Foraging specialization of flower visitors in a grassland community: insights from metabarcoding versus traditional methods

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ABSTRACT

Ecological networks of plant-pollinator interactions at the community level were traditionally studied using natural history-type direct observations as well as microscope-based palynology. Over the last decade, these methods have been complemented by emerging molecular technologies such as metabarcoding of pollen loads on insects which holds the promise to reveal interactions that would be difficult to observe directly. We compared traditional methods of studying plant-pollinator interactions (observational data, palynology) with metabarcoding data to assess the degree of complementarity of the different approaches and whether the molecular-based approach revealed previously unknown interactions. This was done for a community on Mount Gilboa, KwaZulu-Natal, South Africa. In an initial exploratory study, I demonstrated successful amplification of DNA from fuchsin-fixed pollen which opens the possibility of using this method of pollen barcoding from specific sites on pollinator bodies, including fragile archival specimen. My results showed that networks constructed from metabarcoding data were more complex with a higher resolution of ecological interactions than those obtained using the traditional methods of microscopy of pollen-loads and visitor records. A complete community approach combining both the nocturnal and diurnal components showed a complex network comprising 66 pollinator species and 172 plant species, corresponding to a network with low modularity, high nestedness and high linkage density, and which is likely to be ecologically robust owing to high generality within the network. However, because many of the plants and animals in the community have not yet been barcoded and were not available in the DNA barcode reference library, it was difficult to obtain species-specific names for 72% of the putative plant species and 45% of the putative animal species in these networks. These were assigned to the lowest taxonomic level possible after cross referencing against known occurrence records. Nocturnal pollination networks revealed a 2.5-fold increase in interaction diversity, and 4.5-fold increase in pollinator generality when using metabarcoding data in comparison to conventional microscopy. Hyper diverse countries such as South Africa, however, still require significant resources to build comprehensive reference libraries allowing for sequence data to accurately assign species names. I conclude that the greater resolution and throughput obtained through metabarcoding can increase our understanding of complex ecological interactions and networks.

PREFACE

The data described in this thesis were collected in 2020-2021 in the Republic of South Africa. Experimental work was carried out while registered at the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Steven D. Johnson and Prof. Sandi Willows-Munro.

This thesis, submitted for the degree of Philosophy of Science in the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, School of Life Sciences, Pietermaritzburg campus, represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is duly acknowledged in the text.

Melanie B. Streicher

Febraury 2024

I certify that the above statement is correct and as the candidate's supervisor I have approved this thesis for submission.

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Prof Steven D. Johnson

Supervisor

February 2024

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DECLARATION 1 - PLAGIARISM

I, Melanie B. Streicher, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Melanie B. Streicher
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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis.

Publication 1

M. B. Streicher, S. D. Johnson & S. Willows-Munro.

Effect of fuchsin-fixation of pollen on DNA barcode recovery

Published

Author contributions:

MBS conceived paper with SWM and SDJ. MBS collected and analyzed data, and wrote the paper. SWM and SDJ contributed valuable comments to the manuscript.

Publication 2

M. B. Streicher, S. Willows-Munro & S. D. Johnson

How to hitchhike through time: comparing pollen placement on archival and contemporary hawkmoths

Author contributions:

MBS conceived paper with SDJ and SWM. MBS collected and analyzed data, and wrote the paper. SWM and SDJ contributed valuable comments to the manuscript.

Publication 3

M. B. Streicher, S. Willows-Munro & S. D. Johnson

Hawkmoth pollination networks: Increased resolution using metabarcoding versus traditional palynology

Author contributions:

MBS conceived paper with SDJ and SWM. MBS collected and analyzed data, and wrote the paper. SWM and SDJ contributed valuable comments to the manuscript.

Publication 4

M. B. Streicher, S. Willows-Munro & S. D. Johnson

A plant-pollinator network based on metabarcoding of pollen loads of flower visitors in a South African biodiversity hotspot

Author contributions:

MBS conceived paper with SDJ and SWM. MBS collected and analyzed data, and wrote the paper. SWM and SDJ contributed valuable comments to the manuscript.

Signed: .....

Melanie Streicher

February 2024

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Psalm 19

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Of the insect pollinators included in this study, all carried pollen detectable by metabarcoding methodology. I recorded 675 mutualistic interactions occurring between 66 pollinator species and 172 flowering plant species. The most prolific flower visitor was *Precis Octavia* (Nymphalidae, Lepidoptera) which visited 49 plant species, while *Lampides sp.*, (Lycaenidae, Lepidoptera) only reflected one plant species in its pollen load. The three most generalist pollinator species, and three top plant 'hub' species are given in Table 5.1. It is worth noting that pollinators can appear particularly generalist because of the pooling of individuals. This caveat can artificially increase host ranges, yet at the species level, is it still possible to determine host range. Additionally, *Precis octavia* was a top generalist with 49 floral links, yet only 4 individuals were pooled. In contrast *Apis mellifera*, a well-known generalist, was linked

CHAPTER 1

INTRODUCTION

1.1 Background and problem statement

Like many other aspects of ecosystem functioning, pollination systems have been brought to the forefront of research due to a multitude of threats facing them (Vanbergen 2013; Nicholson & Egan 2020). Pollination systems have been intensively researched because of the value they provide to agriculture and ecosystem health (Southwick & Southwick Jr 1992; Chagnon *et al.* 2015; Potts *et al.* 2016). Despite depending on the pollinator communities for their service, it is the increase in agricultural practices that has directly caused the loss of high-quality natural habitats necessary to pollinators, as well as deteriorated the semi-natural land and communities adjacent to farmlands (Benton, Vickery & Wilson 2003; Bongaarts 2019). The current estimate is that more than 20% of plant species are facing an extinction risk (Brummitt *et al.* 2015). Species extinction is thought to be directly proportional to habitat loss, and because of the exacerbated anthropogenic change in natural landscapes, this has become the signature conservation crisis of the 21st century (Millennium Ecosystem Assessment 2005; Barnosky *et al.* 2011; He & Hubbell 2011). The alarming rate of loss of species and the unpredictability of extinction patterns, in combination with the decrease of ecological services rendered (Valiente-Banuet *et al.* 2015), has resulted in a proliferation of research on species interactions.

1.2 Species-centred (flagship and umbrella) vs. community/ecosystem (interactions) conservation

Conservation research is a natural parallel to the burgeoning research on taxonomy, biodiversity and the effect changing environments had on the natural world. However, with the intensification of research into the rate of species' loss as human activity impacted on

extinction rates, the urgency and scope of conservation has increased exponentially. Because species interact with a cohort of other species in often complex networks, whole system studies require a high level of multidisciplinary expertise. Yet, for simplicity-sake, research has needed to 'ignore' these intertwined systems and instead had to focus on single species as the threat arose (Tylianakis *et al.* 2010). The use of few, focal species to concentrate conservation efforts on became a popular model in that many of the, sometimes unnecessary, delays and expenses were bypassed in favour of expediency. The definition for "flagship" and "umbrella" species arose and these were useful for conservation in that efforts could be streamlined. Where a surrogate species was used for conservation, public support could additionally be garnered when this was a charismatic species, such as China's giant pandas. Public perception and financial support can be lobbied where an emotional link to a species can be invoked. Although there is debate about the ultimate efficacy of these "flagship" species (Li & Pimm 2016), it is generally believed that conservation goals are not being adequately met. The applicability of implementing these definitions also differs between terrestrial and aquatic environments (Kalinkat *et al.* 2017). For example, the critical landscape that was deemed sufficient for giant pandas did not cover the critical resources that are required for sympatric species that were intended to be under the blanket protection of the initiative (Li & Pimm 2016; Wang *et al.* 2021). The stipulations for an umbrella species, on the contrary, are to exhibit complex spatiotemporal behaviour, as well as to occupy a large and diverse range which encompasses much co-occurring diversity. These umbrella species, however, are often uncharismatic species in inaccessible habitats which makes studying them cumbersome, time-consuming, and expensive (Kalinkat *et al.* 2017).

Studies of species-specific interactions have time and cost limitations that prevent a comprehensive analysis of entire systems. Conservation has therefore gradually progressed to an ecosystem function, community-level focus, from previously being species-centric

(Memmott, Waser & Price 2004; Heleno, Devoto & Pocock 2012; King, Ballantyne & Willmer 2013). This has been an almost complete paradigm shift from focussing on single species, to protecting the intrinsic value of an ecosystem and especially those functions which benefit humans. Yet, community-wide research could be at risk of being oversimplistic, or too reduced to adequately assess the ecological importance of biological interactions. There is inherent variability within natural communities and simply assigning a 'presence' or 'absence' of a particular interaction when the strength of such links cannot be assessed, is a downfall (Kay & Schemske 2004).

Pollination ecology too has relied chiefly on single clade analyses (Tylianakis *et al.* 2010). There are inherent complications with focussing on a single taxon when a wider community analysis approach is necessary, as conservation goals are often conflicting. As an example: the eradication of one invasive species may directly threaten the survival of a native species through the removal of an essential resource (Lampert *et al.* 2014). Or, conservation may even require more drastic measures of 'sacrificing' one native species for a more threatened species (Courchamp *et al.* 2011). Cascading trophic effects are also an unintended and catastrophic effect when only simplistic trophic interactions are considered (Dexter *et al.* 2013). Single-clade studies also heavily rely on time and taxonomic expertise (Douglas *et al.* 2012) which are major impediments preventing rapid analysis and thus progress in this field.

Advances in identifying plant taxa using genetic methods (such as DNA barcoding) have made it possible to address these problems in an alternative manner, saving time and costs. 'Plant-pollinator networks' are a popular approach to studying plant-pollinator communities (Blüthgen 2010). Genomic techniques, like barcoding and metabarcoding, have been integrated into plant-pollinator research, and are suggested as additional or alternative methods to observational data and microscopy-based pollen analysis. High sequence similarity thresholds (96%-99%) are generally appropriate for taxonomic assignment of animals where the

mitochondrial cytochrome oxidase subunit I barcode has proven to be highly successful (Bucklin *et al.* 2021). Lower limits are sometimes tolerated for more specific markers (85%–96%) (Govender *et al.* 2022; Bonin, Guerrieri & Ficetola 2023; Westerduin *et al.* 2023), yet selecting the most appropriate threshold for taxonomic assignment requires careful examination given the research aims. For plants though, where species boundaries are inherently less easily and well defined in comparison to animals (Fazekas *et al.* 2009) many barcodes of varying success rate have been designed (Cheng *et al.* 2016), yet extensive *post-hoc* refinement of species identities is usually required. This downfall is further compounded by the lack of a comprehensive and well-curated plant barcode database (23%; Cheng *et al.* 2016), in which southern Africa's endemic plant diversity is particularly poorly represented (Hoveka *et al.* 2020). Beyond several quantitative descriptive statistics (Tylianakis *et al.* 2010) being developed to compare between communities and ascribe a meaningful 'health' value to them, networks have also become a focal method for analysing resilience within a system. Yet pollinator abundance does not perfectly correlate to pollination success (Sahli & Conner 2006), nor does a single flower visitor guarantee that pollination occurs. Caution needs to therefore be taken when constructing networks based on a visitation metric as these links may not be those representing true pollination events (Memmott, Waser & Price 2004).

1.3 Tools for monitoring biodiversity

Observational – palynology – barcoding – metabarcoding

Both plants and pollinators need accurate identification– another time-consuming activity requiring a panel of different specialists. Pollen loads from pollinators can be studied microscopically and this is informative, but this method is also riddled with taxonomic and time difficulties. Pollen loads taken from pollinators are often of mixed origin. As pollinators can visit multiple plant taxa in a single day, they may thus carry pollen from a number of

different plant species. Because of the cumbersome task of sorting through this information, pollen loads are seldom used for community-wise analyses and are an under-tapped resource for studying plant-pollinator network interactions.

The ability to use genetic methods which result in faster identification of pollen origins allows interactions to be studied without intensive sampling and without the necessity for pollen morphology expertise. Although there are yet several pitfalls to metabarcoding, such as the inability to precisely correlate sequence count to biomass (Bell *et al.* 2019), barcoding and metabarcoding hold much promise for biodiversity assessment and surveillance. DNA barcoding is the process of identifying an organism based on a standardized short segment of DNA, and has been used successfully since the early 2000's to taxonomically identify animals and plants (Hebert *et al.* 2003). This process has advanced considerably over the years and is now used in conjunction with high-throughput sequencing (HTS). Mixed origin samples destined for HTS are typically taken from the environment and consist of many different types of organisms that cannot be separated out easily (Hajibabaei *et al.* 2011). Metabarcoding emerged as a result of developing the HTS process: multiple specimens found in one sample could now be sequenced simultaneously (Shokralla *et al.* 2012). Metabarcoding could be used for a wide variety of purposes; not only in the field of taxonomy but also in other research fields such as ecology (Valentini, Pompanon & Taberlet 2009). Assisted by new sequencing technologies and ever improving bioinformatic tools, DNA metabarcoding has proven to be an invaluable addition to the research field of ecology. As reference libraries are 'curated' and methodologies improved, simplified, and economised, this method will continue to be integrated in a growing number of ecological studies with greater reliability and reproducibility (Taberlet *et al.* 2012; Deiner *et al.* 2017).

Due to chiefly time and expertise limitations mentioned previously, conventional plant-pollinator network analyses have been coupled with genetic approaches- specifically

metabarcoding. Metabarcoding does however, rely on a comprehensive reference library. Unfortunately, barcode reference libraries for plant and animal taxa in hyper diverse developing countries such as South Africa are typically incomplete, meaning that traditional methods of identification (Cheng *et al.* 2016), in conjunction with *post-hoc* verification, are still required to be used in conjunction with barcoding. Additionally, metabarcoding data is typically heavily 'cleaned' and reduced before assigning an interaction such that a species identity is not assigned by a single sequence, and many potential incidental interactions are removed. Thus, many false positives can be eliminated from the resultant network. A further complication is the current inability to adequately quantify biomass using metabarcoding. Sequence counts have been proposed as a means to semi-quantify relative abundance in mixed samples (Lamb *et al.* 2019; Ershova *et al.* 2021). Despite being highly contested (Bell *et al.* 2019), new methodologies have increased quantification power of metabarcoding, and further advancements are likely to continue this trend as the field advances (Bell *et al.* 2021; Stapleton *et al.* 2022; Shelton *et al.* 2023).

Host-plant range, specialization and generalisation

Host range, or realised host range, is typically described as the array of plant species available and acceptable as hosts, despite other potential and suitable species existing contemporarily (Bernays & Chapman 1994). For plant-pollinator interactions this concept can be applied to the set of plants available for foraging on (pollinators), and for pollination service (plants). Flowering plant communities are dynamic systems with ever-changing combinations of flowering species over time and between seasons and years (CaraDonna, Iler & Inouye 2014). The pollinating insects of such communities then need to match and track this fluctuating environment. The oscillation hypothesis has been offered as a theory to explain the plasticity present in host-plant ranges (Janz & Nylin 2008).

As new methodologies and evidence have emerged, the pervasively held view that pollinating systems tend toward specialization is being replaced by the more widely accepted tenet that generalization at the level of pollinator species, but not necessarily higher-level pollinator functional groups, is in fact the norm (Waser *et al.* 1996; Brosi 2016). However, certain plant families, such as the Orchidaceae and Iridaceae, have more specialized pollination systems than do other families (Johnson & Steiner 2003; Ackerman *et al.* 2023). The plasticity in diet breadth is seen not only in pollinators more often foraging on a diverse cohort of host plants and rarely being specialized on a single plant species, but also in that diet breadth is highly labile across temporal and geographical scale (Brosi 2016). In a generalized system, the range of plants visited will include an array of alternative host plants which may be suboptimal for either the plant or the pollinator, yet this flexibility can facilitate speciation if the inclusion of a new plant species into the typical range of hosts is important enough for increasing fitness of either partner, which would then be underpinned by genetic changes. This is a likely mechanism where environmental change, or colonization has occurred (Janz & Nylin 2008). Despite possible local specialization being an outcome of such a case, species generally retain wide host ranges. In an ecological context, diversity can be maintained by broad niche-widths of generalists foraging in such 'oscillating' behaviours (Janz & Nylin 2008). Species coexistence, and thus biodiversity, is facilitated by ecological specialization (niche breadth) and complementarity (niche differentiation) (Levine & HilleRisLambers 2009; Phillips *et al.* 2020). Palynology and metabarcoding are both methods appropriate for studying attributes of plant-pollinator systems (Macgregor *et al.* 2019). Metabarcoding, however, has been purported to reveal more complex and biodiverse networks with hidden interactions being revealed (Baksay *et al.* 2022; Encinas-Viso *et al.* 2022; Lowe *et al.* 2022). With its increased resolution and ability to simultaneously identify species in mixed samples, metabarcoding provides the ability to understand fine-scale ecological interactions which was previously not possible

(Lowe *et al.* 2022). I will be using this method to investigate plant-pollinating networks in a South African context. This research provides an opportunity to improve the understanding of fine-scale ecological interactions, as well as contribute to current perceptions of the generalization-specialization spectrum.

The level of generalization in the foraging behaviour of pollinators (also termed diet breadth or host range) is a key aspect of pollinator ecology (Smith 2019; Endres *et al.* 2021) and is particularly difficult to estimate for nocturnal species. Because pollen grains can remain attached to floral visitors for long periods of time, analyses of pollen loads can provide insight into floral host ranges of species which are logistically awkward to study (Bosch *et al.* 2009). Pollen identification using light microscopy has several caveats. Expertise and a thorough knowledge of the flowering community is a primary requirement for identification. Even with expertise and a well-curated reference collection, pollen identification can still be difficult which can lead to reduced discriminatory power at lower taxonomic levels (Devoto, Bailey & Memmott 2011; Galimberti *et al.* 2014). Using the Sphingidae model of nocturnal pollination in a South African grassland ecosystem, I test for the sensitivity of metabarcoding in comparison to traditional pollen analysis.

Value of archival specimens

Archival and museum specimens hold immense value and are repositories of an abundance of ecological and genetic information (Arbeláez-Cortés *et al.* 2017) beyond being a preserved collection of rare and interesting natural specimens intended for public viewing. Well-curated museum collections contain spatial and temporal data which can be used to infer patterns of diversity, and how these may have responded to environmental changes over time. This type of data is particularly useful for public health and safety in identifying emergent diseases and tracking the history of infectious diseases, identifying sources and reservoirs (Suarez & Tsutsui

2004) and for biodiversity studies to track changes in species diversity and community structure over time (Suarez & Tsutsui 2004; Tsai *et al.* 2020). For ecological purposes, a well-curated library of specimens with the accurate sample details can readily be used to reconstruct plant and insect species' distributions, range limits and expansions and diet breadths. Using again the pollen loads of Sphingidae, as a model of nocturnal pollination within a hyper diverse grassland ecosystem, I analyzed nocturnal interactions using pollen load which are likely to remain undetected by observation data alone. Diet breadths of hawkmoths using both archival and newly collected hawkmoth specimens are included in this sample set, and differences in pollen composition by anatomical placement (Morales & Traveset 2008; Muchhala & Thomson 2012) are also investigated.

Appropriate and best preparation and storage of specimens has changed over time (Baars 2010), with a trend to use less toxic and harmful preservatives. For molecular approaches to studying natural systems, methods of gathering and storing biological material can influence the success of downstream analyses (Dillon, Austin & Bartowsky 1996). Both the quantity and quality of template DNA and therefore the resultant PCR efficiency, can be decreased by substandard methods of collection and storage. Early-stage PCR misincorporations, resulting in less reliable final sequences, are more likely to occur when template DNA is low grade (low concentration, possible contamination, damaged/degraded DNA) (Dillon, Austin & Bartowsky 1996; Casbon *et al.* 2011)**.** Yet success with extracting genetic material from museum tissue has had success (Ivanova & Kuzmina 2013; Gous *et al.* 2019). Killing method (ethyl acetate, cyanide and freezing) and post-mortem storage of insect specimens has been shown not to hazard downstream DNA recovery for insect taxa (Willows-Munro & Schoeman 2015). Fuchsin jelly is routinely used on disposable glass slides as a mounting medium to view pollen loads of insects (Jia *et al.* 2021). These mini-pollen libraries are usually discarded. The non-toxicity of fuchsin dye and the characteristics of the robust pollen exine raises the possibility

that pollen collected and embedded in fuchsin jelly may also be suitable for later DNA analysis. The utility of pollen embedded in fuchsin jelly has not yet been considered for downstream molecular applications, nor has it been tested.

The loss of network-partners

Stable interactions between plants and their insect partners are labile and fluctuate within and between seasons, and even over shorter time spans. These changes are linked to flowering phenology (Olesen *et al.* 2008), habitat disturbance (Aizen, Morales & Morales 2008) and environmental variability (Petchey, Brose & Rall 2010) leading to an inherently dynamic system. Although the interactions comprising the network are highly labile, the overall topological features of the network across years are maintained by properties such as functional redundancy and complementarity (Dupont *et al.* 2009; Bramon Mora *et al.* 2020). Despite the plastic nature of network connections through 'rewiring' of network connections, a major concern is the phenological mismatches which could occur as a result of long-term climate change (Burkle & Alarcón 2011). This temporal stability of networks is predicted to erode with the increased frequency and intensity of climate-change associated disturbances (Alarcón, Waser & Ollerton 2008). The global pollinator crisis describing widespread population declines is well documented. Yet there is concern over the large-scale loss of biodiversity and ecosystem functions as complex networks, despite their potential resilience, are pushed beyond the limits of their flexibility. Species extinctions undermine ecosystem functioning, which can eventually lead to a collapse of the system. Several methods of detecting the resilience of networks have been proposed and debated. I used the network index, robustness, to model the sensitivity, and simulate the potential effect of species loss within the system. Robustness, R, estimates the proportion of species in a trophic level that remains in a network after sequential extinctions of species in the other trophic levels. It assumes that a species goes

extinct when all partners to whom it is connected are lost. R ranges from 0 to 1, with values closer to 1 indicating higher robustness of the system, meaning that coextinction rates are slower (Burgos et al., 2007).

1.4 South Africa (Mt. Gilboa, Karkloof) as a case study

South Africa is well-known for its biodiversity, with three Conservation International global hotspots declared within its borders; namely the Succulent Karoo, the Cape Floristic Province (Mittermeier *et al.* 1998) and the Maputaland-Pondoland-Albany region (Steenkamp *et al.* 2004). Complex ecosystems are present across the country, and various different taxa have become interdependent on one another for survival. Pollination is an important ecosystem service provided to humans by animals from these ecosystems and contributes directly to roughly a third of the food we consume (Klein *et al.* 2009).

How South Africa has come to harbour such a vast array of floral forms and species has long held the focus of the botanical community and has also been intensely researched. The evolution of angiosperm lineages has been studied and several theories have been offered to account for the sheer diversity. Landscape topography and physical isolation leading to genetic isolation may cause speciation (Cowling, Procheş & Partridge 2009). The theory predicts that a lineage distributed over a topographically complex habitat should diversify at a faster rate than a sister lineage located in an unvarying landscape (Schnitzler *et al.* 2011). Edaphic heterogeneity has also been offered as an explanation for diversification of lineages in close proximity (Linder 2003). Yet, the pollinator-driven ecological speciation model is currently a favoured theory in explaining the high phenotypic and species diversity found in South African (Cowling & Pressey 2001; van der Niet & Johnson 2009; Johnson 2010; Valente *et al.* 2012). South African ecosystems are unique, and the evolution of such assemblages is most often

attributed to long standing and shifting associations between the plant community and their pollinators.

Sustainable provision of the functional role pollinators fulfil in their ecosystem requires a diverse array of pollinators throughout the seasons (Duchenne *et al.* 2020). Seasonal shifts in pollinator services tend to promote generalist associations between pollinators and plants and thus plants have often evolved to be highly plastic and host a selection/cohort of potential pollinators (Noreika *et al.* 2019). The close-fitting relationship between pollinator morphology and floral features resulting in coevolution of the lineages, rather than generalization, is the typically accepted as the exception in pollination systems (Bosch *et al.* 2009), despite the natural communities then are sustained by a diverse mixture of plants and their pollinators, which fall somewhere along the generalist-specialist spectrum, rather than being solely specialist or generalist (Fontaine, Collin & Dajoz 2008).

Pollination research has chiefly studied plant-pollinator communities by analysing a cohort of descriptive statistics which seeks to understand the resilience of the system (Tylianakis *et al.* 2010). Quantifying communities in order to assess the effect of the loss of a pollinator, or plant, or the resources either of these offers, is achieved through several statistics (connectivity, redundancy, modularity, nestedness etc.). Although applying and incorporating these indicator statistics into practical decision making across systems is not well understood. Despite the obvious need to understand fully the complex relationships within a pollination community, studies which examine the sensitivity of natural systems to changes in pollinator assemblages are still lacking.

Mount Gilboa (Karkloof, South Africa) Nature Reserve falls within the Maputaland-Pondoland-Albany Hotspot (MPA), an internationally recognised region of floristic diversity and endemism of southern Africa. The Gilboa Estate contains ca. 1500 ha of natural grassland on sloping terrain, classified as three vegetation types (Mooi River Highland Grassland,

Midlands Mistbelt Grassland, and Drakensberg Foothill Moist Grassland), all have been assigned high conservation value (Mucina & Rutherford 2006). The natural grassland is interspersed with forest patches (indigenous and commercial eucalypt plantations). Many individual plant-pollinator interactions have been researched and described (Johnson 2000; Hargreaves, Johnson & Nol 2004; Peter & Johnson 2008; Stanley, Msweli & Johnson 2020), making Mt. Gilboa an ideal study system for a broader community-type analysis. This site is a remarkably diverse grassland community, where many plant-pollinator interactions have been studied. For example, *Wahlenbergia cuspidata* (Campanulaceae) flower in scattered clumps on the summit and are primarily visited solitary bee *Lipotriches* sp. (Halictidae) and social bee *Apis mellifera* (Apidae) (Welsford & Johnson 2012). Mount Gilboa is home to a Protea community where three species (*P. roupelliae*, *P. caffra* , and *P. simplex*) flower. *Protea roupelliae* (Proteaceae) is largely dependent on birds for pollination, and thus conforms to an "ornithophilous" floral syndrome, although beetles *Atrichelaphinus tigrina* and *Trichostetha fasiciculari* (Scarabaeidae: Cetoniinae) may also be incidental pollinators (Hargreaves, Johnson & Nol 2004). The community comprises several orchid species, some of which rely on deceptive pollination systems (Johnson 2000; Peter & Johnson 2008; Jersáková *et al.* 2012). Long-proboscid fly (Nemestrinidae) (Johnson *et al.* 2002; Jersakova & Johnson 2006; Valentin, Lunau & Johnson 2006) and nocturnal moth pollination of *Satyrium* (Johnson *et al.* 2011; Castaneda-Zarate, Johnson & van der Niet 2022), and hawkmoth pollination of *Zaluzianskya* and *Gladiolus* (Iridaceae) represent other key pollination system on Mt. Gilboa (Alexandersson & Johnson 2002).

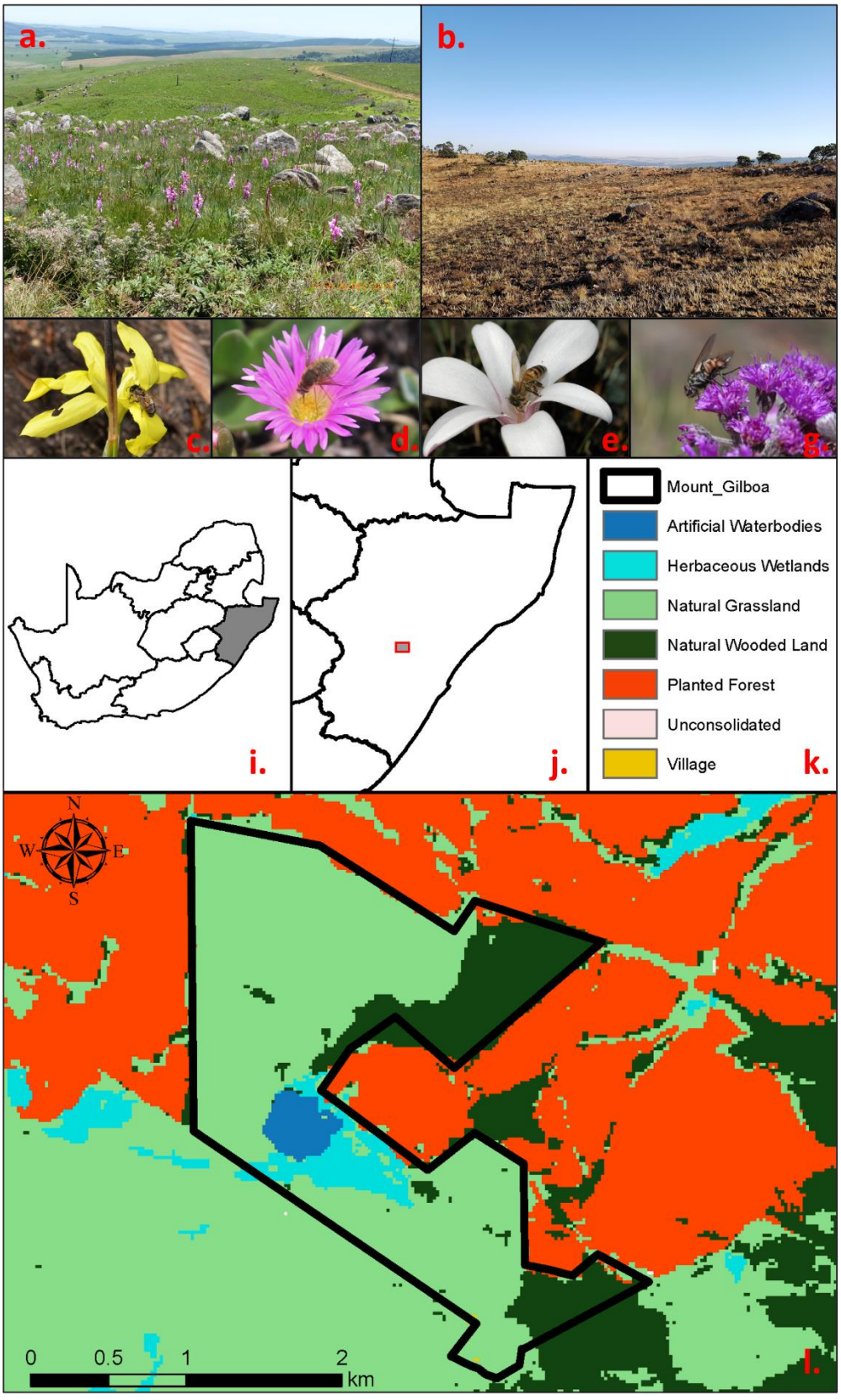

FIGURE 1.1 Mount Gilboa study site showing typical wet (a) and dry (b) seasons, typical plant-pollinator interactions: *Apis mellifera* on *Moraea hiemalis* (c), Bombyliid sp. on *Delosperma sutherlandii* d) *Apis mellifera* on *Apodolirion buchananii* (e), and Diptera sp. on *Hilliardiella* sp. (f), locality and landcover types (i-l).

1.5 Overarching aim of study

The PhD study seeks to further the field of pollination ecology by shedding fresh light on long-standing ideas in pollination research by investigating an entire pollinating system rather than a species-specific interaction emblematic of the system. With the recent advancements in genetic analysis, greater amounts of information can be gathered in a shorter period of time which can be analyzed in a laboratory. I aim to supplement traditional methods (palynological, observational data) of studying pollination systems with the use of genetic data. The overarching question I aim to answer is whether genetic data can reveal pollination interactions which were not detected using the conventional methods. Creating a blueprint for future studies will allow for further comprehensive research and thus an improvement in our current understanding of pollination systems. By employing this method, I aimed to answer questions on the level of specialization of taxa. I subjected pollen loads collected off pollinators to a comprehensive metabarcode analysis to identify which plants are visited by insect groups. The data gathered can also address questions on pollinator host ranges, and the susceptibility of the network to species loss (Ellner et al. 2020). I also provide commentary on the utility of the current DNA barcode reference library to identify sequence data to species.

Can metabarcoding of pollen loads on insects reveal 'hidden' plant-pollinator interactions that were not detected by conventional network analysis? This project assessed the applicability of using metabarcoding to understand pollination networks more accurately. This metabarcoding data provides a direct link to the plants being visited by pollinators, may reveal unknown functional interactions, and provide information which cannot be detected by traditional methods of camera-trapping, direct observation, or microscopic analysis of pollen loads. Specifically, genetic material will be extracted from pollinators and the pollen gathered off pollinators, purified and analyzed. The research site was Mt. Gilboa, Karkloof, KwaZulu-Natal. Mt. Gilboa is useful as a case study to test the hypotheses as it is a well-researched study

site, and barcoding of the flowering plant species and pollinating insects has potential to extend the currently known plant-pollinator interactions.

1.6 Aims & objectives

Aims

Ecological networks are currently a popular practice for estimating ecosystem resilience to climate change and anthropogenic disturbance. The power of this approach lies is using data from a wide variety of sources. For pollination biology studies, input data may include direct observation of pollinator-plant interactions, pollination network analyses, palynology, and DNA barcoding. Barcoding, and metabarcoding more so, is extremely effective and valuable in simple systems where there is thorough, often taxonomic, foundational knowledge. However, in highly biodiverse systems both traditional methods and now metabarcoding can incur greater uncertainty where baseline data of the system is still lacking. Nevertheless, biomonitoring of ecological networks can be hugely improved by incorporating a metabarcoding approach. In this study I will determine the feasibility of using this approach for South African ecosystems generally, by reviewing data available for Mt. Gilboa, Karkloof, KwaZulu-Natal. This case study was chosen as much research has been conducted there and can use this to determine what sources of data should be prioritized for future research in other regions of South Africa. I include DNA barcode data, pollen morphology data, observational data and network data. The genetic sequences generated in this work will contribute to the record database repository which forms the foundation for future studies on patterns of diversity. The combination of both taxonomic and genetic approaches provides greater clarity and resolution when differentiating at the species level. I aimed to incorporate genetic data into the network-building approach, using the data to derive ecologically meaningful metrics

(specifically pertaining to pollinator diet-breadth), and to assess the ease at which it can be applied in a South African context.

Objectives

The objectives of this project focusing on the pollinator community on Mt. Gilboa, Karkloof, South Africa were as follows:

- To determine whether staining of pollen with fuchsin dye and embedding in gel on a glass slide intended for microscopy, had an effect on the quality of the subsequently extracted genetic barcodes.
- To determine whether hawkmoth pollen load is segregated by anatomical position, and whether there is a difference (in total abundance, pollen species composition and derived network parameters) between contemporary and archival samples.
- To assess the difference between two methods- lights microscopy and metabarcoding- of analyzing pollen load of hawkmoths on Mt. Gilboa, Karkloof.
- To analyze, describe and provide a complete overview of plant-pollinator networks found on Mt. Gilboa (Karkloof, KwaZulu-Natal, South Africa) using metabarcoding data.

1.7 Structure of the thesis

This thesis is structured with a brief introduction, and data chapters that are prepared and formatted for submission (some are already published or in review) to international peer-reviewed journals. Some repetition was, therefore, unavoidable. The chapters are as follows:

Chapter 1: Introduction

Chapter 2: Effect of fuchsin-fixation of pollen on DNA barcode recovery

Chapter 3: How to hitchhike through time- comparing pollen placement on archival and contemporary hawkmoths.

Chapter 4: Hawkmoth host ranges: metabarcoding vs. palynology

Chapter 5: Diurnal pollination networks on Mt. Gilboa, Karkloof

Chapter 6: Conclusion

1.8 References

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CHAPTER 2

Effect of fuchsin-fixation of pollen on DNA barcode recovery

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2.1 Abstract

1. Pollen grains attached to insects are a valuable source of ecological information which can be used to reconstruct visitation networks. Morphological pollen identification relies on light microscopy with pollen usually stained and mounted in fuchsin jelly, which is also used to remove pollen from the bodies of insects. Pollen embedded in fuchsin jelly could potentially be used for DNA barcoding and metabarcoding (large-scale taxonomic identification of complex mixed samples) and thus provide additional information for pollination networks.

2. In this study we determine whether fuchsin-embedded pollen can be used for downstream molecular applications. We evaluate the quality of plant barcode (ITS) sequences amplified from DNA extracted from both fresh (untreated) pollen, and pollen which had been embedded in fuchsin jelly.

3. We show that the addition of fuchsin to DNA extraction does not impact DNA barcode sequence quality during short-term storage. DNA extractions from both untreated and fuchsin-treated pollen produced reliable barcode sequences of high quality.

4. Our findings suggest that pollen which has been collected, stained, and embedded in fuchsin jelly for preliminary microscopy work can be used within several days for downstream genetic analysis, though the quality of DNA from pollen stored in fuchsin jelly for extended periods is yet to be established.

Keywords: fuchsin, pollen identification, barcoding, ITS.

2.2 Introduction

Pollination networks were traditionally studied and constructed by direct observation of interactions (Dupont, Padrón, Olesen & Petanidou 2009; King, Ballantyne & Willmer 2013) or by tracking the transferred pollen-load using palynological techniques (Wood & Roberts 2018; Arceo-Gómez *et al.* 2020). The latter methodology is time-consuming and relies on well-curated and comprehensive pollen reference libraries (Elliott & Jonathan Davies 2014; Kraaijeveld *et al.* 2015). The scale of such research is thus often limited. Although the input costs are presently still high, DNA barcoding and metabarcoding is currently an emerging method for rapid species identification involving plant-pollinator interactions (Keller *et al.* 2015; Arstingstall *et al.* 2021).

The success of DNA-based methods depends on many factors including the gathering, processing and storing of specimens (Dillon, Austin & Bartowsky 1996; Zimmermann *et al.* 2008; Willows-Munro & Schoeman 2015). Metabarcoding methods are also sensitive to contamination and have poor quantitative power (Lamb *et al.* 2019); the taxa amplified depend on primer specificity, data may include sequencing errors, and data may incur false positives or negatives (Bell *et al.* 2017; Cuff *et al.* 2022). It is therefore desirable to use a combination of traditional morphology-based and newer molecular-based methods in order to characterise

network structural properties (Bosch, Martín González, Rodrigo & Navarro 2009; Pornon, Andalo, Burrus & Escaravage 2017).

Barcoding and metabarcoding have the advantage of discriminating pollen of closely-related species, which is seldom possible using light microscopy alone (Sickel *et al.* 2015). Several barcode primers can be 'pooled' to amplify an array of target DNA (e.g., allowing for simultaneous barcoding of pollinator and pollen) at a comparatively minimal increase in cost. It is, however, possible for 'rare' taxa to be underrepresented in molecular data, depending on the chosen markers and quantity of pollen grains (Bell *et al.* 2016a). It is also possible for errors to be incorporated during DNA replication which, once amplified, could significantly distort ecological interpretations (Bandelt, Lahermo, Richards & Macaulay 2001; Kanagawa 2003). False positives and negatives are a concern too (Farrell, Whitmore & Duffy 2021). These inaccuracies are especially problematic in forensic fields (Bandelt, Lahermo, Richards & Macaulay 2001). For ecological studies in regions where a comprehensive barcode reference library linking DNA sequence data to taxonomically identified specimens exists, potential contamination, misidentifications and other sequencing errors can be ruled out. Additionally, both field- and laboratory practices and protocols need to be standardized to eliminate errors arising from inter-personal handling techniques (Raclariu, Heinrich, Ichim & de Boer 2018; Farrell, Whitmore & Duffy 2021) and to allow for the integration of results from different studies. In the case of pollen, DNA extraction methods are destructive, with original samples not available for cross-referencing at a later stage (Bell *et al.* 2016a).

When using traditional microscopy methods, the integrity of the pollinator and pollen remains intact for museum storage and future palynological analyses. Analyzing the pollen-loads by light microscopy and precise, targeted swabbing, allows one to make inferences about specialization and pollen placement (Walton, Sayer, Bennion & Axmacher 2020). This typically involves the use of dye prior to being viewed by light microscopy (Wodehouse 1929;

Beattie 1971; Jia *et al.* 2021). Fixing pollen to slides with fuchsin jelly (Beattie 1971) has become a routine and popular protocol for removing pollen from insects and then visualizing pollen exine morphology (Jia *et al.* 2021). Preparing semi-permanent fuchsin pollen mounts is a cost-effective and non-pathogenic for human use when phenol is eliminated (Umroong 2021). Fuchsin, when used as an exine-specific dye for staining, attaches to the pollen coat which comprises sporopollenin- a chemically inert and robust biopolymer (Mackenzie *et al.* 2015; Jia *et al.* 2021). Although soluble in strong oxidizing agents which renders stored DNA susceptible to damage, the pollen coat does not disintegrate when in contact with other organic and inorganic acids and bases (Southworth 1974).

For molecular approaches to studying natural systems, methods of gathering and storing biological material can influence the success of downstream analyses (Dillon, Austin & Bartowsky 1996). Both the quantity and quality of extracted DNA and thus the PCR efficiency, can be decreased by substandard methods of collection and storage. Early-stage PCR misincorporations, resulting in less reliable final sequences, are more likely to occur when template DNA is of poor quality (low concentration, possible contamination, damaged/degraded DNA) (Dillon, Austin & Bartowsky 1996; Casbon, Osborne, Brenner & Lichtenstein 2011)**.** Contrary to previous assumptions, killing method (ethyl acetate, cyanide and freezing) and post-mortem storage of insect specimens has been shown not to hazard downstream DNA recovery for insect taxa (Willows-Munro & Schoeman 2015). This raises the possibility that pollen collected and embedded in fuchsin jelly may also be suitable for DNA analysis. However, pollen embedded in fuchsin jelly has not previously been considered for downstream molecular applications, nor has it been tested.

Fuchsin jelly contains several ingredients: glycerine; gelatine; crystalline basic fuchsin stain, and sometimes crystalline phenol, which may have an effect on genetic material (Massie & Zimm 1965) and may inhibit downstream PCR-based amplification. Fuchsin staining has

been shown to inhibit PCR DNA amplification only minimally in histological samples (Murase, Inagaki & Eimoto 2000). However, the heating, melting, and setting of the fuchsin jelly may also affect the stability of the pollen-derived DNA.

Depending on the scope of the research, a combination of morphology and molecular methods has the potential to be a powerful diagnostic tool (Sarwar & Takahashi 2014; Laha *et al.* 2017; Leontidou *et al.* 2021; Li *et al.* 2021). The aim of this study was to determine whether pollen can be extracted and amplified successfully after being embedded in fuchsin jelly. Signal strength and sequence quality values were used to evaluate DNA barcode amplification success. Phylogenetic analyses were used to test the reliability of the sequence data recovered.

2.3 Materials and methods

Plant species were collected from the summit at Gilboa Estate (MONDI Forests Ltd) (29° 19′S, 30° 17′E) in the Karkloof mountain range of the KwaZulu–Natal Midlands of South Africa. Five plant species were gathered after the first rains of the 2021 flowering season: *Apodolirion buchanii* Baker (Amaryllidaceae); *Dimorphotheca jucunda* E. Philips (Asteraceae); *Senecio speciosus* Wiild (Asteraceae); *Hypoxis angustifolia* Lam. (Hypoxidaceae), and *Moraea modesta* Killick (Iridaceae). Five flowering individuals were separately bagged and collected from each of the species. Two pollen samples were collected per individual. One sample (referred to as 'untreated') was used directly for DNA extractions. The second sample ('treated') was embedded in fuchsin jelly. Each pollen sample comprised a single anther. In the case of the Asteraceae species, an entire disc floret was removed first and then an anther removed under a dissecting microscope. The single anthers were removed using forceps and placed into individual 1.5-ml plastic Eppendorf microcentrifuge tubes. The anther for the fuchsin-embedded treatment was transferred to a glass slide and embedded in fuchsin jelly (~1 mm3, weight = 18-20 mg) by heating on a heat-block set to 55°C for ~1 minute or until just melted.

With the exception of the omission of crystalline phenol, fuchsin jelly was made following (Beattie 1971). This involves a simple procedure of mixing 150 ml glycerine with 50 g gelatine dissolved in 175 ml distilled water and then adding basic fuchsin crystals until the desired colour is attained. A cover slip was placed over the fuchsin-embedded pollen and left for 48 hours at room temperature, away from direct light.

The fuchsin-embedded pollen was viewed at 40× magnification under a compound microscope to confirm its presence, and then scraped off the glass slide and returned to the Eppendorf microcentrifuge tubes. Extraction of DNA from both pollen treatments was done using the ZYMO Quick-DNA™ Plant/Seed Miniprep Kit (Zymo Research Group, California, USA) with minor modifications suitable to the sample tissue/material: The BashingBead^{TM} Buffer was pipetted directly into the microcentrifuge tubes containing the pollen sample, and not a ZR BashingBeadTM Lysis Tube (2.0 mm). Each pollen sample was crushed with a micropipette tip and allowed to settle for ~10 minutes before rinsing the pipette tip off with the buffer and removing it. The microcentrifuge tube was vortexed briefly, and the subsequent steps in the protocol were followed without amendments. Eluted DNA was stored at -20°C.

Concentrations of the eluted DNA samples were calculated by measuring the absorbance of the sample at 260/280 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, US). Samples were then couriered to a commercial company (Genomyx Laboratories, Johannesburg) for amplification and Sanger sequencing. The plant ITS region was amplified using the primer pair ITSAB101F (5'-ACGAATTCATGGTCCGGTGAAGTGTTCG-3') and 26SE (5'-TAGAATTCCCCGGTTCGCTCGCCGTTAC-3') (Sun, Skinner, Liang & Hulbert 1994). Specimen data and DNA sequences were uploaded into BOLD under the project "Fuchsin-embedded pollen" (FFFCC) (Table S2.1).

Sequences were also Blasted against NCBI (National Centre for Biotechnology Information) GenBank to authenticate and quality-check the data. Forward and reverse fragments were combined to create high-quality consensus sequences. Sequence quality was assessed from trace files using Phred quality scores (Kearse et al. 2012), sequence percentage quality (Kearse et al. 2012), and signal strength (relative fluorescence units; RFU). A Phred score measures the probability that a nucleotide has been correctly labelled. A Phred score of 30 indicates that a base has a 1 in 1000 chance of being incorrect. Phred values fall on a scale ranging from one to 60, with one being highly improbable and 60 indicating the highest accuracy possible (Ewing & Green 1998; Ewing, Hillier, Wendl & Green 1998). Although not applied in all sequencing studies, a Phred score of 40 is required as the minimum threshold for human clinical samples and sequencing (Al Naiemi, Schipper, Duim & Bart 2006). Sequence chromatograms are categorised in Geneious Prime 2022.1 (https://www.geneious.com) by the quality scores assigned to each base (Kearse et al. 2012). Percentage quality identifies the percentage of bases in a consensus sequence which falls into each of the categories. Here sequences were binned into "high", "medium" and "low" set at the default parameter settings. Relative fluorescence values (RFU) increase in proportion to the DNA fragment amplification success. The data were analyzed with a generalized linear mixed model (GLMM) in SPSS 28 (IBM). Phred and RFU scores were analyzed using models with a normal distribution and an identity link function. Plant specimen was treated as a random effect, while species, process (forward vs reverse sequencing), treatment (fuchsin embedded or not) and their interactions were treated as fixed effects. For both Phred scores and RFU values, the final model was determined by the Akaike Information Criterion (AIC). Model degrees of freedom were adjusted using the Kenward-Roger method (Kenward & Roger 1997).

To determine if fuchsin-fixation led to increased levels of nucleotide misincorporation during PCR amplification, the ITS sequences were also used in phylogenetic analyses.

Consensus sequences were aligned using MUSCLE (Edgar 2004) and then the alignment was manually optimised in Bioedit (Hall, Biosciences & Carlsbad 2011) to ensure homology. Hypervariable sections of the final alignment that were difficult to align were removed prior to analyses. Hypervariable regions of the ITS are known to include high rates of homoplasy, which may decrease the accuracy of the reconstructed tree topologies (Barta, Jenkins & Danforth 1991; Barta *et al.* 1997; Ogden & Rosenberg 2006; Dress *et al.* 2008). Maximum likelihood (ML) analyses were performed in Garli version 0.95 (Zwickl 2006). The best-fit model of nucleotide substitution (TIM1 + G) was selected using the AIC in JMODELTEST2 (Darriba, Taboada, Doallo & Posada 2012). For the assessment of nodal support, 1000 bootstrap iterations were performed, and these values were annotated onto the most likely phylogeny using FIGTREE v1.3.10 (Rambaut 2009). Average uncorrected genetic distances were calculated within and among plant species using MEGA version 11.0.10 (Tamura *et al.* 2011).

2.4 Results

The best-supported GLMM model including all possible interactions showed that fuchsin treatment had no effect on Phred scores ($F = 1.36$, $p = 0.26$). Extracted DNA from both treatments produced sequence trace files with high Phred quality scores (Q-values, Figure 2.1). Overall Phred scores for untreated (Q-score = 43.6, Figure 2.1) and fuchsin-treated pollen DNA (Q-score = 40.4, Figure 2.1) are beyond the minimum threshold and were not significantly different. Reverse primer amplification resulted in lower quality and a greater accumulation of erroneous base calls for both treatments across all categories ($F = 35.6$, $p < 0.01$, Figure 2.1). There was a significant effect of species on amplification success as detected by Phred scores ($F = 5.27$, $p < 0.01$). Likewise, RFU value was not significantly different between the treatments ($F = 0.38$, $p = 0.57$, Figure 2.2). Forward primer amplification resulted in higher

RFU scores than reverse amplification ($F = 12.25$, $p < 0.01$). There was no effect of fuchsin on base-call quality (Figure 2.3). A minimally but non-significant improvement was found in the percentages of high-quality base calls between the two treatments (Figure 2.3).

The resulting ML topology was robust. There was no shifting of the taxa, and species replicates grouped together reliably regardless of their pre-extraction treatment, and the monophyly of species was well supported (ML bootstrap > 70, Figure 2.4). On average, the uncorrected genetic distances for the fuchsin-treated among species samples ranged from 19.7% to 41.7%, which is similar to the untreated pollen genetic distances (16.5% - 41.3%) (Table 2.1). Within-species sequence divergence was ≤ 1.6% (Table 2.1). Sequence divergence, irrespective of the effect of fuchsin, was sufficient to separate out individuals belonging to the same species.

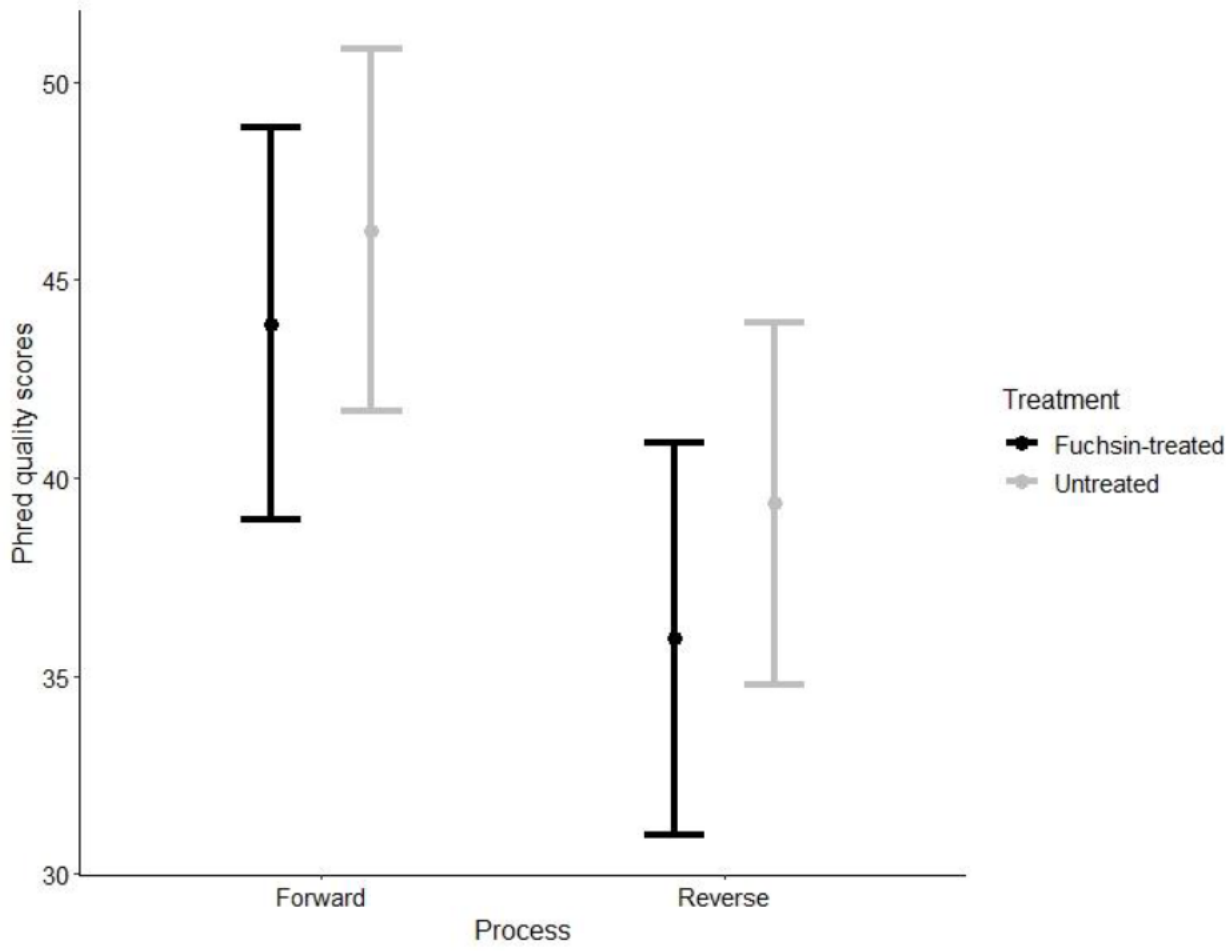

FIGURE 2.1 Estimated marginal means with 95% confidence intervals of Phred quality scores showing no significant difference ($p > 0.05$) between sequences generated using forward and reverse primers from specimens untreated and treated (fuchsin-embedded) pollen.

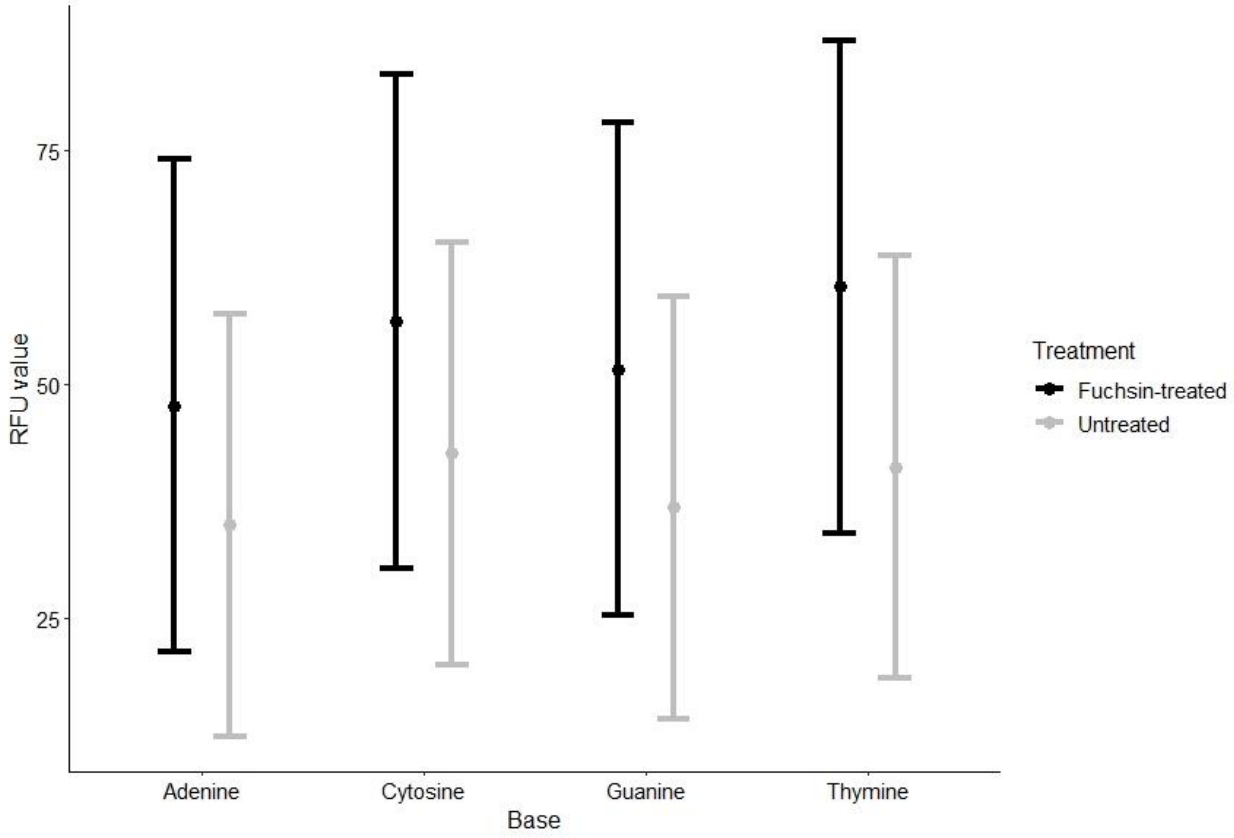

FIGURE 2.2 Estimated marginal means of relative fluorescence unit (RFU) values for each nucleotide for both sequences generated using forward and reverse primers with 95% confidence intervals.

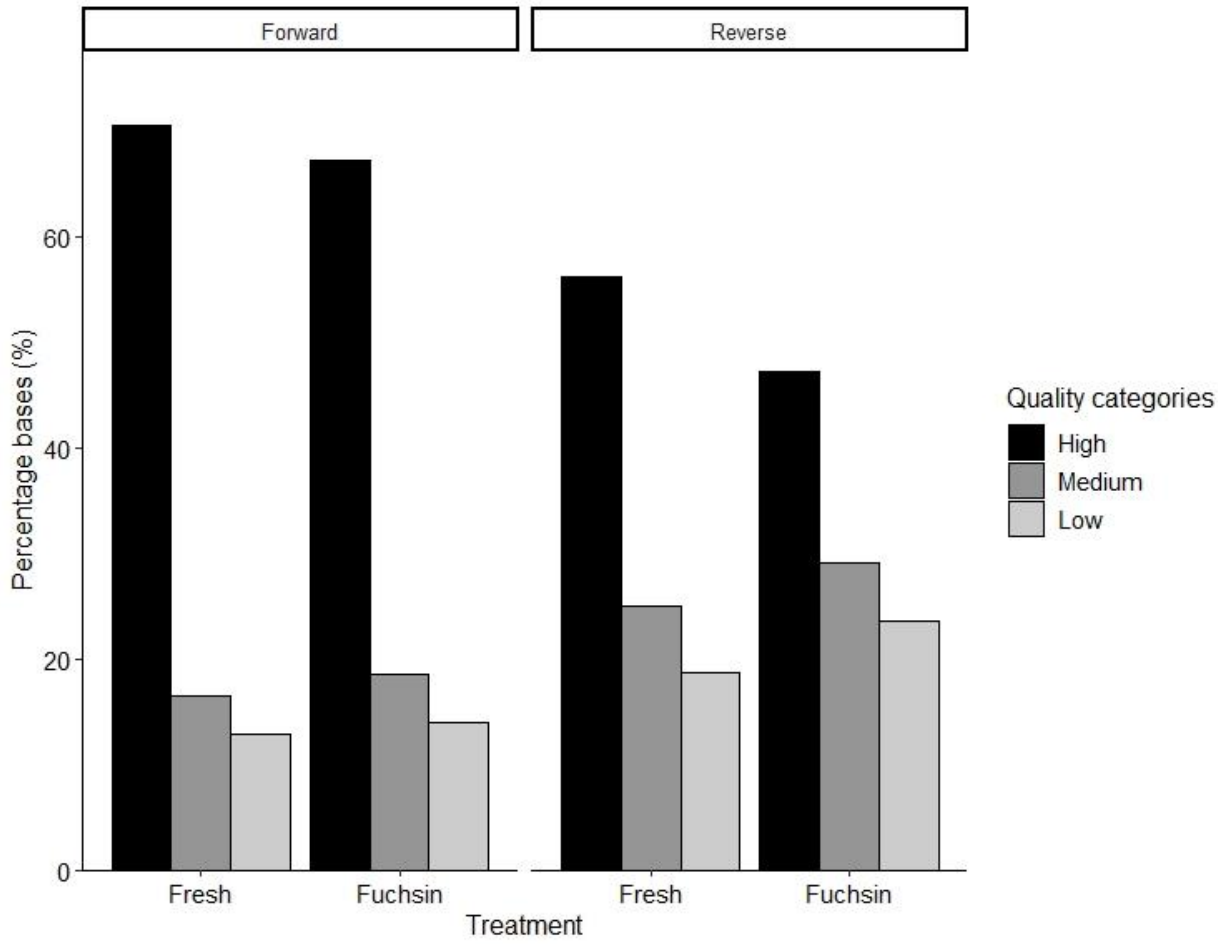

FIGURE 2.3 Mean percentage of base call quality, separated by category (high, medium, low) and treatment.

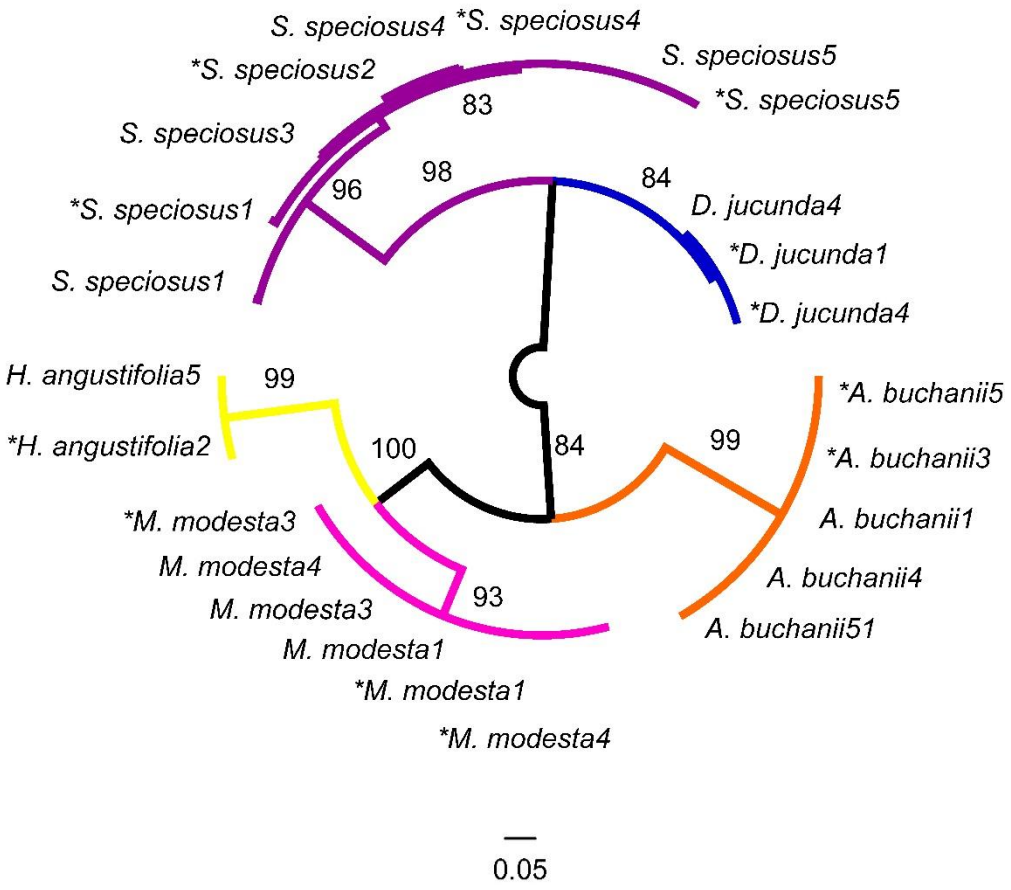

FIGURE 2.4 Mid-point rooted maximum likelihood phylogeny from DNA barcode (ITS) sequences amplified from 24 individuals belonging to 5 species. Bootstrap values are shown on branches. Pollen DNA was extracted from untreated, or after being embedded in fuchsin-treated jelly (marked with an asterisk).

TABLE 2.1. Uncorrected pairwise sequence distances among the species included in this study. Fuchsin-treated samples are below the diagonal, values from untreated pollen are above the diagonal.

	S. speciosus	*A. buchanii*	*D. jacunda*	*M. modesta*	*H. angustifolia*
S. speciosus	**0.016**	0.431	0.192	0.339	0.325
A. buchanii	0.417	**0.000**	0.386	0.241	0.293
D. jacunda	0.197	0.376	**0.000**	0.305	0.307
M. modesta	0.339	0.233	0.303	**0.000**	0.165
H. angustifolia	0.372	0.341	0.371	0.197	**n/c**

average within species pairwise sequence distances for fuchsin-treated pollen are given in bold on the diagonal.

2.5 Discussion

Pollen is an invaluable source of data in the fields of pollination ecology, forensics, invasive species management and others (Bell *et al.* 2016b). An initial step in the process often relies on staining the unique exine structure of pollen retrieved off of target objects (e.g. pollinators, crime scene objects) in order to visualize it. However, the effect of fuchsin-dye on the extraction success of pollen DNA has not yet been assessed. Here, we extracted DNA from untreated pollen and pollen which had been stained with fuchsin to determine if pollen used previously for light microscopy could be used in downstream molecular analyses.

Extracted DNA from both untreated and fuchsin-treated pollen produced DNA sequences of high quality, as seen by both Phred quality scores and RFU values, which did not statistically differ between the treatments. The data from both treatments also produced robust phylogenies, allowing for the reliable identification, and grouping of species. This indicates that the data are accurate (Ewing & Green 1998; Ewing, Hillier, Wendl & Green 1998). Increased DNA damage is associated with increased rates of nucleotide misincorporations during PCR amplification which artificially inflates sequence divergence and impacts the branching pattern seen in phylogenetic trees (Ogden & Rosenberg 2006; Dress *et al.* 2008). We did not find evidence of this in the fuchsin-treated samples. Our results indicate that DNA quality is not significantly

reduced when extracted from pollen embedded on slides with fuchsin jelly, at least not over short-term storage (up to 48h). We did extract DNA from samples that were embedded in fuchsin for over a year (14 months). Absorbance measurement readings for these older fuchsin samples (260/280 absorbance ratio 1.04 - 2.07) were similar to that recovered from the samples that were left in fuchsin jelly for only 48h (260/280 absorbance ratios between 1.6 and 1.8). We tested neither for an effect between the size of the pollen grain and the quality of amplified DNA barcodes, nor was it possible, in this study, to confirm a long-term temporal effect of fuchsin-embedded pollen on the recovery of DNA barcodes. These would be valuable avenues of further investigation, as both the size of the pollen grain and the duration of contact with fuchsin might contribute to diminishing the quality of recovered DNA.

Data accessibility:

Data will be made publicly available on Genbank via BOLD at http://dx.doi.org/10.5883/DS-FFFCC00.

Supplementary data

Table S2.1. Details of samples used in this study Stored at NU Herbarium, University of KwaZulu-Natal

Species name	Family	Geographic locality	Sample tissue	Voucher type	Sample name	BOLD specimen number
Dimorphotheca jucunda	Asteraceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B2.1FP	FFFCC022-22
Moraea modesta	Iridaceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A1.1RP	FFFCC001-22
Apodolirion buchananii	Amaryllidaceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A6.5.1RP	FFFCC017-22
Senecio speciosus	Asteraceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A3.4RP	FFFCC008-22
Senecio speciosus	Asteraceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B3.5FP	FFFCC028-22
Apodolirion buchananii	Amaryllidaceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B6.3FP	FFFCC031-22
Senecio speciosus	Asteraceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A3.3RP	FFFCC007-22
Moraea modesta	Iridaceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B1.1FP	FFFCC018-22
Moraea modesta	Iridaceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B1.3FP	FFFCC019-22
Senecio speciosus	Asteraceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B3.1FP	FFFCC025-22
Senecio speciosus	Asteraceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B3.2FP	FFFCC026-22
Moraea modesta	Iridaceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A1.4RP	FFFCC003-22
Senecio speciosus	Asteraceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B3.4FP	FFFCC027-22
Hypoxis angustifolia	Asparagales	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A4.5RP	FFFCC012-22
Moraea modesta	Iridaceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A1.3RP	FFFCC002-22
Apodolirion buchananii	Amaryllidaceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B6.5FP	FFFCC033-22
Senecio speciosus	Asteraceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A3.1RP	FFFCC006-22
Apodolirion buchananii	Amaryllidaceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A6.4RP	FFFCC015-22
Senecio speciosus	Asteraceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A3.5RP	FFFCC009-22
Moraea modesta	Iridaceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B1.4FP	FFFCC020-22
Dimorphotheca jucunda	Asteraceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B2.4FP	FFFCC023-22
Dimorphotheca jucunda	Asteraceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A2.4RP	FFFCC005-22
Hypoxis angustifolia	Asparagales	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	B4.2FP	FFFCC029-22
Apodolirion buchananii	Amaryllidaceae	South Africa, KwaZulu-Natal, Mt. Gilboa	pollen	Entire sample	A6.1RP	FFFCC013-22

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CHAPTER 3

How to hitchhike through time: comparing pollen placement on archival and contemporary hawkmoths

3.1 Abstract

1. Pollen grains attached to insects are a valuable source of ecological information which can be used to reconstruct visitation networks. Using palynology techniques, I reconstructed the nocturnal pollen-transfer network for a community of hawkmoths on Mt. Gilboa, South Africa.
2. In this study I determine whether the nocturnal pollen-transfer network reveals interactions undetected by observation data. I analyze the pollen loads of both contemporary samples and archival samples to detect whether pollen is lost off pollinators in storage. I also separate out pollen loads by anatomical position to determine whether pollen species composition and abundance varies. Several indices of network structure are calculated to compare between contemporary and archival data on plant-pollinator interactions.
3. Our results suggest that nocturnal pollen-loads reveal more complex interaction and pollen-transfer networks than observations alone. In particular, they show that hawkmoths regularly visit *Protea* and *Lobelia* flowers that do not conform to the moth pollination syndrome. Contemporary samples with minimal handling hold greater species composition and pollen quantity than archival specimens. Anatomical position of pollen attachment is a key determinant of pollen species composition and total pollen grain abundance with the highest species composition and pollen load retention being

held on the probscis. Despite this, the overall 'retrospective' network structure and calculated indices was not severely impacted by the loss of pollen grains in archival specimens.

4. I show that analyzing the pollen load on hawkmoths, both in quantity and species composition adds valuable information that is absent from networks created by observation alone. From these data I can infer that the nocturnal network represents a significant component of pollen transfer in this community. Network reconstructions revealing predominant interactions can still be reliably created from archival specimens.

Keywords: pollen placement, nocturnal pollination networks, palynology, pollen library, hawkmoth.

3.2 Introduction

Archival and museum specimens hold immense value and are repositories of information (Arbeláez-Cortés *et al.* 2017) beyond being a preserved collection of rare and interesting natural specimens intended for public viewing. Spatial and temporal data to infer patterns of species occurrence and diversity, and how these change through time, are contained in well-curated museum collections. This type of data is particularly useful for public health and safety in identifying emergent diseases and tracking the history of infectious diseases, identifying sources and reservoirs (Suarez & Tsutsui 2004) and for biodiversity studies to track changes in species diversity and community structure over time (Suarez & Tsutsui 2004; Tsai *et al.* 2020). With accurate sample metadata, species distributions, range limits and expansions, can also be reliably reconstructed. Without meticulously curated collections of insects agricultural pest control strategies would incur far greater financial costs with reduced efficacy (Suarez &

Tsutsui 2004). With recent advances museum collections have also found added value in being a source of genetic material which makes possible the study of evolution through time (Payne & Sorenson 2002; Holmes *et al.* 2016). Likewise, collections of pollinators can be windows into past community interactions and assemblages by analyzing pollen-load to retrieve data detailing which flowering plants were visited during foraging bouts.

The value of studying pollen loads retrospectively

Analyzing the pollen loads carried by pollinators provides insight into plant-pollinator interactions within in a community. Conservation efforts have recently recognized the importance of conserving species' interactions, as healthy ecosystem functioning depends more on interactions and entire networks rather than single species' contributions (Tylianakis *et al.* 2010). For this end goal of conservation, applying a network-based viewpoint is a powerful tool for informing and directing management (Heleno *et al.* 2010; Tylianakis *et al.* 2010; Devoto *et al.* 2012). The global pollinator decline crisis in the context of climate change has resulted in increased focus being placed on network-level approaches (van der Sluijs 2020; Dicks *et al.* 2021). Tracking pollen movement and its vectors is valuable in conservation efforts targeting the spread of invasive plants (Mathiasson & Rehan 2020) and pathogen transmission (Figueroa *et al.* 2020). While plant-centered analyses find their benefits in increasing the likelihood of detecting novel interactions (Vianna, da Luz & de Matos Peixoto Kleinert 2014), pollinator pollen-load data can provide a more comprehensive overview of community network structure in comparison to a flower-visitor based approach (Jędrzejewska-Szmek & Zych 2013). Additionally, using a pollinator-focused approach often reveals rare interactions which are overlooked in a plant-centric approach, especially for short-term studies (Vianna, da Luz & de Matos Peixoto Kleinert 2014).

Pollinator networks are also known to be highly labile. In contrast to the prevailing perspective, several studies have shown that networks are more dynamic than previously thought (Petanidou *et al.* 2008; Burkle & Alarcón 2011; CaraDonna 2016). Temporal variation in pollinator interactions has been detected across years, seasons and even weeks (CaraDonna 2016). Natural fluctuations in pollinator species abundance and community composition necessitates that network interactions are not temporally static but are able to dis- and reconnect to balance the plasticity (Petanidou *et al.* 2008; CaraDonna 2016; Hahn & Brühl 2016). The flexibility in network interactions is supported and evidenced by the majority of plant species being generalist in their pollinator preferences, rather than specialized (Waser *et al.* 1996; Johnson & Steiner 2000). Plants tend to partner with pollinators who fulfil a functional role, rather than with a single or few specific (and precise) pollinator species, thus networks tend to arrange themselves in a way that facilitates species persistence (Bascompte *et al.* 2003). Environmental perturbations therefore should have less effect on a broadly connected network than a highly specialized network. However, the idea that the majority of plants tend toward being pollinator non-specific is controversial in that it may not hold true for communities found outside of cool temperate ecosystems (Johnson & Steiner 2003). For pollinating community located in southern hemisphere, prevailing network structure could differ vastly. Thus, being able to detect these temporal fluctuations in pollinator networks across spatial scales would be highly prized in conservation efforts.

In contrast to the timed observational and transect studies which suffer a standardization pitfall, a potentially simpler alternative study would be to collect pollen directly off of pollinators. Pollen grains can remain attached to active pollinators over large distances (Garcia Bulle Bueno *et al.* 2022). In identifying pollen grains, it is possible to capture and recreate a more complete and quantitative view of the interacting partners with arguably more biologically useful information (Bosch *et al.* 2009). Using pollen load data, however, requires

the creation and curation of a comprehensive pollen library which, due to the added effort, may not be initially a time-saving solution when compared to observational studies (Elle, Elwell & Gielens 2012). Additionally, plant species identification by light microscopy is limited by the inability to distinguish accurately beyond genus level.

Anatomical pollen attachment

Plants have diversified to attach pollen to specific positions on their pollinators in a way that minimizes wastage (Armbruster *et al.* 2009). This heterospecific pollen deposition is known to be prevalent in species-rich communities, particularly in systems where pollen is limited and thus competition for pollen-transfer occurs (Armbruster, Shi & Huang 2014). In combination with other ecological mechanisms, reproductive barriers are purported to be maintained by the physical segregation of pollen placement on pollinators (Minnaar *et al.* 2019; Moreira-Hernández & Muchhala 2019). Thus, analyzing the pollen species communities carried on these different anatomical positions could provide support of speciation between closely related sister taxa.

Several anatomical placements have been documented for the transfer of pollen by hawkmoths (Sphingidae). Pollinia have been recorded to attach to the eyes (Singer & Cocucci 1997), forelegs (Peter *et al.* 2009; Xiong *et al.* 2020), thorax (Johnson, Balducci & Shuttleworth 2020), the antennae and the proboscides (van der Voort *et al.* 2022). Advancements in methods of removing pollen have also been developed and improved upon such that more precise and selective sampling can be achieved while minimizing damage to both museum specimens and live pollinators (Donald, Bolstridge & Ridden 2022).

In our study, the pollen loads of archival and contemporary hawkmoths were analyzed. I separated out the pollen load by several positions along the hawkmoth anatomy. I pose several questions: (1) is sample age (archival vs. contemporary) a determinant of hawkmoth pollen

load quantity? (2) is there a difference in pollen loads (both in abundance and species composition) depending on anatomical placement? Lastly, I investigated how the resultant visitation networks (contemporary and archival) differed in several indices. *Do archival hawkmoth collections reliably retain pollen such that we can 'reconstruct' plant communities?* I firstly hypothesized that those sections of the body which are more susceptible to handling during storage preparation and subsequent use- such as the abdomen- would retain fewer pollen grains than body parts which are more concealed and less likely to be accidentally removed. Secondly, I expected that archival hawkmoth samples would retain less pollen overall when compared to contemporary samples.

3.3 Materials and methods

Contemporary hawkmoths were gathered from the summit at Gilboa Estate (MONDI Forests Ltd) (29° 19′S, 30° 17′E), in the Karkloof mountain range of the KwaZulu–Natal Midlands of South Africa. Five species - *Agrius convolvuli* (Linnaeus 1758), *Basiothia schenki* (Möschler 1872), *Hippotion celerio* (Linnaeus, 1758), *Theretra capensis* (Linnaeus, 1764), and *Hyles livornica* (Esper, 1780) - totaling 32 individuals, were collected during peak hawkmoth activity over the summer months from February 2020 to February 2021. To eliminate happenchance flower visitors, pollinators were observed and caught only during active foraging bouts. The plant species on which the moth was foraging was recorded. Individual trapping events took place from sundown until ~1.5 hours after sunset when moth activity ceased.

The surrounding flowering plant community was also recorded during trapping events. Pollen samples were taken from flowering plants found in the surrounding area (n=129). These pollen samples were then used to create a reference library. Semi-permanent fuchsin jelly slides of single-origin pollen were made as reference slides of the identified flowering community. These were made by embedding anthers of known plant species in fuchsin jelly (~1 mm3,

weight = 18-20 mg). These were placed on a heat-block set to 55°C for ~1 minute or until just melted. With the exception of the crystalline phenol, fuchsin jelly was made following a standardised recipe (Beattie 1971). This involves a procedure of mixing 150 ml glycerine to 50 g gelatine dissolved in 175 ml distilled water, and then adding basic fuchsin crystals until the desired colour is attained. A cover slip was placed over the fuchsin-embedded pollen. Photographs of the slides were taken using a Zeiss Zen 3.4 (blue edition version 3.91.0) and a Zeiss Axio Lab. A1 (Carl Zeiss Microscopy GmbH, Jena, Germany) microscope at 40X and 100X magnification to compare and match against pollen grains retrieved from the pollinators.

Each individual pollinator was placed in a clean 50 ml Eppendorf and placed on ice. Specimens were then transferred to a freezer until further processing. Glass vials (15 ml) were used to hold specimens while they were placed in silica gel with the vial lids removed. Samples were left to desiccate completely. Each individual was carefully and separately stored so as to avoid contamination and transferal of pollen between individuals. Excessive handling and repositioning of the hawkmoths was kept to a minimum during all stages of hawkmoth processing to avoid pollen loss.

Twenty-one hawkmoths from four species - *Agrius convolvuli (*Linnaeus, 1758), *Basiothia schenki* (Möschler, 1872), *Hippotion celerio* (Linnaeus, 1758), and *Theretra capensis* (Linnaeus, 1764) were selected for the archival samples. These were collected on Mt. Gilboa over the summer in 1999, pinned and kept in storage at the University of KwaZulu-Natal, Pietermaritzburg.

Small cubes of fuchsin jelly (max 1 mm^3, max weight = 18-20 mg) were used to target specific sections of the hawkmoth body including the head, antennae, thorax, abdomen, and the proboscis (coiled and uncoiled). The proboscides were first swabbed in the coiled state. This included swabbing the mouthparts in the immediate vicinity of the proboscis, but the proboscis was not unfurled. The proboscides were then unfurled and swabbed. A humidity

chamber was used to assist in the unfurling of hawkmoth proboscides. The cubes of fuchsin jelly were placed on separate glass slides and melted at 55°C for ~30 seconds, or until just melted. A cover slip was placed over the fuchsin-embedded pollen and left to set. Each slide contained pollen carried by a single individual. The fuchsin-embedded pollen was viewed under a compound microscope at 40x and 100x magnifications, and all pollen grains counted and compared to the pollen library created for this study (see above) for Mount Gilboa. The hawkmoths were returned to the silica gel for storage.

I used linear mixed models to assess the extent to which several variables were related to hawkmoth pollen count information. Using SPSS 28 (IBM), I constructed a model accounting for all possible combinations of the different variables, including an intercept only model. Pollen load count was roughly Gaussian and was thus analyzed using models with a normal distribution and an identity link function. Moth specimen was treated as a random effect, while hawkmoth species, specimen age (archival or contemporary), pollen species, and anatomical pollen position and their interactions were treated as fixed effects. The final model was determined by the Akaike Information Criterion (AIC). Model degrees of freedom were adjusted using the Kenward-Roger method (Kenward & Roger 1997). I accounted for possible correlations between replicate samples by considering each hawkmoth sample as a subject variable.

Quantitative pollination networks were constructed. The function *plotweb* in Bipartite was used to create a visual overview of the network. To determine the difference in structural properties between archival and contemporary interaction networks, the networks were compared using several indices (Table 3.1). Network properties were calculated using the function *networklevel* in the R-package 'bipartite', version 1.02 (Dormann, Gruber & Fründ 2008). The function *H2'* was used to compare specialization (Blüthgen, Menzel & Blüthgen

2006). Several other indices were calculated including robustness, connectance, modularity etc (Table 3.1).

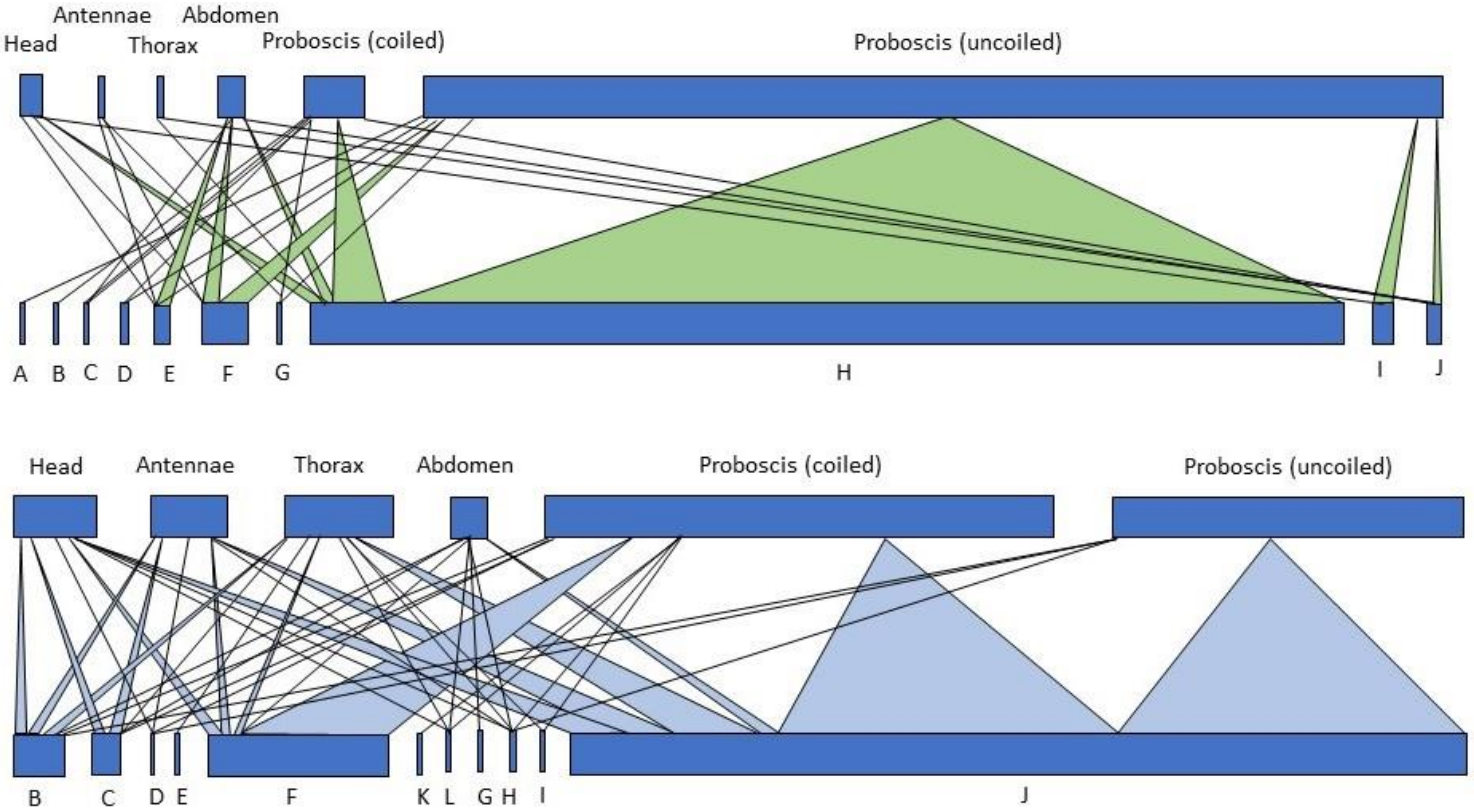
3.4 Results

Pollen load was significantly affected by sample age ($F = 17.03$, $p < 0.01$). Archival samples (mean ± S.E. = 0.8 ± 2.3) carried ~30 times less pollen than contemporary samples (mean ± S.E. = 26.3 ± 2.7). Moth species was not a determinant of the total pollen load ($F = 17.0$, $p = 0.28$), while anatomical position was ($F = 46.6$, $p = 0.00$). Across all specimens, pollen species composition varied significantly in response to specimen age ($F = 35.9$, $p < 0.01$) and anatomical position ($F = 11.50$, $p < 0.01$).

Counting pollen grains on the hawkmoth body revealed that pollen is disproportionately found on certain anatomical sites ($F = 46.6$, $p < 0.01$). The proboscides (both coiled and uncoiled) carried the most pollen grains ($p < 0.01$). The coiled proboscis (mean ± S.E. = 19.1 ± 2.6) held less pollen compared to the uncoiled proboscis (mean ± S.E. = 32.1 ± 3.0). Plant species also differed significantly between contemporary and archival samples ($p < 0.01$). Within the archival data set, all anatomical positions were a significant determinant of plant species composition within the pollen load. The uncoiled proboscis (mean ± S.E. = 4.15 ± 0.82) contained the greatest number of pollen grains. A key result was that *Protea* pollen was the most abundant plant species found on archival samples (mean ± S.E. = 8.4 ± 1.5) potentially revealing a more diverse foraging breadth. The fewest pollen grains were attached to the thorax of archival samples (mean ± S.E. = 0.01 ± 0.82). The contemporary samples followed a similar pattern with the uncoiled proboscides containing the most pollen (mean ± S.E. = 69.7 ± 3.6). *Zaluzianskya* (mean ± S.E. = 115.8 ± 3.7) and *Lobelia* (mean ± S.E. = 16.5 ± 3.7) pollen was the most abundant plant species represented in the pollen load. The coiled proboscides of

contemporary samples held a large proportion of the total pollen load (mean ± S.E. = 40.4 ± 3.3). The abdomen held the fewest pollen grains (mean ± S.E. = 1.3 ± 3.5).

The contemporary pollen-transfer network showed greater generality, connectance, species interaction and functional complementarity. This renders this network more robust, less specialized, and possibly less vulnerable to secondary extinctions (Table 3.1). The network constructed from archival pollen-load showed a general reduction in number of plant-pollinator interactions recovered (Table 3.1, Figure 3.1).



A	*Erica sp.*
B	*Berkheya sp.*
C	*Dierama sp.*
D	*Ledebouria sp.*
E	*Ranunculus sp.*
F	*Lobelia sp.*
G	*Pine sp.*
H	*Protea sp.*
I	*Silene sp.*
J	*Zaluzianskya sp.*
K	*Albuca sp.*
L	*Pentanisia sp.*

FIGURE 3.1 Quantitative hawkmoth pollen-transport networks for archival (top) and contemporary pollen (below) analysis. Each pollen species and anatomical placement is represented by a rectangle. The bars represent the total number of pollen grains carried on each anatomical placement; the triangular links between rectangles represent the proportion of pollen of each plant taxa carried by each anatomical feature; the lower rectangles represent the total number of pollen grains of each plant taxa from all individuals captured.

TABLE 3.1. Bipartite network-level properties analyzed by constructing networks for archival and contemporary communities.

Network property	Archival	Contemporary
Number of floral partners	6 - 10	6 - 11
Nestedness (NODF)	74.69	79.45
Connectance	0.52	0.62
Specialization asymmetry	-0.01	0.07
H2′	0.18	0.25
Extinction slope	3.192	4.288
Robustness	0.76 - 0.84	0.79 - 0.91
Functional complementarity	1826.5 – 1826.7	8043.1 - 10777.0
Niche overlap	0.728	0.895
Modularity	0.066	0.178
Generality	1.54	1.92
Vulnerability	1.492	3.62
Shannon diversity	0.85	2.02
Interaction evenness	0.21	0.48
Shannon diversity	0.85	2.02
Mean no. of shared floral partners/moth	1.8 - 3.1	2.3 - 5.2

3.5 Discussion

Value of museums

In being vast repositories of data, archival pollinator specimens are invaluable for researchers analyzing past ecological interactions (Payne & Sorenson 2002; Suarez & Tsutsui 2004; Holmes *et al.* 2016). Plant-pollinator networks and structural parameters calculated from archival and current specimens can provide useful information for comparisons of ecosystem health through time, and this can inform conservation decisions (Elle, Elwell & Gielens 2012). Although the scale (both temporal and spatial) at which networks are analyzed impacts on the resulting parameters (Prendergast & Ollerton 2022), within the same system, network parameters and extents of diet breadths can be used as baselines from which environmental perturbations can be assessed, and other ecological comparisons made (Burkle & Alarcón 2011). Again, studies such as this highlight the value of museum specimens and the importance of excellency in curation. Although the study system is likely to be similar and the diet breadths

still informative, the contrasts drawn between the network metrics is potentially not because of age, but simply because the hawkmoths had different pollen loads to start off with, having been caught under different conditions and on different host plants.

Value of studying pollen-loads

Adding a pollinator-centered approach to observational records adds complementary data to create a more accurate representation of the plant-pollinator network. Pollen loads are helpful in revealing interactions that are rare, elusive, or problematic to confirm in a field-setting (Cocucci & Sérsic 1998; Cozien *et al.* 2019). An added value of a pollinator-centered approach is that they provide a reliable snapshot of the realized community which can be compared weekly, seasonally, or annually.

The networks constructed from pollen removed from older specimens seem to lose interactions and appear more susceptible to overestimating the vulnerability of the plant-pollinator system to perturbations. Merely because of the potential loss of pollen from pollinators during storage, the apparent loss of these interactions in the recreated network could artificially inflate or underestimate network indices leading to erroneous conclusions of community structure. This has also been recorded for pollination networks analyzed at various spatial scales (Prendergast & Ollerton 2022), although certain indices (nestedness, interaction asymmetry) are more robust for comparison and less prone to vacillate over spatial scale and network size (Nielsen & Bascompte 2007; Elle, Elwell & Gielens 2012). The nestedness value for the contemporary network was minimally higher than for the archival network indicating that both networks will be equally vulnerable to disturbance, and that the network has persisted despite potential environmental change over the last two decades. A pollinating community which is highly nested, as both here were found to be, indicates a tendency of specialist plants to interact with generalist pollinators (and vice versa), as well as the lack of interacting partners being

organized into distinct compartments (Memmott, Waser & Price 2004). High values for both nestedness and redundancy point towards a system that is tolerant to partner-loss and ultimately extinction (Memmott, Waser & Price 2004). Network connectance, despite its reliance on network size, is a robust and valuable index for assessing how interactions are changing over time. Other studies have reported on interaction network weakening in response to anthropogenic climate change (Memmott *et al.* 2007; Burkle, Marlin & Knight 2013). Studies such as this one of Mount Gilboa, Karkloof are important in using long-term museum data to reveal response changes in plant-pollinator communities.

Value of segregating pollen loads by anatomical position

The proboscides in both the contemporary and archival hawkmoths accounted for both the greatest quantity and species composition of pollen. Although this could also reflect that the proboscis is the most common site of pollen deposition on these moths, and not that it is protected from handling per se, many other anatomical placements for pollen deposition such as legs, eyes, wings (Johnson, Balducci & Shuttleworth, 2019; Butler & Johnson, 2020) have been identified in hawkmoths. Within the contemporary samples, the thorax held the most pollen grains after the proboscides. Although observationally the foraging bouts of the contemporary hawkmoths appeared to feed on *Zaluzianskya* alone, the pollen load composition reflects a more complex interaction pattern. Hawkmoths chiefly visited *Zaluzianskya* flowers, but also foraged to a large extent on *Lobelia* and *Berkheya*. This finding reflects the polyphagous nature of hawkmoths (Johnson *et al.* 2017). Hawkmoths are known to be important pollinators of several orchid species in southern Africa (Johnson 1995; Peter *et al.* 2009). Interestingly, no orchid pollen was found on the Hawkmoths despite species of *Habenaria* and *Satyrium* flowering in the area contemporaneously. The highest species-rich pollen load was removed from *Basiotha schenkii*, *Hippotion celerio* and *Agrius convolvuli* (in

that order). Fewer plant species were represented in the pollen loads of *Hyles livornica* and *Theretra capensis*. A distinct lack of an *Agrius*-*Gladiolus* link was evident. Seasonality may be the simple reasons as *Gladiolus* mainly flower in November and the hawk moths seemed to be absent until December, January when they were collected.

The realized pollination niche is constrained by community-level interactions (competition for pollinators, pollinator preferences etc.) while being limited by the necessary biotic and abiotic factors required for effective pollination (Phillips *et al.* 2020). Such niches can therefore be expected to be a product of time and space, and likewise the resulting topology of plant-pollinator networks. Interactions within networks can be organized into compartments of strongly- or weakly-interacting species (Olesen *et al.* 2007) which reflects the extent to which nodes (here plant species) in a compartment are more likely to use the same anatomical placement for pollen. Here I found the contemporary network to be more modular in comparison to the archival network. As modularity is likely to be influenced by the size of networks (and hence the loss of pollen from archival specimens), our findings corroborate that of others' conclusions and cautions against using this index to compare networks across temporal scales (Elle, Elwell & Gielens 2012; Prendergast & Ollerton 2022).

Nocturnal pollinators are underappreciated and underrepresented in research (Devoto, Bailey & Memmott 2011; Knop *et al.* 2018; Macgregor & Scott-Brown 2020; Walton *et al.* 2020), and plant-pollinator networks are generally poorly studied in the Africa (representing only 4% of studies) (Archer *et al.* 2014; Ollerton 2021). Nocturnal pollination services also incur added costs such as the requirement for thermogenicity which may become important in light of climate change (Macgregor & Scott-Brown 2020). Nocturnal pollinators also have added stressors, such as ALAN (artificial light at night) (Kalinkat *et al.* 2017), which diurnal pollinators needn't deal with directly yet may have negative impacts on the overall plant reproductive success (Macgregor *et al.* 2017). In the absence of properly resolved nocturnal

networks, conservation efforts would be misinformed and possibly ill-applied. In a South Africa context, I find that the nocturnal network contributes a substantial portion to pollination networks as evidenced by the richness and abundance of pollen species transported in pollen loads. This adds support to the increasingly accepted view that nocturnal pollinators are a vital and complementary component of pollination which needs to be considered in conservation.

3.6 References

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CHAPTER 4

Hawkmoth pollination networks: Increased resolution using metabarcoding versus traditional palynology

4.1 Abstract

Premise. Networks of interactions between nocturnal pollinators and plants are difficult to estimate using direct observations. Palynology has traditionally been used to infer the floral host breadth of nocturnal pollinators such as hawkmoths, but pollen morphology is not always reliable for identification to species level. Metabarcoding of pollen therefore holds promise for improved detection of the floral hosts of nocturnal pollinators.

Methods. I estimated the structure of a hawkmoth pollination network in South Africa using data from both light microscopy and DNA metabarcoding obtained by comparing the pollen loads of hawkmoth individuals using these two analytical methods.

Results. In comparison with microscopy, DNA metabarcoding of pollen from the same hawkmoth individuals detected a three-fold greater number of plant species. Regardless of the method of analysis, floral host diversity did not differ significantly among five different hawkmoth species with tongue lengths varying from 4 to 10 cm. Networks constructed using metabarcoding data showed more complexity (e.g., higher linkage density and generality) than those constructed using pollen identified by microscopy. Nonetheless, both analyses revealed that hawkmoths frequently visit flowers that do not conform to a moth pollination syndrome.

Conclusions. Metabarcoding is a powerful method for establishing the range of floral hosts visited by nocturnal pollinator species such as hawkmoths that are difficult to observe directly. However, data from metabarcoding is mostly qualitative and is thus most effective when used in conjunction with other methods such as direct observations, camera-trapping and palynology. In support of previous research using alternate methods, metabarcoding confirms that hawkmoths are indeed more polyphagous than previously thought. Although network complexity is increased with the addition of molecular data, other important network metrics such as connectance and robustness remained largely unchanged.

Keywords: pollen load, diet breadth, nocturnal pollination networks, palynology, pollen library, hawkmoth.

4.2 Introduction

The functional role of pollinators is a key topic of research, given their importance for plant reproduction in natural ecosystems as well as food production (Potts *et al.* 2010; Potts *et al.* 2016). However, there are considerable difficulties in studying ecological networks of interactions involving nocturnal pollinator species (Macgregor *et al.* 2015; Buxton *et al.* 2022).

The level of generalization in the foraging behaviour of pollinators (also termed diet breadth or host range) is a key aspect of their ecology (Smith *et al.* 2019; Endres *et al.* 2021) and is particularly difficult to estimate for nocturnal species. Pollinators are mostly generalist in their foraging behaviour as their adult phenology is often longer than that of flowering of any given plant species and because plant communities have variable species composition (CaraDonna, Iler & Inouye 2014). Hawkmoths (Family Sphingidae) are highly polyphagous foragers (Martins & Johnson 2013; Barták 2022). While short-tongued hawkmoths are limited to foraging from floral partners with shorter tube-lengths in order to receive nectar rewards,

long-proboscid hawkmoths are not and therefore are able to forage on a much larger variety of species. These guilds of hawkmoths differing in proboscis length correspond to a bi- or multimodal distribution in floral tube length (Martins & Johnson 2013; Johnson *et al.* 2017). In most plant communities, hawkmoths are known to typically occupy distinct pollination-niches corresponding to their proboscis length (Johnson 2017, Martins & Johnson 2013). Within southern Africa, two hawkmoth guilds have been identified: one of tongue-lengths ca. 4cm, and the other of ca. 10cm (Johnson *et al.* 2017). Plant species conforming to these pollination niches characteristically have long floral tubes, are pale/cream coloured, and emit scent during crepuscular/nocturnal hours (Martins & Johnson 2013). However, visitation studies have revealed that hawkmoths don't necessarily abide by these floral characteristics (Martins & Johnson 2013). Further studies involving field observations, light trapping, camera surveillance and pollen load analysis have confirmed the pollination of many more long-tubed native plant species by hawkmoths (Johnson & Raguso 2016). Particularly for the longer-tongued hawkmoths, such as *Agrius convolvuli*, their propensity to visit many plant species falling outside these 'syndrome' emphasizes the highly generalist foraging nature of long-proboscid moths (Johnson *et al.* 2017).

Palynology and DNA metabarcoding of insect pollen loads are methods that have been used to estimate the host range of pollinators (Macgregor *et al.* 2019). Because pollen grains can remain attached to floral visitors for long periods of time, analyses of pollen loads can provide clues about floral host ranges (Bosch *et al.* 2009). Pollen identification using light microscopy has several caveats. Expertise and a thorough knowledge of the flowering community is a primary requirement for identification. Even with expertise and a well-curated reference collection, pollen identification can still be difficult which can lead to reduced discriminatory power at lower taxonomic levels (Devoto, Bailey & Memmott 2011; Galimberti *et al.* 2014). Additionally, the input cost and effort per sample for upscaling biodiversity

analysis is comparatively low (Schmidt *et al.* 2013; Deiner *et al.* 2017; Porter & Hajibabaei 2018; Liu *et al.* 2020). DNA barcoding has emerged as an alternate means to identify species based on short, standardized segments of DNA (DNA barcodes). As an extension of this, DNA metabarcoding is able to simultaneously identify multiple taxa from a complex, pooled community sample of mixed origins. In contrast to morphological pollen identification where any additional samples added increases the time and cost input linearly, these costs in metabarcoding are negligible or even reduced as more samples are incorporated. As much of laboratory protocols can be standardized, metabarcoding is reportedly less ambiguous and less prone to bias than traditional palynology. The analysis of sequence data from pollen collected from pollinators reveals more complex and biodiverse networks where cryptic or hidden interactions are revealed (Baksay *et al.* 2022; Bell *et al.* 2022; Lowe *et al.* 2022). Despite the advantages of metabarcoding, input costs are still high, the method yet lacks full standardization and is impeded by the incomplete database of reference records to which the amplified sequences can be compared. However, these impediments are lessening as standardized methods are developed and globally accessible databases such as NCBI GenBank and Barcode of Life Database (BOLD; www.boldsystems.org.) are improving.

I used both traditional palynology and metabarcoding to identify the floral host plants of hawkmoths in a South African grassland ecosystem. My chief aim was to identify and compare hawkmoths range of host plants using both metabarcoding and microscopic pollen load analyses. My further aims were to construct qualitative networks and to assess the sensitivity of the two methods by comparing several network indices.

4.3 Materials and methods

Field sampling

Hawkmoths were collected using hand-nets from the summit at Gilboa Estate (MONDI Forests Ltd) (29° 19′S, 30° 17′E), in the Karkloof mountain range of the KwaZulu–Natal Midlands of South Africa. The plant species on which each moth was foraging when it was netted was recorded. Collecting took place from sundown until ~1.5 hours after sunset when moth activity largely ceased (Martins & Johnson 2013; Johnson *et al.* 2020). Five species – *Agrius convolvuli* (Linnaeus 1758), *Basiothia schenki* (Möschler 1872), *Hippotion confirm* (Linnaeus, 1758), *Theretra capensis* (Linnaeus, 1764), and *Hyles livornica* (Esper, 1780) - totaling 32 individuals, were collected during peak hawkmoth activity over the summer months of February 2020 to February 2021.

Each pollinator was retained individually in a clean 50 ml Eppendorf and euthanized on ice. Specimens were then transferred to a -80°C freezer until further processing. Glass vials (15 ml) were used to hold specimens while they were placed in silica gel with the vial lids removed. Samples were left to desiccate completely. Each individual was carefully and separately stored so as to avoid contamination and transfer of pollen between individuals. Excessive handling and repositioning of the hawkmoths was kept to a minimum during all stages of hawkmoth processing to avoid pollen loss.

Light microscopy

Small cubes of fuchsin jelly (max 1 mm^3, max weight = 18-20 mg) were used to gather pollen from the hawkmoths. With the exception of the crystalline phenol, fuchsin jelly was made following a standardized recipe (Beattie 1971). Hawkmoths were visually divided equally along the sagittal plane, with one side being randomly chosen for pollen-swabbing. Fuchsin jelly cubes were used to thoroughly swab a half-section of the hawkmoth's body. As a single curling event of proboscides has been found to be sufficient to distribute pollen across the entire length of the proboscis (Smith *et al.* 2022), the proboscides were swabbed at regular intervals

in a systematic fashion. After swabbing with fuchsin jelly, the hawkmoths were returned to silica gel for storage, and subsequent metabarcoding processing. The pollen load of the remaining, un-swabbed sections of the hawkmoth were used for the metabarcoding sample.

The cubes of fuchsin jelly were placed on separate glass slides and melted at 55°C for ~30 seconds, or until just melted. A cover slip was placed over the fuchsin-embedded pollen and left to set. Each slide contained pollen carried by a single individual. The fuchsin-embedded pollen was viewed under a compound microscope Zeiss Axio Lab. A1 (Carl Zeiss Microscopy GmbH, Jena, Germany) at 40× and 100× magnifications, and all pollen grains counted and compared to the pollen library. To establish the pollen library semi-permanent fuchsin jelly slides of single-origin pollen were made as reference slides of the identified flowering community. The pollen library comprised 129 plant species which were sampled from the surrounding area across all seasons. Of these, 93 were successfully barcoded (Table S5.2). These samples were used to create a pollen reference library. These were made by embedding anthers of known plant species in fuchsin jelly (~1 mm3, weight = 18-20 mg) which was melted as described above and enclosed beneath a cover slip.

Photographs of the slides were taken at 40× and 100× magnification using a Zeiss Axiocam Icc 5 camera and processed digitally using Zeiss Zen 3.4 software (blue edition version 3.91.0) to compare and match against pollen grains retrieved from the pollinators. Pollen identified to genus level represented groupings that could not be unambiguously separated to a lower taxonomic level and might have contained pollen from several species belonging to the same genus.

DNA metabarcoding of hawkmoth samples

The Dneasy Plant MiniKit (QIAGEN®) was used for all DNA extractions. The standard protocol for extracting genetic material was followed, with the following modifications:

volumes of buffer AP1 and Rnase A stock solutions were scaled up by four times due to size of specimens. Hawkmoths were manually crushed- all items used in the crushing process were autoclaved prior to use. Quantification of DNA was performed with the Qubit BR DNA Assay kit and Qubit Flex Fluorometer (Invitrogen/ThermoFisher). Extracted DNA was diluted, and equimolar pools prepared according to manufacturer's protocol for the appropriate sequencer. Pooled DNA samples were couriered to MRDNA (Texas, USA) for high-throughput sequencing which was performed using the Illumina MiSeq system (Illumina, Inc., San Diego, CA, USA).

Reference Plant DNA barcode protocol

A reference library for pollen DNA sequence matching was constructed by collecting plant material from representatives of 129 plant species flowering at the study site, of which 93 were successfully barcoded (Table S5.2). Voucher individuals for each species were curated and are stored at the University of KwaZulu-Natal herbarium (NU). DNA was extracted from silica-dried leaf tissue retrieved from the voucher specimens, and sequenced at Genomyx Laboratories, Johannesburg. All plant specimen details are available on the online BOLD© repository under the project FFVCC (https://www.boldsystems.org). ITS1 and ITS2 were amplified for every specimen and sequenced using Sanger sequencing. The plant ITS region was amplified using the primer pair ITSAB101F (5'-ACGAATTCATGGTCCGGTGAAGTGTTCG-3') and 26SE (5'-TAGAATTCCCCGGTTCGCTCGCCGTTAC-3') (Sun *et al.* 1994). Final sequences were compiled into FASTA files for reference library construction.

Pollen DNA bioinformatics

Demultiplexed paired FASTQ files were run through the DADA2 algorithm via QIIME2/2022.2 (Bolyen *et al.* 2019; Xia & Sun 2023) an initial quality-control check where: chimeras are removed; amplicons are filtered; primers are trimmed; forward and reverse reads are truncated, and paired-end reads are merged. Reference sequences of all amplicon sequences variants (ASVs) for both ITSp3/ITSu4 (n = 38) and ITSp5/ITSu2 (n = 282) were manually queried against GenBank (Benson *et al.* 2015) and BOLD (Ratnasingham & Hebert 2007). The sequence returning the highest percentage similarity was selected and records of its distribution checked against the GBIF database (https://www.gbif.org/developer/occurrence). After applying a minimum threshold (50 and 100 sequence reads for ITSp3/ITSu4 and ITSp5/ITSu2). ASV clusters identifying the same species were merged into single taxonomic units by summing reads (n = 4 and 22 species for ITSp3/ITSu4 and ITSp5/ITSu2, respectively). Chi-square analysis was used to statistically compare frequencies of the two detection methods (microscopy versus metabarcoding).

Network analyses

For each method of pollen species detection, I constructed a qualitative pollen-transport network (Figure 4.1) based on presence and absence of pollen species found. Networks were constructed, and network properties calculated, using the function *plotweb* and *networklevel* in R-package 'bipartite', version 1.02 (Dormann, Gruber & Fründ 2008). The function *H2'* was used to compare specialization (Blüthgen, Menzel & Blüthgen 2006). Several other indices were calculated including robustness, connectance and modularity (Table 4.3).

4.4 Results

Pollen was detected on all 32 hawkmoth individuals using both metabarcoding and microscopy methods. Microscopy yielded pollen from nine plant species while metabarcoding detected 26

plant species (Figure 4.1 and Table 4.2). Of these, only one species (*Watsonia lepida*) was detected by both methods (Table 4.1). Microscopy-assigned pollen from *Zaluzianskya* sp. Could not be unambiguously identified to species level as several species of *Zaluzianskya* flower simultaneously and could not be distinguished by light microscopy. *Agrius convolvuli* carried the greatest pollen species richness as identified by metabarcoding analysis, whereas microscopy detected little interspecies difference in the species richness of pollen loads (Table 4.2). A significant difference in species detected was observed for the two methods of pollen species detection (χ^2=8.26, p=0.004). No significant difference in plant species detected by metabarcoding was found across all species of hawkmoths (χ^2=0.77, p=0.943, Table 4.2).

TABLE 4.1. Matrix of interactions detected in the present study. Open and black circles indicate interactions detected only by microscopy and metabarcoding, respectively, and half-black–half-white circles were interactions detected by both methods.

Family	Species ID consensus	*Agrius convolvuli*	*Basiothia schenki*	*Hippotion celerio*	*Hyles livornica*	*Theretra capensis*
Aizoaceae	*Aizoaceae sp. 5*		●	●	●	●
Amaryllidaceae	*Nerine sp.*		●	●	●	
Apiaceae	*Alepidea sp.*	●	●	●	●	●
Apiales	*Pimpinella caffra*	●				●
Asparagaceae	*Albuca setosa*		○			
	Leobordea corymbosa					●
	Ledebouria sandersonii		○	○		
Asteraceae	*Asteraceae sp.*	○	○		○	○
Boraginaceae	*Mysotis sp.*		●		●	
Brassicaceae	*Crucifereae sp. 2*		●	●	●	
Caryophyllaceae	*Silene burchellii*	○		○	○	○
Cleomaceae	*Cleome sp.*			●		
Euphorbiaceae	*Acalypha sp. 2*	●				
	Acalypha sp. 3	●				
Fabaceae	*Psoralea sp.*	●		●	●	
	Tephrosia sp. 2		●	●	●	
	Quercus robur			○		
Iridaceae	*Watsonia lepida*	●	◑	●	◑	●
Lobelioideae	*Lobelia flaccida*	○		○		○
Myrtaceae	*Syzygium sp.*					●
Orchidaceae	*Satyrium longicauda*	●	●	●	●	
Poaceae	*Megathyrsus maximus*					●
	Triticum sp. 1	●	●	●		●
Proteaceae	*Protea sp. 3*	●	●	●		●
Rubiaceae	*Pentanisia angustifolia*	○				○
	Rothmannia capensis	●		●		

Scrophulariaceae	*Hebenstretia sp.*	●		●		●
	Chaenostoma floribunda	●	●	●	●	●
	Zaluzianskya sp.	◑	◑	●	◑	◑
Solanaceae	*Datura sp.*				●	
	Datura stramonium		●	●	●	●
	Lantana camara	●		●		●
Verbenaceae	*Volkameria sp.*	●				●

TABLE 4.2. Comparisons between DNA metabarcoding and microscopy approaches in detecting pollen species on all hawkmoths and separated out by hawkmoth species.

Method	Plant species detected					
	Combined	*Agrius convolvuli*	*Basiotha schenkii*	*Hippotion celerio*	*Hyles livornica*	*Theretra capensis*
Metabarcoding	26	15	13	17	13	15
Microscopy	9	5	5	4	5	5

Construction and analysis of networks

Several network metrics (Table 4.3) differed markedly between the two methods, specifically linkage density and generality, which were much lower in the microscopy-based network than the metabarcoding network (Figure 4.1, Table 4.3). Connectance was minimally higher for the metabarcoding network leading to a slightly reduced modularity in comparison to the microscopy network, but other metrics describing the overall structure did not differ vastly (Table 4.3). Unexpectedly, specialization (H2') was the same for both networks.

TABLE 4.3. Network metrics calculated for each pollen detection method.

Network metrics	Metabarcoding	Microscopy
Linkage density	9.14	3.87
Connectance	0.29	0.28
Generality	14.75	3.09
H2' (specialization)	0.50	0.50
Robustness	0.72	0.78
Modularity	0.23	0.27

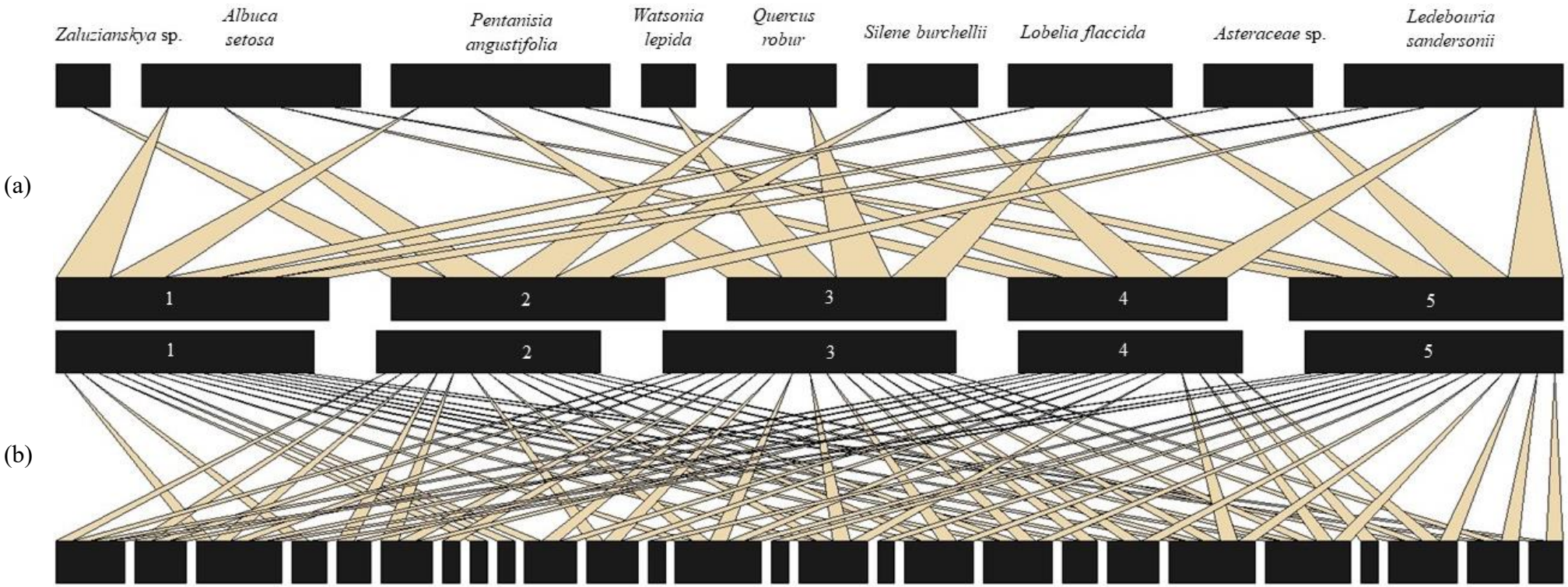

FIGURE 4.1 Networks constructed using microscopy (a) and DNA metabarcoding (b) from matched samples of Sphingid moths. Moth species are numbered sequentially following this order: *Agrius convolvuli* (1), *Basiothia schencki* (2), *Hippotion confirm (3)*, *Hyles livornica* (4) and *Theretra capensis* (5). Species interactions for (b) follow the pollen species from Figure 4.1. in order from left to right.

4.5 Discussion

Metabarcoding of hawkmoth pollen loads confirmed that hawkmoths are highly polyphagous, and forage on plant species falling outside of the accepted moth pollination syndrome. Metabarcoding revealed a highly diverse and complex network with increased linkage between nodes in comparison to microscopy-based networks (Baksay *et al.* 2022; Bell *et al.* 2022). Although I expected structure to be changed by the greater accuracy of metabarcoding, several network metrics estimating overall network stability were not affected by the improved detectability of this method. Both metabarcoding and traditional microscopy however revealed interactions which were not recorded by human observers during moth capturing. This improvement in species detectability methods (observational to microscopy to metabarcoding) and its effect on network structure is corroborated by Encinas-Viso et al. (2022) finding a significant difference in overall network topology when comparing metabarcoding to observational data.

Advantages and disadvantages of each method

Fuchsin swabbing and conventional microscopy is typically a less efficient method as the entire pollinator is never swabbed completely, rather anatomical sections of interest are targeted. Even with possibly low relative abundance, metabarcoding can detect pollen of species which may be overlooked by microscopy. Despite the exine morphology of pollen grains usually being species-specific (Wang & Dobritsa 2018), pollen grains cannot be identified unambiguously without higher quality microscopy, such as SEM, which shows greater detail. As the exine structure is mostly phylogenetically constrained with closely related species producing pollen with similar pollen wall patterns (Wang & Dobritsa 2018), identification by exine morphology cannot always distinguish between closely related species. Misidentification and lumping of pollen-types in morphospecies grouping may lead to an underrepresentation of

the diversity in a system when applying microscopy alone. Metabarcoding does not have this downfall and is thus able to detect more plant-pollinator interactions and provide a detailed overview of complex ecological networks. In comparison to the microscopy network, the linkage density of our metabarcoding network reflects the inherent network complexity which has been one of the foremost challenges as well as motivations for ecological network analysis. Research is hampered by the inability to identify species accurately and timeously, especially across larger geographic scales. Here I show that metabarcoding is a viable solution to supplement studies based on visitation or traditional palynology. I found that many species remained undetected by conventional microscopy (compound) analysis alone implying that this method, based only on exine morphology of pollen load, is viable as an initial and brief overview but is unable to present a complete and accurate representation of ecological interactions. As metabarcoding is more sensitive in distinguishing species, the resulting community network is inherently more linked, and therefore generality is increased. While the estimated generality of our microscopy network here is comparative to that of the grassland network (visitation) calculated by Johnson et al. (2016), generality estimated by our metabarcoding data is almost five times as much.

A further major drawback for network analysis by conventional microscopy is that time investment increases linearly for each sample added, which would restrict this methods applicability at larger scales (Macgregor *et al.* 2019). This time impediment may however be significantly reduced with the burgeoning field of artificial intelligence (AI). AI is already used to accurately discriminate between fragmented fingerprints (Chauhan, Sharma & Siddhartha, 2022), and the potential application of this technology in the field of palynology could be highly advantageous. Erroneous species assignment is a major downfall of metabarcoding. However, this will become less of a concern as online databases are continually increasing and improving as records are uploaded. While I used presence-absence data, metabarcoding based on this

method alone may not be advisable as rare taxa are overemphasized while abundant taxa are devalued (Lowe *et al.* 2022). Metabarocding is however unable to quantify the pollen in mixed samples, where with microscopy this is possible. Although not without its downfalls, recently, sequence counts have been shown to provide a more realistic, semi-quantitative approach of species interaction frequency (Baksay *et al.* 2022). The aim of the study, which was to construct a pollination network using metabarcoding data to identify links possibly overlooked by other methods, was satisfied. To this end, quantifying the strength of those interactions was not required.

Pollen transport by hawkmoths

These results confirm that hawkmoths are highly polyphagous and that long-tongued species feed on both long- and short-tubed flowers (Martins & Johnson 2013; Barták 2022). The data collected here are useful in describing diet at a clearer, finer resolution. Metabarcoding data reveals interactions which are unexpected and might wildly defy the typical pollination syndromes, such as *Poaceae* and *Quercus* in this study. Although these species are typically wind-pollinated, ambophilous-systems have been observed in certain grass species (Schulze-Albuquerque *et al.* 2020; Gous *et al.* 2021). The presence also of *Rothmannia capensis* and other exotic garden plants points towards long distance flight of the moths from forests, plantations and a nearby cultivated garden, Benvie Open Garden, which is roughly 8 km away from Mt. Gilboa summit.

The convolvulus hawkmoth *Agrius convolvuli* particularly, has been identified as the most important 'keystone pollinator' species for particular groups of long-tubed African plants (Johnson & Raguso 2016). Interestingly, metabarcoding identified *Hippotion confirm* as the most prolific visitor of flower species in our study. *Agrius convolvuli* has a proboscis ranging from 96mm to 108mm, which is ~2.5 times longer than the proboscis of *Hippotion confirm*

(41.2 mm to 41.5 mm) (Martins & Johnson 2013). Yet despite this difference, our results do not show that the added length corresponds to a greater foraging breadth. In contrast to earlier research proposing a trend of greater polyphagy in longer-tongued hawkmoths (Martins & Johnson 2013), our data clearly show that both long- and short-tongued hawkmoths are equally polyphagous and forage from many plant species. However, results from the long-tongued convolvulus hawkmoth may even be an underestimate in this study as the pollen from short-tubed flowers might not be represented in the pollen load as long-tongued hawkmoths could fail to make contact with the anthers of short-tubed flowers (Martins & Johnson 2013). Our results add support to earlier research proposing that non-native invasive plant species (such as *Lantana*) with long-tubed flowers which, although known to be predominantly butterfly pollinated (Negi *et al.* 2019) and not characteristically fitting in the typical moth syndrome, could be outcompeting native plant species by being more attractive to opportunistic long-tongued hawkmoth feeders (Johnson & Raguso 2016).

The repercussions for network ecologists in drawing comparative conclusions between networks derived from different data types (microscopy vs metabarcoding) would lead to misinformed decision making and should be cautioned against. Using microscopy alone produces a network three-fold less complex than is apparent, and underestimating the true number of ecological connections within a community is problematic. In a simple system where time is not a constraint, I advocate for the incorporation of both methodologies in providing a comprehensive network overview. This study follows others highlighting the need for a thorough understanding of complex ecological networks and their responses (Evans *et al.* 2016; Pornon *et al.* 2017), but to our knowledge this is the first community pollen-transfer study set in a South African context. The results from the metabarcoding analysis are encouraging as the potential applicability of this method in fields beyond pollination ecology

are still underexploited in a South African context (Elsaied *et al.* 2021; Pereira-da-Conceicoa *et al.* 2021).

Communities are dynamic and highly labile to external factors with species adjusting their interactions in context-specific ways. Understanding how they respond is likely to become important for species persistence (Hervías-Parejo *et al.* 2023; Valdovinos, Dritz & Marsland 2023). Particularly for protecting biodiversity hotspots, scalable methods such as metabarcoding will become critical for understanding the ecological processes that govern them.

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CHAPTER 5

A plant-pollinator network based on metabarcoding of pollen loads of flower visitors in a South African biodiversity hotspot

Running header: pollination network of eastern South Africa

5.1 Abstract

1. Metabarcoding is a valuable tool in ecological community analysis, particularly network reconstruction, in being able to solve several difficulties pertaining to time investment and network resolution, associated with network reconstruction using the conventional methods. With the advantage of speed and the potential of upscaling, metabarcoding has also shown to provide a more accurate, more complex, and more refined network than others constructed from microscopy of pollen-load or visitation data alone.
2. I used metabarcoding of insect pollen-loads to describe the network of interactions between plants and the pollinator community of Mount Gilboa, a species-rich grassland site in South Africa. DNA from pollen mixtures was isolated and sequenced against two primer pairs from the ITS gene regions in single runs on the Illumina MiSeq platform.
3. Pollen loads of 66 pollinator species revealed interactions with 172 flowering plant species. The reconstructed qualitative bipartite network showed a highly generalist and organised system of interacting partners, with pollinators being more generalist than their floral counterparts. Pollinators and plants interacted with an average of 10.23 and 3.92 corresponding partners, respectively. Plant diversity was high in comparison to pollinator diversity (network asymmetry = -0.45). Metabarcoding revealed an average 5-fold increase in the number of floral links detected per insect order. No pollinator

grouping (Hymenoptera, Diptera, Lepidoptera (neither nocturnal or diurnal), Coleoptera) contributed disproportionately to overall network coherence, nor was any grouping distinctly more specialized/generalized. Metabarcoding greatly increased the known diet breadth of several important pollinators within this community.

4. Species removal simulations revealed a network resilient to species loss in its overall topology with no sudden network collapse nor increased rate of secondary extinctions. Links per species and modularity were not severely altered by species removal although nestedness increased gradually in response to the removal of interacting trophic partners. Plant and pollinator visitation networks were highly robust, tolerating partner extinctions beyond 50% and up to ~80%, respectively, despite the removal of generalist species in both layers.

Keywords: diurnal pollination networks, South African pollinators, pollinator specialist, pollinator generalist, network cascade effects.

5.2 Introduction

The fate of biodiversity and associated ecosystem services in the context of global change is becoming increasingly precarious, especially in understudied regions home to high levels of diversity, such as the Maputaland-Pondoland-Albany biodiversity hotspot. Understanding this biodiversity is a primary step in assessing and managing the interactions which govern communities. The dramatic increase in research focussed on pollinator-services is a necessary parallel to the accumulating evidence of their global decline (Leather 2017) and the associated loss of ecological interactions (Valiente-Banuet *et al.* 2015). This is of particular importance in view of the effect that ever-changing environments continue to have on the natural world, and particularly so when considering the advancing Anthropocene (Sanderson, Walston &

Robinson 2018) and associated global climate change (Potts *et al.* 2010; Vasiliev & Greenwood 2021). Plant-pollinator networks describe the temporally and geographically constrained complex associations of mutualistic interactions between co-occurring flowering plants and their pollinators (Bascompte & Jordano 2013). As a consequence of the recognized importance of pollinators to agriculture and ecosystem health (Kearns, Inouye & Waser 1998; Deguines *et al.* 2014), research investment into plant-pollinator interactions and what influences them has increased (Porto *et al.* 2020). These networks are known to be inherently labile, with interacting partners changing in response to pollinator abundance and flowering phenology leading to high turnover in any one community (Valverde, Gómez & Perfectti 2016; CaraDonna & Waser 2020). Understanding how these ecological networks respond to external factors necessitates continued monitoring. Network metrics have been developed to describe how the interacting partners within communities are linked, the relative strength of these interactions, what the levels of specialization are, whether the interacting species form subnetworks of interacting 'modules', and whether the extent to which the network is robust, asymmetrical, and nested. The 'health' and resilience of pollination networks can be inferred from several network metrics including robustness, centrality measures, linkage, or connectance. Measures of interaction strength, paired difference index (PDI) which quantifies a relative measure of partner interactions, and 'd' are indices measuring the level of specialization (Dormann 2011), while indices such as centrality give an indication of network structure and stability (González, Dalsgaard & Olesen 2010). The use of metrics to describe ecological networks has also provided a means to decipher the structure of communities which can be used to interpret meaningful ecological and evolutionary mechanisms, such as functional complementary, mutualistic adaptation and coevolution of interacting partners (Blüthgen & Klein 2011; Lomáscolo *et al.* 2019; Thompson 2019).

Plant-pollinator community resilience

Besides the well documented decline in global pollinator species populations there is also concern over how entire pollinating communities will respond to environmental pressures as species are connected in networks of mutualistic and antagonistic interactions (Memmott *et al.* 2007). Past research centered mostly on describing ecological communities using conventional descriptors such as species richness yet these have been shown to be inadequate for detecting system perturbations (Tylianakis, Tscharntke & Lewis 2007; Schleuning, Fründ & García 2015). Global diversity loss is threatened by a more insidious danger. With the predicted species loss, there is a concern over the loss of ecosystem functions which may result in an accelerated biodiversity loss rather than a predictable linear decline (Valiente-Banuet *et al.* 2015). Although the loss of directly interacting trophic links is well recorded where entire systems have been eradicated, there is much uncertainly over a system which is in the initial stages of being degraded. Where this 'tipping point' is located remains unknown and contentious. Typically, hypothetical systems are modelled with the removal of the most linked species, or the species which is the most abundant. As an extinction debt is often experienced by species where there is a time delay between the effector and the species' ultimate disappearance, an arguably more realistic situation is where the response of stepwise removal of species comprising a community is modelled. This allows for the interaction between indirect links to be modelled in a more biologically realistic approach.

Utility of metabarcoding data in pollination networks

Considering the well-documented declines in many pollinator species, a better understanding of plant-pollinator relationships is urgently needed. Metabarcoding is a taxonomic identification method where species can be identified simultaneously from a mixed (bulk) sample which have been amplified by PCR targeting a chosen barcode and sequenced on a high

throughput platform (Taberlet *et al.* 2012; Cristescu 2014). Metabarcoding has been applied to the study of pollination networks (Bell *et al.* 2017; Pornon *et al.* 2017; Macgregor *et al.* 2019; Arstingstall *et al.* 2021). In particular metabarcoding has demonstrated its utility in being an invaluable tool in detecting rare and logistically difficult to study species (Bosch *et al.* 2009; Hawkins *et al.* 2015; Macgregor *et al.* 2019; Thomsen & Sigsgaard 2019). Not negating the inherent caveats of metabarcoding (Arstingstall *et al.* 2021), this high-throughput sequencing (HTS) method has advantages in efficiency and resolution (Lowe *et al.* 2022b) and is thus a powerful tool which can improve our understanding of ecological interactions.

Globally important biodiversity hotpots harbour disproportionate concentrations of species richness, many of which are characterized by high levels of endemism and vulnerability to extinction (Myers et al. 2000). Short-term climate stability and homogeneous landscape structure, such as is found in the Maputaland-Pondoland-Albany hotspot in South Africa, equates to a greater susceptibility to climate change (Trew & Maclean 2021). Global conservation efforts target biodiversity hotspots as they match the conservation planning requirements of irreplaceability and vulnerability (Myers et al. 2000; Mittermeier et al. 2011). These biodiverse zones are conventionally designated based on a species-centric approach (Myers et al. 2000; Ceballos & Ehrlich 2006). This is likely to offer a partial solution to conservation as efforts have primarily focused on vertebrate species richness, while omitting certain taxa, in addition to overlooking important facets of biological diversity, such as phylogenetic diversity (Davies & Cadotte 2011; Daru, van der Bank & Davies 2015). Strong taxonomic and geographical sampling biases points towards an incomplete measurement of the diversity present in southern Africa (Hoveka *et al.* 2020). Further, the recent realization of the loss of functional diversity provided by interactions increases the urgency for conservation efforts to include community and function-centred approaches. Without being able to measure biodiversity adequately, conservation efforts will not meet their target. Metabarcoding, enabled

by fine-scale resolution and rapid analysis turnover (Mathon *et al.* 2021), is a current tool that can address this knowledge gap in one of South Africa's hyper-diverse and insufficiently protected regions (Gous *et al.* 2021).

The aims of the study were two-fold: firstly, I aimed to use metabarcode data to describe the network of plant-pollinator interactions in an environment situated in a biodiversity hotspot. Specifically, I asked 1) how metrics of the overall network differed from those obtained at other sites using comparable methods, 2) what levels of specialization characterize plant and pollinator species in the network, 3) whether metabarcoding would reveal previously unknown interactions and 4) to investigate network stability and how perturbations in the form of removal of the most-connected pollinator and plant network nodes might affect the overall network.

5.3 Materials and methods

Study site

Fieldwork sampling took place at Mount Gilboa summit, Karkloof (MONDI Forests Ltd) (29° 19′S, 30° 17′E), Karkloof Nature Reserve, South Africa. Mount Gilboa Private Nature Reserve is a protected area that forms part of the Karkloof Nature Reserve depicted by grasslands classified as Drakensberg Foothill Moist Grassland (Mucina & Rutherford 2006). Karkloof Nature Reserve falls within a center of floristic endemism (CFE) where Mount Gilboa is situated, is characterized by extremely low levels of formal protection and poor ecological connectivity, coupled with high levels of land transformation and intensive utilization (Carbutt, 2023).

Reference DNA barcode construction

I constructed a plant reference library for DNA sequences by removing leaf material from 129 plant species flowering at the study site from 2020-2021 (Table S5.2). Voucher specimens for each species were curated and are stored at the University of KwaZulu-Natal herbarium (NU). DNA was extracted from silica-dried leaf tissue retrieved from the voucher specimens, and sequenced at Genomyx Laboratories, Johannesburg. All specimen details are available online on the BOLD© repository under the project "Foraging specialisation of flower visitors in a community construct" (FFVCC) (https://www.boldsystems.org/). ITS primers were amplified for every specimen and sequenced using Sanger sequencing. The plant ITS region was amplified using the primer pair ITSAB101F (5'-ACGAATTCATGGTCCGGTGAAGTGTTCG-3') and 26SE (5'-TAGAATTCCCCGGTTCGCTCGCCGTTAC-3') (Sun *et al.* 1994). Of the 129 plant species gathered for reference sequences, 72.1% amplified successfully. Final sequences were compiled into FASTA files for database construction.

Sampling of flower visitors

Flower visitors were collected between 2019 and 2021, spanning two summer seasons. Transects of the flowering community were walked at weekly intervals during peak pollinator activity in the mornings until midday. Biweekly surveys were conducted during the winter months. Only insects that appeared to be actively foraging were netted and the plant species on which the animals was collected was recorded, by writing down the sample number corresponding to the herbarium voucher which could later be confirmed or identified by experts. Each specimen captured was placed in a separate sterile Eppendorf tube to prevent pollen cross-contamination between insects. Insects were stored in ice-filled polystyrene cooler boxes in the field, and then transferred to −20°C freezers in the laboratory until further

processing. I sorted the flower visitors into families and morphospecies. Morphospecies were identified and sorted based on visual comparisons and assigned a species identity to the best taxonomic resolution with the help of accessible keys. I used geographical ranges from GBIF (https://www.gbif.org/), morphological and genetic barcode identities. Several morphospecies groupings were only assigned a more precise identity after being processed using various channels of information (photographs, genetics barcode from BOLD and GenBank, species range). In total 65 of the 68 pollinator morphospecies could be assigned species-level identities based on their genetic barcodes, two samples were discarded as their barcodes did not match the initially assigned specimen identity, and the morphological identity (*Hyles livornica*, Sphingidae) for a single hawkmoth specimen was chosen over a barcode assigned identity (*Rhodafra marshalli*, Sphingidae).

DNA metabarcoding

The DNeasy Plant MiniKit (QIAGEN®) was used for all DNA extractions. The standard protocol for extracting genetic material was followed, with the following modifications: volumes of buffer AP1 and RNase A stock solutions were scaled up by four times due to the size of the specimens. DNA for each of the pollinator specimens was extracted separately. Pollinators were manually crushed- all items used in the crushing process were autoclaved prior to use. Individual specimens were crushed whole rather than removing pollen loads from specific anatomical positions using a precision method as a complete overview of the visitation network was the target, as well as attempting to avoid a potentially incompletely sampled pollinator. Additionally, in 39 cases, there was only a single representative specimen for the morphospecies so assisted by *post-hoc* verification of genetically assigned identities and photographed images, I chose to make use of barcode identifications. Quantitation of sample

DNA concentration was performed with the Qubit BR DNA Assay kit and Qubit Flex Fluorometer (Invitrogen/ThermoFisher). As the principal aim was to determine host range at the species level and because the cost of metabarcoding is high (project budget prohibited processing the pollinators individually), morphospecies were pooled for DNA extraction. Morphospecies that were collected in either the 'wet' or 'dry' season were kept as separate samples. Extracted DNA was diluted, and equimolar pooled samples were prepared according to the manufacturer's protocol for the appropriate sequencer. Pooled DNA samples were couriered to MRDNA (Texas, USA) for sequencing which was performed on an Illumina MiSeq system (Illumina, Inc., San Diego, CA, USA). The length of standard plant gene regions is currently beyond the reach of many HTS technology platforms (e.g., the Illumina MiSeq platform) that are limited in read length (Marquina, Andersson & Ronquist 2019). Pollinator samples were then sequenced to target the internal COI primer, mlCOIintF (cytochrome oxidase subunit I) (Hebert *et al.* 2003) such that a species name could be attached to the genetic barcode. The use of shorter DNA fragments, called mini-barcodes (100–250 bp), may be used in place of full-length barcodes to overcome DNA degradation for samples with poor DNA preservation (Hajibabaei *et al.* 2006; Meusnier *et al.* 2008). Therefore, the ITS plant primer pairs chosen for identifying pollen in this study were ITSp3/ITSu4 and ITSp5/ITSu2 (Cheng *et al.* 2016), which have high amplification success rates across land plant groups (Cheng *et al.* 2016).

Pollen DNA bioinformatics

Demultiplexed FASTQ files, which are text files containing raw data from paired-end sequences, were run through the DADA2 algorithm via QIIME2/2022.2 (Bolyen et al. 2019; Xia & Sun 2023) in an initial quality-control check where chimeras are removed; amplicons are filtered; primers are trimmed; forward and reverse reads are truncated, and paired-end reads

are merged. Reference sequences of all amplicon sequences variants (ASVs) for both ITSP3 (n = 611) and ITSP5 (n = 3148) were manually queried against GenBank (Benson *et al.* 2015) and Barcode of Life Database (Ratnasingham & Hebert 2007).

ASV clusters identifying the same species were merged into single taxonomic units by summing reads (n = 18 and 154 species for ITSP3 and ITSP5, respectively). A further threshold was applied to both ITSP3 and ITSP5 ASV clusters. ASVs that fell below 50 and 100 sequence counts for ITSP3 and ITSP5 respectively, were not included in further analysis. This lower threshold was set to prevent potential contaminations or happenchance visits as far as possible, which were not representative of true mutualisms. Counts of values above this threshold I took as proof of a true link to that species. These limits are low compared to other metabarcoding studies (Pornon *et al.* 2017), yet were realistic given the range of sequence counts for each of the plant barcodes across all pollinators (Bonin, Guerrieri & Ficetola 2023; Westerduin *et al.* 2023). Filtering the raw data does impact the descriptive network properties, yet it is recognized as an important precaution which results in better description of interactions, therefore leading to reliable and uncompromised ecological interpretations (Tommasi *et al*. 2021).

Taxonomic assignment of amplicon sequence variants

For generalist barcode markers, high similarity thresholds (96%-99%) are generally appropriate for taxonomic assignment of animals, while lower limits are acceptable for more specific markers (85%–96%) (Govender *et al.* 2022; Bonin, Guerrieri & Ficetola 2023; Westerduin *et al.* 2023), yet selecting the most appropriate threshold for taxonomic assignment requires careful examination given the research aims. Particularly for plants, where species boundaries are inherently less easily and well defined in comparison to animals (Fazekas *et al.* 2009), extensive *post-hoc* refinement of species identities is required. This downfall is further compounded by the lack of a comprehensive and well curated plant barcode database (Cheng

et al. 2016)), in which southern Africa's endemic plant diversity is particularly poorly represented (Hoveka *et al.* 2020). Using a threshold of 95% sequence similarity yielded 144 detected plant species, but this declined to 87 species at a stricter 99% threshold. Twenty-eight species fell between 75% and 95% sequence similarity. Of the 28 plant species identified at the lowest threshold, several are known to occur and flower in the area. Using genetic barcodes led to the correct identity of 44 (25.6%) and 145 ASVs (84.3%) to species and genus level, respectively. The remaining plant ASVs could not be identified below family level. Species detected by metabarcoding, irrespective of the sequence similarity, that were out of geographical range (absent from GBIF database) were assigned to the lowest taxonomic resolution possible. For all taxonomic assignments, whether for pollen or pollinator, I used the sequence similarity in combination with the GBIF website (https://www.gbif.org/) as well as local knowledge (pers. coms.) on occurrence records to inform the final species assignment. ASVs were given the lowest taxonomic assignment possible when all channels of information agreed. Ideally a barcode (such as I had for *Hypocharis radicata*), when searched against the NCBI database, should yield a precise match with 100% query cover and high percent identity (100%) to the query sequence. When checked against GBIF for occurrence records as well as being collected and represented in the projects pollen reference database, this is a clear match. Unfortunately for geographic regions where the barcode database poorly reflects the rich local diversity, this is not always the case. In some cases (such as I had for *Nerine bowdenii*) where the top NCBI match (94.13) is less than <95% despite a 100% query cover and the species occurring generally in the region, but where no recorded individual is known to flower in the near vicinity, I hence chose to allocate this entity to *Brunsvigia radulosa*, which despite having a lower NCBI sequence percent identity (90.93%), is well recorded on Mount Gilboa within close proximity to where pollinator sampling was done. In this case, both geographical and

local knowledge informed the chosen identity despite not being the first and top NCBI blast match.

DNA was extracted from 301 pollinator individuals belonging to Hymenoptera (n = 135), Diptera (n = 70), Coleoptera (n = 13) and Lepidoptera (n = 83). These were initially pooled into 80 morphospecies with Hymenoptera comprising 14 morphospecies, Diptera 23 morphospecies, Coleoptera 5 morphospecies and Lepidoptera 38 morphospecies. After confirming morphospecies identities using all available information sources, several of the classified morphospecies samples were 'duplicates' and their data were therefore combined, thus reducing the network pollinator nodes from 80 to 66 species. Forty-five samples consisted of individual specimens, while the remaining samples contained between two and 44 individuals pooled into a single sample.

When considering network completeness, there is a tradeoff between sampling completeness and precise accuracy. The effects of seasonality (typically wet summer months are when abundance is highest and can therefore be more completely described) and spatial sampling cannot be ignored. In plant–pollinator networks there is distinct variation in plant and pollinator abundance and richness throughout and across years. Pooling across seasons can address some of these sampling issues, but it also can introduce bias in network metrics (Ings *et al*. 2008). As I collected individuals randomly, I chose to pool like-species together to decrease sampling bias and to create a more complete network to determine host range at the species level.

Species and network descriptors

To characterise the matrix, I used the bipartite package 2.18 in R v.4.3.1 statistical environment (Dormann *et al.* 2009; Dormann 2011) to estimate the following set of descriptive indices: number of plant species, number of pollinator species, total number of binary links observed

between plants and pollinator species (degree). Species strength provides a quantitative measure of the importance of a species within the network. It is the sum of dependencies of each species and provides an index of the species' relevance across all its interacting partners (Bascompte, Jordano & Olesen 2006). It is calculated as the sum of the dependencies of all the partners on that species (Bascompte, Jordano & Olesen 2006). As a measure of interaction strength, I determined species' linkage level, connectance, interaction push-pull (asymmetry), and two measures of specialization, d' and closeness centrality (CC). Species interaction asymmetry is an index of the unevenness between the effect that a species has on its partners and the dependence of that species on its partners. The push-pull index (Vázquez et al. 2007) ranges from -1 to 1 with positive values indicating a "pusher" species with a disproportionate influence over its interacting "puller" partners which have negative values indicating a weaker reciprocal effect. To evaluate the generalization-specialization continuum, I used generality, d', and number of links as a measure of specialization, and for a measure of network centrality, I used weighted closeness and betweenness centrality. The metric d', ranging from 0 (generalist) to 1 (specialist) and measuring the level of specialization of a species, is a scale-independent index to characterize specialization (Blüthgen, Menzel & Blüthgen 2006). Centrality indices in a network describe the position of an interacting partner within its network. Closeness centrality (CC) measures the path length of a node to all other nodes in the network (Freeman 1979), i.e. nodes with high values hold weight over the network and can rapidly affect other nodes. Betweenness centrality (BC) is complementary to CC but quantifies the importance of a species as a connector to otherwise sparsely linked areas of the network (Newman 2004; González, Dalsgaard & Olesen 2010).

Nestedness describes the hierarchical organization where specialists interact with species that form a subsets of the species with which generalists interact (Almeida-Neto *et al.* 2008). ie. there is an "over-representation of shared mutualistic interactions between specialist and

generalist species" (Song, Rohr & Saavedra 2017). Low values of nestedness correlate to a highly nested and organized network, whereas high values indicate no organizational pattern ie. chaos. I used the weighted NODF (Nestedness metric based on Overlap and Decreasing Fill) index as a measure of nestedness (Almeida-Neto *et al.* 2008). SPSS 29 (IBM) was used to compare between pollinator order for total number of links to floral partners using one-way ANOVA. Data were log transformed.

Stepwise species removal

I simulated the response of network properties to the loss of species from each of the trophic layers beginning with the highest species' strength node. I chose a suite of metrics to describe the overall network stability (Bascompte, Jordano & Olesen 2006). These include linkage density and links per species as a measure of connectivity; modularity as a metric of compartmentalisation, and nestedness to infer stability. The stability of the network is inferred by robustness, extinction slope, and vulnerability metrics. I also tracked the number of nodes lost in the alternate trophic level due to stepwise species removal. Robustness ranges between 0 and 1 and corresponds to the resilience of the overall system. An index of $R < 0.5$ corresponds to a weakly resilient network where the removal of a small proportion of species results in a high number of secondary extinctions (Burgos *et al.* 2007; Rivera-Hutinel *et al.* 2012). A highly robust network has R-vales greater than 0.5. The extinction slope refers to the secondary extinction sequence in that trophic level, following extermination of species in the other trophic level. Vulnerability is a weighted index of the mean effective number of pollinator species per plant species. Modularity is a network property describing the extent to which densely connected nodes can be separated into distinct components. Linkage density is simply a weighted index of the diversity of interactions per species (Bersier, Banašek-Richter & Cattin

2002). Networks with ‘stronger’ values of these indices show greater resilience which is thought to buffer against the effects of species’ loss (Gaiarsa & Guimaraes Jr 2019).

5.4 Results

Pollen DNA high-throughput sequencing

The sequence counts of both ITSp3/ITSu4 and ITSp5/ITSu2 assigned to the flowering plants class Magnoliopsida varied between samples. A total number of 2,402,164 sequence counts were obtained for ITSp3/ITSu4 and 3,132,641 for ITSp5/ITSu2 across the 84 mixed pollen samples. This is on average 56,522 reads for ITSp3/ITSu4 and 73,709 reads for ITSp5/ITSu2 per sample, respectively. A total number of 2,759,553 sequence counts were obtained for COI, with an average of 32,852 sequence counts per sample. Median sequence lengths for both forward and reverse reads for ITSp3/ITSu4 were: forward median length = 293 bp; reverse median length = 301 bp. Median sequence lengths for both forward and reverse reads for ITSp5/ITSu2 were: forward median length = 271 bp; reverse median length = 279 bp. Median sequence lengths for both forward and reverse reads for COI were: forward median length = 225 bp; reverse median length = 224 bp. A total of 41.5% and 51.4% sequences of ITSp3/ITSu4 and ITSp5/ITSu2, respectively, identified to Magnoliopsida.

Of the insect pollinators included in this study, all carried pollen detectable by metabarcoding methodology. I recorded 675 mutualistic interactions occurring between 66 pollinator species and 172 flowering plant species. The most prolific flower visitor was *Precis Octavia* (Nymphalidae, Lepidoptera) which visited 49 plant species, while *Lampides sp.*, (Lycaenidae, Lepidoptera) only reflected one plant species in its pollen load. The three most generalist pollinator species, and three top plant ‘hub’ species are given in Table 5.1. Although the correlation was moderate (Pearson $r = 0.496$, $p < 0.002$), it is worth noting that pollinators can appear particularly generalist because of the pooling of individuals. This caveat can

artificially increase host ranges, yet at the species level, is it still possible to determine host range. Additionally, *Precis octavia* was a top generalist with 49 floral links, yet only 4 individuals were pooled. In contrast *Apis mellifera*, a well-known generalist, was linked to just less than half the number of floral partners despite 79 individuals being pooled (TABLE 5.1.).

Pollinators had an average of 2.0 floral links, while plants were linked with 2.6 pollinating species. When pooled, Diptera contributed the greatest number of diurnal plant-pollinator interactions (32%), although the combined diurnal and nocturnal Lepidoptera floral-links outweighed this (46%) (Figure 5.1). Nocturnal lepidopterans (52.8%) contributed slightly more than diurnal lepidopterans (47.2%) in terms of plant-pollinator links (Figure 5.1). Diptera, Hymenoptera and Coleoptera links accounted for 32%, 16% and 6% respectively (Figure 5.1). This contributed to a network which was modestly modular (0.38) and robust (0.63), showing relatively high linkage density (14.89), and tending towards being unnested (nestedness =5.48). Top specialist and generalist species for both the pollinator and plant network were identified. Pollination by Lepidoptera comprised the greatest proportion of links in the network (46%, Figure 5.1). No grouping of pollinators was distinctly more (or less) specialized or centrally (or remotely) placed in the network (Figure 5.2 and Figure 5.3). Pollinator cohorts contained both specialized and generalised interacting nodes (Figure 5.2).

TABLE 5.2. Top generalist and specialist pollinators and plants. The top three plant and pollinator species by interaction count (degree) are shown below. There are 172 plant species in total, so *Precis octavia* with a total of 49 interactions is connected to ~30% of the flowering community. In top generalist plant family, Scrophulariaceae, *Hebenstretia* sp. is connected to 36 out of 66 possible pollinator species. Many plant species were only represented by links to a single pollinator and so top pollinators are shown only.

Network layer	Family	Species (n)	Degree	Number of specimens pooled
Pollinators	Nymphalidae	*Precis octavia*	49	4
	Muscidae	Muscidae sp. 1	34	8
	Apidae	*Apis mellifera*	27	79
	Noctuidae	Noctuidae *sp. 5*	25	3
	Nemestrinidae	*Prosoeca* sp. 2	25	10
	Nymphalidae	*Pseudonympha trimenii*	23	8
	Anthomyiidae	*Anthomyiidae* sp. 1	22	4
	Pieridae	*Pontia helice*	21	2
	Nemestrinidae	*Prosoeca* sp. 5	16	6
	Nemestrinidae	*Prosoeca* sp.	16	9
	Apidae	Amegilla sp.	15	1
	Noctuidae	Noctuidae sp. 9	15	3
	Lycaenidae	*Aloeides trimeni*	15	2
	Tabanidae	*Philoliche aethiopica*	15	13
	Halictidae	*Lasioglossum* sp. 2	14	3
	Scarabaeidae	*Atrichelaphinus tigrina.*	13	3
	Megachilidae	*Coelioxys* sp. 2	12	2
	Noctuidae	Noctuidae sp. 8	12	2
	Pieridae	*Belenois aurota*	12	6
	Anthomyiidae	*Anthomyiidae* sp. 2	11	4
	Syrphidae	*Eristalinus taeniops*	10	1
	Syrphidae	Syrphidae sp.	10	1
	Megachilidae	Megachile sp.	10	1
	Sphingidae	*Agrius convolvuli*	10	1
	Sphingidae	*Hippotion celerio*	9	11
	Notodontidae	*Notodontidae* sp.	9	1
	Noctuidae	Noctuidae sp. 11	9	1
	Sphingidae	*Nephele comma*	9	3
	Halictidae	*Lasioglossum* sp. 1	9	29
	Nymphalidae	*Vanessa cardui*	9	3
	Noctuidae	Noctuidae sp. 3	8	1
	Noctuidae	*Cucullia* sp. 2	8	1
	Syrphidae	*Betasyrphus intersectus*	8	1
	Scarabaeidae	*Cyrtothyrea marginalis*	8	1
	Geotrupidae	*Tribe Hopliini*	8	7
	Scarabaeidae	*Trichostetha fascicularis ssp. prunipennis*	8	1
	Noctuidae	Noctuidae sp. 4	7	1
	Syrphidae	*Eristalinus fuscicornis*	7	1
	Megachilidae	*Coelioxys* sp. 1	7	6

	Apideae	Hymenoptera sp.	7	1
	Geometridae	*Pseudolarentia megalaria*	7	1
	Tabanidae	*Philoliche bivirgulata*	6	2
	Nemestrinidae	*Prosoeca umbrosa*	6	1
	Noctuidae	*Cucullia* sp.	6	1
	Noctuidae	Noctuidae sp. 6	6	1
	Sphingidae	*Basiothia schenki*	6	15
	Megachilidae	*Heriades* sp.	6	4
	Muscidae	Muscidae sp. 2	5	3
	Nemestrinidae	*Prosoeca robusta*	5	1
	Diptera sp.	Diptera sp.	5	1
	Noctuidae	Noctuidae *sp. 1*	5	1
	Sarcophagidae	*Sarcophaga* sp. 2	5	1
	Noctuidae	Noctuidae sp. 2	5	1
	Scarabaeidae	*Leucocelis amethystina*	4	1
	Sphingidae	*Hyles livornica*	4	1
	Nymphalidae	*Aeropetes tulbaghia*	4	1
	Noctuidae	*Thysanoplusia* sp. 1	4	1
	Noctuidae	*Noctuidae* sp. 10	4	1
	Sarcophagidae	Sarcophagidae sp.	4	1
	Nemestrinidae	*Prosoeca* sp. 12	3	1
	Sarcophagidae	*Sarcophaga* sp. 1	3	1
	Hesperiidae	*Gegenes niso*	3	1
	Noctuidae	Noctuidae sp. 7	2	2
	Apidae	*Anthophora* sp.	2	5
	Geometridae	Geometridae sp.	2	1
	Lycaenidae	*Lampides* sp.	1	1
	Scrophulariaceae	*Hebenstretia* sp.	36	n/a
Plants	Scrophulariaceae	*Zaluzianskya* sp.	34	n/a
	Iridaceae	*Watsonia lepida*	29	n/a

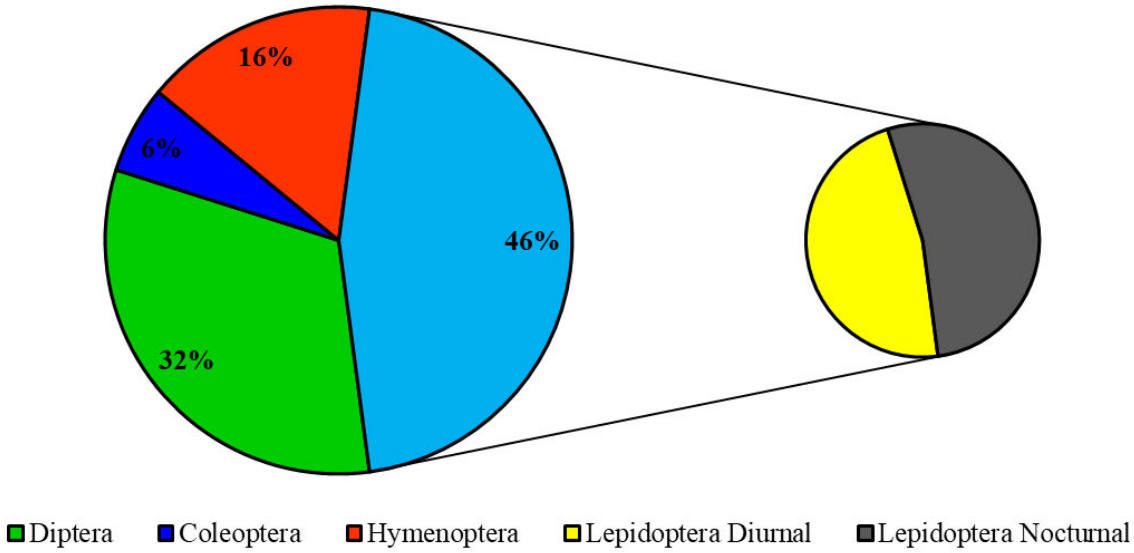

FIGURE 5.1 Species degree (sum of links/species) grouped by pollinator order.

Species and network descriptors

Pollinator species varied greatly in their relative contribution to visitation network structure as given by floral links. In each order, there was a range of total floral links, with no pollinator group outperforming another (Figure 5.3). The strength and degree of the pollinator species is shown in Figure 5.2. Correspondingly, the most-linked pollinator species, Nymphalidae, *Precis Octavia*, was also involved in the strongest interactions, had the greatest species strength, is a central node to the network, yet is not the top connector species (Figure 5.2). The top connector node in the network was *Muscidae* sp. 1, also having high species strength, linkage and influence on the network topology. Neither of these species, despite showing high linkage and connectance, were the most generalised. It is interesting that diurnal Lepidopterans showed to be both the most (Lycaenidae, *Lampides sp.*) and least (Geometridae, *Geometridae sp.*) specialized (Table 5.1; Figure 5.2). Among insect orders, Lepidoptera were the most generalist network node with connections to 119 floral partners (d' = 0.33) (Table S5.1; Figure S5.1d).

Coleoptera were linked to 28 plant species and were thus the least specialized (d' = 0.41) (Table S5.1; Figure S5.1b). When separating out Diptera into long- and short-tongue flies, short-tongue flies were linked with 124 plant species (average = 10.3) while long-tongued flied had links to 92 plants (average of 11.5). Interesting, airborne pollen (*Poaceae*) species were represented in the pollen loads, yet these may represent true links as some species are known to visit wind-pollinated plants (Pornon *et al.* 2016; Pornon *et al.* 2017; Bertrand *et al.* 2019; Lowe *et al.* 2022b). The number of floral links detected by metabarcoding increased by an average of 5 times from that of 'observational' data gathered at sampling events (Figure S5.5). The greatest, and least, discrepancy between these methods was evident in Lepidoptera (8.5) and Hymenoptera (1.2) (Figure S5.2). Although not significant (F=0.287, p=0.835, df=3), by order, Hymenoptera (mean = 10.90; 95% CI = 5.6-16.2) had the most floral links, followed by Diptera (mean = 10.80; 95% CI = 7.0-14.6), Lepidoptera (mean = 9.97; 95% CI = 6.9-13.0) and Coleoptera (mean = 8.20; 95% CI = 0.7-15.7).

I analyzed species asymmetry with the push-pull index. Most plant species were "pullers" (had negative index values), indicating they had a weak effect on their pollinators and were highly dependent on them. Accordingly, most pollinator species were "pushers", influencing strongly their floral counterparts while being affected by them minimally (Figure 5.2). The majority of species had BC values > 0 indicating that they play a key role as connectors. 52% of plants in the network (BC>0) played a key role as connectors. This high level of centrality and generalism is important to the overall networks functioning, structure, and resilience, and may be integral to network cohesiveness. Only three species of pollinators- Nemestrinidae, *Prosoeca* sp. 12; Lycaenidae, *Lampides sp.* and Noctuidae sp. 7- were not considered connector nodes in the web and these corresponded to areas of 'stability' in the 'network collapse simulation' as their removal was inconsequential to the network stability (Figure 5.4). These species were also amongst the most specialized in the web. Tabanidae, *Philoliche bivirgulata*,

despite being comparatively poorly connected and similarly specialised, had a strong "pulling" effect on the overall network.

Stepwise species removal

I simulated the response of network properties to the loss of species from both the pollinator and plant trophic levels. To simulate the 'worst' case scenario, I removed species according to their species' strength index. Removing pollinator species resulted in more rapid, albeit steady, decline of interacting plant partners. The removal of the first several highest strength plant species led to a rapid decline in the vulnerability metric and linkage density indices of the pollinator network, despite the number of pollinator partners remaining stable. Linkage density in both levels expectedly decreased as the network decreased in size with the removal of interacting partners. Network nestedness temperature increased with the removal of both pollinators and plants such that both networks became markedly less organised (chaotic) with the accrued removal of interacting partners. The loss of network (nestedness) structure was more severely experienced in the pollinator trophic level after ~a third of the plants had been removed. This corresponded with a greater rate of loss of pollinator species. Plants experienced greater secondary extinction rates than the pollinating trophic level. This should be anticipated given the greater generality in the pollinator-level in comparison to the lower-level nodes. Biodiversity naturally decreased with the loss of species, but in the simulation, most network properties and overall structure tended to remain stable. That is, a sudden collapse of network structure in the simulations of species loss did not occur. Both trophic levels were correspondingly robust to secondary extinctions, with robusticity declining rapidly only as corresponding node removal accumulated until a threshold was ultimately reached.

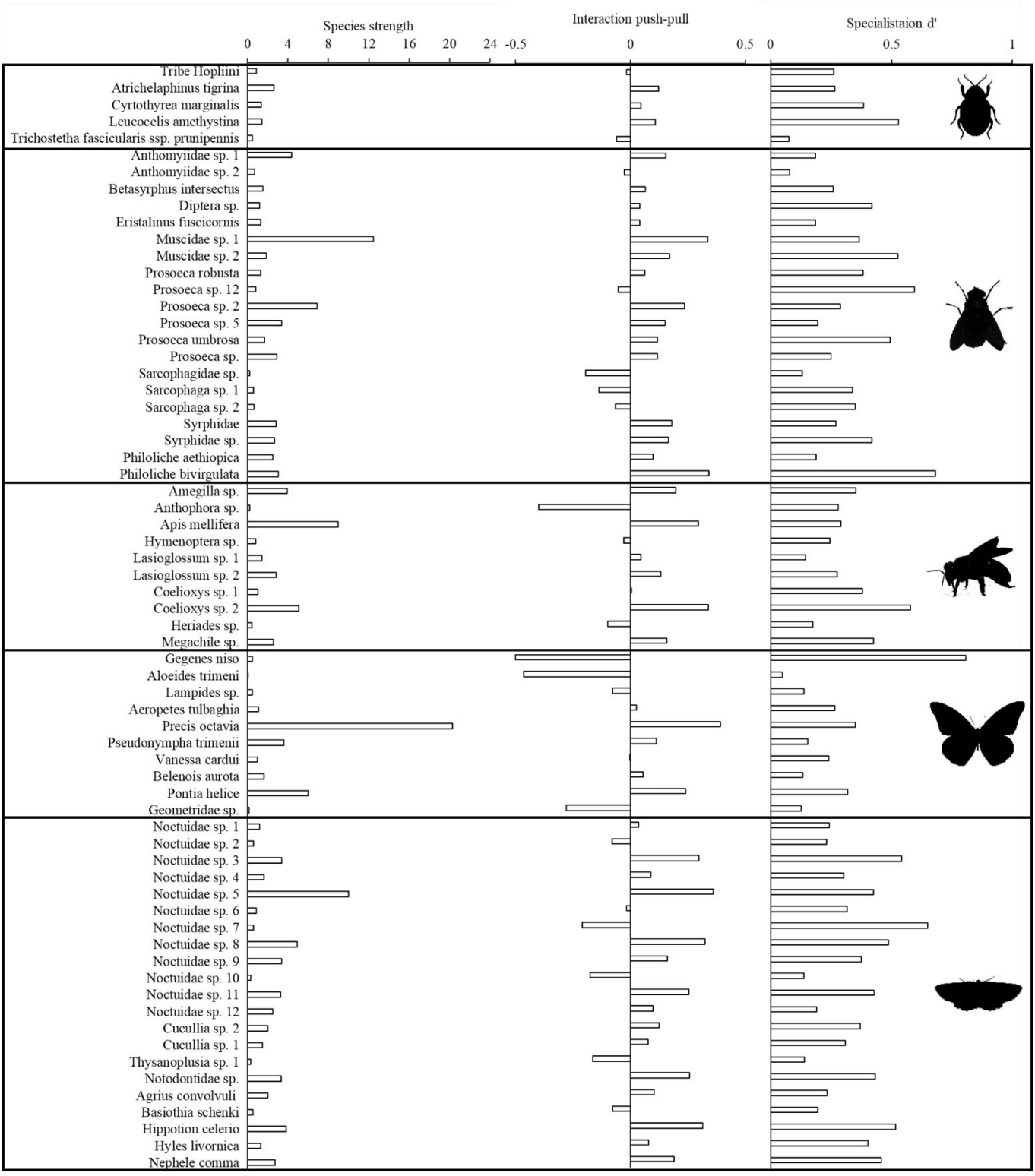

FIGURE 5.2 Specialization metrics for each species given by d', species strength and interaction push-pull.

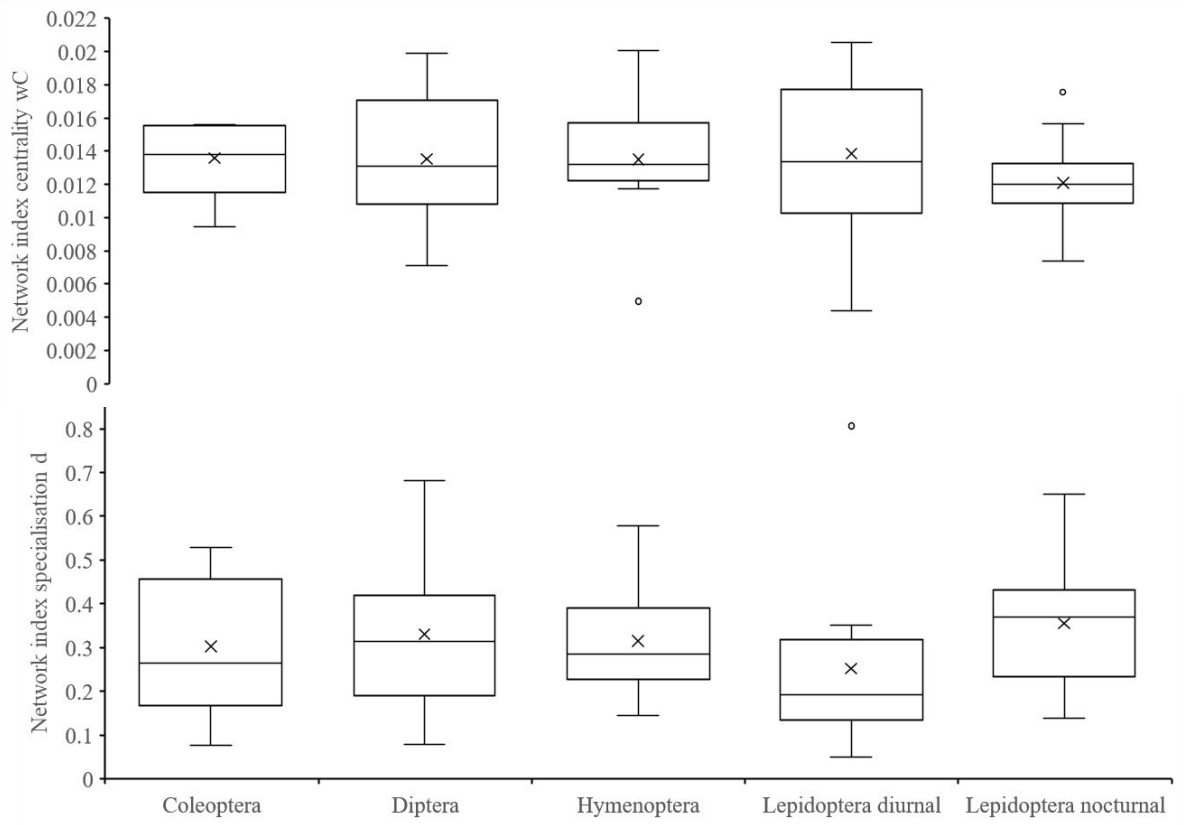

FIGURE 5.3 Spread of data across major pollinator cohorts for two measures of specialization: weighted closeness and d'. The lower and upper box boundaries represent 25th and 75th percentiles, respectively, line inside box median, outliers and the mean are represented by '○' and 'x'.

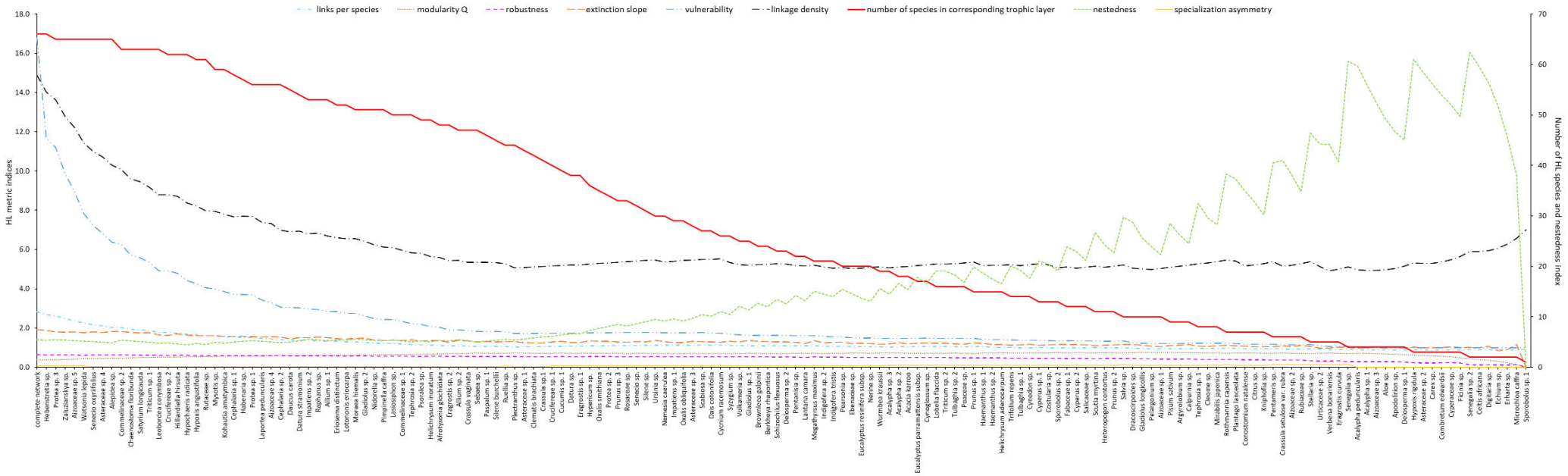

FIGURE 5.4 Network metrics in response to simulations of species loss from the pollinator trophic levels.

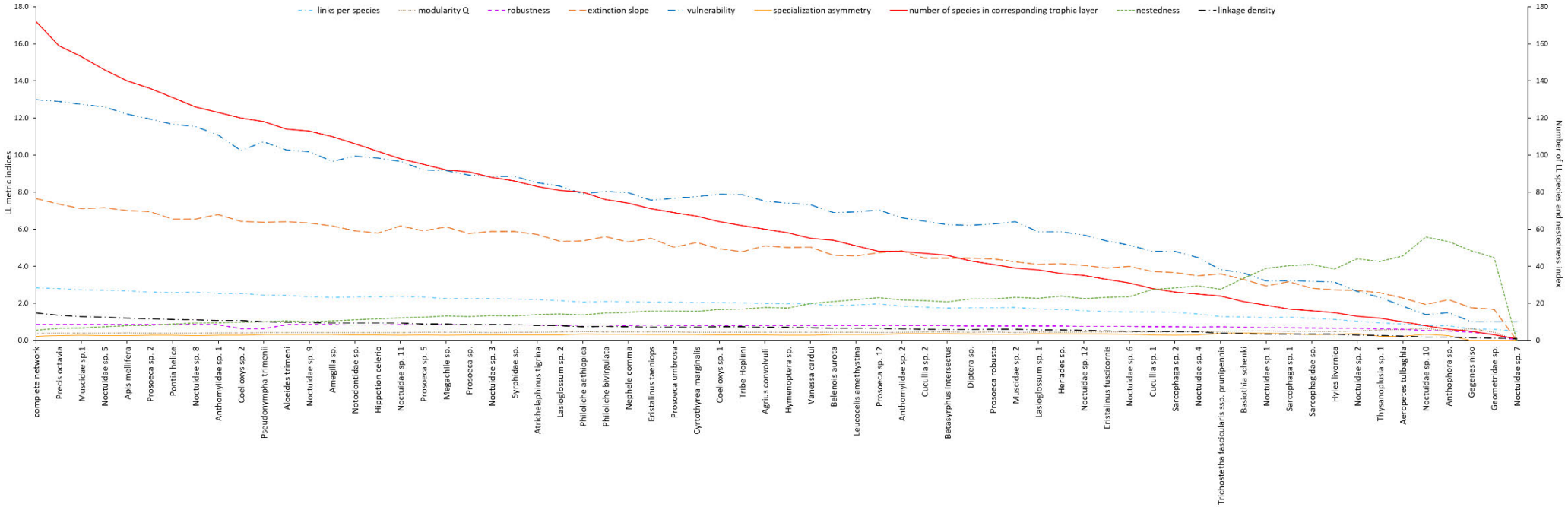

FIGURE 5.5 Network metrics in response to simulations of species loss from the plant trophic levels.

5.5 Discussion

I used metabarcoding to describe the network of plant-visitor interactions in a grassland community in the Maputaland-Pondoland-Albany region, one of South Africa's biodiversity hotspots. Although not all plant-visitors are effective pollinators, and flower visitation alone is a poor proxy of the service of successful pollination (Theodorou *et al*. 2016), the presence of pollen DNA does provide a closer, more convincing floral link. In line with my first aim, using metabarcoding data I described the diverse plant-pollinator network of Mount Gilboa, Karkloof, South Africa as one that is highly diverse, and robust against extinction cascades for the pollinator- and plant trophic level, respectively. Negative web asymmetry indicated greater diversity of lower-trophic level species which corresponds to the pollinator-level being supported by many plant species, thus being more robust. On average, pollinator species shared many more plant species (1.88) than the reverse (0.36), leading to a higher generality within the pollinator (16.8) than the plant layer (12.9). However, sampling of a natural community is likely not to be fully representative of the entire network, and therefore some interactions may be undetected and thus metrics could be underrepresented by false negatives in an interaction matrix (Blüthgen 2010). Yet, diet breadths reported here, and the levels of generalism, are larger than previously reported. Using metabarcoding to describe diet breadths was increased by an average of 5-fold in comparison to observational data. Additionally, due to the inability of plant primers to distinguish easily between closely related and rapidly radiating plant species (Fazekas *et al.* 2009), some interactions may have been overlooked which may have the potential to impact such network metrics. South African pollination systems are purported to have remarkably high levels of specialization, with many plants often relying of pollination by a single pollinator (Johnson & Steiner 2003; Johnson 2004). In contrast, the use of metabarcoding substantially increases the link between pollinators and plants. In my metabarcoding network, plants had links to 3.92 pollinator species, and pollinators in turn visited on average 10.23 plant species. I assessed the levels of generalism and specialization in

the network using the d' metric and the number of links between species. Specialization given by d' has been critiqued as the visits from a particular pollinator may not be unexpected given its general importance as a plant visitor within a community ie. A plant with many pollinators may still return a high d' because many of its pollinators may not be recorded on any other plant (Pauw & Stanway 2015). I did not find such a clear discord between the indices. Lycaenidae, *Lampides sp.*, was the most specialized pollinator node in the network as predicted by both d' and the number of links. However, the most generalist species, Nymphalidae, *Precis Octavia*, though being linked to 49 plant species, did not have the lowest d' value. And several of the species with the fewest links did not always have the highest d' value. Here I also pooled data from morphospecies which were sampled across two flowering seasons. Although this may lead to the creation of 'forbidden' links due to seasonality and phenological uncoupling (Olesen et. Al 2011), no links detected in the dry season were unique (i.e., They are duplicates of interactions occurring in the wet season). The use of qualitative data in networks has been cautioned against due to rare taxa being overstated and abundant taxa devalued (Lowe *et al.* 2022b). However, to avoid any bias caused by possible differences in pollen retrieval, DNA extraction, amplification and sequencing I used qualitative data (presence/absence) for the network analysis and investigation of differences in pollen loads (Lucas *et al*. 2018). In spite of using qualitative data, metabarcoding significantly improves the spatiotemporal observation window of pollination interactions (Pornon *et al.* 2016), and the result of understanding broad diet breaths of pollinators and the levels of specialization and generalization within a network, is still achievable (Petanidou *et al.* 2008; Bosch *et al.* 2009). The promise of linking sequence counts to plant-pollinator interaction strength holds great promise for future metabarcoding of ecological interactions. Many species which appear to be specialists in one season may be keystone generalists another year which then also influences overall network structural parameters, such as generalization (Petanidou *et al.* 2008). Greater levels of generalization can indicate a higher potential for functional redundancy amongst species which is valuable in

assigning conservation priority (Pornon *et al.* 2017). As my work was primarily an analysis at the community level, pooling of this data provides the clearest overview of the complete network. Interestingly all insect orders were similarly generalist in behaviour. High levels of generalization within the network are thought to be vital to its structure, functioning and persistence. Long-proboscid fly pollination is also known to be a specialized system with pollinators visiting plants with a long "cylindrical floral tube … a perianth of specific colours and marking, a floral reward of nectar, and lack floral fragrance" (Goldblatt & Manning 2000). Yet here long-proboscid flies were linked to many plant species not limited to this pollination syndrome (e.g. Many Asteraceae species). Similarly, long-tubed flowers of *Zaluzianskya* (Scrophulariaceae) species are specialized for pollination by hawkmoths and flies with correspondingly long proboscis (Johnson *et al.* 2002). Metabarcoding revealed that links exist between *Zaluzianskya* beyond the expected Sphingidae and long-tongued fly pollinators, such as with Syrphidae and many Noctuid moths. *Philoliche aethopica* is known to visit *Watsonia lepida* (Iridaceae), *Cycnium racemosum* (Scrophulariaceae) (Johnson 2000) and *Dais cotonifolia* (Thymelaeaceae) (pers. comms.) on Gilboa and this is confirmed by the metabarcoding data. Again, whereas *Aeropetes tulbaghia* is known to be attracted entirely to large red flowers (Goldblatt & Manning 2002), metabarcoding links to *Hebenstretia* (likely *H. dura*) (Scrophulariaceae), *Crassula* and *Watsonia lepida* do not conform to this. *Hebenstretia dura* (36 links) interacted with both nocturnal and diurnal lepidoptera pollinators and is possibly a keystone species for lepidoptera. This appears concordant with the removal simulations- where extinction of *Hebenstretia sp.* was simulated, this led to a dramatic increase in the vulnerability of the system.

Metabarcoding extends several known plant-pollinator links identified previously (Johnson, Harris & Procheş 2009). *Gladiolus longicollis*, pollinated by hawkmoths, is linked also to Noctuidae and *Apis mellifera*. Interestingly, metabarcoding did not detect a link between hawkmoths and *G. longicollis* which could reflect a mismatch between the timing of flowers

of this species and the collection of hawkmoths. Although metabarcoding extends diet breadth resolution, incomplete sampling can still be a pervasive issue especially if the flowering season is short and flower abundance is low (Chacoff *et al*. 2012). *Watsonia lepida*, pollinated by long-tongued flies and long-tongue bees, has many more links (29) including long-tongued nocturnal pollinators (Sphingidae and Noctuidae), as well as several Apidae and Sarcophagidae links. Metabarcoding adds Noctuidae and short-tongued fly links to known *Eriosema distinctum* (Fabaceae) pollinator links. *Satyrium longicauda* is known to be pollinated by hawkmoths. Yet here I found only links to Noctuidae and *Apis mellfera*, and no visible pollinia were attached to any insect. This genetic link could possibly be due to residual remains of the pollinia on the moths (such as a viscidium). Here, metabarcoding revealed links to several day-active Lepidoptera (Nymphalidae, *Precis octavia* and *Pseudonympha trimenii*; Pieridae*, Belenois aurota* and *Pontia helice*; Lycaenidae, *Aloeides trimeni*) as well as Halictidae, Lasioglossum sp., *Prosoeca* sp. 5 and Coleoptera Scarabaeidae,s *Trichostetha fascicularis ssp. prunipennis*. Whereas Johnson et al. (2009) report on butterfly, long-tongue fly and long-tongue bee pollination syndromes for *Pentanisia* sp. (likely, *P. prunelloides*), metabarcoding only detected two links (Nymphalidae, *Precis octavia* and Acartophthalmidae, *Acartophthalmus nigrinus*). Lastly, *Zaluzianskya* sp. (likely, *Z. natalensis*), known to be pollinated by hawkmoths, has many more links detected by metabarcoding including both night- and day-active insect groups. Two floral links with *Apis mellifera* (*Hilliardiella hirsuta* and *Watsonia lepida*) only were overlapping between metabarcoding and visitation networks (Stanley, Msweli & Johnson 2020), despite many of the listed species flowering contemporaneous. *Protea* pollination by cetoniine beetles is another well-known syndrome on Mount Gilboa. Because of their abundance, size and relatively pure pollen loads, cetoniine beetles are the most important pollinator of Protea species (Steenhuisen & Johnson 2012). Here, metabarcoding found many links between the cetoniid beetle *Atrichelaphinus tigrina*, and flowering species not

conforming to this syndrome. *Protea roupelliae* additionally had several links with many Lepidoptera, Diptera and Hymenoptera species.

Because of the greater resolution that metabarcoding provides, generalism appears to be more pervasive within networks than previously thought. The high level of generalization within the network is vital to its structure, functioning and persistence, as generalist species are integral to the cohesiveness and continuation of communities in event of disturbance (González, Dalsgaard & Olesen 2010). Both the pollinator- and plant-level network was relatively tolerant to extinction of species in the alternate layer. High functional complementarity (low specialization) in an entire community, as in this network, may be ensuring community and species persistence (Blüthgen & Klein 2011).

Understanding how biotic components of ecological communities are arranged is important to the interpretation of network structure and its link to ecosystem functioning and stability (Bjerke & Ostdahl 2004; Bosch *et al.* 2009). Mutualistic interactions do not occur in isolation but are organized into highly complex networks which can be described by indices including interaction strength, richness, modularity and generalization/specialization (Bascompte *et al.* 2003; Petanidou *et al.* 2008; Bosch *et al.* 2009). Network patterns, as described and compared by network metrics, can facilitate the coexistence and stability of species, and may provide insight into meaningful ecological and evolutionary mechanisms (Song & Feldman 2014; Lomáscolo *et al.* 2019). DNA metabarcoding has become a powerful tool in biodiversity research owing to its cost-effectiveness, lack of bias, increased resolution, and scalability as a bioassessment tool (Taberlet *et al.* 2012; Compson *et al.* 2020; Bell *et al.* 2022; Lowe *et al.* 2022b). In pollination studies, metabarcoding has been employed to detect cryptic and nocturnal pollination networks (Macgregor *et al.* 2019), to understand temporal changes in diet breadth (Lowe *et al.* 2022a), to interpret historic host ranges of pollinators (Gous *et al.* 2021), to understand how pollination networks respond to habitat heterogeneity (Encinas-Viso *et al.* 2022), and to study the effects of global climate change on pollination networks (Burkle &

Alarcón 2011; Benson et al. 2015; Tommasi et al. 2022). However, it is important to consider that metabarcoding has several pitfalls. Firstly, although mini-barcodes amplify with greater success than full-length barcodes, the taxonomic discriminatory power of a mini-barcode is lower than that of a standard full-length barcode (Little 2014). Using a combination of several mini-barcodes can assist in decreasing this limit. A further impediment to metabarcoding is the current lack of a fully comprehensive record database. Species coverage for land plants is still very limited (23% (Cheng *et al.* 2016)), yet with advancements of molecular techniques and improvement in primer design and primer combination (Lahaye *et al.* 2008), reference databases and analysis methods will only improve.

South Africa boasts one of the world's richest assemblages of biodiversity (Kepe, Saruchera & Whande 2004), with three Conservation International global hotspots declared within its borders; namely the Succulent Karoo, the Cape Floristic Province (Mittermeier *et al.* 1998) and the Maputaland-Pondoland-Albany region (Steenkamp *et al.* 2004). South African ecosystems are unique, and the evolution of such assemblages is most often attributed to long standing and shifting associations between plant communities and their pollinators (Dodd, Silvertown & Chase 1999; Vamosi & Vamosi 2010). Global ecological change reduces biodiversity (Pereira *et al.* 2010) leading to the more threatening consequence of the loss of ecological interactions, the loss of evolutionary and ecological process that support it (Valiente-Banuet *et al.* 2015) and ultimately functional homogenization of the natural world (Clavel, Julliard & Devictor 2011). DNA metabarcoding is an important tool with high throughput capabilities which is essential to characterize plant-pollination communities at an appropriate scale, speed and resolution which was previously not possible (Bell *et al.* 2022).

Species loss is a probable and likely scenario in the event of global change to natural environments. Community resilience can be modelled and described by network metrics such as robustness, modularity, linkage density and vulnerability (Vanbergen *et al.* 2017). I effected the 'worst case' scenario in this natural community by simulating the stepwise loss of the

highest strength interacting partners for both the pollinator and plant level of the network. Stepwise removal of pollinators led to an expected and linear decline of the interacting plant species community. Similar to previous research, I also found no worse than a linear and predictable decline of species. A linear decline was expected in networks of low modularity and connectance of the entire web (Memmott, Waser & Price 2004). Removing pollinator species resulted in a more severe prediction of network stability as opposed to the removal of interacting plant partners. This can be explained by the greater generality in this trophic level describing a network of many plants depending on fewer pollinating species. The effect of stepwise removal in the plant-level, due to the disproportionate dependence of pollinator species on many plant species, resulted in a more extreme change in species loss and network topology. Although these simulations ignore the possibility of real-life likelihood of network rewiring and recolonization events (Memmott, Waser & Price 2004; Vizentin-Bugoni *et al.* 2020). In the event of extreme habitat fragmentation or other climate associated change, it is possible that nodes from both levels are likely to be lost simultaneously and thus this 'worst case scenario' may even be an underrepresentation. However, Bain et al. (2022) experimentally removed plant species from a network to assess the change in network topology. They found that networks became more specialized, less nested, and less robust to subsequent species loss, concluding that such disturbances to network structure were preferentially driven by species turnover rather than by interaction rewiring (Bain *et al.* 2022; Sandacz *et al.* 2023). Similarly, both trophic levels in this network steadily became more specialized, less nested and more modular as species were removed. A second study found that co-extinction following species loss was less likely in networks with low connectance, and that network size was inversely proportional to the risk of large co-extinction cascades (Vanbergen *et al.* 2017). This stands in contrast to earlier work which attributed strong indirect facilitation within pollination networks to the persistence of highly nested and connected pollination networks (Lever *et al.* 2014). Although this was peculiar to a disturbed habitat, the pattern identified by Vanbergen et al.

(2017) seems to hold for this study site too. Campbell and colleagues (2012) also reported that up to slightly less than 50% of the total number of species need to be lost in order for stable networks to collapse completely (Campbell *et al.* 2012). Here, plant and pollinator visitation networks were highly robust, tolerating partner extinctions beyond 50% and up to ~80%, respectively. With the stepwise removal of species, the most important pollinator node in the network switched between diurnal and nocturnal pollinating species, emphasising the importance of both diel networks acting synergistically to accomplish pollination of the entire system. The extinction of highly generalist species undermines ecosystems' functional stability as these nodes have a disproportionate influence on the network (Biella *et al.* 2019). Here, the simulated removal of several highly generalist plants, such as *Hebenstretia* sp. and *Zaluzianskya* sp., had no disproportionate effect on increasing predicted pollinator extinctions or sudden network collapse. The low connectance, large network, and the high proportion of generalists in both plants and pollinators in this particular network might be acting as a buffer against an earlier and more severe extinction cascade.

Our current ability to identify metabarcodes and assign error-free species identities with certainty from a diverse sample is currently limited, particularly in a hyper-diverse environment such as South Africa (Furlan, Davis & Duncan 2020). However, as DNA barcoding and databases are expected to become more representative as research continues, these limitations will diminish. Those amplicon sequence variants (ASVs) which I could not confidently assigned to species level are still useful for biodiversity analyses and community level research in identifying biological units (Porter & Hajibabaei 2020). Metabarcoding can assess biodiversity on a larger scale and provide greater resolution particularly for plant-pollinator networks where many species go undetected using conventional methods of observation or pollen-load analysis. With metabarcoding analysis, and in-line with previous research, I could greatly increase the known diet breadths of pollinating insect species (Bush *et al.* 2020), and the effect of including a greater number of interactions to these networks necessarily alters

network metrics such as levels of specialization. However, metabarcoding requires the careful assessment of sequence similarity thresholds and extensive *post-hoc* verification of species assignments particularly in areas where databases are not representative of the diversity present (Govender *et al.* 2022; Zaiko *et al.* 2022). Corroborating occurrence and distribution records to identify inaccuracies was vitally necessary in this study particularly as South Africa is an understudied area where reference databases do not match the local diversity. Metabarcoding has both the advantage and disadvantage of upscaling. Metabarcoding can very easily be upscaled (in comparison to observational/palynology studies) to assess species richness across large geographical scales. However, metabarcoding across large scales where comprehensive reference databases are lacking can prove to be very difficult as one is likely to be including many congenerics. In such cases, research over a smaller geographic scale would be suitable, even in the absence of a complete database, as there is less likelihood of many sister species, and therefore *post-hoc* verification would have a higher success rate. Within a community context, as here, metabarcoding is an effective means of assessing an entire community. Still, I highly recommend that future research make use of every available source of information, such as the use of high-quality photography (particularly where a single specimen represents an entire sample), local observations, and retaining a type specimen where possible, in order to simplify and expedite *post-hoc* verification.

Pollination networks are highly labile in time and space, with different functional roles being assumed by different species across ecoregions (Kovács-Hostyánszki *et al.* 2019). Network ecology, coupled with metabarcoding, is a powerful tool in assessing pollination networks in the current context of habitat loss and degradation, with the end goal of conserving biodiversity (Sandacz *et al.* 2023). Revealing overlooked yet integral nodes in plant-pollinator

networks is a further benefit of incorporating metabarcode data. Changes to network structure can have direct impacts on the persistence of native plant populations (Gómez, Perfectti & Jordano 2011). Therefore identifying keystone nodes in plant-pollinator networks, and how this might relate to species invasion and the reorganizing of community interactions (Vanbergen, Espíndola & Aizen 2018) or the transfer of pathogens and microbes through the network (Arstingstall *et al.* 2021; Sandacz *et al.* 2023) is another key avenue where metabarcode data can assist conservation efforts in high priority biodiverse areas.

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Supplementary Information

TABLE S5.1 Network metric for major pollination groups

Order	Links per species	Total number of floral partners	Generality	Specialization d'
Coleoptera	1.24	28	9.20	0.41
Hymenoptera	1.68	55	14.80	0.30
Diptera	1.89	94	16.86	0.38
Lepidoptera	2.06	119	18.46	0.33

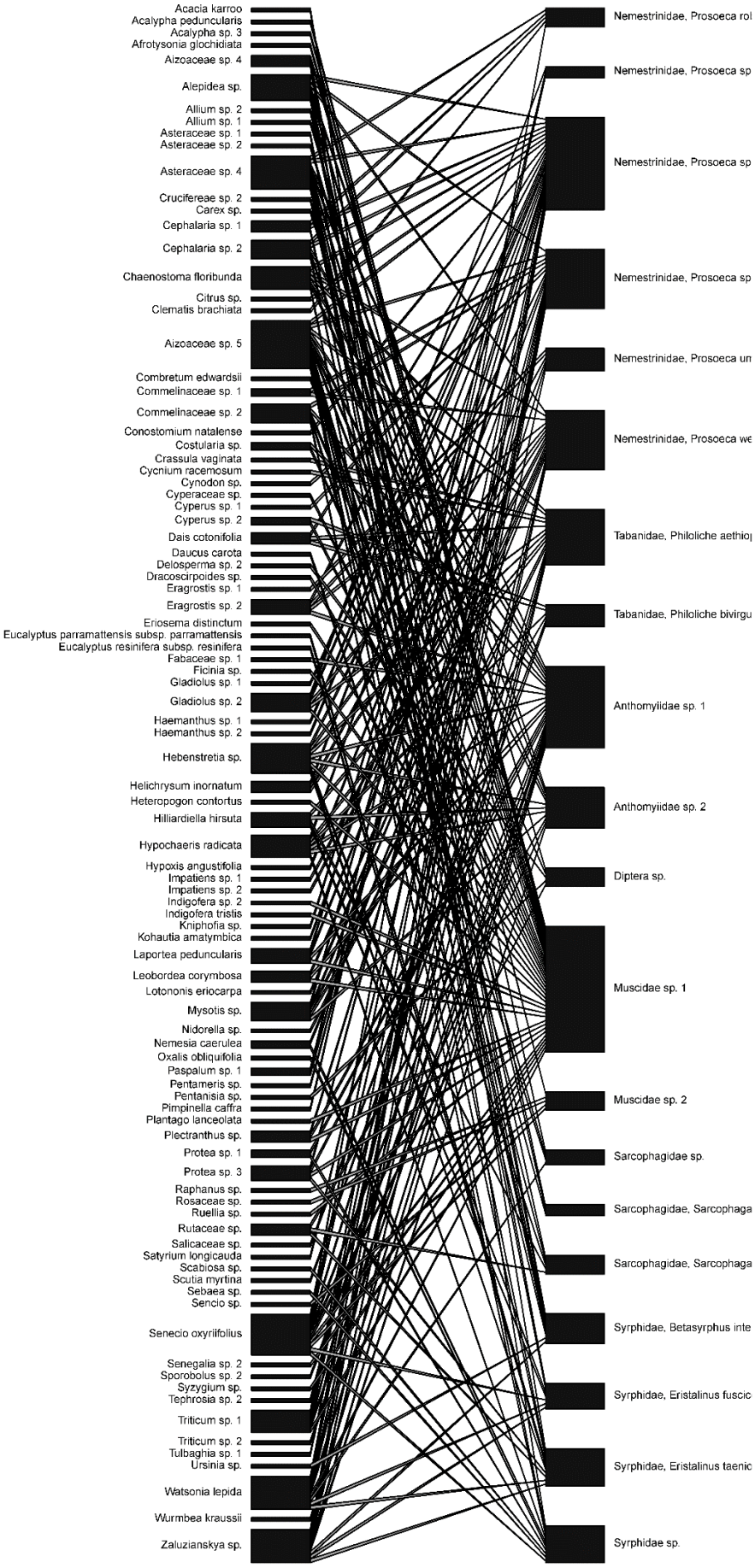

FIGURE S5.1 Visitation network for Diptera

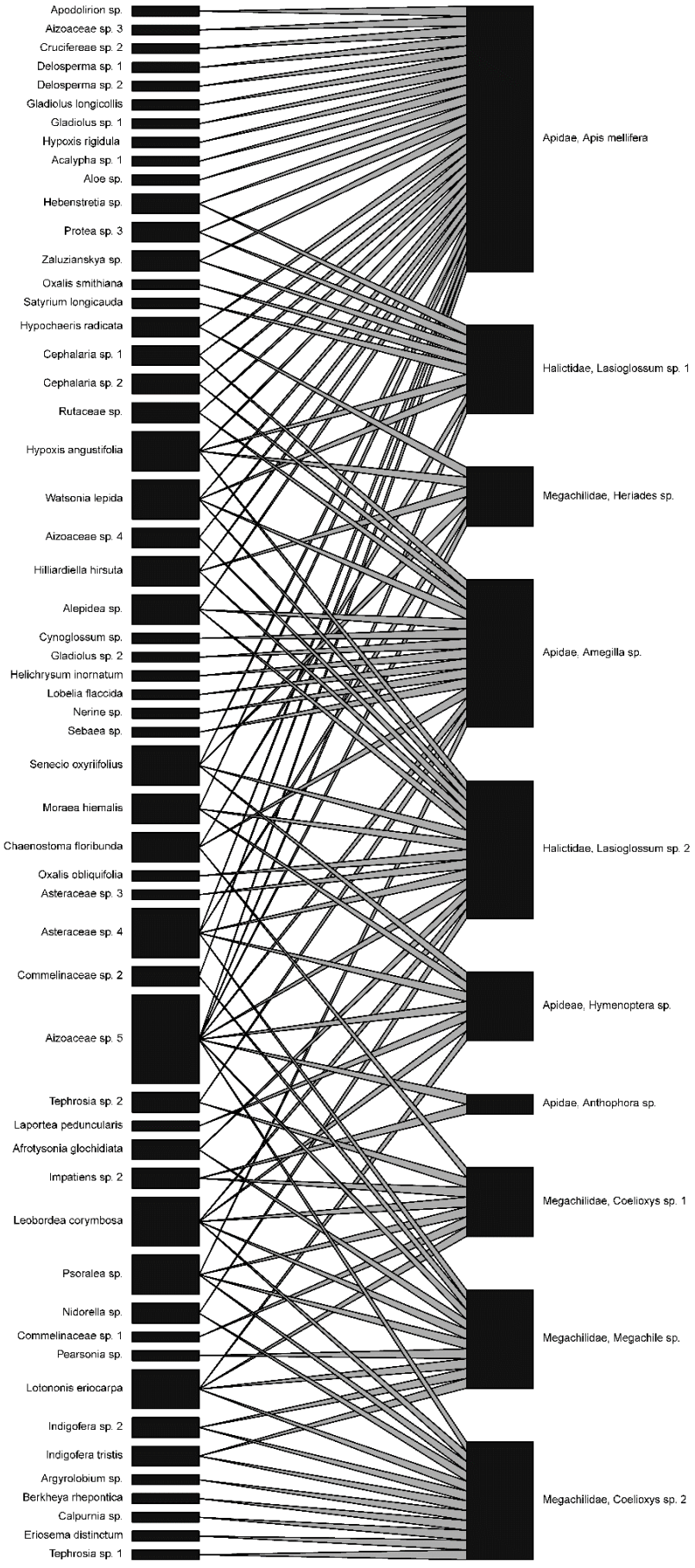

FIGURE S5.2 Visitation network for Hymenoptera

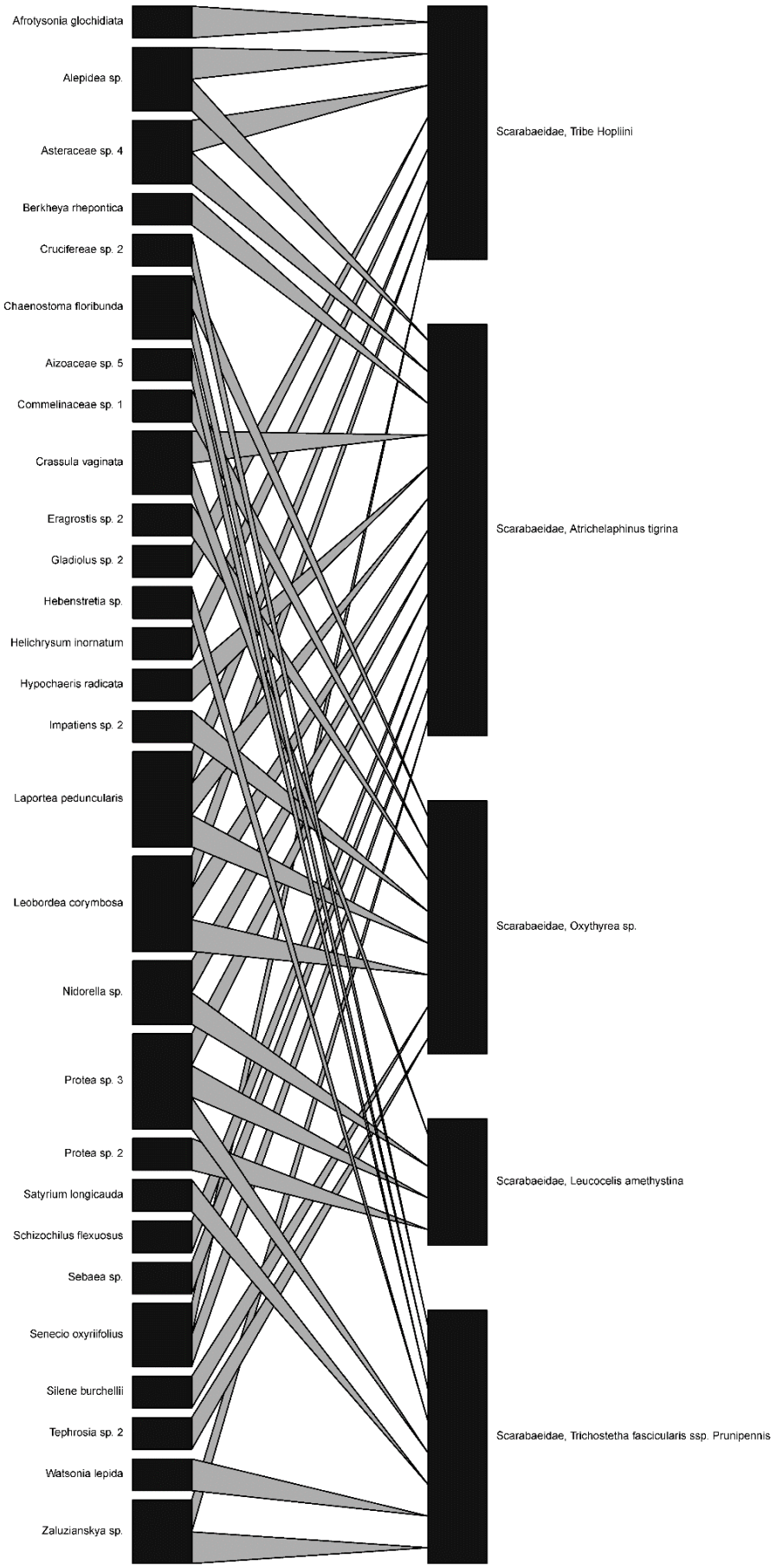

FIGURE S5.3 Visitation network for Coleoptera

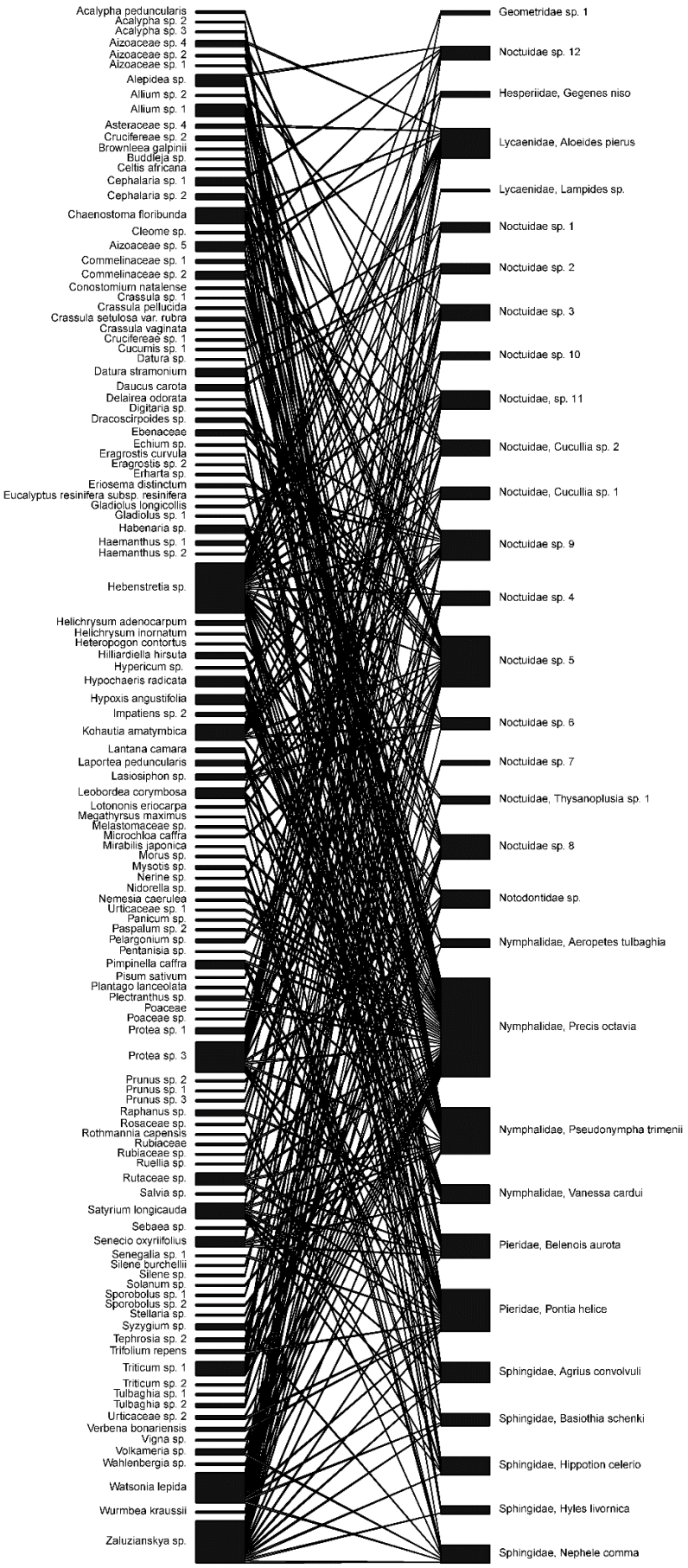

FIGURE S5.4 Visitation network for Lepidoptera, diurnal and nocturnal

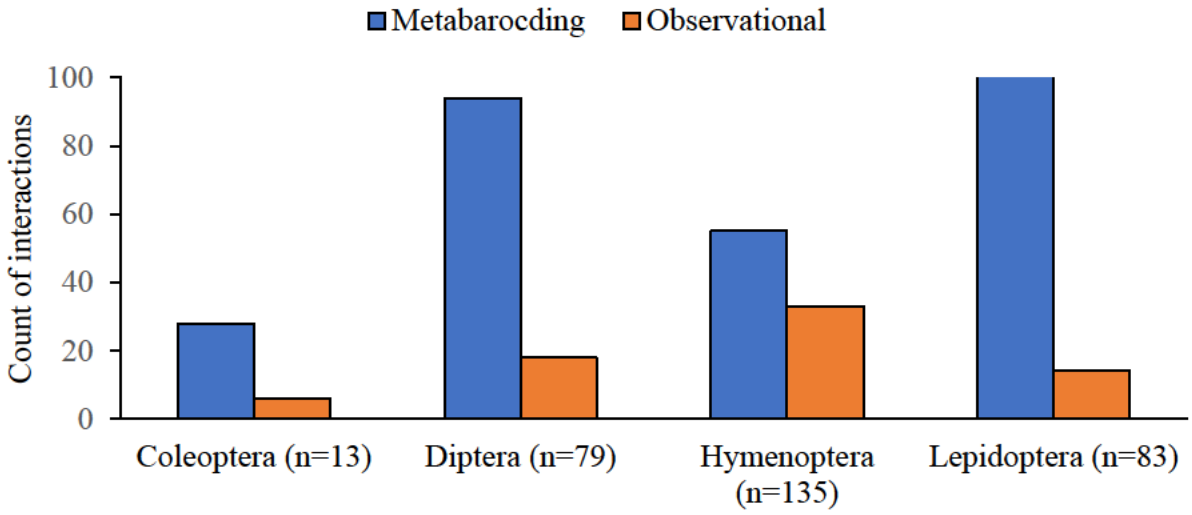

FIGURE S5.5 Number of floral links detected from capture events and by metabarcoding. Sample numbers given in parentheses.

TABLE S5.2 Details of plant specimens collected on Mount Gilboa, Karkloof, South Africa

Sample name	Date	Family	Genus & species	Successfully barcoded	Latitude	Longitude	Alt. (m)	Common name
MS 1	30 April 2019	Asteraceae	*Helichrysum adenocarpum*	Yes	-29.2877	30.2928	1747	Pink Everlasting
MS 1a	24 October 2019	Asteraceae	*Senecio sp. 1*	No	-29.2875	30.2922	1642	Ragworts
MS 2	30 April 2019	Asteraceae	*Berkheya echinaceae*	No	-29.2878	30.2926	1747	
MS 3	30 April 2019	Campanulaceae	*Lobelia flaccida*	Yes	-29.2829	30.2929	1750	Floppy Lobelia
MS 4	30 April 2019	Geraniaceae	*Geranium schlechteri*	Yes	-29.2876	30.2929	1752	
MS 5	30 April 2019	Scrophulariaceae	*Sutera floribundum*	Yes	-29.2876	30.2929	1752	Curry Skunkbush
MS 6	30 April 2019	Asteraceae	*Cineraria spp. (aspera)*	Yes	-29.2875	30.2928	1754	
MS 7	30 April 2019	Proteaceae	*Protea roupelliae roupelliae*	Yes	-29.2876	30.2928	1753	Tall Silver Sugarbush
MS 8	30 April 2019	Asteraceae	*Hypochaeris radicata*	Yes	-29.2875	30.2929	1754	Hairy Wild Lettuce
MS 9	30 April 2019	Asteraceae	*Helichrysum confertiflorum*	Yes	-29.2857	30.2925	1755	
MS 10	30 April 2019	Campanulaceae	*Wahlenbergia pallidiflora*	Yes	-29.2857	30.2924	1754	
MS 11	30 April 2019	Asteraceae	*Nidorella sp.*	Yes	-29.2830	30.2914	1738	
MS 12	30 April 2019	Lamiaceae	*Leonotis leonurus*	Yes	-29.2764	30.2931	1703	Common Lionspaw
MS 13	24 May 2019	Rubiaceae	*Conostomium natalense*	Yes	-29.2845	30.2916	1742	Wild Pentas
MS 14	24 May 2019	Ericaceae	*Erica cooperi cooperi*	Yes	-29.2845	30.2917	1743	
MS 15	24 May 2019	Asteraceae	*Cirsium vulgare*	Yes	-29.2800	30.2900	1750	Bull Thistle
MS 16	24 May 2019	Solanaceae	*Solanum mauritianum*	Yes	-29.2777	30.2942	1695	Bugweed
MS 17	16 August 2019	Asteraceae	*Senecio speciosus*	Yes	-29.2836	30.2911	1736	Beauty Ragwort
MS 18	16 August 2019	Asteraceae	*Othonna natalensis*	No	-29.2831	30.2922	1742	Natal Baboon cabbage
MS 19	16 August 2019	Iridaceae	*Moraea hiemalis*	Yes	-29.2831	30.2922	1742	
MS 20	16 August 2019	Amaryllidaceae	*Apodolirion buchananii*	No	-29.2831	30.2922	1744	Natal Crocus
MS 21	16 August 2019	Asteraceae	*Ursinia nana*	No	-29.2841	30.2927	1747	Little Paraseed
MS 22	16 August 2019	Hypoxidaceae	*Hypoxis angustifolia*	Yes	-29.2841	30.2927	1747	Narrow Stargrass
MS 23	16 August 2019	Iridaceae	*Moraea modesta*	No	-29.2860	30.2920	1744	Shy Uintjie
MS 24	16 August 2019	Iridaceae	*Dierama pendulum*	Yes	-29.2838	30.2913	1738	Fairy Wand
MS 25	16 August 2019	Iridaceae	*Moraea inclinata*	No	-29.2858	30.2912	1744	Nodding Wild Moraea
MS 26	16 August 2019	Asteraceae	*Dimorphotheca jucunda*	Yes	-29.2859	30.2913	1745	Cape Daisy

MS 27	6 September 2019	Colchicaceae	*Wurmbea kraussii*	Yes	-29.2823	30.2916	1740	
MS 28	6 September 2019	Scrophulariaceae	*Hebenstretia dura*	Yes	-29.2831	30.2914	1738	Eastern Shrubby Slugwort
MS 29	6 September 2019	Asteraceae	*Ageratina Ageratina*	Yes	-29.2756	30.2886	1642	Snakeroots
MS 30	1 October 2019	Asteraceae	*Helichrysum cooperi*	Yes	-29.2842	30.2918	1714	Drakensberg Yellow Everlasting
MS 31	1 October 2019	Asteraceae	*Senecio oxyriifolius*	Yes	-29.2842	30.2923	1717	Nasturtium Ragwort
MS 32	1 October 2019	Iridaceae	*Gladiolus longicollis*	Yes	-29.2851	30.2926	1710	Long Afrikaner
MS 33	1 October 2019	Scrophulariaceae	*Nemesia caerulea*	Yes	-29.2868	30.2927	1727	Skyblue Lionface
MS 34	1 October 2019	Amaryllidaceae	*Tulbaghia leucantha*	Yes	-29.2860	30.2931	1735	Highveld Wild Garlic
MS 35	1 October 2019	Hypoxidaceae	*Rhodohypoxis platypetala*	Yes	-29.2865	30.2929	1724	
MS 36	1 October 2019	Asteraceae	*Gerbera ambigua*	Yes	-29.2876	30.2928	1729	Butter Gerbera
MS 37	1 October 2019	Rubiaceae	*Kohautia amatymbica*	yes	-29.2857	30.2925	1716	Charm Trembletop
MS 38	1 October 2019	Fabaceae	*Leobordea corymbosa*	Yes	-29.2858	30.2923	1718	
MS 39	1 October 2019	Apocynaceae	*Schizoglossum flavum*	Yes	-29.2857	30.2923	1719	
MS 40	1 October 2019	Asparagaceae	*Scilla natalensis*	No	-29.2875	30.2922	1642	Blouberglelie
MS 41	16 October 2019	Rosaceae	*Rubus sp,*	Yes	-29.2875	30.2922	1642	
MS 42	16 October 2019	Aizoaceae	*Delosperma sandersonii*	No	-29.2828	30.2916	1642	Fire Sheepfig
MS 43	16 October 2019	Asparagaceae	*Ledebouria sandersonii*	No	-29.2828	30.2917	1642	Fairy African Hyacinth
MS 44	16 October 2019	Asteraceae	*Senecio sp. 2*	No	-29.2863	30.2924	1642	
MS 45	16 October 2019	Hypoxidaceae	*Hypoxis parvula*	Yes	-29.2867	30.2927	1642	Mini White Stargrass
MS 46	24 October 2019	Fabaceae	*Eriosema distinctum*	Yes	30.2921	-29.2839	1642	Scarlet Eriosema
MS 47	24 October 2019	Asteraceae	*Hilliardiella hirsuta*	Yes	30.2922	-29.2861	1642	Quiltleaf Vernonia
MS 48	24 October 2019	Asteraceae	*Hilliardiella oligocephala*	Yes	30.2924	-29.2871	1642	Bicolour Vernonia
MS 49	24 October 2019	Hypoxidaceae	*Hypoxis angustifolia (duplicate)*	Yes	30.2926	-29.2867	1642	Narrow Stargrass
MS 50	24 October 2019	Orobanchaceae	*Graderia scabra*	Yes	30.2923	-29.2863	1642	Wild Penstemon
MS 51	6 November 2019	Euphorbiaceae	*Acalypha punctata*	Yes	-29.2876	30.2929	1765	Sticky Brooms and Brushes
MS 52	6 November 2019	Ranunculaceae	*Ranunculus multifidus*	Yes	-29.2875	30.2930	1762	Wild Buttercup
MS 53	6 November 2019	Orchidaceae	*Eulophia aculeata*	Yes	-29.2875	30.2928	1766	Common Pointed Harlequin
MS 54	6 November 2019	Oxalidaceae	*Oxalis smithiana*	Yes	-29.2875	30.2927	1763	Windmill Sorrel
MS 55	6 November 2019	Orobanchaceae	*Cycnium racemosum*	No	-29.2875	30.2927	1761	Large Mountain Ink Flower
MS 56	18 November 2019	Iridaceae	*Aristea ecklonii*	Yes	-29.2818	30.2920	1732	Star Capeblue
MS 57	18 November 2019	Molluginaceae	*Psammotropha myriantha*	Yes	-29.2834	30.2919	1743	Ungent Stackleaf

MS 58	18 November 2019	Iridaceae	*Watsonia lepida*	Yes	-29.2851	30.2926	1748	Lonesome Watsonia
MS 59	18 November 2019	Asparagaceae	*Ornithogalum graminifolium*	Yes	-29.2876	30.2929	1770	Grass Chink
MS 60	18 November 2019	Lamiaceae	*Stachys nigricans*	Yes	-29.2878	30.2930	1760	Black Tea Stachys
MS 61	18 November 2019	Caprifoliaceae	*Valeriana capensis*	No	-29.2878	30.2930	1762	Cape Valerian
MS 62	18 November 2019	Hypericaceae	*Hypericum aethiopicum ssp. sonderi*	Yes	-29.2875	30.2929	1755	Small Hypericum
MS 63	18 November 2019	Fabaceae	*Eriosema kraussianum*	Yes	-29.2828	30.2913	1733	Pale Eriosema
MS 64	28 November 2019	Iridaceae	*Hesperantha baurii*	No	-29.2869	30.2925	1756	Grassveld Eveninglily
MS 65	28 November 2019	Asteraceae	*Senecio pentactinus*	Yes	-29.2875	30.2926	1767	
MS 66	28 November 2019	Fabaceae	*Indigofera hilaris*	Yes	-29.2875	30.2926	1761	Cheery Indigo
MS 67	28 November 2019	Asteraceae	*Helichrysum inornatum*	Yes	-29.2876	30.2926	1760	
MS 68	28 November 2019	Asteraceae	*Berkheya speciosa*	Yes	-29.2877	30.2926	1759	Pretty African Thistle
MS 69	28 November 2019	Caprifoliaceae	*Scabiosa sp.*	No	-29.2877	30.2926	1759	
MS 70	28 November 2019	Apocynaceae	*Xysmalobium tysonianum*	No	-29.2877	30.2926	1758	
MS 71	20 December 2019	Orchidaceae	*Disa chrystostachya*	Yes	-29.2871	30.2921	1751	Torch Orchid
MS 72	20 December 2019	Campanulaceae	*Wahlenbergia cuspidata*	No	-29.2872	30.2924	1750	
MS 73	20 December 2019	Orchidaceae	*Satyrium longicauda (duplicate)*	Yes	-29.2877	30.2922	1753	Longtail Satyre
MS 74	20 December 2019	Orchidaceae	*Disa versicolor*	Yes	-29.2872	30.2921	1752	Browning Disa
MS 75	20 December 2019	Fabaceae	*Psoralea rhizotoma*	Yes	-29.2881	30.2923	1736	
MS 76	20 December 2019	Iridaceae	*Dierama luteoalbidum*	No	-29.2881	30.2923	1745	
MS 77	20 December 2019	Asphodelaceae	*Aloe neilcrouchii*	No	-29.2876	30.2930	1768	
MS 78	20 December 2019	Rosaceae	*Rubus ludwigii ludwigii*	Yes	-29.2876	30.2931	1769	Common Silver Bramble
MS 79	20 December 2019	Lamiaceae	*Ajuga ophrydis*	No	-29.2869	30.2925	1754	Bugle Plant
MS 80	20 December 2019	Orchidaceae	*Disa stachyoides*	Yes	-29.2821	30.2918	1733	Wormwood Disa
MS 81	3 January 2020	Amaryllidaceae	*Agapanthus campanulatus*	No	-29.2870	30.2923	1754	Bell Bluelily
MS 82	3 January 2020	Scrophulariaceae	*Zaluzianskya microsiphon*	No	-29.2879	30.2926	1759	Short-tube Drumsticks
MS 83	3 January 2020	Apiaceae	*Alepidea natalensis*	No	-29.2878	30.2925	1751	Natal Ministar
MS 84	3 January 2020	Fabaceae	*Indigofera hedyantha*	Yes	-29.2878	30.2925	1751	Regret Indigo
MS 85	3 January 2020	Fabaceae	*Lotononis eriocarpa*	Yes	-29.2880	30.2930	1760	
MS 86	3 January 2020	Rubiaceae	*Pentanisia angustifolia*	Yes	-29.2874	30.2929	1759	Smooth Pentanisia
MS 87	3 January 2020	Iridaceae	*Tritonia disticha rubrolucenss*	No	-29.2872	30.2918	1744	
MS 88	3 January 2020	Amaryllidaceae	*Brunsvigia undulata*	No	-29.2867	30.2923	1754	Candelabras

MS 89	3 January 2020	Asparagaceae	*Albuca setosa*	No	-29.2869	30.2925	1761	Thick Slime-Lily
MS 90	3 January 2020	Commelinaceae	*Cyanotis speciosa*	No	-29.2869	30.2925	1755	Showy Blue Ear
MS 91	11 January 2020	Caryophyllaceae	*Silene burchellii*	Yes	-29.2869	30.2926	1765	African Catchfly
MS 92	11 January 2020	Proteaceae	*Protea sp.*	No	-29.2864	30.2919	1765	Grassveld Sugarbushes
MS 93	11 January 2020	Fabaceae	*Pearsonia grandifolia*	Yes	-29.2875	30.2931	1764	
MS 94	11 January 2020	Lamiaceae	*Ajuga ophrydis (duplicate)*	No	-29.2875	30.2922	1642	Bugle Plant
MS 95	16 January 2020	Iridaceae	*Moraea brevistyla*	No	-29.2867	30.2912	1751	Shortstyle Uintjie
MS 96	16 January 2020	Lamiaceae	*Stachys natalensis*	No	-29.2865	30.2919	1760	White Stachys
MS 97	16 January 2020	Campanulaceae	*Craterocapsa tarsodes*	Yes	-29.2865	30.2920	1752	Northern Bergbell
MS 98	16 January 2020	Campanulaceae	*Lobelia flaccida (duplicate)*	Yes	-29.2865	30.2920	1751	Floppy Lobelia
MS 99	16 January 2020	Crassulaceae	*Crassula brachypetala*	yes	-29.2865	30.2920	1751	
MS 100	16 January 2020	Rutaceae	*Agathosma ovata*	Yes	-29.2875	30.2929	1770	False Buchu
MS 101	28 January 2020	Ericaceae	*Erica aestiva*	Yes	-29.2880	30.2919	1642	Summer Heath
MS 102	28 January 2020	Orchidaceae	*Schizochilus angustifolius*	Yes	-29.2880	30.2919	1642	Alpine Splitlip Orchid
MS 103	28 January 2020	Crassulaceae	*Crassula vaginata*	Yes	-29.2877	30.2922	1642	White Stonecrop
MS 104	28 January 2020	Gentianaceae	*Sebaea natalensis*	Yes	-29.2877	30.2922	1642	Natal Yellowwort
MS 105	28 January 2020	Asteraceae	*Helichrysum monticola*	No	-29.2878	30.2922	1642	
MS 106	28 January 2020	Hypericaceae	*Hypericum lalandii*	Yes	-29.2877	30.2920	1642	
MS 107	13 February 2020	Campanulaceae	*Wahlenbergia appressifolia*	No	-29.2867	30.2926	1642	
MS 108	13 February 2020	Lamiaceae	*Plectranthus calycinus*	No	-29.2874	30.2916	1642	Upland Flybush
MS 109	13 February 2020	Campanulaceae	*Wahlenbergia fasciculata*	Yes	-29.2877	30.2918	1642	
MS 110	13 February 2020	Caprifoliaceae	*Cephalaria pungens*	No	-29.2881	30.2924	1642	African Mock Scabious
MS 111	13 February 2020	Asteraceae	*Berkheya rhapontica*	No	-29.2879	30.2928	1642	
MS 112	13 February 2020	Orchidaceae	*Disa patula*	Yes	-29.2818	30.2918	1642	Lurelip Disa
MS 113	13 February 2020	Asphodelaceae	*Kniphofia laxiflora*	Yes	-29.2818	30.2918	1642	Slender Poker
MS 114	13 February 2020	Orchidaceae	*Satyrium longicauda*	Yes	-29.2818	30.2920	1642	Longtail Satyre
MS 115	21 February 2020	Orchidaceae	*Habenaria dives*	Yes	-29.2853	30.2927	1642	Death Orchid
MS 116	21 February 2020	Orobanchaceae	*Alectra sessiliflora*	Yes	-29.2854	30.2928	1642	Yellow Paintflower
MS 117	21 February 2020	Orchidaceae	*Disa brevicornis*	Yes	-29.2873	30.2922	1642	Monodisa
MS 118	24 February 2020	Scrophulariaceae	*Zaluzianskya distans*	Yes	-29.2793	30.2941	1642	
MS 119	26 February 2020	Scrophulariaceae	*Zaluzianskya natalensis*	Yes	-29.2865	30.2927	1642	Natal Drumsticks

MS 120	10 March 2020	Orchidaceae	*Habenaria laevigata*	Yes	-29.2880	30.2924	1642	Smooth Ghost Orchid
MS 121	5 March 2020	Orchidaceae	*Disperis fanniniae*	Yes	-29.2864	30.2927	1642	Fanny Kappie
MS 122	5 March 2020	Iridaceae	*Hesperantha sp.*	No	-29.2875	30.2927	1642	Evening Lilies
MS 123	10 March 2020	Orchidaceae	*Brownleea galpinii*	Yes	-29.2877	30.2927	1642	Common Purplespot False-Disa
MS 124	10 March 2020	Solanaceae	*Solanum retroflexum*	No	-29.2878	30.2929	1642	Nightshades
MS 125	10 March 2020	Orchidaceae	*Habenaria lithophila*	Yes	-29.2880	30.2925	1642	Rock Ghost Orchid
MS 126	10 March 2020	Orchidaceae	*Disa fragrans*	Yes	-29.2880	30.2917	1642	Fragrant Disa
MS 127	18 March 2020	Orchidaceae	*Disa fragrans (duplicate)*	Yes	-29.2875	30.2922	1642	Fragrant Disa
MS 128	7-9 January 2021	Commelinaceae	*Commelina africana*	Yes	-29.2875	30.2922	1642	Yellow Dayflower
MS 129	19 January 2021	Orchidaceae	*Pterygodium magnum*	Yes	-29.2875	30.2922	1642	Big Bonnet
MS 130	7-9 January 2021	Iridaceae	*Moraea trifida*	No	-29.2875	30.2922	1642	Trifid Uintjie
MS 131	7-9 January 2021	Orchidaceae	*Disperis renibractea*	No	-29.2875	30.2922	1642	Kidney Kappie
MS 132	26 January 2021	Scrophulariaceae	*Diclis reptans*	No	-29.2875	30.2922	1642	Dwarf Koenana
MS 133	26 January 2021	Asparagaceae	*Dipcadi sp.*	Yes	-29.2875	30.2922	1642	Dainty Bells

CHAPTER 6

CONCLUSIONS

6.1 Introduction

Pollinators are declining worldwide with implications for biodiversity and agricultural food production (Potts *et al.* 2010; Aizen *et al.* 2019). Ecological diversity research is often bottlenecked by the inability to provide reliable and cost-effective results within a reasonable timespan (Kim & Byrne 2006). Biodiversity research has therefore often been limited in space and time, mostly relying on accumulation of single-species or small-scale research (Hortal *et al.* 2015; Folk & Siniscalchi 2021). The Anthropocene and global climate change demands research which can be conducted both across larger scales and at higher resolutions (Rasmussen *et al.* 2013; Bell *et al.* 2016). The need for repeatability and time expediency is also a priority. Specifically, the global pollinator crisis, popularised by the windshield phenomenon (Vogel, 2017), has stimulated much research into individual plant-pollinator interactions as well highlighted the need for entire pollinator network studies (Borchardt *et al.* 2021). Whereas pollination ecology research has often relied on traditional palynology methods (observation, pollen transfer networks using light microscopy), DNA metabarcoding has the potential to address both time and scale constraints in ecological research (Arstingstall *et al.* 2021). We initially determined the possibility of using DNA extracted from fuchsin-fixed pollen for species assignment using the ITS barcode (**chapter 2**), as the quality of DNA may be degraded by fuchsin staining (Murase, Inagaki & Eimoto 2000) yet visualising pollen grains as an initial step in research is valuable and applicable in certain circumstances. Assessing the state of plant-pollinator systems and their response to global change requires an understanding of 'baseline' data to which we can compare current day networks (Bartomeus *et al.* 2019). Museum collections provide these invaluable long-term data sets (Lister 2011). In **chapter 3**, I compare the difference in nocturnal plant-pollination network structure using traditional methods of

exine staining and light microscopy. **Chapters 4** and **5** investigate the applicability of using metabarcoding in pollination ecology research with the specific goals of assessing diet breadths of pollinator groups (specialization and generalisation) and simulating the networks sensitivity to species loss (**chapter 5**).

6.2 Research findings

My introductory chapter tracked the progress and applicability of genetic techniques in ecological fields, while highlighting the lack of investment in southern Africa, a global biodiversity hotspot. Coupled with the global and drastic decline in insect pollinator abundance and richness, research has gradually changed focus from a species-centric to ecosystem-functions approach. Barcoding and metabarcoding as alternative tools to other data gathering methods (observational/traditional palynology) has found its place in managing the increased need and demand for fast and accurate measures of biodiversity.

My second objective was to test whether one could reliably use pollen that had been fixed in fuchsin gel to extract DNA from. Fuchsin gel is a commonly used technique to fix pollen to glass slides in order to view the exine morphology of pollen grains under a compound microscope and assigned an identification for plant-pollinator network studies. However, DNA is known to be degraded when exposed to light as well as certain chemicals. It is possible that the DNA, while being fixed with fuchsin, stored, and viewed under a microscope, would diminish the quality of the extracted DNA barcode, and thus the species assignment from the extracted barcode would be less certain. However, if the DNA is indeed of high enough quality, this would open the possibility of using genetic data from fuchsin-fixed pollen in downstream analysis. The results from our test showed that, within a short period of time, pollen fixed in fucshin gel was found to be viable in reliably assigning identities and distinguishing between species.

Using the well-studied model of hawkmoth pollination, my third objective was to compare pollen placement and diet breadth of hawkmoth pollinators across time using traditional palynology. I found that nocturnal pollen-loads reveal more complex interaction and pollen-transfer networks than observation alone. Archival pollinator specimens are valuable as a source of past interactions. Although pollen is indeed lost during storage and handling of specimens, pollen loads from anatomical positions such as the proboscis can still be used to reconstruct bygone communities. Despite pollen loss, the overall retrospective network structure and calculated indices are not severely impacted by the loss of pollen grains in archival specimens. This type of data can be used to assess community responses across time and space by tracking the change in diet breadth.

My next objective was to establish the range of floral hosts for hawkmoths contrasting light microscopy and metabarcoding results, and in this show the utility of metabarcoding for elusive species which are logistically problematic to study. The highly polyphagous nature of hawkmoths it confirmed, and metabarcoding is proved to be a valuable method to decipher nocturnal pollination networks, as well as confirming that nocturnal networks contribute vastly to pollen-transfer and the persistence of the system as a whole.

My last objective was to provide a complete overview of the pollination network on Mt. Gilboa, KwaZulu-Natal, a well-studied flowering community located in the Maputo-Pondoland Albany biodiversity hotspot. Using metabarcoding, I describe and decipher the complex diurnal and nocturnal pollen transfer networks sustaining that system. I provide network metrics and place it in context of similar studies. I find that this network is complex, and highly nested despite minimal structure which should be conferred by modularity. Using the network metric robustness, I provide a sensitivity measure of the system simulating the hypothetical removal of species beginning with the most relevant and pivotal species and gradually working down to the least effectual node. No sudden community collapse occurs

despite the gradual erosion of descriptive network metrics which purportedly confer network functionality and stability, particularly ‘nestedness’.

6.3 Future research

My research has tested and answered several questions on the applicability of metabarcoding in a South African context. The use of fuchsin gel in pollination ecology research is a still a widely used method. I showed that genetic data in the form of pollen embedded in fuchsin gel need not be discarded but can be used, within a limited timeframe, for DNA extraction and downstream genetic analysis. An initial further research pursuit would be to modify and improve upon this protocol, as well as test it across a greater number of species including many more plant families.

Metabarcoding revealed an increase in network complexity by identifying more species than would have been included based on observation or microscopic analysis of pollen loads alone. The increase in biodiversity corresponds to an increase in the number and complexity of interactions. These reconstructed interactions need to be carefully curated and sifted through such that the network still reflects a biologically meaningful community. In our context, where we have the blessing and curse of living in a biodiversity hotspot, this remains a mammoth task as South African diversity is not well represented in global databases such as Genbank. Where records do exist, these are often poorly curated and may lack a herbarium specimen which provides validity and reputability to the genetic barcode. This immediately necessitates and calls for greater funding to be supporting and underpinning ‘foundational’ research which seeks to bulk up poorly barcoded plant and pollinator families. As other researchers have called for, there is an obvious and clear need to standardise genetic methodologies, especially as pertains to metabarcoding and similar methodologies which can easily and significantly be influenced by minor differences. Simple measures in the standardization of both laboratory and analysis methods would go a far way in permitting the comparison of results across space and time. A

constantly changing environment means that results are specific to locality and time. With standardisation, metabarcoding can provide greater scope with meaningful comparisons. Biodiversity conservation is limited by what is chosen to be measured (Ji *et al.* 2013). The vastly greater information which metabarcoding provides enables this limit to be extended such that largescale biodiversity trends can be monitored. Lastly, as an example, not only are there interspecific differences between pollinators, individual differences within species also exist, e.g. sex-differences (Tourbez *et al.* 2023), which potentially alters ideas and methods of pollinator management. With the correct methodologies and databases in place, the scope of applying metabarcoding to investigate ecological interactions within communities is potentially endless. Until such quantifying abilities advance, an intermediate solution would be to sample and analyze more individuals of each species, but this still relies on the costs of high throughput sequencing to be significantly reduced.

6.4 Concluding remarks

This dissertation offers new insights into the use of genetic methodologies in the field of pollination ecology, with the prospect of a wider application in many other fields relying on high turnover, reliable and unbiased biological species assignments. Pollination networks using metabarcoding as a data source reveal ecological interactions far more diverse and complex that previously assumed. With this tool, one is able to “see” into the hidden interactions and intricate workings of ecological networks with greater clarity. The foundation has been set- biological research began with naming and classifying species. Subsequent research has built upon this foundation, and we now know that ecological interactions is a pivotal sustaining force behind the ecological services which humanity relies on. Throughout history many powerful figures have admonished that “with great wisdom (knowledge) comes great responsibly”, and this is echoed by the admonition that "from everyone who has been given much, much will be demanded; and from the one who has been entrusted with much,

much more will be asked." The ability to detect biodiversity quicker and at a higher resolution must be accompanied by an urgency to protect this biodiversity and arrive at protective measures with as much speed and accuracy as possible. It is both our individual and collective responsibility.

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