Received: 15 November 2022 | Revised: 9 February 2023 | Accepted: 14 February 2023

DOI: 10.1002/edn3.399

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences

WILEY

ORIGINAL ARTICLE

Monitoring the birds and the bees: Environmental DNA metabarcoding of flowers detects plant–animal interactions

Joshua P. Newton[1,2] | Philip W. Bateman[1,3] | Matthew J. Heydenrych[2] | Joshua H. Kestel[1,2,4] | Kingsley W. Dixon[5] | Kit S. Prendergast[3] | Nicole E. White[2] | Paul Nevill[1,2]

[1]Minesite Biodiversity Monitoring with eDNA (MBioMe) Research Group, School of Life and Molecular Sciences, Curtin University, Perth, Western Australia, Australia

[2]Trace and Environmental DNA Laboratory, School of Life and Molecular Sciences, Curtin University, Perth, Western Australia, Australia

[3]Behavioural Ecology Lab, School of Molecular and Life Sciences, Curtin University, Perth, Western Australia, Australia

[4]Molecular Ecology and Evolution Group (MEEG), School of Science, Edith Cowan University, Joondalup, Western Australia, Australia

[5]ARC Centre for Mine Site Restoration, School of Molecular and Life Sciences, Curtin University, Perth, Western Australia, Australia

Correspondence
Joshua P. Newton, Minesite Biodiversity Monitoring with eDNA (MBioMe) Research Group, School of Life and Molecular Sciences, Curtin University, GPO Box U1987, Perth, WA 6102, Australia.
Email: joshua.p.newton@postgrad.curtin.edu.au

Funding information
Mineral Resources Limited

Abstract

Animal pollinators are vital for the reproduction of ~90% of flowering plants. However, many of these pollinating species are experiencing declines globally, making effective pollinator monitoring methods more important than ever before. Pollinators can leave DNA on the flowers they visit, and metabarcoding of these environmental DNA (eDNA) traces provides an opportunity to detect the presence of flower visitors. Our study, collecting flowers from seven plant species with diverse floral morphologies, for eDNA metabarcoding analysis, illustrated the value of this novel survey tool. eDNA metabarcoding using three assays, including one developed in this study to target common bush birds, recorded more animal species visiting flowers than visual surveys conducted concurrently, including birds, bees, and other species. We also recorded the presence of a flower visit from a western pygmy possum; to our knowledge this is the first eDNA metabarcoding study to simultaneously identify the interaction of insect, mammal, and bird species with flowers. The highest diversity of taxa was detected on large inflorescence flower types found on *Banksia arborea* and *Grevillea georgeana*. The study demonstrates that the ease of sample collection and the robustness of the metabarcoding methodology has profound implications for future management of biodiversity, allowing us to monitor both plants and their attendant cohort of potential pollinators. This opens avenues for rapid and efficient comparison of biodiversity and ecosystem health between different sites and may provide insights into surrogate pollinators in the event of pollinator declines.

KEYWORDS
avian metabarcoding assay, insect eDNA, plant–animal interactions, pollination, vertebrate eDNA

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2023 The Authors. *Environmental DNA* published by John Wiley & Sons Ltd.

1 | INTRODUCTION

Close to 90% of all 400,000 flowering plant species are dependent on animals for pollination (Ollerton et al., 2011). The taxonomic identities and ecology for many of these pollinators remains, however, an area of on-going discovery (Forister et al., 2019; Knight et al., 2018). Concerningly, many of these potential pollinating species (scientifically described and undescribed) are experiencing rapid declines around the world (Rafferty, 2017; Regan et al., 2015; Sánchez-Bayo & Wyckhuys, 2019). For example, wild pollinator diversity has more than halved in some areas in Europe (see Biesmeijer et al., 2006) and some pollinator species in North America have fallen in relative abundance by over 95% (Jacobson et al., 2018). Concurrently, native plant communities globally are showing community shifts that are altering the availability of foraging and nesting resources for wild pollinating species (Scheper et al., 2014). For instance, native plant species in America have shown range declines between 3 and 50% (see MacKenzie et al., 2019), while some areas in Europe have seen over 125 plant species lost (see Finderup Nielsen et al., 2019). The decline in native foraging resources threatens the resilience of remaining native pollinators, especially those with specialized plant relationships (Mola et al., 2021; although see Simanonok et al., 2021). These declines in wild insects and other animals, as well as the plants they interact with are driven by a combination of environmental and anthropogenic stressors (i.e., habitat loss, invasive species, and climate change; Potts et al., 2010). Consequently, there is an ever-growing need to increase our understanding of plant–animal interactions to ensure informed management decisions are made for species conservation and habitat restoration.

Insects have historically been the most frequently studied pollinators, with a bias toward managed insects such as the European honey bee (*Apis mellifera*). Out of nearly 4000 studies relating to pollination ecology, 65% cited insects (Millard et al., 2020). Many other vertebrate taxa, including birds (Krauss et al., 2018; Whelan et al., 2008), mammals (bats, primates, rodents, and marsupials; Carthew & Goldingay, 1997; Kunz et al., 2011), and reptiles (lizards; Olesen & Valido, 2003) are recognized as having important pollination impacts and effects on plant mating systems. Such diversity is especially pronounced for the South-Western Australia's Floristic Region (SWAFR), a globally recognized biodiversity hotspot with over 8379 vascular plant species and high levels of endangerment (Gioia & Hopper, 2017). Here, approximately 15% of all plant species are pollinated by vertebrates (Brown et al., 1997; Keighery, 1980) with insects also providing key pollination services (i.e., Lunau et al., 2021; Menz et al., 2013). As with elsewhere in the world, pollinators of native species in the SWAFR are experiencing declines in abundance, geographic range, and genetic connectivity due to habitat loss and fragmentation, climate change and associated increase in fire frequency (Bezemer et al., 2019; Campbell et al., 2022; Phillips et al., 2010; Prendergast, 2022). Due to the pace of decline in pollinators (Potts et al., 2010) there is a need to develop fast and accurate methods to detect animal visitors to flowers which, until now, relied on time-intensive field observation by specialists.

Conventional approaches for the identification of insect visitors and pollinators have relied on morphological identification after capture/observation, including pan, vane and camera traps, targeted netting, trap-nests, and observational records (O'Connor et al., 2019; Saunders et al., 2013). However, the accuracy of each method can vary between animal taxa and may miss certain groups, creating sampling bias (Prendergast et al., 2020; Prendergast & Hogendoorn, 2021). For instance, there are limitations in the ability of visual surveys to detect reclusive pollinators (Krauss et al., 2017). While camera traps can overcome this limitation and can provide valuable behavioral data (Droissart et al., 2021), they require a substantial time commitment not only in the field but also through the screening process as a result of technological complications such as false triggers (Krauss et al., 2017). In addition, the reliance on taxonomic expertise for morphological identification of species, a field that is in decline globally (Pearson et al., 2011), represents a substantial bottleneck when insects are involved in pollination (Stork, 2018). Direct observational approaches are also limited as the field time commitment may not capture all insect visitors/pollinators (Blüthgen, 2010). Therefore, to determine plant–animal interactions and to construct pollinator networks on multiple plant species within a highly biodiverse landscape, using conventional methods would be challenging. Instead, a variety of techniques and expertise are required to obtain reliable classifications and accurate information.

Environmental DNA (eDNA) metabarcoding is a recently developed molecular approach that may provide an opportunity to rapidly detect the presence of all floral visitors and pollinators. During plant–animal interactions, DNA can be deposited on flowers in detectable amounts (Evans & Kitson, 2020). eDNA metabarcoding involves the extraction of this DNA and high-throughput sequencing of taxonomically informative genome regions, known as "barcode regions," that have been amplified using polymerase chain reaction (PCR) technology (Taberlet et al., 2012). These approaches have successfully been tested as a means to detect flower-visiting insects (Thomsen & Sigsgaard, 2019). Similarly, eDNA has been used to detect a variety of vertebrates including birds (Ushio et al., 2018), mammals (Ishige et al., 2017) and reptiles (Kucherenko et al., 2018), from substrates such as soil (Andersen et al., 2012; Ryan et al., 2022), water (Ushio et al., 2018), scat (Van Der Heyde et al., 2021), and saliva deposited on plant material by browsing animals (Nichols et al., 2012). Thus, eDNA metabarcoding offers an exciting opportunity to rapidly detect both insect and vertebrate visits from multiple flowering plant species simultaneously.

While the ability to detect insect visits to flowers using eDNA shows promise (Evans & Kitson, 2020; Gamonal Gomez et al., 2022; Harper et al., 2022; Thomsen & Sigsgaard, 2019), the technique has only recently been used to identify vertebrate flower visitors (Jønsson et al., 2023; Walker et al., 2022). Furthermore, while eDNA metabarcoding surveys have been shown to outperform the use of both visual (Barata et al., 2020) and camera trap (Leempoel et al., 2020) techniques in other contexts, there are limitations of the method, including limited knowledge on the deposition and

persistence of eDNA on floral surfaces (Barnes & Turner, 2016; Harrison et al., 2019; Valentin et al., 2021). To better understand any limitations of this novel approach, we compared eDNA metabarcoding of flowers with conventional plant–animal interaction survey methods. We tested this technique using flowers from seven plant species, with diverse floral morphologies, within a pristine natural environment (Helena and Aurora Range) that is home to more than a dozen rare, short-range endemic, and threatened plant species (Gibson et al., 2012). Three eDNA barcoding assays were used to determine if eDNA metabarcoding can reliably detect plant–animal interactions verified with conventional pollinator visual surveys. Ease of data collection and the robustness of the metabarcoding methodology has profound implications for future management of biodiversity, allowing us to monitor both plants and their attendant cohort of potential pollinators. This opens avenues for us to compare biodiversity and ecosystem service health rapidly and efficiently between different sites.

2 | METHODS

2.1 | Study site

Our site was within the Helena and Aurora Range (Kalamaia name: "Bungalbin"), in the Goldfields-Esperance region of Western Australia (Figure 1). We undertook two visits: one in spring time September 2020, when visual pollinator surveys for insect, bird and mammal pollinators were conducted for six focal flowering species - *Acacia adinophylla*, *Eremophila clarkei*, *Eremophila oppositifolia*, *Grevillea georgeana*, *Leucopogon spectabilis* and *Tetratheca aphylla* subsp. *aphylla*, followed by the collection of flowers for eDNA analysis, and one in autumn May 2021, when the same procedures were repeated for *Banksia arborea*, which was not flowering on the first visit. These plants represented a range of species with different flower morphologies and different assumed pollinators. Furthermore, many of the plant species sampled are of conservation concern, with little information on pollinating taxa currently available (Figure 2; Appendix S5).

2.2 | Flower sample collection

A total of 175 samples were taken from the same individual plants surveyed for bird and insect pollinators and subsequently used in the eDNA metabarcoding study. Based on morphology and size, flowers were assigned to one of three categories: large inflorescence, small inflorescence, or single flower (Figure 2). From the seven plant species, five complete flowers or inflorescences were collected from five individual plants totaling 25 flowers/inflorescences per species. To prevent contamination, single-use sterile nitrile gloves were worn during sampling and all flowers were collected in individual sterile plastic tubes. All samples were then stored on ice in a well-insulated polystyrene ice box (60-mm walls) after sampling and stored below −20°C after returning from the field (within 48 h) until DNA extraction.

2.3 | Bird surveys

Bird pollinator surveys were undertaken during peak foraging times for birds: before 1000 and after 1500 (Gilpin et al., 2017). Each plant was surveyed once during each time period, with all visible inflorescences on each focal plant observed simultaneously for 20 min from a distance of approximately 20 m to minimize disturbance (Gilpin et al., 2017). However, where it was not possible to survey from this distance due to vegetation and terrain restraints, surveys were undertaken from no <10 m and as close to 20 m as possible. The specific plant and observation time for each plant was chosen at random to avoid temporal bias, and the species and number of birds that interacted with flowers recorded. A full list of the taxa observed are listed in Table S1.

2.4 | Insect surveys

Insect pollinators were surveyed on the same individual plants surveyed for bird pollinator surveys during daylight hours when temperatures were warm enough for bee activity (approx. 0900–1600, >16°C) by a single experienced entomologist (K.S.P). Surveys were conducted on each plant for 10 min, approximately 1 m away from the plants, except for flora that were inaccessible (*L. spectabilis*). Visiting insect taxa were classified to the lowest taxonomic level possible via visual observation in the field. For the purposes of analyses, insect taxa observed were collapsed to family level; a full list of the taxa observed are listed in Table S2.

2.5 | Nocturnal surveys

Nocturnal surveys were conducted approximately 40 min after sunset using torches for 60 min throughout the study site on two consecutive nights to detect nocturnal mammal pollinators. No fauna was seen.

2.6 | Bird primer design

Searches for available mitochondrial DNA sequences of 134 common bush birds of south-western Australian (SWWA) were conducted using NCBI's nucleotide database (Altschul et al., 1990). The mitochondrial gene NADH dehydrogenase subunit 2 (ND2) provided the most extensive number of available sequences (120 species, each ~1040 bp length; Appendix S7, Table S10). The identified avian ND2 sequences were downloaded from NCBI (Altschul et al., 1990) and aligned using the MUSCLE plugin (default settings) in Geneious v11.0.12 (Kearse et al., 2012). Based

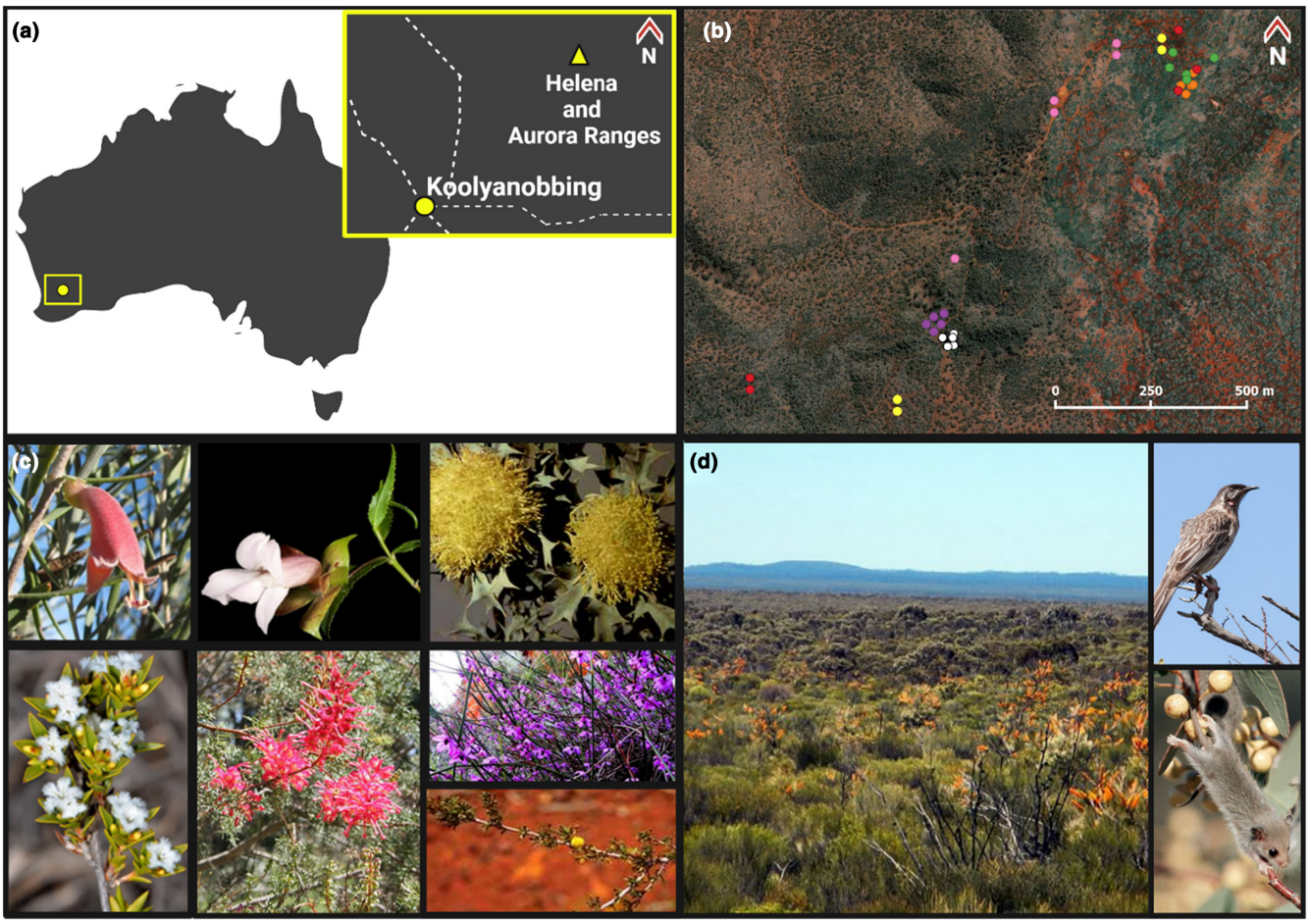


FIGURE 1 (a) Helena and Aurora Range is a 2.5-billion-year-old Banded Iron Formation located in the south west of Western Australia. (b) Sampling sites within the Helena and Aurora Range; yellow = *Acacia adinophylla*, green = *Banksia arborea*, orange = *Eremophila clarkei*, pink = *Eremophila oppositifolia*, red = *Grevillea georgeana*, white = *Leucopogon spectabilis*, and purple = *Tetratheca aphylla* subsp. *aphylla*. (c) Target species, clockwise from top left: *E. oppositifolia*, *E. clarkei*, *B. arborea*, *T. aphylla* subsp. *aphylla*, *A. adinophylla*, *G. georgeana*, and *L. spectabilis*. (d) Typical landscape in Helena and Aurora Range (top) red wattlebird (*Anthochaera carunculata*) and (bottom) western pygmy possum (*Cercartetus concinnus*). Panel (c and d) Images courtesy of Wikimedia Commons and Florabase. Graphic made in Biorender.

on the alignments, candidate universal primer sets were designed using Primer3 within Geneious (Koressaar & Remm, 2007; Untergasser et al., 2012). Candidate primers were then visually assessed to determine the best sites for primer pairing and maximum taxonomic resolution within the internal amplified regions. To allow primer annealing to polymorphic sites, both the forward and reverse primers were modified to include degenerate bases, and primer characteristics were analyzed (G/C content, temperature, secondary structures, primer pair compatibility) to further improve the choice of primer using both Primer3 within Geneious (Koressaar & Remm, 2007; Untergasser et al., 2012) and the online bioinformatics tool Sequence Manipulation Suite: PCR Primer Statsn (Stothard, 2000). The following ND2 assay was designed to generate an approximately 229 bp amplicon (excluding primers): BirdND2F 5′ CCATTCCACTTYTGRTTYCC 3′ and BirdND2R: 5′GGGAGATDGADGARAADGC 3′.

In silico analysis was then performed to test the specificity of the priming sites for target taxa and resolution of internal amplified regions. Specificity of the primer set was tested through in silico PCR using the "search_PCR" command in USEARCH v11.0.667 (Edgar, 2010), targeting both the newly generated target bird species list and custom generated full-length mitochondrial DNA reference databases (Sakata et al., 2022). These DNA reference databases were comprised of 18,283 mammals, 10,594 fish, 4510 birds, 1461 reptiles, and 746 amphibian sequences (35,597 vertebrate sequences). In silico PCR was performed under the following parameters: min amplicon size: 100, max amplicon size 1000, mismatch 0, 1, 2, 3. To determine the dissimilarity of internal amplified regions generated from both the custom target taxa database and full-length mitochondrial bird database the "dist.dna" function within the "ape" package (Paradis & Schliep, 2019) was used in R v.4.0.3 (R Core Team, 2013).

To assess PCR amplification, efficiency, sensitivity, and optimal annealing temperature, in vitro testing was conducted using the gDNA of two bird species: Red-tailed black cockatoo (*Calyptorhynchus banksii*) and rainbow lorikeet (*Trichoglossus moluccanus*). A temperature gradient from 51 to 61°C, in combination with seven 10-fold gDNA dilutions from 1:10 to $1:10^{12}$, inclusive

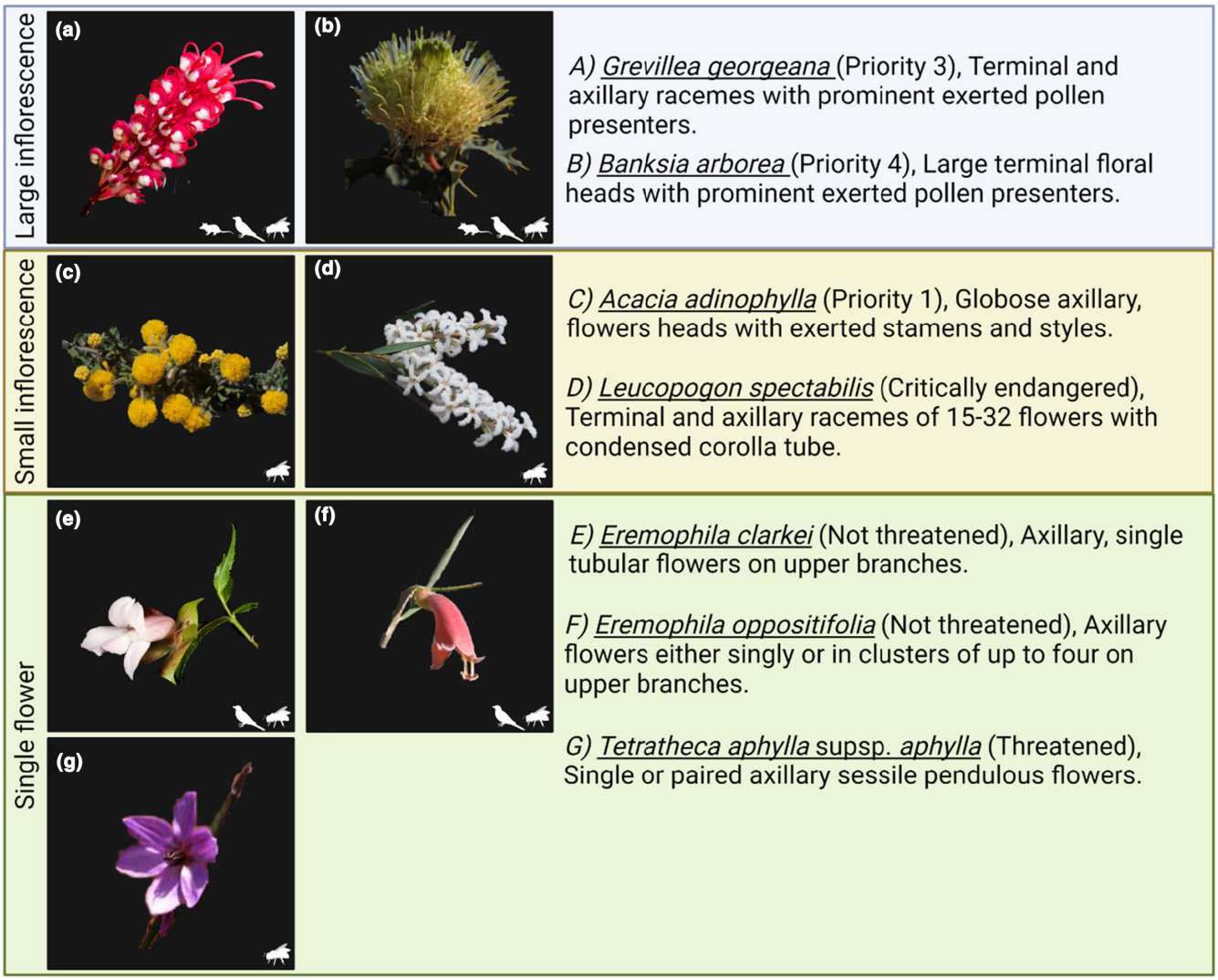


FIGURE 2 Plant species surveyed and sampled for eDNA metabarcoding within the Helena and Aurora Range. Plant species are grouped by flower type and likely pollinators described in the literature represented (insect, bird, and mammal; see Appendix S5). Information on inflorescence/flower structure and conservation status under the Biodiversity Conservation Act 2016 (WA) provided. Images modified from Wikimedia Commons and Florabase. Graphic made in Biorender.

of non-template controls (NTCs), were evaluated. The temperature gradient showed 53°C as the optimal annealing temperature with NTCs undetected and gDNA detected at all levels of dilution from the quantitatuve PCR (qPCR) assay.

To determine the efficacy of the newly developed assay, in vitro testing was conducted on bird bath water (n = 8; 500 mL each) from Perth, Western Australia, and a mixed positive genomic DNA (gDNA) sample containing red-tailed black cockatoo (*C. banksii*), rock dove (*Columba livia*), and southern boobook (*Ninox novaeseelandiae*) gDNA. Bird bath water samples were filtered through a Pall 0.45 μm GN-6 Metricel® mixed cellulose ester membranes using a Pall Sentino® Microbiology pump (Pall Corporation, Port Washington, USA) and frozen at −21°C prior to extraction. Filtered birdbath samples were extracted and amplified alongside mock tissue samples using birdND2 and 12S-V5F2/R2 (Riaz et al., 2011) assays for comparison and then sequenced as described below.

2.7 | Sample processing and DNA extraction

All laboratory processes were conducted in dedicated laboratories within the Trace and Environmental DNA (TrEnD) Laboratory, Curtin University, Perth, Western Australia. Large inflorescences (*B. arborea*, *G. georgeana*) were individually placed in decontaminated plastic containers and covered with purified distilled water (~500 mL) for 10 min, manually agitated twice for 30 seconds at 5-min intervals. Water samples were then individually filtered across Pall 0.45 μm GN-6 Metricel® mixed cellulose ester membranes using a Pall Sentino® Microbiology pump (Pall Corporation) and frozen at −21°C prior to extraction. For all but *A. adinophylla* samples, DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen) with lysis performed by either adding a small flower, small inflorescence, or half filter membrane to a 2-mL plastic tube, adding 60 μL of Proteinase K, 540 μL of ATL lysis buffer and digesting for 17-h at 56°C. For *A.*

adinophylla inflorescences, all extraction parameters remained the same except for 120 μL of Proteinase K and 1080 μL of ATL lysis buffer added due to the absorbent property of the flowers. DNA was then extracted from the digest supernatant using the QIAcube extraction platform (Qiagen). To detect possible cross-contamination during sample filtering, 500 mL of bleach solution, used for rinsing the pump equipment and containers between samples, was filtered and processed in the same way as field samples ($n = 2$). Similarly, digest ($n = 1$) and extraction controls ($n = 4$) were processed with each batch of samples.

2.8 | Assessment of DNA extracts

Two assays were used to amplify eDNA from all flowers ($n = 175$), fwhF2/fwhR2n (F: 5'-GGDACWGGWTGAACWGTWTAYCCHCC-3′; R: 5′- GTRATWGCHCCDGCTARWACWGG-3′; Vamos et al., 2017), hereafter referred to as fwh, designed to amplify a 205 bp fragment of the arthropod cytochrome c oxidase I (COI) gene region and birdND2 (229 bp) designed to target local bird species (see above). On all *B. arborea* ($n = 25$) and *G. georgeana* ($n = 25$) flowers, the 16Smam1/2 assay (F: 5'-CGGTTGGGGTGACCTCGGA-3′; R: 5'-GCTGTTATCCCTAGGGTAACT-3′; Taylor, 1996), hereafter referred to as 16Smam, designed to amplify a ~130 bp fragment of mammalian 16 S ribosomal gene region was also used to detect small mammals known to pollinate these taxa.

To assess the quality and quantity of DNA in each extract, and determine the optimal level of DNA input, qPCR was carried out on three PCR replicates per sample at neat, 1:10, and 1:100 dilutions (Murray et al., 2015). All qPCR reactions were performed on a StepOnePlusTM Real-Time PCR system (Applied Biosystems) with reaction volumes totaling 25 μL. Reactions contained 1× PCR Gold buffer (Applied Biosystems), 2.5 mM MgCl2 (Applied Biosystems), 0.4 mg/mL BSA (Fisher Biotec), 0.25 mM of each dNTPs (Astral 204 Scientific Australia), 0.4 μM forward and reverse primer, 1 U AmpliTaq Gold (Applied Biosystems), 0.6 μL SYBR Green (Life Technologies) and 2 μL of template eDNA. Cycling conditions were initial denaturation at 95°C for 5 min, followed by 55 cycles at 95°C for 30 s, then 30 s at primer specific annealing temperature (50°C for fwh, 53°C for birdND2 and 55°C for 16Smam), and 45 s at 72°C, ending with 10 min elongation at 72°C. To detect possible contamination a non-template control (reagents only) was used, and a positive control to ensure efficacy of PCR reagents and cycling conditions.

2.9 | DNA amplification and sequencing

Based on the qPCR amplification (as detailed above), DNA extracts with sufficient DNA quantity and quality were assigned a unique 6–8 bp multiplex identifier tag (MID tag) for each birdND2, 16Smam and fwh primer set. Independent MID tag qPCRs were prepared in a physically separate DNA-free laboratory, in duplicate, on each potentially positive extract and control with qPCR solutions containing unique combinations of MID tag (fusion) primers with the same reagents and cycling conditions described above. Template eDNA volumes varied based on the qPCR amplification ΔRn values, CT values, and melt curves generated, resulting in reaction volumes ranging from 25–35 μL containing 2–12 μL of template eDNA (Table S3). MID tag amplicons were then pooled together with other MID-tag amplicons according to ΔRn values. Pooled samples were then quantified using the QiAxcel Advanced System (Qiagen) and concentrations (ng/μL) used to combine all pools in appropriate equimolar ratios creating a DNA sequence library. Size selection of amplicons (200–400 bp) was then performed using a PippinPrep (Sage Science) before samples were purified using a QIAquick PCR Purification Kit (Qiagen), and re-quantified using a Qubit Fluorometer (Invitrogen). Sequencing was then performed on an Illumina MiSeq platform (Illumina), as per Illumina protocols for single-end sequencing (MiSeq® v2 Reagent Kit 300 Cycles PE), with a final library molarity of 6 pM containing 7% PhiX.

2.10 | Sequence analysis (filtering and taxonomic assignment)

Sequence filtering and taxonomic assignment were conducted using the eDNAFlow bioinformatics pipeline (Mousavi-Derazmahalleh et al., 2021) via the supercomputer Magnus, based at the Pawsey Supercomputing Centre in Kensington, Western Australia. Quality checking of sequence reads was performed using FASTQC (Andrews, 2010), and quality filtered (minimum Phred quality score of 20), including trimming sequences with Ns using AdapterRemoval v2 (Schubert et al., 2016). Demultiplexing was achieved using obitools (ngsfilter; Boyer et al., 2016) and sequences under 60 bp removed (obigrep). Dereplication and the creation of zero-radius operational taxonomic units (ZOTUs; min sequence abundance = 4) and ZOTU tables were then generated using the USEARCH unoise3 algorithm (Edgar, 2016). The ZOTUs were queried using the following parameters: % identity ≥ 97, evalue ≤ 1 e−3, % query cover 100, max target sequences = 10 via a BLASTN search (Altschul et al., 1990) against NCBI's GenBank nucleotide database (accessed in January 2022). Erroneous ZOTUs were identified and removed using the post clustering curation method LULU with the minimum threshold of sequence similarity at 95 (Frøslev et al., 2017). A custom python script (Mousavi-Derazmahalleh et al., 2021) was then used to taxonomically assign ZOTUs by the lowest common ancestor (LCA) approach, with taxonomic assignment collapsed to the LCA if the percent identity of two hits with 97% query cover and 95% identity, differed by <1%.

Further filtering of the metabarcoding data was performed using the "phyloseq" package (McMurdie & Holmes, 2013) in R v.4.0.3 (R Core Team, 2013). Initially, sequence counts in eDNA samples below the threshold value of 5 were discarded. Following this, ZOTUs present in negative controls, common PCR contaminants (e.g., human and ungulate sequences), and taxonomic assignments higher than family level were removed from the data set. Species likely not present at the sample site were also removed from the data set

or collapsed to genus or family level based on species distribution data (e.g., *Cymbacha similis* to Cymbacha sp. and *Exorista deligata* to *Exorista* sp.). Genera *Colluricincla* and *Manorina* were then reassigned to *Colluricincla harmonica* (grey shrike-thrush) and *Manorina flavigula* (yellow-throated miner), respectively, based on existing species distribution data. ZOTUs from all metabarcoding assays were combined for further analysis and individual flower samples agglomerated to plants. The ZOTU table was then transformed to presence–absence data for community comparisons.

2.11 | Statistical analysis

Statistical analysis was performed in R v.4.0.3 (R Core Team, 2013). Initially, animal ZOTU richness was calculated for each flowering plant species using the "phyloseq" package (McMurdie & Holmes, 2013). Species accumulation curves were then calculated using the number of observed ZOTUs by the number of flower samples using the "accumcomp" function in the "BiodiversityR" package (Kindt & Coe, 2005) with *A. adinophylla* and *L. spectabilis* removed due to the detection of a taxa on only a single plant. Observed ZOTU richness was then square-root transformed to meet the assumption of normality and homogeneity of variance and an analysis of variance (ANOVA) was used to compare the square-root transformed ZOTU richness detected between plant species (factor = plant species, level 6). A Tukey-HSD post-hoc test was then run to assess the significant differences between plant species. Data were visualized using the package "ggplot2" (Wickham, 2016).

A Kruskal–Wallis rank-sum test was used to compare the observed ZOTU richness between flower types (factor = sample type, levels 3; large inflorescence, small inflorescence, single flower). A pairwise Wilcoxon post-hoc test was then run to assess the significant differences between flower types with a Benjamini–Hochberg correction for multiple comparisons with visual surveys.

Data were then transformed to presence–absence data and a one-way permutational MANOVA (PERMANOVA) was conducted to compare the ZOTU presence/absence composition between the different species using the "vegan" package (Oksanen, 2009) with Jaccard similarity and 9999 permutations. Pairwise comparisons were performed using the "PairwiseAdonis" (Arbizu, 2020) package with a Benjamini–Hochberg correction for multiple comparisons. A Sankey plot was created using R package network3D (Allaire et al., 2017) to show the links between plants and all taxa detected within this study.

To make direct comparisons with visual surveys, while bird and mammal species identifications were retained at species level, all arthropods detected using eDNA metabarcoding were collapsed to family level (see insect survey methods outlined above). The similarity between eDNA metabarcoding and conventional visual surveys was then calculated between species presence/absence matrices per plant (matrices of species x plants surveyed) using a Mantel test (Pearson method, 999 permutations) on dissimilarity indices calculated using the Jaccard method, computed with the function "mantel" of the "vegan" package (Oksanen, 2009).

3 | RESULTS

3.1 | Evaluation of birdND2 metabarcoding assay

The specificity of each newly developed birdND2 assay was evaluated through in silico PCR, with avian taxa showing the highest amplification levels (Appendix S7, Table S11). A distance matrix (Appendix S7, Table S12) showed the number of bases that differ within internal amplified regions of target taxa varied between 0 and 74 bp, with a mean of 49.9 bp (SE = 9.3). Only three closely related species pairs (*Anas castanea* and *Anas gibberifrons gracilis*, *Cacatua sanguinea* and *Cacatua pastinator*, *Calyptorhynchus baudinii*, and *Calyptorhynchus latirostris*) contained identical amplicon regions and therefore would not be able to be resolved to species level if detected via eDNA metabarcoding.

The newly developed birdND2 avian and existing 12S-V5 general vertebrate metabarcoding assays were evaluated in vitro using both a mock community and filtered water obtained from local bird baths, to infer their ability to detect avian taxa. A total of 899,914 avian sequences were generated from the birdND2 and 12S-V5 assays, yielding 612,188 and 287,726 quality-filtered sequences, respectively. The newly developed birdND2 data set included a total of 17 avian ZOTUs, 14 assigned to species, and three to genus (Figure 3). BirdND2 outperformed the 12S-V5 assay which identified a total of five avian ZOTUs, two assigned to species, three assigned to genius and one assigned to a family. However, the birdND2 assay failed to detect Australian wood duck (*Chenonetta jubata*) and galah (*Eolophus roseicapilla*) successfully detected by the 12S-V5 assay (Figure 3).

3.2 | eDNA metabarcoding of flowers and visual surveys

In total, 8,028,614 metabarcoding reads were generated by the three assays used to detect animal DNA from flower samples, with 3,676,391 sequences remaining after quality filtering. Of these, the birdND2 assay yielded 2,601,729 sequences from 21 samples (mean = 123,892, SD = 21,505), the fwh assay yielded 1,074,431 sequences from 30 samples (mean = 35,814 SD = 14,569), and the 16Smam assay yielded 1118 sequences from 5 samples (mean = 224, SD = 106). Prior to filtering, over 90% of sequences generated from the 16Smam assay were *Homo sapiens*, with a further six contaminant mammal species identified (*Canis sp.*, *Ovis sp.*, *Bos taurus*, *Sus scrofa*, *Pan troglodytes*, *Felis* sp.), all of which were removed from the data set (Appendix S6, Table 9). Similarly, two arthropod species likely not present at the sample site, *Liposcelis bostrychophila* (booklouse) and *Parosteodes fictiliaria*, were also removed from the data set. Three non-target plant species (*Lotus japonicus*, *Telopea speciosissima*, and Bignoniaceae sp.) were also detected using the BirdND2 assay and subsequently removed. In total, across all three assays, eDNA metabarcoding detected 59 unique taxa (Figure 4) from the classes Mammalia ($n = 5$), Aves ($n = 7$), and Arthropoda ($n = 47$), for a total of 41 families detected within 16 orders. Of these taxa, 26 were

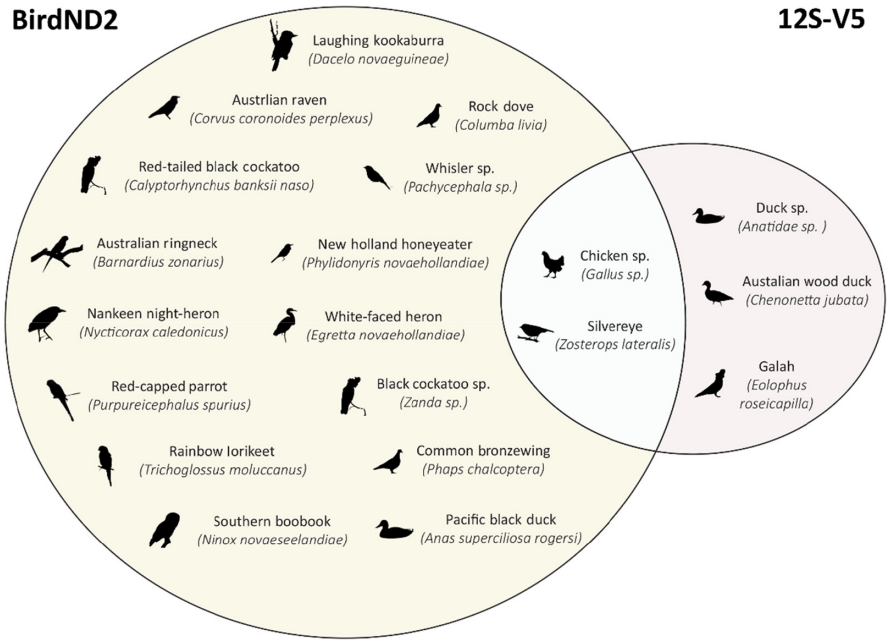


FIGURE 3 Bird taxa detected for birdND2 and 12S-V5 metabarcoding assays from both mock community ($n = 1$) and bird bath samples ($n = 8$). Where ZOTUs had multiple possible assignments, the higher taxonomic order was retained and labeled "sp."

assigned to species level (Mammalia = 3, Aves = 7, Arthropoda = 16), 21 to genus (Mammalia = 1, Arthropoda = 20), and 12 to family (Mammalia = 1, Arthropoda = 11).

3.3 | Differences in taxa detected by plant species

eDNA metabarcoding of flowers detected arthropods on all plant species, while vertebrate pollinators were detected solely on *B. arborea*, *E. clarkei*, and *G. georgeana* (Figure 5). An ANOVA test on ZOTU richness found a significant difference in the number of ZOTUs detected between plant species ($F = 7.151$, df = 6, $p < 0.001$). Total ZOTU richness was highest for *B. arborea* and *G. georgeana*, with the Tukey-HSD test indicating a significant difference between *B. arborea* and all species except *G. georgeana* (Figure 5). Conversely, no significant difference was found between *G. georgeana* and all species (Figure 5). No significant difference was seen between all other plant species (Figure 5; Table S5). A Kruskal–Wallis rank-sum test showed a significant difference between flower types (X2 = 17.535, df = 2, $p < 0.001$) with a greater number of ZOTUs detected on large inflorescences (*B. arborea* and *G. georgeana*) than both small inflorescences and single flowers (Table S4). No difference in the number of ZOTUs was seen between small inflorescences and single flower samples (Table S4). The Sankey plot shows that the highest diversity of taxa was also detected on the large inflorescence flower types found on *B. arborea* and *G. georgeana* (Figure 6). Conversely, only a single termite genus (*Amitermes sp.*) was detected on the relatively small *A. adinophylla* inflorescences. eDNA metabarcoding of the specialized, likely buzz-pollinated *T. aphylla* subsp. *aphylla* flowers (see Ladd et al., 2019) only detected feral honey bees (Apidae) and failed to detect Australian bee species known to be present in the area. Of the animal species detected on *L. spectabilis* only those in the insect order Diptera (*Heterotrissocldius sp.*, *Coenosia octopunctata*, and *Ochlerotatus camptorhynchus*) could be considered potential pollinators (Keighery, 1996).

A PERMANOVA detected a significant difference in animal community composition between plant species ($F = 2.702$, df = 6, $R^2 = 0.283$, $p > 0.001$) with a pairwise comparison indicating the species composition of *B. arborea* significantly differed from *E. oppositifolia*, *G. georgeana* and *T. aphylla* subsp. *aphylla*. Similarly, *E. clarkei* significantly differed from *G. georgeana*. (Table S6). NMDS did not show clear segregation between plant species (Figure S1).

Although multiple flowers ($n = 5$) were taken from each plant surveyed, eDNA metabarcoding did not amplify animal DNA from all the flower samples. eDNA metabarcoding only detected taxa on a single flower for both *A. adinophylla* and *L. spectabilis*. In contrast, taxa were detected on 21 *B. arborea* and 14 *G. georgeana* inflorescences surveyed. ZOTU richness did not reach an asymptote for any species as the number of flowers sampled increased (Figure 7).

3.4 | Visual survey diversity recorded

Visual surveys resulted in the detection of 14 arthropods and two bird taxa, with no mammal detections, culminating in a total of 13 families across four orders. All bird taxa detected were assigned to species level. Of the arthropod taxa detected, one was assigned to species level, five to genus, seven to family, and one to order.

3.5 | Taxonomic composition differences between visual surveys and eDNA

A Mantel test found no significant correlation between the taxa detected on plants by visual surveys and eDNA metabarcoding of flowers (Mantel statistic = 0.065, $p = 0.902$). Of the 35 individual plants

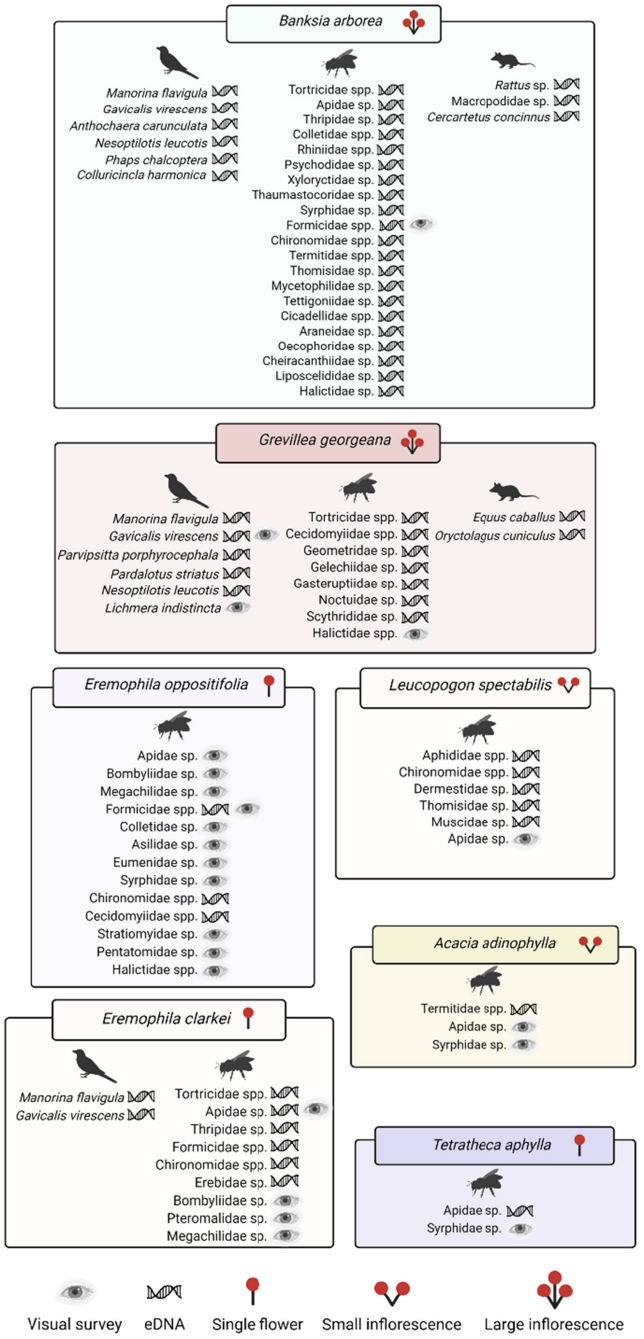


FIGURE 4 Animal diversity identified from visual surveys and eDNA metabarcoding of flowers collected from the Helena and Aurora Range. Arthropods detected using eDNA metabarcoding are collapsed to family level. Graphic made in BioRender.

surveyed, eDNA metabarcoding and visual surveys detected taxa on 19 and 23 plants, respectively. However, eDNA metabarcoding of flowers detected a greater number of taxa ($n = 59$) compared with visual surveys ($n = 16$; Figure 4). Of the taxa detected by both eDNA and visual surveys, only Apidae sp., Formicidae sp. and the singing honeyeater (*Gavicalis virescens*), were detected on the same plant using both survey methods (Figure 4). While eDNA metabarcoding detected a variety of arthropod species, the most abundant arthropod family detected in visual surveys Apidae (represented solely by the introduced European honey bee; *Apis mellifera*), detected on 13 out of 35 plants surveyed, was only detected on four plants using eDNA metabarcoding. Similarly, eDNA metabarcoding failed to detect the brown honeyeater (*Lichmera indistincta*), despite this species being recorded in visual surveys. However, an additional assay targeting the 12S-V5 mitochondrial region was run on selected flowers with positive visual sightings (see Appendix S4 for methods) and successfully amplified brown honeyeater's DNA. Only eDNA surveys detected mammal species including the successful detection of the western pygmy possum (*Cercartetus concinnus*). Similar to eDNA metabarcoding, visual surveys failed to detect potential buzz pollinators on *T. aphylla* subsp. *aphylla*, although visual surveys did successfully detect potential pollinators on *A. adinophylla* (European honey bee and hover fly [Syrphidae] species; Figure 4). Visual surveys also identified significant native bee fauna diversity (e.g., Megachilidae, and oligolectic *Euhesma* [Colletidae]) on *Eremophila* species that were undetected using eDNA metabarcoding. While both eDNA and visual surveys detected a variety of potential pollinator species, eDNA metabarcoding of flowers also detected various non-pollinator species including phytophagous insects (e.g., thrips and aphids), gall inducers (e.g., Cecidomyiidae), predators (e.g., spiders), and large mammals (e.g., kangaroo and horse families).

4 | DISCUSSION

In this study, we successfully detected the DNA of both invertebrates and vertebrates on flowers, including the detection of both bird and mammal flower visitors using eDNA metabarcoding. This suggests that eDNA metabarcoding of flowers can offer a strong complement to conventional plant–animal interaction monitoring techniques, increasing the capacity to identify a more complete set of floral visitors. Furthermore, this study demonstrates the utility of this survey method for understudied and rare plant species that may be difficult to monitor if they grow in remote regions, receive relatively few pollinator visits, or are visited by cryptic/reclusive animal species.

4.1 | eDNA metabarcoding survey

The metabarcoding of eDNA from flowers detected a wide range of organisms including three mammal species, eight bird species, and 57 arthropod taxa, with different assemblages of animals detected on each plant species. Multiple bird pollinator species, including the singing honeyeater (*Gavicalis virescens*) and the yellow-throated miner (*Manorina flavigula*) were detected on *E. clarkei*, *B. arborea*, and *G. georgeana*, all of which have related taxa that are known to be bird pollinated (e.g., *E. glabra* ssp. *glabra*, Elliott, 2009; *B. menziesii*, Krauss et al., 2018; *G. macleayana*, Whelan et al., 2009). A cryptic mammal species, the western pygmy possum, was also detected on *B. arborea* flowers—indicating its presence in the area and role as a potential pollinator. Furthermore, many of the arthropod

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences
WILEY

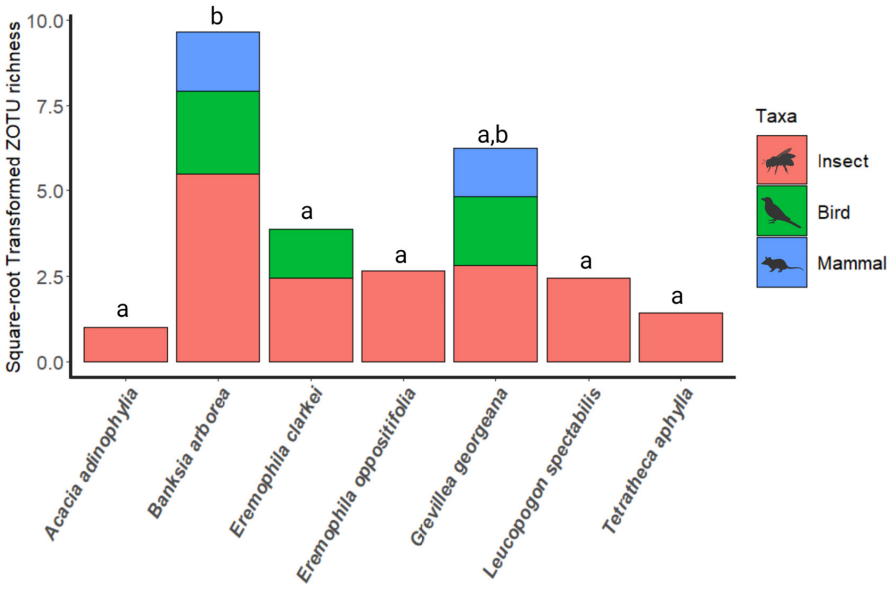


FIGURE 5 Square-root transformed observed ZOTU richness per plant species. The letters show the results from Tukey-HSD test, indicating statistically different ZOTU richness values between seven plant species sampled within the Helena and Aurora Ranges in Western Australia.

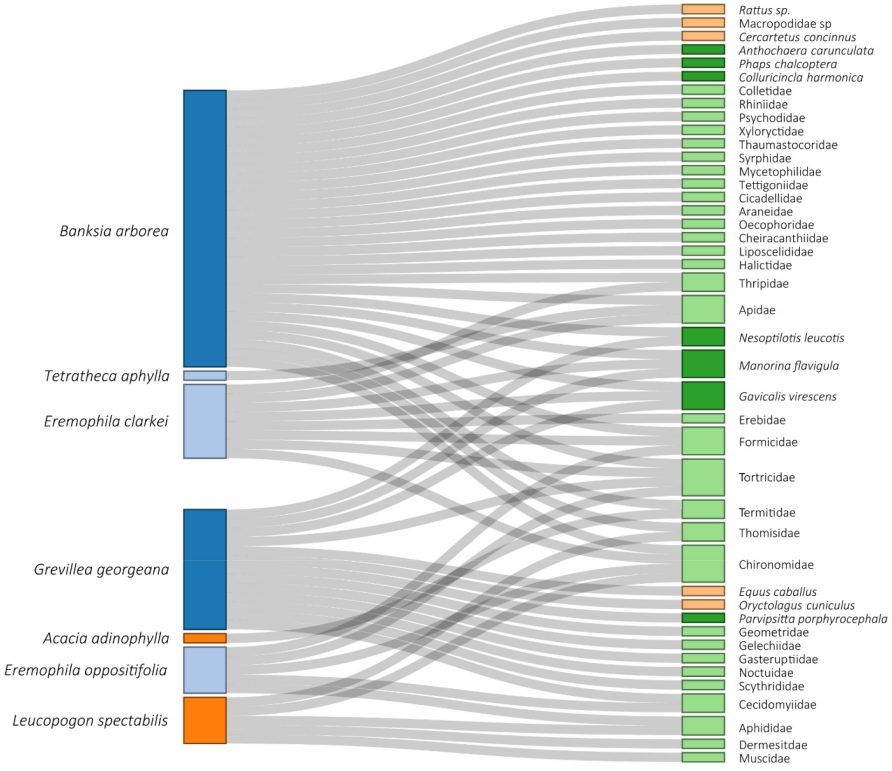


FIGURE 6 Taxa identified on each plant species using eDNA metabarcoding of flowers. Where ZOTUs had multiple possible assignments, the higher taxonomic order was retained and labeled "sp." Plant taxa names color denote sample type (dark blue: large inflorescence, orange: small inflorescence, and light blue: single flower). Taxa name color denotes taxonomic class (light green: arthropoda, peach: mammalia, and dark green: aves).

families identified in this study using eDNA metabarcoding have not previously been recorded as flower-visitors. The moth families Oecophoridae, Xyloryctidae, and Tortricidae have not previously been recorded as flower-visitors for the *Banksia* genus, nor have the moth families Noctuidae, Geometridae and Tortricidae been previously recorded as floral-visitors for the *Grevillea* genus (Table S8). Until now, no studies have provided community descriptions for the focal flower visitors for many of these study species; here however, using a relatively non-invasive molecular technique, we identified flower-visiting taxa that may provide pollination services to these conservation priority plant species. As such, this study indicates that eDNA metabarcoding of flowers offers a survey method able to detect past interactions and increase the understanding of plant–animal interactions of commonly understudied species.

Supporting Thomsen and Sigsgaard (2019), we found that inflorescence size had the greatest impact on the number of taxa detected using eDNA metabarcoding. Larger inflorescences attracted an array of species, and we detected a significantly greater number

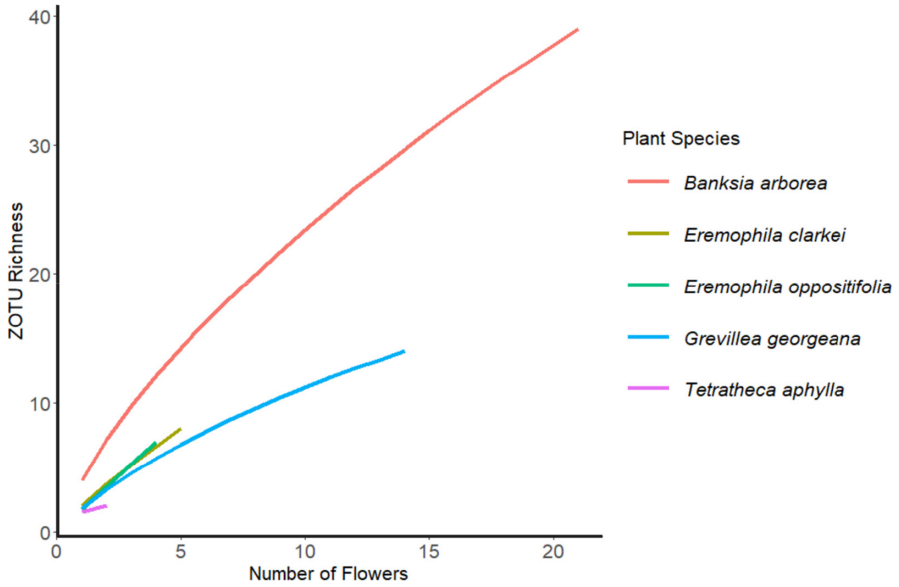


FIGURE 7 ZOTU accumulation curve showing the number of ZOTUs by the number of flower samples collected within the Helena and Aurora Ranges, Western Australia. *Acacia adinophylla* and *Leucopogon spectabilis* were removed as eDNA metabarcoding only detected taxa on a single flower sample.

of animal species on these flower types than we did on small inflorescences and single flowers (Figure 6). Of these smaller specialized flowers, eDNA metabarcoding of flowers was unsuccessful at detecting many expected pollinators. For example, eDNA metabarcoding of flowers from *T. aphylla* subsp. *aphylla* failed to detect Australian bee fauna known to "buzz" pollinate other *Tetratheca* species with similar flower morphology (e.g., *Lasioglossum* species, which pollinate *Tetratheca paynterae* subsp. *paynterae*; Ladd et al., 2019). Similarly, native bee species that are considered major pollinators of *Leucopogon* species were not detected on *L. spectabilis* (Keighery, 1996). While this may in part be due to sample size, some of our study species (e.g., *T. aphylla* subsp. *aphylla* and *A. adinophylla*) where few animal taxa were detected using eDNA metabarcoding, also showed a similar result with visual surveys, suggesting a lack of pollinator activity at the time of sampling. This emphasizes the need to adapt sampling methodologies to the plant species sampled to detect the full range of taxa. For all flower types, but particularly smaller flowers, an increase in sample size (both the number of flowers and the number of plants sampled) may increase the likelihood of detecting pollinator taxa. Similarly, sampling at multiple time points may also increase the likelihood of detecting pollinators absent at the time of sampling.

A limitation of eDNA metabarcoding of flowers to detect pollinators is its inability to distinguish between animals that simply come into contact with flowers versus those that act as pollinators. We detected not only a variety of potential pollinators (bees, birds and butterflies) but also non-pollinator taxa such as phytophagous insects (e.g., thrips and aphids), gall inducers (e.g., Cecidomyiidae), predators (e.g., spiders), and large mammals (e.g., kangaroo and horse). While this suggests opportunities for further applications of eDNA metabarcoding such as the detection of agricultural pests (Kestel et al., 2022), explicitly determining pollinators, requires exclusion experiments with plant-centric sampling that targets floral organs specific to reproductions (i.e., stigmas and anthers), as well as insect-centric sampling to target pollen collected during foraging.

4.2 | Comparison of eDNA metabarcoding and visual surveys

The ability to detect pollinators through direct visual observations is limited by the time allocated per survey and the capacity to detect cryptic/reclusive species (Petanidou et al., 2008). In this study, some common diurnal pollinators such as the European honey bee and singing honeyeater were detected with both eDNA and visual surveys; however, other common pollinators such as the Red Wattlebird were not observed during visual surveys. eDNA metabarcoding also detected nocturnal species such as the western pygmy possum and a range of arthropod pollinators including moth species. Nocturnal species such as moths are commonly underrepresented in visual surveys (see Macgregor et al., 2019), especially for plant species that lack the characteristics typically associated with moth pollination (i.e., white floral morphology and/or crepuscular scent production; Pellmyr, 2002). Various alternative survey techniques could be used to detect potential pollinators such as autonomous sound recording devices (birds; e.g, Darras et al., 2019), a camera trap array (mammals; e.g., Ortmann & Johnson, 2021) or a pitfall/trap grid (mammals; e.g., Waudby et al., 2019), and can offer advantages over eDNA metabarcoding, mostly in regards to abundance metrics. However, all require considerable field effort and have ethical requirements. As such, eDNA metabarcoding of flowers represents a major advance, providing a valuable complement to conventional monitoring surveys, able to detect common visitor/pollinator species and, perhaps most importantly, difficult-to-observe species that are often missed with traditional trapping and monitoring techniques.

The inability of eDNA metabarcoding of flowers to detect some species observed during visual surveys highlights common limitations present in eDNA studies. For example, there is a highly diverse fauna of *Euhesma* bees that are oligolectic on *Eremophila* (Exley, 1998) that were observed in the visual surveys, but not detected with eDNA metabarcoding. While eDNA metabarcoding is a powerful monitoring tool, its ability to accurately reflect total biodiversity is commonly

affected by the ability of primers to amplify all taxa present (Alberdi et al., 2018). For example, in our study, eDNA metabarcoding using the birdND2 assay failed to detect Brown Honeyeater observed on *G. georgeana* plants sampled despite sequences present on NCBI's nucleotide database. While further development and validation of the birdND2 assay may alleviate this issue, it highlights the difficulty in designing an assay not only broad enough to target a variety of taxa but also specific enough to differentiate between closely related taxa such as those of the Honeyeater family (Meliphagidae). However, the subsequent detection of this species through the use of a second assay further highlights the benefits of including additional assays and targeting different gene regions to increase the detection and/or specificity of eDNA metabarcoding surveys (Alberdi et al., 2018; Newton et al., 2022).

Incomplete reference databases also commonly reduce the resolution of taxonomic identifications in eDNA metabarcoding studies (Margaryan et al., 2021). With reference barcode sequences currently not available for all species, this not only affects the ability to detect potential pollinator species but also prevents identification of all taxa to species level, which would be vital if appropriate plant-pollinator associations are to be made. This low taxonomic resolution, particularly for insect taxa, then hinders our ability to properly understand and conserve plant–pollinator interactions (Prendergast & Hogendoorn, 2021). However, as more reference sequences are generated and made publicly available, this problem should be alleviated. In addition, by complementing eDNA detections with visual surveys, there is an opportunity to identify which species are missed by eDNA metabarcoding and target them for locally relevant DNA sequence reference database generations.

While eDNA metabarcoding also detected a greater number of taxa compared with visual surveys, the two methods did not perfectly overlap. eDNA metabarcoding detected 84% of the total species detected and visual surveys 27%, with eDNA metabarcoding failing to observe confirmed interactions with plants surveyed such as native bee species (*Megachile* species). This finding is similar to those of previous studies comparing eDNA metabarcoding with conventional survey methods, (e.g., camera traps) which show each to detect unique, but not all visiting taxa (Gogarten et al., 2020; Leempoel et al., 2020; Ryan et al., 2022). Therefore, eDNA metabarcoding used in conjunction with conventional survey methods is recommended to yield the highest species diversity.

4.3 | Conservation implications

Surveying animal assemblages in remote locations and identifying the species associated with rare or infrequently visited plants can be challenging (Gilpin et al., 2014; Ladd et al., 2019; Smith & Gross, 2002). For example, remote locations present a logistical challenge to thoroughly survey in a relatively short timeframe (i.e., Ladd et al., 2019), and it is difficult to capture the full flower-visitor cohorts for rarely visited flowering plants with short visual surveys (i.e., 15 min; see Whelan et al., 2008). Here, eDNA collected from flowering material provided a complementary tool to help survey and monitor plant–animal interactions (Figure 4). Although not all of these organisms detected are likely to affect pollination, these data do provide a general overview of the flower-visitor community (and potential pests) for plant species of interest (see Thomsen & Sigsgaard, 2019), and may provide information that would otherwise not be available. We do note, however, that eDNA does not universally deliver more complete community descriptions than visual observations. For example, eDNA detections accounted for only 21% of all animals visiting *E. oppositifolia* (Figure 4).

4.4 | Future directions

Further baseline studies are necessary to establish eDNA metabarcoding of flowers as a robust tool for assessing flower-visiting animals. To date, few studies have examined the relevant factors (i.e., temperature, UV, and rainfall) that may influence DNA degradation on plant material (although see Valentin et al., 2021), with no studies, to the best of our knowledge, examining the factors that influence deposition of eDNA on flowers. Therefore, it is currently impossible to determine if relatively low flower-visiting animal diversity is a result of few visitations (as suggested by visual surveys in our study) or DNA degradation due to environmental factors (Evans & Kitson, 2020; Goldberg et al., 2018).

We also caution that a baseline understanding is necessary of the organisms likely to affect pollination for the plant species under study. As mentioned above, not all of the species detected using eDNA metabarcoding are likely to affect pollination, as any organism that moves across a flower surface (or potentially secretes DNA into plant tissue, that is, sap-sucking organisms; see Thomsen & Sigsgaard, 2019) could be detected. Therefore, a species or genus understanding of the animal visitors likely to affect pollination in the plant species of interest is a necessary starting point before interpreting flower-visiting communities derived from eDNA metabarcoding. With these caveats, we predict that eDNA could provide rapid and reliable community-level descriptions for a wide range of flowering plant species.

AUTHOR CONTRIBUTIONS

JN conducted the study and wrote the initial manuscript. JN, PB, PN, NW, JK, and MH were involved in the experimental design; Primers were designed and tested by JN, NW, and MH; Samples were collected by JN, PB, PN, and KP and processed by JN and MH; bioinformatics work was performed by JN; JN analyzed data with assistance from JK; the manuscript was edited by all authors.

ACKNOWLEDGMENTS

We acknowledge the traditional owners of the land on which the research presented here was undertaken and pay our respects to Elders past, present, and emerging. This work was funded by Mineral Resources Limited (MRL; https://www.mineralresources.com.au/) and bioinformatic resources provided by the Pawsey

Supercomputing Research Centre with funding from the Australian Government and the Government of Western Australia. The collection of flowers and insects was conducted under collection permits from the Department of Biodiversity, Conservation, and Attractions. We would also like to thank all the members of the TrEnD laboratory for their assistance during the metabarcoding workflow and bioinformatics phases of this project, and Celine Magnon and Kyle Hodgson from MRL for their support throughout the project.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Sequencing data are available at the Dryad Digital Repository https://doi.org/10.5061/dryad.pg4f4qrv5.

ORCID

Joshua P. Newton https://orcid.org/0000-0002-6269-1789
Philip W. Bateman https://orcid.org/0000-0002-3036-5479
Matthew J. Heydenrych https://orcid.org/0000-0002-8426-3400
Joshua H. Kestel https://orcid.org/0000-0002-3159-5559
Kit S. Prendergast https://orcid.org/0000-0002-1164-6099
Paul Nevill https://orcid.org/0000-0001-8238-0534

REFERENCES

Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, *9*(1), 134–147.

Allaire, J., Gandrud, C., Russell, K., & Yetman, C. (2017). Vegan: community ecology package. R package version 1. 15-4. Available from: http://CRAN.R-project.org/package=vegan.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410.

Andersen, K., Bird, K. L., Rasmussen, M., Haile, J., Breuning-Madsen, H., Kjaer, K. H., Orlando, L., Gilbert, M. T. P., & Willerslev, E. (2012). Meta-barcoding of 'dirt'DNA from soil reflects vertebrate biodiversity. *Molecular Ecology*, *21*(8), 1966–1979.

Andrews, S. (2010). FastQC: A quality control tool for high throughput sequence data. In *Babraham bioinformatics*. Babraham Institute.

Arbizu, M. (2020). pairwiseAdonis: Pairwise multilevel comparison using Adonis. R package version 0.4. In.

Barata, I. M., Griffiths, R. A., Fogell, D. J., & Buxton, A. S. (2020). Comparison of eDNA and visual surveys for rare and cryptic bromeliad-dwelling frogs. *Herpetological Journal*, *31*(1), 1–9.

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, *17*(1), 1–17. https://doi.org/10.1007/s10592-015-0775-4

Bezemer, N., Krauss, S. L., Roberts, D. G., & Hopper, S. D. (2019). Conservation of old individual trees and small populations is integral to maintain species' genetic diversity of a historically fragmented woody perennial. *Molecular Ecology*, *28*(14), 3339–3357.

Biesmeijer, J. C., Roberts, S. P., Reemer, M., Ohlemuller, R., Edwards, M., Peeters, T., Schaffers, A., Potts, S. G., Kleukers, R., & Thomas, C. (2006). Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science*, *313*(5785), 351–354.

Blüthgen, N. (2010). Why network analysis is often disconnected from community ecology: A critique and an ecologist's guide. *Basic and Applied Ecology*, *11*(3), 185–195.

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., & Coissac, E. (2016). Obitools: A unix-inspired software package for DNA metabarcoding. *Molecular Ecology Resources*, *16*(1), 176–182.

Brown, E., Burbidge, A., Dell, J., Edinger, D., Hopper, S., & Wills, R. (1997). *Pollination in Western Australia: A database of animals visiting flowers*. Perth.

Campbell, T., Bradshaw, S. D., Dixon, K. W., & Zylstra, P. (2022). Wildfire risk management across diverse bioregions in a changing climate. *Geomatics, Natural Hazards and Risk*, *13*(1), 2405–2424.

Carthew, S., & Goldingay, R. (1997). Non-flying mammals as pollinators. *Trends in Ecology & Evolution*, *12*(3), 104–108.

Darras, K., Batáry, P., Furnas, B. J., Grass, I., Mulyani, Y. A., & Tscharntke, T. (2019). Autonomous sound recording outperforms human observation for sampling birds: A systematic map and user guide. *Ecological Applications*, *29*(6), e01954.

Droissart, V., Azandi, L., Onguene, E. R., Savignac, M., Smith, T. B., & Deblauwe, V. (2021). PICT: A low-cost, modular, open-source camera trap system to study plant–insect interactions. *Methods in Ecology and Evolution*, *12*(8), 1389–1396.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461.

Edgar, R. C. (2016). UNOISE2: Improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv*, *081257*. https://doi.org/10.1101/081257

Elliott, C. P. (2009). Isolation and characterization of microsatellites in the bird-pollinated, autohexaploid, Eremophila glabra ssp glabra (R. Br.(Ostenf.)). *Molecular Ecology Resources*, *9*(4), 1242–1246.

Evans, D. M., & Kitson, J. J. (2020). Molecular ecology as a tool for understanding pollination and other plant–insect interactions. *Current Opinion in Insect Science*, *38*, 26–33.

Exley, E. M. (1998). New Euryglossa (Euhesma) bees (hymenoptera: Colletidae: Euryglossinae) associated with the Australian plant genus Eremophila (Myoporaceae). *Records-Western Australian Museum*, *18*, 419–437.

Finderup Nielsen, T., Sand-Jensen, K., Dornelas, M., & Bruun, H. H. (2019). More is less: Net gain in species richness, but biotic homogenization over 140 years. *Ecology Letters*, *22*(10), 1650–1657.

Forister, M. L., Pelton, E. M., & Black, S. H. (2019). Declines in insect abundance and diversity: We know enough to act now. *Conservation Science and Practice*, *1*(8), e80.

Frøslev, T. G., Kjøller, R., Bruun, H. H., Ejrnæs, R., Brunbjerg, A. K., Pietroni, C., & Hansen, A. J. (2017). Algorithm for post-clustering curation of DNA amplicon data yields reliable biodiversity estimates. *Nature Communications*, *8*(1), 1–11.

Gamonal Gomez, N., Sørensen, D. H., Chua, P. Y. S., & Sigsgaard, L. (2022). Assessing flower-visiting arthropod diversity in apple orchards through metabarcoding of environmental DNA from flowers and visual census. *Environmental DNA*, *5*(1), 117–131.

Gibson, N., Meissner, R., Markey, A. S., & Thompson, W. A. (2012). Patterns of plant diversity in ironstone ranges in arid South Western Australia. *Journal of Arid Environments*, *77*, 25–31.

Gilpin, A.-M., Ayre, D. J., & Denham, A. J. (2014). Can the pollination biology and floral ontogeny of the threatened acacia carneorum explain its lack of reproductive success? *Ecological Research*, *29*(2), 225–235.

Gilpin, A.-M., Collette, J. C., Denham, A. J., Ooi, M. K., & Ayre, D. J. (2017). Do introduced honeybees affect seed set and seed quality in a plant adapted for bird pollination? *Journal of Plant Ecology*, *10*(4), 721–729.

Gioia, P., & Hopper, S. D. (2017). A new phytogeographic map for the southwest Australian floristic region after an exceptional decade of collection and discovery. *Botanical Journal of the Linnean Society*, *184*(1), 1–15.

Gogarten, J. F., Hoffmann, C., Arandjelovic, M., Sachse, A., Merkel, K., Dieguez, P., Agbor, A., Angedakin, S., Brazzola, G., & Jones, S. (2020). Fly-derived DNA and camera traps are complementary

tools for assessing mammalian biodiversity. *Environmental DNA*, *2*(1), 63–76.

Goldberg, C. S., Strickler, K. M., & Fremier, A. K. (2018). Degradation and dispersion limit environmental DNA detection of rare amphibians in wetlands: Increasing efficacy of sampling designs. *Science of the Total Environment*, *633*, 695–703.

Harper, L. R., Niemiller, M. L., Benito, J. B., Paddock, L. E., Knittle, E., Molano-Flores, B., & Davis, M. A. (2022). BeeDNA: Microfluidic environmental DNA metabarcoding as a tool for connecting plant and pollinator communities. *Environmental DNA*, *5*(1), 191–211.

Harrison, J. B., Sunday, J. M., & Rogers, S. M. (2019). Predicting the fate of eDNA in the environment and implications for studying biodiversity. *Proceedings of the Royal Society B*, *286*(1915), 20191409.

Ishige, T., Miya, M., Ushio, M., Sado, T., Ushioda, M., Maebashi, K., Yonechi, R., Lagan, P., & Matsubayashi, H. (2017). Tropical-forest mammals as detected by environmental DNA at natural saltlicks in Borneo. *Biological Conservation*, *210*, 281–285.

Jacobson, M. M., Tucker, E. M., Mathiasson, M. E., & Rehan, S. M. (2018). Decline of bumble bees in northeastern North America, with special focus on Bombus terricola. *Biological Conservation*, *217*, 437–445.

Jønsson, K. A., Thomassen, E. E., Iova, B., Sam, K., & Thomsen, P. F. (2023). Using environmental DNA to investigate avian interactions with flowering plants. *Environmental DNA*. https://doi.org/10.1002/edn3.393

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., & Duran, C. (2012). Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647–1649.

Keighery, G. (1980). Bird pollination in South Western Australia: A checklist. *Plant Systematics and Evolution*, *135*(3), 171–176.

Keighery, G. (1996). Phytogeography, biology and conservation of Western Australian Epacridaceae. *Annals of Botany*, *77*(4), 347–356.

Kestel, J. H., Field, D. L., Bateman, P. W., White, N. E., Allentoft, M. E., Hopkins, A., Gibberd, M., & Nevill, P. (2022). Applications of environmental DNA (eDNA) in agricultural systems: Current uses, limitations and future prospects. *Science of the Total Environment*, *157556*, 157556.

Kindt, R., & Coe, R. (2005). *Tree diversity analysis: A manual and software for common statistical methods for ecological and biodiversity studies*. World Agroforestry Centre.

Knight, T., Ashman, T. L., Bennett, J., Burns, J., Passonneau, S., & Steets, J. (2018). Reflections on, and visions for, the changing field of pollination ecology. *Ecology Letters*, *21*(8), 1282–1295.

Koressaar, T., & Remm, M. (2007). Enhancements and modifications of primer design program Primer3. *Bioinformatics*, *23*(10), 1289–1291.

Krauss, S. L., Phillips, R. D., Karron, J. D., Johnson, S. D., Roberts, D. G., & Hopper, S. D. (2017). Novel consequences of bird pollination for plant mating. *Trends in Plant Science*, *22*(5), 395–410.

Krauss, S. L., Roberts, D. G., Phillips, R. D., & Edwards, C. (2018). Effectiveness of camera traps for quantifying daytime and nighttime visitation by vertebrate pollinators. *Ecology and Evolution*, *8*(18), 9304–9314.

Kucherenko, A., Herman, J. E., III, Everham, E. M., & Urakawa, H. (2018). Terrestrial snake environmental DNA accumulation and degradation dynamics and its environmental application. *Herpetologica*, *74*(1), 38–49.

Kunz, T. H., De Torrez, E. B., Bauer, D., Lobova, T., & Fleming, T. H. (2011). Ecosystem services provided by bats. *Europe*, *31*, 32–38.

Ladd, P., Yates, C., Dillon, R., & Palmer, R. (2019). Pollination ecology of Tetratheca species from isolated, arid habitats (banded iron formations) in Western Australia. *Australian Journal of Botany*, *67*(3), 248–255.

Leempoel, K., Hebert, T., & Hadly, E. A. (2020). A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity. *Proceedings of the Royal Society B*, *287*(1918), 20192353.

Lunau, K., Scaccabarozzi, D., Willing, L., & Dixon, K. (2021). A bee's eye view of remarkable floral colour patterns in the south-west Australian biodiversity hotspot revealed by false colour photography. *Annals of Botany*, *128*(7), 821–824.

Macgregor, C. J., Kitson, J. J., Fox, R., Hahn, C., Lunt, D. H., Pocock, M. J., & Evans, D. M. (2019). Construction, validation, and application of nocturnal pollen transport networks in an agro-ecosystem: A comparison using light microscopy and DNA metabarcoding. *Ecological Entomology*, *44*(1), 17–29.

MacKenzie, C. M., Mittelhauser, G., Miller-Rushing, A. J., & Primack, R. B. (2019). Floristic change in New England and New York: Regional patterns of plant species loss and decline. *Rhodora*, *121*(985), 1–36.

Margaryan, A., Noer, C. L., Richter, S. R., Restrup, M. E., Bülow-Hansen, J. L., Leerhøi, F., Langkjær, E. M. R., Gopalakrishnan, S., Carøe, C., & Gilbert, M. T. P. (2021). Mitochondrial genomes of Danish vertebrate species generated for the national DNA reference database, DNAmark. *Environmental DNA*, *3*(2), 472–480.

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, *8*(4), e61217.

Menz, M. H., Phillips, R. D., Dixon, K. W., Peakall, R., & Didham, R. K. (2013). Mate-searching behaviour of common and rare wasps and the implications for pollen movement of the sexually deceptive orchids they pollinate. *PLoS One*, *8*(3), e59111.

Millard, J. W., Freeman, R., & Newbold, T. (2020). Text-analysis reveals taxonomic and geographic disparities in animal pollination literature. *Ecography*, *43*(1), 44–59.

Mola, J. M., Richardson, L. L., Spyreas, G., Zaya, D. N., & Pearse, I. S. (2021). Long-term surveys support declines in early season forest plants used by bumblebees. *Journal of Applied Ecology*, *58*(7), 1431–1441.

Mousavi-Derazmahalleh, M., Stott, A., Lines, R., Peverley, G., Nester, G., Simpson, T., Zawierta, M., De La Pierre, M., Bunce, M., & Christophersen, C. T. (2021). eDNAFlow, an automated, reproducible and scalable workflow for analysis of environmental DNA sequences exploiting Nextflow and singularity. *Molecular Ecology Resources*, *21*(5), 1697–1704.

Murray, D. C., Coghlan, M. L., & Bunce, M. (2015). From benchtop to desktop: Important considerations when designing amplicon sequencing workflows. *PLoS One*, *10*(4), e0124671.

Newton, J. P., Bateman, P. W., Heydenrych, M. J., Mousavi-Derazmahalleh, M., & Nevill, P. (2022). Home is where the hollow is: Revealing vertebrate tree hollow user biodiversity with eDNA metabarcoding. *Environmental DNA*, *4*(5), 1078–1091.

Nichols, R. V., Koenigsson, H., Danell, K., & Spong, G. (2012). Browsed twig environmental DNA: Diagnostic PCR to identify ungulate species. *Molecular Ecology Resources*, *12*(6), 983–989.

O'Connor, R. S., Kunin, W. E., Garratt, M. P., Potts, S. G., Roy, H. E., Andrews, C., Jones, C. M., Peyton, J. M., Savage, J., & Harvey, M. C. (2019). Monitoring insect pollinators and flower visitation: The effectiveness and feasibility of different survey methods. *Methods in Ecology and Evolution*, *10*(12), 2129–2140.

Oksanen, J. (2009). *Vegan: community ecology package*. R package version 1. 15-4. Available from: http://CRAN.R-project.org/package=vegan.

Olesen, J. M., & Valido, A. (2003). Lizards as pollinators and seed dispersers: An island phenomenon. *Trends in Ecology & Evolution*, *18*(4), 177–181.

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos*, *120*(3), 321–326.

Ortmann, C., & Johnson, S. (2021). How reliable are motion-triggered camera traps for detecting small mammals and birds in ecological studies? *Journal of Zoology*, *313*(3), 202–207.

Paradis, E., & Schliep, K. (2019). Ape 5.0: An environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics*, *35*(3), 526–528.

Pearson, D. L., Hamilton, A. L., & Erwin, T. L. (2011). Recovery plan for the endangered taxonomy profession. *Bioscience*, *61*(1), 58–63.

Pellmyr, O. (2002). Pollination by animals. Plant–animal interactions. *Herrera CM & Pellmyr O*, 157–184.

Petanidou, T., Kallimanis, A. S., Tzanopoulos, J., Sgardelis, S. P., & Pantis, J. D. (2008). Long-term observation of a pollination network: Fluctuation in species and interactions, relative invariance of network structure and implications for estimates of specialization. *Ecology Letters*, *11*(6), 564–575.

Phillips, R. D., Hopper, S. D., & Dixon, K. W. (2010). Pollination ecology and the possible impacts of environmental change in the southwest Australian biodiversity hotspot. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1539), 517–528.

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

Prendergast, K. S. (2022). Assessing climate change impacts on pollinators. In P. K. A. S. W. Chan (Ed.), *Promoting pollination and pollinators in farming*. Burleigh Dodds Science Publishing.

Prendergast, K. S., & Hogendoorn, K. (2021). Methodological shortcomings and lack of taxonomic effort beleaguer Australian bee studies. *Austral Ecology*, *46*(5), 880–884.

Prendergast, K. S., Menz, M. H., Dixon, K. W., & Bateman, P. W. (2020). The relative performance of sampling methods for native bees: An empirical test and review of the literature. *Ecosphere*, *11*(5), e03076.

Rafferty, N. E. (2017). Effects of global change on insect pollinators: Multiple drivers lead to novel communities. *Current Opinion in Insect Science*, *23*, 22–27.

Regan, E. C., Santini, L., Ingwall-King, L., Hoffmann, M., Rondinini, C., Symes, A., Taylor, J., & Butchart, S. H. (2015). Global trends in the status of bird and mammal pollinators. *Conservation Letters*, *8*(6), 397–403.

Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P., & Coissac, E. (2011). ecoPrimers: Inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Research*, *39*(21), e145.

Sakata, M. K., Kawata, M. U., Kurabayashi, A., Kurita, T., Nakamura, M., Shirako, T., Kakehashi, R., Nishikawa, K., Hossman, M. Y., & Nishijima, T. (2022). Development and evaluation of PCR primers for environmental DNA (eDNA) metabarcoding of Amphibia. *Metabarcoding and Metagenomics*, *6*, e76534.

Sánchez-Bayo, F., & Wyckhuys, K. A. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological Conservation*, *232*, 8–27.

Saunders, M. E., Luck, G. W., & Mayfield, M. M. (2013). Almond orchards with living ground cover host more wild insect pollinators. *Journal of Insect Conservation*, *17*(5), 1011–1025.

Scheper, J., Reemer, M., van Kats, R., Ozinga, W. A., van der Linden, G. T., Schaminée, J. H., Siepel, H., & Kleijn, D. (2014). Museum specimens reveal loss of pollen host plants as key factor driving wild bee decline in the Netherlands. *Proceedings of the National Academy of Sciences USA*, *111*(49), 17552–17557.

Schubert, M., Lindgreen, S., & Orlando, L. (2016). AdapterRemoval v2: Rapid adapter trimming, identification, and read merging. *BMC Research Notes*, *9*(1), 1–7.

Simanonok, M. P., Otto, C. R., Cornman, R. S., Iwanowicz, D. D., Strange, J. P., & Smith, T. A. (2021). A century of pollen foraging by the endangered rusty patched bumble bee (*Bombus affinis*): Inferences from molecular sequencing of museum specimens. *Biodiversity and Conservation*, *30*(1), 123–137.

Smith, J., & Gross, C. L. (2002). The pollination ecology of grevillea beadleana McGillivray, an endangered shrub from northern New South Wales, Australia. *Annals of Botany*, *89*(1), 97–108.

Stork, N. E. (2018). How many species of insects and other terrestrial arthropods are there on earth? *Annual Review of Entomology*, *63*, 31–45.

Stothard, P. (2000). The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *BioTechniques*, *28*(6), 1102–1104.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., & Willerslev, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, *21*(8), 2045–2050.

Taylor, P. G. (1996). Reproducibility of ancient DNA sequences from extinct Pleistocene fauna. *Molecular Biology and Evolution*, *13*(1), 283–285.

Team, R. C. (2013). *R: A language and environment for statistical computing*. Team, R.C.

Thomsen, P. F., & Sigsgaard, E. E. (2019). Environmental DNA metabarcoding of wild flowers reveals diverse communities of terrestrial arthropods. *Ecology and Evolution*, *9*(4), 1665–1679.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3—New capabilities and interfaces. *Nucleic Acids Research*, *40*(15), e115.

Ushio, M., Murata, K., Sado, T., Nishiumi, I., Takeshita, M., Iwasaki, W., & Miya, M. (2018). Demonstration of the potential of environmental DNA as a tool for the detection of avian species. *Scientific Reports*, *8*(1), 1–10.

Valentin, R. E., Kyle, K. E., Allen, M. C., Welbourne, D. J., & Lockwood, J. L. (2021). The state, transport, and fate of aboveground terrestrial arthropod eDNA. *Environmental DNA*, *3*(6), 1081–1092.

Vamos, E. E., Elbrecht, V., & Leese, F. (2017). *Short COI markers for freshwater macroinvertebrate metabarcoding (2167–9843)*.

Van Der Heyde, M., Bateman, P., Bunce, M., Wardell-Johnson, G., White, N., & Nevill, P. (2021). Scat DNA provides important data for effective monitoring of mammal and bird biodiversity. *Biodiversity and Conservation*, *30*(12), 3585–3602.

Walker, F. M., Sanchez, D. E., Froehlich, E. M., Federman, E. L., Lyman, J. A., Owens, M., & Lear, K. (2022). Endangered nectar-feeding bat detected by environmental DNA on flowers. *Animals*, *12*(22), 3075.

Waudby, H. P., Petit, S., & Gill, M. J. (2019). The scientific, financial and ethical implications of three common wildlife-trapping designs. *Wildlife Research*, *46*(8), 690–700.

Whelan, C. J., Wenny, D. G., & Marquis, R. J. (2008). Ecosystem services provided by birds. *Annals of the new York Academy of Sciences*, *1134*(1), 25–60.

Whelan, R. J., Ayre, D. J., & Beynon, F. M. (2009). The birds and the bees: Pollinator behaviour and variation in the mating system of the rare shrub Grevillea macleayana. *Annals of Botany*, *103*(9), 1395–1401.

Wickham, H. (2016). *Package 'ggplot2': Elegant graphics for data analysis*. Springer-Verlag. doi: 10,978–970.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Newton, J. P., Bateman, P. W., Heydenrych, M. J., Kestel, J. H., Dixon, K. W., Prendergast, K. S., White, N. E., & Nevill, P. (2023). Monitoring the birds and the bees: Environmental DNA metabarcoding of flowers detects plant–animal interactions. *Environmental DNA*, *5*, 488–502. https://doi.org/10.1002/edn3.399