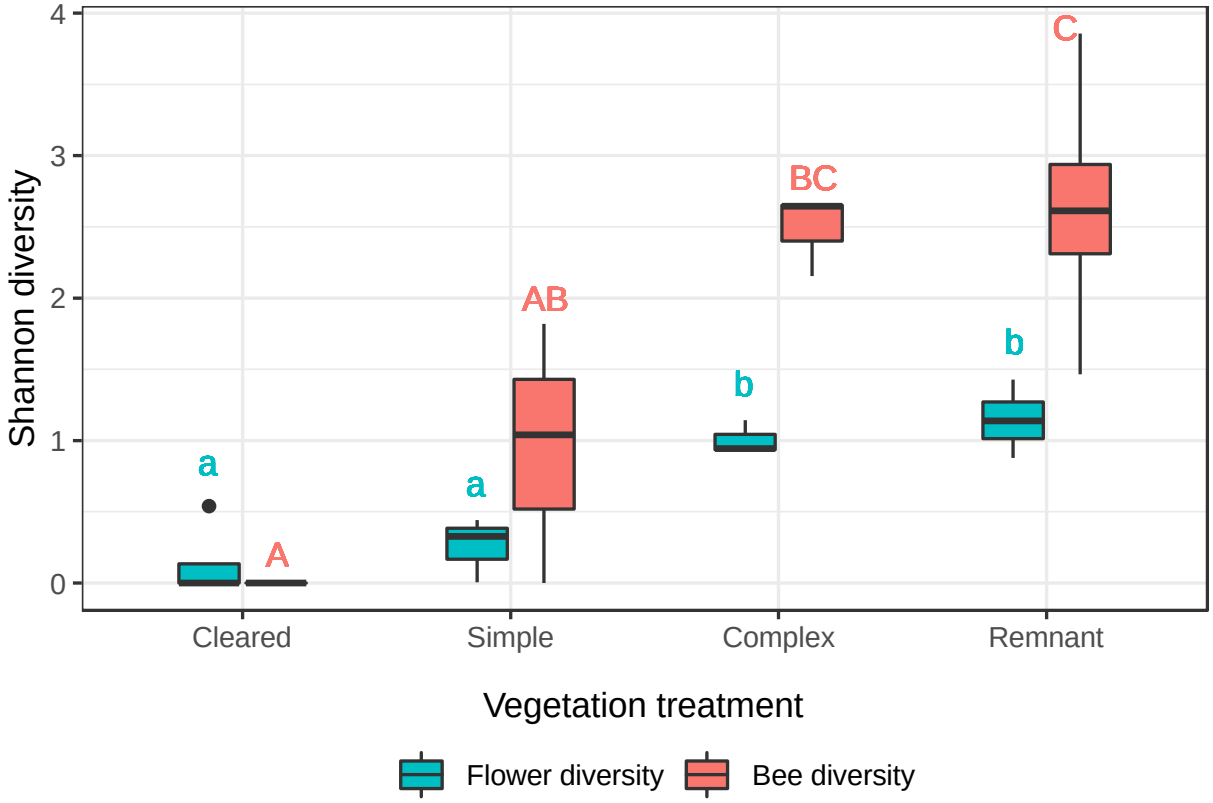

Figure 2. Box plot of Shannon's species diversity in four vegetation treatments: Simple and Complex revegetation, with negative (Cleared) and positive (Remnant) controls. Bars depict survey results for flower diversity (teal, left), and bee diversity (red, right). Letters indicate significance between treatments determined with Tukey's post hoc tests.

Network results

No network or network metrics were calculated for the cleared treatment, as no bees were captured.

The pollination networks calculated for the three treatments where bees were observed were clearly different between the Simple, Complex and Remnant vegetation treatments, increasing in size and complexity respectively (Fig. 3). The colours of the interaction bars reflect the comparison to null models, where blue indicates weaker, white indicate consistent, and red indicate stronger interactions than expected compared to null models.

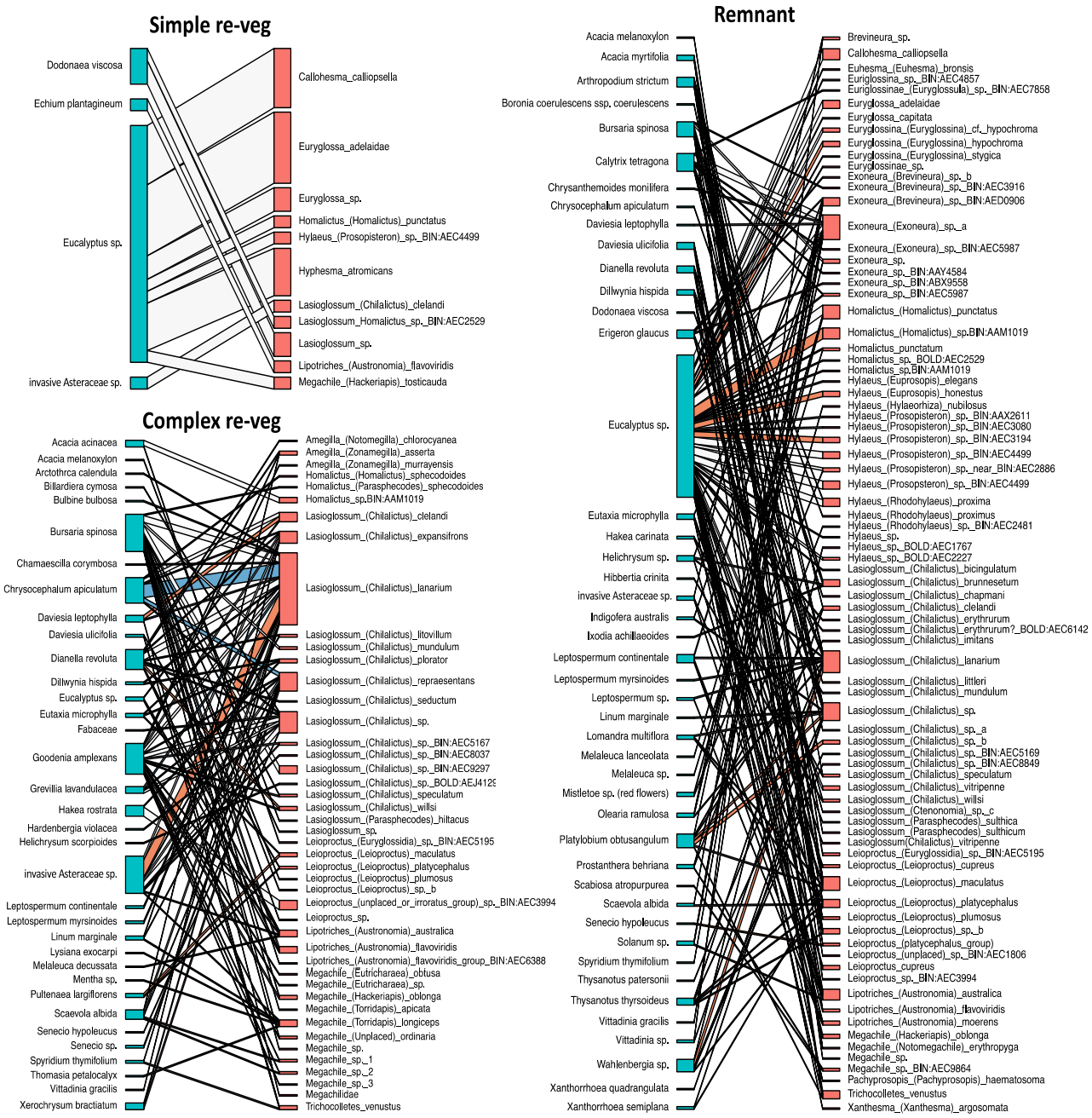

Figure 3. Mutualistic network between bees and flowering plants in Simple revegetation (re-veg), Complex revegetation, and Remnant vegetation, merged across four field sites in South Australia. Blue bars indicate weaker, white indicate consistent, and red bars indicate stronger interactions, compared to the null model.

The network metrics calculated for Simple revegetation were the same for the observed and null results. Significance between the observed and null results, except for weighted nestedness which could not be calculated (Table 2; Table S5-A).

The observed network metrics calculated for the Complex revegetation were lower than the null result for all metrics apart from interaction evenness, which was the same between the observed and null model, however, weighted connectance was significantly lower in the observed result (Table 2; Table S5-B). This indicates that there were fewer links realised than expected by chance.

The observed network metrics calculated for the Remnant vegetation were significantly lower than the null result for all metrics except for H2, which had no significant difference (Table 2; Table S5-C).

Table 2. Summary of results of observed mutualistic network metrics for simple revegetation, complex revegetation and remnant vegetation. * indicates values that are significantly lower than expected on the basis of the null model

Observed metrics	**Simple re-veg**	**Complex re-veg**	**Remnant**
weighted nestedness	NA	0.58	0.64*
linkage density	2.86	6.07	9.81*
weighted connectance	0.19	0.08*	0.08*
interaction evenness	0.89	0.93	0.95*
H2	1.00	0.29	0.30
Network size	15	80	127
num bee species	11	45	82
num plant species	4	35	45

Apart from network specialisation (H2), all metrics of remnant vegetation differed from the null model, whereas in the complex revegetation, only connectance differed from the null model. In addition, the observed weighted nestedness and linkage density were substantially higher in the remnant than in the complex revegetation.

Discussion

Pollination networks were re-constructed in revegetated sites categorised into simple tree plantings, and more complex biodiversity plantings, with positive and negative site controls. A novel hybrid capture DNA metabarcoding method was used to identify the pollen for building the networks, which revealed many interactions in addition to those observed in the field. The pollination networks increased in size and complexity

with vegetation quality, in particular in simple verses complex revegetation. This finding is in line with floral and bee diversity, and previously measured pollination services within the same sites (Kireta *et al.*; unpublished; Chapter 2). The network robustness increased from very low to high in simple and complex revegetation compared with remnant vegetation (indicated by linkage density and weighted nestedness), indicating that high planting diversity is needed to restore resilient interactions between plants and bees.

Higher weighted nestedness in the remnant vegetation compared with complex revegetation suggests that remnant sites are the most robust (Mariani *et al.* 2019). In a study modelling network decay through habitat loss, the authors found that more nested systems persisted longer with increasing habitat loss than communities with only random interactions (Fortuna and Bascompte 2006). Furthermore, networks with higher nestedness were more able to withstand species loss, where losses of the most generalised species resulted in larger declines in the network (Memmott *et al.* 2004). This suggests that the simple revegetation studied here had poor robustness, since the network was so small that nestedness could not be measured. The average number of links per species increased by a third from simple to complex revegetation, and from complex revegetation to remnant vegetation. This means that bees in complex revegetation and remnant vegetation were visiting more flower species. This was to be expected, since there was increasing floral diversity, which allowed bees access to more species. However, it leads to increased redundancy in the system, and to stronger and more stable networks.

In both complex revegetation and remnant vegetation, the links per species and weighted connectance was lower than in null models, indicating that bees had stronger preferences for plants than if their interactions were based on species availability alone.

Lower interaction evenness is likely due to the presence of more specialists in remnant vegetation, which is expected in more intact habitats, especially with the presence of *Eucalyptus* which host many specialist bees (Michener 1965; Houston 2018). In contrast, lower interaction evenness could also indicate that remnant habitats were somewhat disturbed, since this metric has been found to be lower in more disturbed habitats (Tylianakis *et al.* 2007; Bluthgen *et al.* 2008). This idea somewhat fits with our results, as interaction evenness was higher in the less disturbed habitats. Furthermore, although the remnant sites we chose were the best reference sites available, they were not pristine environments, discussed further below. Few if any completely undisturbed environments exist in the Adelaide hills, and the sites we had access to had experienced recent fires (Millbrook in 2014), historic farming (Craigburn farm), and general over-grazing from the overabundant Kangaroo population (DEW 2018).

Pollinator richness has been shown to increase network stability (Potts *et al.* 2009). The higher complexity in the pollination networks in higher quality sites (compared

with simple revegetation) can be attributed to the higher floral diversity found within them, which likely led to higher bee diversity. The higher bee diversity in turn led to higher levels of pollination services (Kireta *et al.*, unpublished; Chapter 2). This supports the results found by a study in alpine Australia, where the authors found that habitat heterogeneity drove diversity in pollination networks (Encinas-Viso *et al.* 2022). Few studies have explored pollination networks within restoration sites, but those that have also came to similar conclusions. In a comparison of two natural heathlands, pollination networks were larger in the site undergoing restoration, with higher species richness and abundance (Kaiser-Bunbury *et al.* 2009). Bee richness at restored mine sites converted to wildflower meadows recovered quickly to levels equal with established meadows. Networks within these restored meadows had more links between bees and plants, and greater nestedness, suggesting pollination services stabilise over time (Novotny and Goodell 2020). A further study that manipulated floral diversity in restoration sites on a reclaimed strip mine found that pollinator diversity declined with distance from remnant vegetation, and that pollinator diversity was reduced in low diversity plots far from remnant vegetation. However, high floral diversity compensated for the loss of pollinators in plots far from remnant vegetation by attracting generalist pollinators, which increased network connectivity, and resulted in more robust systems (Cusser and Goodell 2013). This compares to our results, where complex revegetation with higher floral diversity had a more diverse bee population, and larger and more complex pollination network.

The specialisation indices measured suggest that the networks were similarly specialised in complex revegetation and remnant vegetation. Low specialisation has been linked to high functional redundancy (Lucas *et al.* 2018) which is a desired trait as it leads to higher network robustness and resilience (Encinas-Viso *et al.* 2022). For our study, this indicates that simple revegetation, returning a high specialisation level, had poor redundancy, and may be less resilient to future change and stressors, and therefore may have a less sustainable outcome in the future. Conversely, complex revegetation may persist as well as remnant vegetation given the same stressors in the future.

One large distinction between restoration sites and remnant vegetation was the presence of flowering *Eucalypts. Eucalypts* occurred in all sites, but due to drought and very low spring rainfall which influenced flowering, plus extreme heatwaves, the *Eucalypts* did not flower in the first field season, and only some individuals in some sites flowered in the second season (Law *et al.* 2000; Australian Bureau of Meteorology 2020, 2021). *Eucalypts* are generalists, they are serviced by many specialist bee species, and Australia has many bees known to be *Eucalyptus* specialists (Michener 1965; Houston 2018). These were one of the significant groups in the pollination networks, with the most bee visitors in Simple and Remnant sites where they did flower, and at least five species of bee had a significant preference for it. This

effect could have been influenced by the sheer abundance of resources *Eucalyptus* trees provide. But regardless of the underlying cause, which is likely a combination of the quantity and quality of resources, the networks highlight their importance in the landscape. We suggest that revegetation projects ensure the establishment of some *Eucalypt* individuals, however, attention should be given to planting density, as very dense and heavily shaded vegetation fails to support mid and lower canopy species which provide a great deal of the flowering resources bees require.

Project limitations

This project faced some challenges which limited the data collection and metabarcoding for species ID. Difficult environmental conditions (extreme heat and low rainfall, discussed above) likely led to a flowering shortage, likely lowering sample sizes, and limited the opportunities for species interactions. It is possible that in other years, such as the very wet La Niña seasons directly following this study, the patterns found could vary, and thus these patterns could be reflective of dryer conditions. Furthermore, availability of our focal species in publicly accessible reference databases was limited, and made confidently identifying some species difficult. A possible solution for future studies is to generate in house reference sequences, customized to the species encountered in the study. However, this approach is a large, expensive, and time-consuming undertaking, which is likely to constrain many projects. We incorporated 200 custom references from a parallel project (unpublished) into our database, and this addressed a number of missing taxa that we encountered, but we were nevertheless limited in scope and several species were still ultimately missing from the database. The issue of incomplete DNA reference libraries heavily impedes many studies relying on plant and invertebrate identification, which we will not detail here, but we re-iterate the point that this is an avenue of research well deserving of attention and funding. Lastly, the barcoding approach used here, while providing many benefits through cost cutting and high throughput data generation, also had some drawbacks. Large levels of false positives are a consequence of highly sensitive methods such as the one used here (Bell *et al.* 2017), and while the high sensitivity of the method meant that rare taxa were likely discovered, they may have been hidden by the large numbers of false positives. A consequence of netting bees was that the net provided an unavoidable contamination source, regardless of the care used to reduce in field contamination. There is no practical solution to this other than having fresh nets for each sample, which is unfeasible. Some lab contamination also occurred. An example is the case with *Spyridium parvifolium*, which was recorded flowering during one sampling time point (September 2019 in Remnant vegetation at Clarendon), a single bee was captured visiting this species, yet it was detected in 43 bee pollen samples following metabarcoding, in both seasons. To control for the issues discussed here, we would draw attention to the importance of having good knowledge of the study sites. Given our extensive flower surveys, and knowledge of the flora

found in South Australia through ALA records (https://www.ala.org.au/), we were able to create a more customized barcode reference database to begin with, and we were further able filter out many of the erroneous barcoding results.

Management implications and conclusion

Bee diversity increased with floral diversity, and more florally diverse, complex revegetation achieved levels of both floral and bee diversity on par with remnant vegetation. In contrast, simple revegetation was not different to cleared sites regarding floral diversity, and pollination networks in this revegetation type were small. Pollination networks were largest and most complex in remnant vegetation, even when similar levels of floral diversity were achieved in complex revegetation. These networks were also the most stable and robust, and most likely to withstand future stressors. Therefore, to safeguard existing plant-bee relationships it is vitally important to protect and appropriately manage remnant vegetation, and this should be a high priority for land management practitioners. To restore healthy and robust pollination networks, and thus functionally stable ecosystems, complex and florally diverse revegetation is necessary, but our data show that complex revegetation only partly restores the network attributes. It is possible that these networks will continue to improve with time, and re-sampling such revegetation sites across time would be a valuable avenue for future study. There are some species such as *Eucalypts* which are particularly important to include. Resources should be focused on plant diversity rather than revegetation size to restore ecosystem functionality.

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Supplementary information: Restoration of pollination networks in revegetated sites using pollen metabarcoding

Table S1. Comprehensive list of all *Eucalyptus* species with occurrence records (from the Atlas of Living Australia, https://spatial.ala.org.au/, accessed 20/6/2023) or with planting records, across all study sites.

Site	Treatment	*Eucalyptus* species
Clarendon	Low Div	*Eucalyptus obliqua* *Eucalyptus fasciculosa*
	High Div	*Eucalyptus leucoxylon ssp. leucoxylon* *Eucalyptus viminalis ssp. cygnetensis* *Eucalyptus macrocarpa* *Eucalyptus fasciculosa*
	Remnant	*Eucalyptus microcarpa* *Eucalyptus camaldulensis ssp. camaldulensis* *Eucalyptus leucoxylon ssp. leucoxylon* *Eucalyptus fasciculosa*
Onkaparinga	Low Div	*Eucalyptus porosa* *Eucalyptus fasciculosa* *Eucalyltus leucoxylon ssp. leucoxylon* *Eucalyptus microcarpa*
	Remnant	*Eucalyptus fasciculosa* *Eucalyptus macrocarpa* *Eucalyptus porosa*
Craigburn	High Div	*Eucalyptus leucoxylon ssp. leucoxylon* *Eucalyptus macrocarpa* *Eucalyptus camaldulensis ssp. camaldulensis* *Eucalyptus obliqua*
Millbrook	High Div	*Eucalyptus leucoxylon ssp. leucoxylon* *Eucalyptus fasciculosa* *Eucalyptus goniocalyx* *Eucalyptus camaldulensis ssp. camaldulensis*
	Remnant	*Eucalyptus goniocalyx* *Eucalyptus camaldulensis ssp. camaldulensis* *Eucalyptus leucoxylon ssp. leucoxylon* *Eucalyptus obliqua*
Unique list across all sites		*Eucalyptus obliqua* *Eucalyptus camaldulensis ssp. camaldulensis* *Eucalyptus fasciculosa* *Eucalyptus goniocalyx* *Eucalyptus leucoxylon ssp. leucoxylon* *Eucalyptus macrocarpa*

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	Eucalyptus microcarpa *Eucalyptus porosa* *Eucalyptus viminalis ssp. cygnetensis*

Table S2: Species lists of plants used to create DNA databases

Acacia acinacea	*Brassica rapa*
Acacia ligulata	*Brassica tournefortii*
Acacia longifolia	*Brunonia australis*
Acacia melanoxylon	*Bulbine bulbosa*
Acacia myrtifolia	*Burchardia umbellata*
Acacia paradoxa	*Bursaria spinosa*
Acacia provincialis	*Bursaria spinosa ssp. spinosa*
Acacia pycnantha	*Caesia calliantha*
Acacia rupicola	*Callistemon rugulosus*
Acacia verticilata	*Callistemon sieberi*
Acaena echinata	*Callistemon teretifolius*
Acaena novae-zelandiae	*Calocephalus citreus*
Actinidia deliciosa	*Calytrix tetragona*
Allocasuarina muelleriana ssp.	*Carex tereticaulis*
Allocasuarina verticillata	*Carpobrotus rossii*
Alyogybe sp.	*Centaurea melitensis*
Amyema miquelii	*Chamaescilla corymbosa*
Anagallis arvensis	*Chamaescilla corymbosa var. corymbosa*
Arctotheca calendula	*Cheiranthera alternifolia*
Arthropodium strictum	*Chondrilla juncea*
Asparagus asparagoides	*Chrysanthemoides monilifera ssp. monilifera*
Asparagus asparagoides f. asparagoides	*Chrysocephalum apiculatum*
Asparagus officinalis	*Chrysocephalum semipapposum*
Atriplex cinerea	*Cicer arietinum*
Austrodanthonia geniculata	*Cirsium vulgare*
Austrostipa elegantissima	*Citrullus lanatus*
Baeckea crassifolia	*Clematis microphylla var. microphylla*
Banksia marginata	*Comesperma volubile*
Banksia ornata	*Convolvulus arvensis*
Baumea juncea	*Convolvulus remotus*
Billardiera cymosa	*Correa decumbens*
Billardiera cymosa ssp. cymosa	*Craspedia glauca complex*
Billardiera heterophylla	*Crataegus monogyna*
Boronia coerulescens ssp coerulescens	*Crataegus monogyna ssp. azarella*
Brachyscome ciliaris var. ciliaris	*Cucumis melo*
Brachyscome diversifolia	*Cucurbita sp.*
Brassica napus	*Cullen australasicum*

Cynoglossum suaveolens
Daucus glochidiatus
Daviesia brevifolia
Daviesia leptophylla
Daviesia ulicifolia
Daviesia ulicifolia ssp. aridicola
Daviesia ulicifolia ssp. incarnata
Dianella revoluta
Dianella revoluta var. revoluta
Dichondra repens
Dillwynia hispida
Dillwynia sericea
Diplotaxis tenuifolia
Dittrichia graveolens
Diuris sp
Divisia leptofyla
Dodonaea viscosa
Dodonaea viscosa ssp. spatulata
Dodonea viscosa
Drosera sp
Drosera whittakeri
Drosera whittakeri ssp. whittakeri
Echium plantagineum
Elymus scaber var. Scaber (Large)
Elymus scaber var. Scaber (sm)
Eremophila scoparia
Erica arborea
Erigeron glaucus
Eucalyptus baxteri
Eucalyptus camaldulensis var. camaldulensis
Eucalyptus cladocalyx
Eucalyptus cladocalyx ssp. cladocalyx
Eucalyptus cosmophylla
Eucalyptus diversifolia ssp. diversifolia
Eucalyptus fasciculosa
Eucalyptus gracilis
Eucalyptus incrassata
Eucalyptus largiflorens
Eucalyptus leptophylla
Eucalyptus leucoxylon
Eucalyptus leucoxylon ssp. leucoxylon
Eucalyptus leucoxylon ssp. megalocarpa
Eucalyptus leucoxylon ssp. pruinosa
Eucalyptus microcarpa
Eucalyptus obliqua
Eucalyptus oleosa ssp. ampliata
Eucalyptus oleosa ssp. oleosa
Eucalyptus ovata var. grandiflora
Eucalyptus ovata var. ovata
Eucalyptus phenax ssp. phenax
Eucalyptus porosa
Eucalyptus sp.
Eucalyptus viminalis ssp. cygnetensis
Eucalyptus viminalis ssp. viminalis
Eufaxia diffusa
Euphorbia terracina
Eutaxia diffusa
Eutaxia microphylla
Exocarpos cupressiformis
Fabaceae sp.
Foeniculum vulgare
Freesia sp
Fumaria capreolata
Gahnia sieberiana
Galium gaudichaudii
Geranium retrorsum
Glischrocaryon behrii
Glossodia major
Gompholobium ecostatum
Gonocarpus mezianus
Gonocarpus tetragynus
Goodenea sp.
Goodenia amplexans
Goodenia blackiana
Goodenia pinnatifida
Grevillea ilicifolia ssp. ilicifolia
Grevillea lavandulacea
Grevillea sp.
Hakea carinata
Hakea rostrata
Hakea rugosa
Halgania andromedifolia
Hardenbergia violacea
Helianthus annuus

Helichrysum leucopsideum	*Malus pumila*
Helichrysum rutidolepis	*Medicago sativa*
Helichrysum scorpioides	*Melaleuca brevifolia*
Helminthotheca echioides	*Melaleuca decussata*
Hibbertia crinita	*Melaleuca halmaturorum*
Hibbertia devitata	*Melaleuca lanceolata*
Hibbertia exutiacies	*Melaleuca uncinata*
Hibbertia riparia	*Melilotus indica*
Hibbertia sericea	*Melilotus indicus*
Hibbertia sp.	*Mentha australis*
Hibbertia glabriuscula (D.J.Whibley 9012)	*Mentha diemenica*
Hibbertia virgata	*Mentha piperita*
Hypochaeris glabra	*Mentha piperita var. citrata*
Hypochaeris radicata	*Mentha piperita var. piperita*
Hypochaeris sp.	*Microlaena stipoides var. stipoides*
Indigofera australis	*Microseris lanceolata*
Indigofera australis ssp. australis	*Mistletoe sp. (red flowers)*
Indigofera australis ssp. hesperia	*Myoporum insulare*
Indigofera australis var. australis	*Myoporum montanum*
Ixodia achillaeoides	*Myoporum parvifolium*
Juncus pallidus	*Myoporum petiolatum*
Juncus subsecundus	*Myoporum viscosum*
Kennedia prostrata	*Oenothera stricta ssp. stricta*
Kunzea pomifera	*Olea europaea ssp. europaea*
Lagenophora huegelii	*Olearia ramulosa*
Lens culinaris	*Oxalis pes-caprae*
Leptorhynchos squamatus	*Passiflora edulis*
Leptorhynchos squamatus ssp. squamatus	*Patersonia occidentalis*
Leptospermum continentale	*Persea americana*
Leptospermum coriaceum	*Persoonia juniperina*
Leptospermum lanigerum	*Philotheca angustifolia ssp. angustifolia*
Leptospermum myrsinoides	*Pisum sativum*
Leptospermum sp.	*Pittosporum angustifolium*
Leucopogon virgatus var. virgatus	*Plantago lanceolata*
Limonium lobatum	*Plantago lanceolata var. lanceolata*
Linum marginale	*Platylobium obtusangulum*
Lomandra multiflora ssp. dura	*Poa labillardieri (Large)*
Lotus australis	*Poa labillardieri (Med)*
Lucopogon sp.	*Poa labillardieri var. labillardieri*
Lycium ferocissimum	*Prostanthera behriana*
Lycopersicon esculentum	*Prunella vulgaris*
Lysiana exocarpi ssp. exocarpi	*Prunus armeniaca*

Prunus avium
Prunus cerasus
Prunus domestica
Prunus dulcis
Prunus persica var. nectarina
Prunus persica var. persica
Pultenaea acerosa
Pultenaea daphnoides
Pultenaea largiflorens
Pultenaea laxiflora
Pultenaea pedunculata
Pultenaea tenuifolia
Pultenaea trinervis
Pyrus communis
Ranunculus lappaceus
Raphanus raphanistrum
Rapistrum rugosum ssp. rugosum
Reichardia tingitana
Ricinus communis
Rosa rubiginosa
Rubus anglocandicans
Rubus rubritinctus
Salvia verbenaca var. verbenaca
Santalum acuminatum
Scabiosa atropurpurea
Scaevola albida
Schinus molle
Senecio hypoleucus
Senecio lautus
Senecio picridioides
Senecio pterophorus
Senecio quadridentatus
Senecio sp.
Senecio spanomerus
Senna artemisioides ssp. artemisioides
Sinapis sp.
Sisymbrium erysimoides
Sisymbrium irio
Sisymbrium orientale
Solanum elaeagnifolium
Solanum laciniatum
Solanum lycopersicum
Solanum nigrum
Sonchus oleraceus
Spyridium parvifolium
Spyridium thymifolium
Stackhousia monogyna
Stellaria media
Suaeda australis
Templetonia egena
Templetonia retusa
Tetratheca pilosa ssp. pilosa
Thelymitra antennifera
Thomasia petalocalyx
Thysanotus patersonia
Thysanotus patersonii
Tricoryne elatior
Trifolium campestre
Trifolium sp.
Ulex europaeus
Urospermum picroides
Velleia arguta
Velleia paradoxa
Verbascum virgatum
Vicia faba
Viola betonicifolia
Vittadinia blackii
Vittadinia cuneata
Vittadinia gracilis
Vittadinnia blackii
Vittadinnia cuneata
Wahlenbergia litticola
Wahlenbergia luteola
Wahlenbergia sp
Wahlenbergia sp.
Wahlenbergia stricta ssp. stricta
Xanthorrhoea quadrangulata
Xanthorrhoea semiplana ssp. semiplana
Xanthorrhoea semiplana
Xerochrysum bractiatum
Zieria veronicea ssp. veronicea

Table S4: Tukey's pairwise comparison statistics for means of Shannon's diversity indices for flower and bee surveys in four vegetation treatments. LCL and UCL are lower and upper 95% confidence limits.

	Flower diversity				**Bee diversity**			
contrast	Est.	LCL	UCL	P value	Est.	LCL	UCL	P value
Low Div - Cleared	0.12	-0.41	0.65	0.89	0.95	-0.65	2.55	0.32
High Div - Cleared	0.87	0.34	1.40	**<0.001**	2.49	0.88	4.09	**<0.005**
Remnant - Cleared	1.01	0.52	1.50	**<0.0005**	2.64	1.15	4.12	**<0.005**
High Div - Low Div	0.75	0.18	1.31	**<0.05**	1.53	-0.18	3.24	0.083
Remnant - Low Div	0.89	0.36	1.42	**<0.005**	1.68	0.08	3.28	**<0.05**
Remnant - High Div	0.14	-0.39	0.67	0.85	0.15	-1.45	1.75	0.99

Table S5-A. Mutualistic network metrics for Simple revegetation. CL is the Confidence Limit, Test indicates whether there is significant differences between the Observed and Null result, SES is the Standardized Effect Size for the difference between the observed and null result, NA means result Not Available and NaN indicate Not A Number. NA and NaN results occurred when a value could not be calculated.

Simple re-veg	**Observed**	**Null**	**Lower.CL**	**Upper.CL**	**Test**	**SES**
weighted nestedness	NaN	NaN	NA	NA	NA	NaN
linkage density	2.86	2.86	2.86	2.86	NA	NaN
weighted connectance	0.19	0.19	0.19	0.19	NA	NaN
interaction evenness	0.89	0.89	0.89	0.89	NA	NaN
H2	1.00	1.00	1.00	1.00	NA	NaN

Table S5-B. Mutualistic network metrics for Complex revegetation. CL is the Confidence Limit, Test indicates whether there is significant differences between the Observed and Null result, SES is the Standardized Effect Size for the difference between the observed and null result, ns means not significant, Lower means significantly less than the null model.

Complex re-veg	**Observed**	**Null**	**Lower.CL**	**Upper.CL**	**Test**	**SES**
weighted nestedness	0.58	0.63	0.58	0.68	ns	-2.06
linkage density	6.07	6.13	5.90	6.34	ns	-0.55
weighted connectance	0.08	0.09	0.08	0.09	Lower	-4.48
interaction evenness	0.93	0.93	0.93	0.94	ns	1.18
H2	0.29	0.30	0.27	0.32	ns	-0.47

Table S5-C. Mutualistic network metrics for Remnant vegetation. CL is the Confidence Limit, Test indicates whether there is significant differences between the Observed and Null result, SES is the Standardized Effect Size for the difference between the observed and null result, ns means not significant, Lower means significantly less than the null model.

Remnant	**Observed**	**Null**	**Lower.CL**	**Upper.CL**	**Test**	**SES**
weighted nestedness	0.64	0.74	0.70	0.77	Lower	-5.27
linkage density	9.81	10.57	9.91	11.17	Lower	-2.36
weighted connectance	0.08	0.09	0.09	0.10	Lower	-4.80
interaction evenness	0.95	0.96	0.95	0.96	Lower	-2.06
H2	0.30	0.32	0.28	0.36	ns	-0.82

CHAPTER 5: Concluding Remarks

Summary

Continuing investments into restoration to reverse habitat decline are providing opportunities to remedy pollinator and pollination service loss, while simultaneously ensuring the success and longevity of restoration programs. Yet, ecological or biodiversity plantings have so far not been assessed for their impact on bee diversity and pollination services. Limitations to pollination research are driving the development of new molecular methods that can identify pollen and describe pollination networks, but the quantification of pollen mixtures still remains difficult and unreliable. The results from this thesis can directly inform practitioners wishing to restore native bees and pollination services. They also indicate strong support for the protection of remnant vegetation in Australia, and can be used to further support this interest.

In this thesis, the differences in floral and bee diversity, pollination services, and pollination networks in established revegetated landscapes differing in planting design and quality were explored. The major conclusion of this project is that high diversity revegetation is needed to restore bee diversity and associated pollination services. It is likely that this revegetation practice would also support non-bee pollinators which similarly rely on high floral diversity (Garibaldi *et al.* 2014; Kremen *et al.* 2015; Rader *et al.* 2016). However, that these metrics do not recover to levels found in remnant vegetation using current revegetation methodologies, within 8 years of planting. Furthermore, the pollination services provided by native versus non-native bees differed, and native bees were more dependent on high habitat quality restoration that in turn supported higher levels of pollination services.

I additionally explored a novel molecular approach to identify pollen, and demonstrated that hybrid capture DNA metabarcoding with high throughput sequencing can successfully be used to identify and quantify relative proportions of pollen within artificial mixtures. The strength of hybrid capture lies in the ability to target multiple genomic regions, potentially utilising more informative loci without prior knowledge about the target taxa. However, there were some limitations to the method, and I was unable to apply it in its entirety to quantify pollination networks. Database quality and choice had a large effect on pollen metabarcoding result accuracy. When pollen identity was not in question the relative abundances of pollen found were quite accurate, but as it stands there was too much ambiguity in the ID of unknown samples to effectively quantify them. However, with appropriate localised site information from floral surveys and local species lists, it was possible to customise the reference databases to the point where it was still possible to generate genus level

ID at minimum, and thus still proved a useful tool for reconstructing pollination networks. Pollination networks explored using this pollen metabarcoding method, within the same revegetation sites, supported the diversity and pollination service results. In low diversity revegetation sites, networks were small and lacked resilience. In the smaller high diversity revegetation sites, the robustness and complexity of the networks was similar to those in remnant vegetation, suggesting these sites could be more resilient to future stressors.

The findings support previous research that emphasizes the crucial role of high floral diversity in supporting bee diversity and pollination services (Morandin and Kremen 2013; Garibaldi *et al.* 2014; Rader *et al.* 2016). The positive relationship between floral diversity and bee abundance and diversity has been well-documented in numerous studies (Morandin and Kremen 2013; Blaauw et al. 2014; Kremen et al. 2015). The differences observed in pollination services provided by native versus non-native bees corroborate previous research highlighting the unique contributions of native pollinators to plant reproductive success (Garibaldi *et al.* 2013). Furthermore, the limitations and challenges encountered in pollen metabarcoding reflect the need for further research to enhance the accuracy and reliability of these molecular methods (Richardson *et al.* 2015; Bell *et al.* 2021; Bell *et al.* 2022).

Project limitations

This project faced some challenges which limited data size, and aspects of the metabarcoding, however, this lends opportunities for future studies.

Of most notable difficulty was managing the consequences of the extreme weather conditions preceding and during this study, which made sampling difficult (Australian Bureau of Meteorology 2019, 2020, 2021). Extreme heat, low rainfall, and a drought leading up to the sampling years, likely led to a shortage of floral resources, which led to smaller bee populations and small sample sizes, and limited the opportunities for species interactions. It is possible that in other years, such as the very wet La Niña seasons directly following this study (2021-2023), the patterns found could vary, and thus these patterns could be reflective of dryer conditions. The extreme heat that occurred in South Australia very early in season 1 (November 2018) also severely impacted the pollination experiment. The experiment was planned to coincide with the bee surveys across both field seasons, however, in the first year there was close to 50% plant mortality due to the heat, after only one week in the field. In the second season, a watering system was employed to keep the plants continually hydrated in the field, since twice weekly watering in the first season was insufficient. The new field set up was successful, and the plants survived at much higher rates for approximately two months in the field. Unfortunately, it meant that for chapter 2, although two seasons of floral and bee survey data were available, one data season was used, to allow comparison between pollination services and diversity. Future projects could

aim to extend the sampling performed in this study area and could reveal if the results found are specific to dry conditions, or if they are general trends.

To increase sample sizes, many other studies use additional trapping methods such as pan traps, to supplement catch numbers. However, I opted not to use that method because it is becoming increasingly clear that traps are more attractive to bees when there are fewer floral resources in the landscape, and that they are very biased in the taxa they attract (Prendergast and Hogendoorn 2021). Furthermore, the pollen collected by the bees was required for chapter 3, so other trapping methods were not appropriate as they would have introduced contamination.

Some statistical challenges were encountered due to the small sample numbers on a per site basis. A common but unsound practice when reconstructing pollination networks is to merge data across sampling replicates. This is often necessary to generate statistically sound network metrics, but it introduces forbidden links. Due to phenological and spatial mismatches, some links between bees and plants cannot exist. An unrealised link that cannot exist (forbidden link, e.g. bee present but plant not flowering) is fundamentally different from an unrealised link that can exist (i.e. bee and flowering plant present, but bee does not visit plant). Therefore, the analyses of combined networks can lead to conclusions about the network structure (lots of unrealised links) that are unwarranted. To avoid this problem, I attempted to calculate network metrics individually for each site and compare these between treatments. However, there was not enough data per site for robust network calculations. Instead, used the econullnetr R package where forbidden links can be specified (Vaughan *et al.* 2017). The package calculates network metrics for both the null and observed network, and an iterative approach is used to estimate the probability that observed metrics are different from the null model. This approach is not used often, but can lead to a more accurate understanding of the structure and function of ecological networks, and is strongly recommended.

Future direction

Bee restoration success and thus pollination services may have been influenced by a number of other factors not tested here, and this opens up interesting avenues for future research. The Island Biogeography Theory states that the number of species colonising an island (in this case revegetation site) is determined by the immigration and extinction of species, which in turn depend on the distance to the source population (in this case remnant land), and the size of the island (revegetated land) (MacArthur and Wilson 2009). This means that, everything else being equal, the closer the revegetation is to remnant land, and the larger the area planted, the more species will colonise it through targeted dispersal, and by random chance. Hence, with time and larger plantings, species diversity could continue to increase. The role of time, the

area revegetated and the landscape context, in the restoration of the bee fauna are therefore interesting and important avenues for future study.

In this study, the effect of time since restoration was not considered, which could have had an impact on the findings since some of the revegetation sites were relatively young. Researching the effect of time since restoration on bee diversity and pollination services is therefore an important extension to fully understand restoration potential.

Testing the effect that the revegetated area has on bee restoration could involve exploring the effects in relation to bee size and habitat requirements of the species. Large, and specialist bees often require a larger area to meet their needs. However, exploring the effect of size is possibly more challenging, as it would require larger replicated sites to be established, and these may be rare, and are usually beyond the control of researchers.

There are also vast gaps to explore in the context of the hybrid capture metabarcoding of pollen. This project touched on the application of a new molecular method, but much more research is needed to reliably analyse unknown pollen mixtures. For example, expanding the number of taxa used in pollen mixtures, may allow us to understand whether other taxa behave in the same way, in terms of detection and quantification, and identify the occurrence and possible causes of biases. An interesting approach could be to use different species from the same families, and/or to add these additional species to the mix, although this may be made difficult by pollen availability issues in Australia. There are no companies selling research grade pollen within Australia, and it is against customs rules to import it from overseas companies. In relation to the bioinformatic approach, there is still no applicable method to combine multiple barcodes in a single analysis. While the RefSeq chloroplast library produced better results than a single *matK* barcode library, there were far fewer plastid sequences available compared with single barcode sequences, and missing certain taxa in the database could lead to issues with downstream quantification. Conversely, when the taxa present were known and the database restricted to just those present, the *matK* barcode library resulted in relatively accurate and highly correlated sequence proportions compared with input pollen. The issue of incomplete DNA reference libraries heavily impedes many studies relying on plant and invertebrate identification, and is an avenue of research well deserving of attention and funding. However, there is often good taxon coverage across individual barcodes, and the development of a method to combine information from multiple barcodes would greatly advance this avenue of research. Nevertheless, identifying pollen in a large number of unknown species is likely to remain a mathematically and computationally challenging endeavour.

Management implications

Bee diversity increases with floral diversity, and more florally diverse, complex revegetation achieved levels of both floral and bee diversity on par with remnant vegetation. In contrast, simple revegetation was not different to cleared sites regarding floral diversity, and pollination networks in this revegetation type were small. Pollination networks were largest and most complex in remnant vegetation, even when similar levels of floral diversity were achieved in complex revegetation. These networks were also the most robust, and most likely to withstand future stressors. Therefore, to safeguard existing plant-bee relationships it is vitally important to protect and appropriately manage remnant vegetation, and this should be a high priority for land management practitioners, and is in line with previous findings (Olynyk *et al.* 2021). To restore healthy and robust pollination networks, and thus functionally stable ecosystems, complex and florally diverse revegetation is necessary. However, the data in this study show that complex revegetation only partly restores the network attributes. Notably, the bee assemblies differed between remnant habitat and areas which had undergone diverse revegetation, and the latter specifically lacked a number of specialist bees. There are some species such as *Eucalyptus* and native pea species, which may be slow growing, however they are particularly important to include. Resources could be focused on plant diversity designed to restore ecosystem functionality.

This work provides important insight into how current revegetation methods restore bee pollination networks. In addition, it provides evidence that may guide pollinator targeted restoration, which is of interest to the agricultural industry, especially with the recent outbreak of the *Varroa destructor* mite in 2022, the largest in Australia to date. This work is also particularly important in light of extreme climatic conditions experienced in Australia in recent years, and which are predicted to worsen in the future. Extreme heat and drought were encountered during this project, and sadly, shortly after the completion of fieldwork, bushfires in 2020 burned some of the revegetation sites studied. This is a stark reminder of the many other threats bees and their habitats face. Aside from human land use, climate change, heatwaves and drought seriously threaten native bee populations. To ensure diverse bee communities survive in the future, multi-faceted management is necessary, and with the continuing damage environments face, evidence-based restoration practices will become ever-more important.

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