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Environmental DNA profiling for detecting plant-insect interactions in endangered and native flora

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Abstract

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Environmental DNA (eDNA) is an established technique for studying plant-insect interactions, that has so far had very limited use in exploring flower-visiting insect communities. This study provides important evidence of the effectiveness of eDNA for studying flower-visiting insects, proving its ability to provide a comprehensive overview of pollinator communities beyond traditional observational methods. Our data revealed a surprising diversity of flower-visiting insects, including both expected pollinators and possible non-pollinating species utilising pollen and/or nectar as a nutritional resource. Native bees, such as *Leioproctus* spp., and various flies, including those with uncertain roles in pollination, were detected. This study also shed light on the underexplored area of nocturnal pollination, providing evidence of native moth involvement in pollinating plant species. While there was no definitive evidence of rare insects visiting mānuka (*Leptospermum scoparium*) or *Lophomyrtus* spp., this study did reveal the importance of these plant species and the resources the flowers provide not just to pollinators, but insects with other key roles in the ecosystem.

Keywords

conservation, eDNA, *Leptospermum, Lophomyrtus*, myrtle rust, pollination

Introduction

Pollination of New Zealand's native plants is generally not well studied or understood (Newstrom and Robertson, 2005). Assumptions such as little dependence on pollinators, few specialised pollinators, and low self-incompatibility have long been the basis of understanding native plant pollination in New Zealand (Newstrom and Robertson, 2005; Pattemore, 2013). Most native plants that do require pollinators are thought to rely on generalist native bees (Hymenoptera), but may also be pollinated by insects from other orders such as Diptera, Coleoptera, and Lepidoptera (Newstrom and Robertson, 2005). However, this assumption needs to be tested with studies of individual plant species (Pattemore, 2013).

The Myrtaceae is a globally distributed plant family. In New Zealand, members of the family have distinct functional traits and fill roles that underpin ecosystem health (Jo et al., 2022). Native Myrtaceae provide key resources and habitat for various bird and insect species (e.g., Affeld et al., 2009; McKenzie et al., 1999; Newstrom and Robertson, 2005), and have important ecological, economic, and cultural values and uses (Teulon et al., 2015; Black et al., 2019).

The fungus *Austropuccinia psidii*, cause of myrtle rust, is a widespread invasive species. Myrtle rust was first

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discovered in New Zealand in 2017 on the remote Raoul Island, and later in the same year on the mainland (Ho et al., 2019). It has spread rapidly and is now established in all climatically suitable regions in New Zealand. *Austropuccinia psidii* infects young actively growing parts of the plant, including leaves, buds, flowers and fruit (Beresford et al., 2020). Severe disease can cause defoliation, loss of flowers and fruit, plant dieback, with repeated attacks leading to mortality (Sutherland et al., 2020; Fensham and Radford-Smith, 2021). This disease has been recorded affecting approximately 450 Myrtaceae species globally (Soewarto et al., 2019), including at least 19 species or hybrids native to New Zealand (Soewarto et al., 2019; MPI, 2024). This includes culturally and economically significant species such as pōhutukawa (*Metrosideros excelsa*), mānuka (*Leptospermum scoparium*), ramarama (*Lophomyrtus bullata*) and rōhutu (*Lophomyrtus obcordata*) (Teulon et al., 2015; Sutherland et al., 2020).

The ongoing and realised impacts of myrtle rust in invaded areas are well recorded (e.g., Cannon et al., 2022; Carnegie et al., 2016; Soewarto et al., 2018) and the direct impacts in New Zealand for some Myrtaceae are now well documented. While the impacts on mānuka have been minor so far, on *Lophomyrtus* spp. they have been severe and could lead to localised extinctions (Beresford et al., 2019; Sutherland et al., 2020). In contrast, the indirect impacts of myrtle rust on the ecosystem are poorly understood. Sutherland et al. (2020) found that there were 109 species that have an association with *Lophomyrtus* spp., and additional field surveys and searches of herbarium specimens for associated flora and mycobiota have increased this to 221 taxa (Prasad et al., 2022). For the invertebrates, there are a small number of records of native herbivores feeding on native Myrtaceae, including stick insects (Phasmidae), leafrollers and looper caterpillars (Tortricidae and Geometridae), scale insects (Eriococcidae and Diaspididae), weevils (Curculionidae), and thrips (Thripidae) (PlantSynz, 2024). Native pollinators in the Colletidae and Halictidae families have been recorded visiting the flowers of *Lophomyrtus* spp., as well as the exotic honeybee (Apis mellifera) and common bumble bee (*Bombus terrestris*) (PlantSynz, 2024).

Environmental DNA (eDNA) refers to genetic material shed by organisms into their environment. This genetic material can be collected from the environment (e.g., water, soil, plant surfaces) with the aim of obtaining the maximum DNA-based taxonomic or functional information about the study system (Taberlet et al., 2012; Taberlet et al., 2018). The eDNA metabarcoding approach results in the identification, to varying levels of taxonomic resolution, of the organisms that have been present in a given environment (Taberlet et al., 2012). While eDNA is now a well-established method, it has not yet been widely utilised in identifying pollinator communities (Harper et al., 2023), although it has begun to be used successfully to characterise arthropod flower visitation (Thomsen and Sigsgaard, 2019) and validating eDNA results against observations has begun (Stothut et al., 2024).

Due to the impact of myrtle rust on *Lophomyrtus* spp. in New Zealand and the lack of baseline data prior to the disease arriving, it is critical to learn as much as possible about the ecology of these ecologically important plants to inform their overall conservation strategy. Utilising eDNA metabarcoding and field observations, we aimed to investigate and characterise invertebrate flower visitation of mānuka (*Leptospermum scoparium*) and *Lophomyrtus* spp.

Methods

Study locations

Peri-urban Mānuka & *Lophomyrtus bullata*

Flowering mānuka L. bullata were selected in a peri-urban planted site, established in late 2019 in Rotorua, New Zealand (−38.158983°, 176.263342°) as part of a trial investigating vulnerability of five native Myrtaceae species to natural *A. psidii* infection (Beresford et al., 2021). The surrounding area is a mixture of exotic grasses and shrubs, as well as early successional native plants.

Table 1. Summary of collection locations, sample type and number

Location	Sample type collected	Number of samples collected
Peri-urban Mānuka & *Lophomyrtus bullata*	Insects and flowers	Insects from Mānuka: 6 Flowers from Mānuka: 13 Flowers from Lophomyrtus: 4
Rotorua Lakes site *Lophomyrtus* spp.	Flowers only	Flowers: 9
Kaimai-Mamaku Ranges *Lophomyrtus bullata*	Insects and flowers	Insects: 9 Flowers: 30

Rotorua Lakes site *Lophomyrtus* spp.

These plants were located in a natural forest site in the Rotorua Lakes area, on the eastern side of a lake edge and forest boundary. The impact of myrtle rust on *Lophomyrtus* trees in this area has been tracked and reported by Sutherland et al. (2020) as "Site B" and Soewarto et al. (In Prep) as "Site 2". The coordinates for this location cannot be shared due to agreements with mana whenua (Māori territorial authority). The surrounding area is dominated by regenerating native vegetation with a small pocket of regenerating mānuka and kānuka (*Kunzea ericoides*). Myrtaceae are notable in the area with pōhutukawa, both *Lophomyrtus* spp. and hybrids, mānuka and kānuka, all being present within the surrounding areas.

Kaimai-Mamaku Ranges *Lophomyrtus bullata*

A third location in the Kaimai Mamaku conservation park was sampled, where the impact of myrtle rust on *L. bullata* trees in this area has been tracked and reported by Soewarto et al. (In Prep) as "Site 3". The coordinates for this

location cannot be shared due to agreements with mana whenua (Māori territorial authority). *Lophomyrtus bullata* trees were located at the forest edge along a riverbank. This location is a secondary native forest that suffered extensive kauri (*Agathis australis*) logging in the late 1800's and early 1900's. Native plants in the surrounding sampling area included kauri, beech (*Fuscospora* spp.), hangehange (*Geniostoma ligustrifolium* var. *ligustrifolium*), rewarewa (*Knightia excelsa*), native ferns and various other native plants. Exotic plants present included *Acacia melanoxylon*, and weeds such as gorse (*Ulex europaeus*) and ragwort (*Jacobaea vulgaris*).

Day and night pollinators

The trial with mānuka ran for five days in late November 2023. Prior to flower anthesis, mānuka flower buds were counted (n = 199) and divided approximately equally between treatments and controls (range = 46–52) across five replicates, with mixed treatments on each tree. Each group of replicates and treatments were labelled with white waterproof plant labels. The positive and negative control, and two treatments were 1) no pollination exclusion bag (positive control), 2) 24h pollination exclusion bag (negative control), 3) no bag during the day, and 4) no bag during night. Flower buds were bagged prior to flowering using white 'organza' bags (165 mm L × 125 mm W). Once the trial began, day and night treatment bags were applied or removed within 15 minutes of sunrise or sunset each day.

Sample collection

Peri-urban Mānuka & *Lophomyrtus bullata*
During each evening bag change, and during a calm period during the daytime, a 10-minute insect observation was undertaken at each treatment tree. Insects observed visiting the open flowers were caught with a sweep net and frozen in individual vials at –80 °C for pollen eDNA analysis, alongside recorded observations of other insects in the vicinity. Once the trial was complete, 13 mānuka flowers were individually collected, four from treatments one and four, and five flowers from treatment three (Table 1). Flowers were collected using nitrile gloves, and a pair of sterilised scissors for each treatment, prior soaked in a 10% bleach solution for 20 minutes and rinsed with DI water. Exclusion bags were then applied to all treatments so that pollination rates could be later examined. Six weeks after the trial ended, the seed set was counted in each bag.

A trial examining crepuscular pollination with *L. bullata* in the same location was also attempted, unfortunately this could not be completed due to a very staggered timing of flowering and compromised plant health. However, four flowers were individually collected using nitrile gloves and sterile scissors for eDNA insect visitation analysis. Sample C22 was removed from subsequent analysis, as the extraction or PCR amplification failed and it only contained 167 reads after filtering.

Rotorua Lakes site *Lophomyrtus* spp.
In December 2023, prior to flower anthesis, flower buds were counted (n = 292) and divided approximately equally between treatments and controls. Due to flowering timing, a restricted range of data was collected. Insect flower visitation data was collected as above, with nine flowers individually collected from the positive control treatment using nitrile gloves and sterile scissors (Table 1). Five 10-minute insect observations were carried out for each plant during the flowering period, but no flower visitors were observed, nor insects caught, but general observations of insects in the vicinity were made.

Kaimai-Mamaku Ranges *Lophomyrtus bullata*
Observations were made during 10-minute sampling periods at the six *L. bullata* trees that had the most flowers. Observations at this site were limited by the remote nature of the site and permit collection restraints. Insects visiting the flowers were individually collected with sweep nets and observations were also made of the insects in the general vicinity. Flowers were collected from six different *L. bullata* plants (up to six flowers from each of six plants), with the same methods described above.

DNA extraction and sequencing

Whole intact samples were sent to external providers for extractions and sequencing. Genomic DNA extractions were performed by a commercial service provider (Slipstream Automation, Palmerston North, New Zealand). All samples were freeze-dried prior to extraction for a minimum of 48 hours. Once freeze-dried, steel beads were loaded into the plate well and the plates heat-sealed. Material was extracted using a CTAB-chloroform method in an automated robotic workflow. Positive and negative controls were included, but not sent for sequencing. No obvious DNA contamination occurred between samples in this project, or from samples in other concurrent projects.

PCR primers for the COI gene region for insects (Vamos et al., 2017) and chloroplast trnL intron (Taberlet et al., 2007) gene region for plants were used in a 30–35 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 95 °C for 5 minutes, followed by 30–35 cycles of 95 °C for 30 seconds, 53 °C for 40 seconds and 72 °C for 1 minute, after which a final elongation step at 72 °C for 10 minutes was performed. After amplification, PCR products were checked using a 2% agarose gel to determine the success of amplification and the relative intensity of amplification. Samples were multiplexed using unique dual indices and pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare an Illumina DNA library. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq platform following the manufacturer's guidelines.

Bioinformatics

The OBITools3 package ((Boyer et al., 2016), https://git.metabarcoding.org/obitools/obitools3) was used to align paired-end reads, remove low quality alignments, trim primers, deduplicate sequences, remove sequences shorter than 10 bp, remove singletons, and remove sequences derived from PCR errors. Taxonomic assignment was undertaken using blastn (Camacho et al., 2009) against the NCBI nt database, keeping all hits with a minimum e-value of 1e-5. Hits were then sorted by query, then bitscore, then e-value then percentage identity in that order. A consensus taxon assignment was then computed with a custom script keeping all hits with a percentage identity value 0.5% below the best hit, and cut-off values of <97% to assign to family, <90% to assign to phylum, and <80% to set as unassigned. Assignments to taxa not having a defined family were ignored in order to avoid assignment to spurious references. All non-target sequences were removed. Rarefaction curves were checked for each sample using the vegan R package. Samples with less than 100 reads were removed from subsequent analysis. Taxa abundance analysis and ordination plots were then done in R using the packages phyloseq (McMurdie and Holmes, 2013) and microViz (Barnett et al., 2021) packages.

Results

The pollination of mānuka flowers was highest in the no cage treatment (37.3% seed set), where potential pollinators always had access to the flowers. The 24-hour insect exclusion treatment had the next highest pollination success (19% seed set), possibly due to self-compatibility or due to a failure of pollinator exclusion, although no insects were observed in the exclusion cages. The flowers available during the day had relatively low pollination success (15.2%), while the flowers available at night had the least successful pollination (5.8%).

From the observations made at the mānuka plants during the day and evening, the insects in the immediate vicinity were largely Diptera, with relatively few observations of Hymenoptera, and Lepidoptera. This is mirrored by the five insects collected from the mānuka plants, three flies (C18, 19, 23), one wasp (C20), and one moth (C21) (Fig. 1). The flies were identified as belonging to either the Tachinidae family, which are arthropod parasitoids, or to Dolichopodinae, a subfamily containing many predators of other invertebrates. Two bees were identified to genus, *Leioproctus* sp. (Colletidae). A further three samples were identified to species level; the wasp (C20) was identified as an introduced potter wasp (*Ancistrocerus gazella* (Vespidae)), the moth (C21) was identified as the native *Strepsicrates ejectana* (Totricidae), and one weevil as the exotic *Sitona lepidus* (Curculionidae).

At the Rotorua Lakes site, Diptera (flies) were commonly observed in the vicinity of *Lophomyrtus* spp., but no flower visitation was observed. The insects collected from *Lophomyrtus bullata* (Fig. 1) at the Kaimai-Mamaku ranges site belonged to a range of insect groups including the Halictidae (includes native and some introduced bees), *Leioproctus* spp. a genus of native bees, weevils (includes native and introduced), a small longhorn beetle, and a Zopheridae beetle, which are known mostly from rotting wood and fungi.

Analysis of the plant DNA barcodes detected from

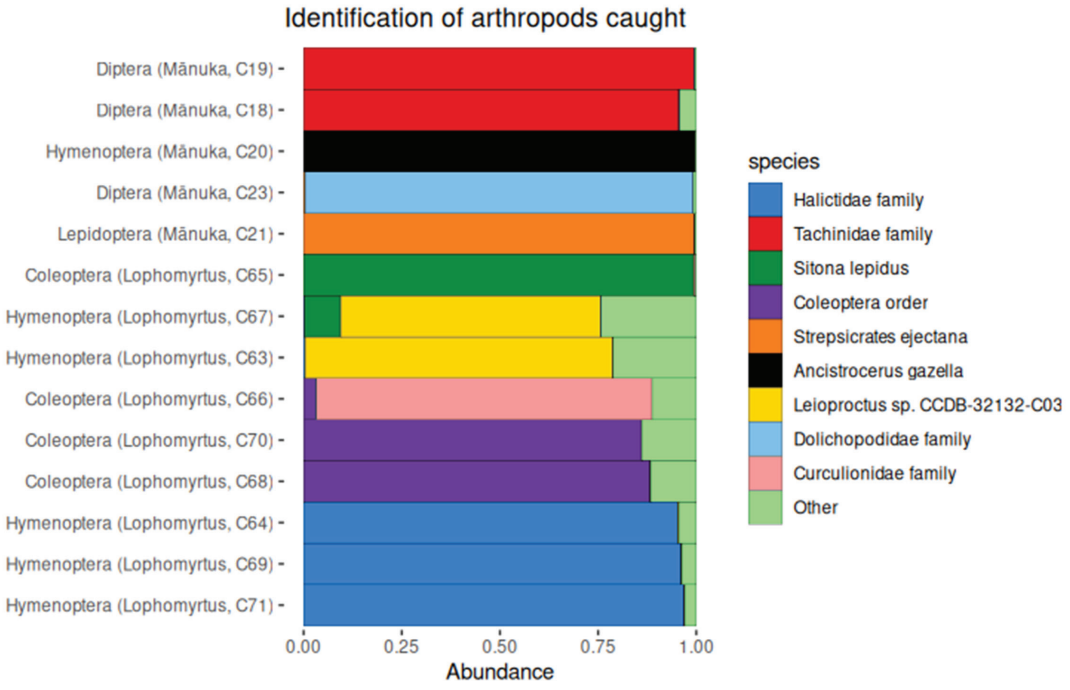

Fig. 1. The frequency of DNA barcodes for each insect specimen collected from mānuka or *Lophomyrtus* spp. Samples C18-23 are from peri-urban mānuka, and C63-71 from Kaimai-Mamaku *Lophomyrtus bullata*.

the insects shows that DNA in the Myrtaceae was predominant (Fig. 2), with a number of different plant genera and families present in smaller quantities. Only two specific species were positively detected, the native *Leptospermum scoparium* (Mānuka) and the endemic *Dacrydium cupressinum* (Rimu). The remainder are a mixture of native and exotic plant groups. Two samples, C20 (*A. gazella*) and C68 (longhorn beetle) had an even spread of plant DNA compared to other samples. We were unable to determine if this is due to an ecological reason, or sequencing bias.

We recorded a wide variety of insect and other invertebrate visitors to mānuka and *Lophomyrtus* spp. flowers (Fig. 3). The arthropod DNA most frequently detected on Mānuka was a native moth (*Strepsicrates ejectana*), Collembola, and predatory flies (Dolichopodinae). On *Lopho*

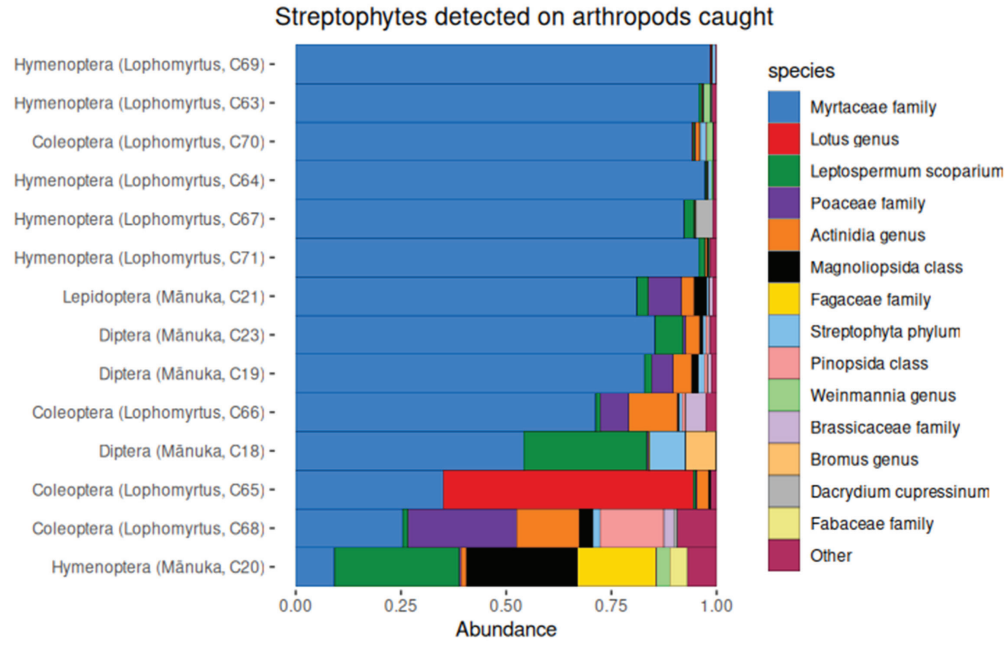

Fig. 2. The frequency of plant DNA barcodes from the insects collected on mānuka or *Lophomyrtus* spp.. Samples C18-23 are from peri-urban mānuka, and C63-71 from Kaimai-Mamaku *Lophomyrtus bullata*.

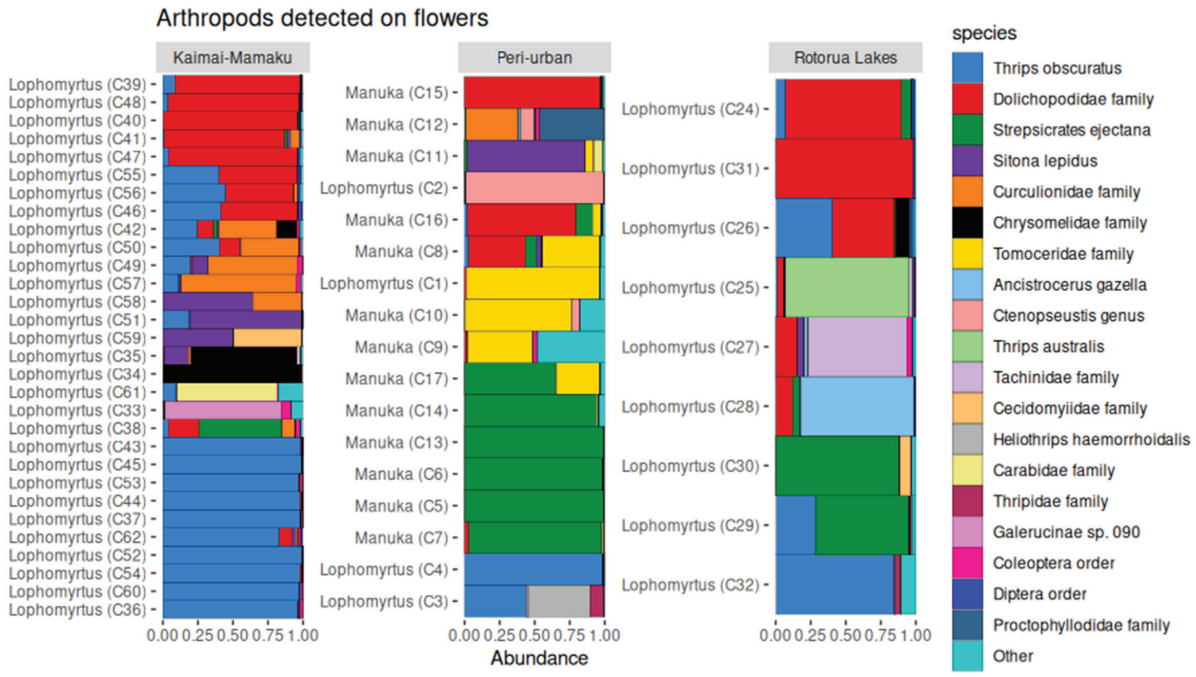

Fig. 3. The frequency of insect visitor DNA barcode sequences on *Lophomyrtus* spp. and mānuka flowers by location.

myrtus spp. flowers, we recorded the DNA of a different arthropod community, including native thrips (*Thrips obscuratus*), predatory flies (Dolichopodinae), native leafroller moths (*Ctenopseustis* spp. and *Strepsicrates ejectana*), weevils (Curculionidae and *Sitona* spp.), and leaf beetles (Chrysomelidae), as well as a range of other native and exotic insects and insect-relatives. The insect visitor communities on *Lophomyrtus* spp. were significantly different between locations (PERMANOVA test P = 0.0457) (Supplementary material – Fig. S1).

Discussion

Environmental DNA is already a useful tool in studying plant-arthropod interactions (Johnson et al., 2023), although it has not yet been widely applied to the study of flower visiting insect communities (Thomsen and Sigsgaard., 2019). The eDNA approach is particularly adept at not just detecting pollinators, but a range of plant or flower visitors (Johnson et al., 2023). This provides a wider ecosystem view than can be achieved from observational studies, which generally only cover short timescales (Knop et al., 2018) and are heavily biased towards daytime hours (Knop et al., 2018).

Plant pollinators come from a range of arthropod groups, and this study found evidence of flower visitation by a diverse and somewhat unexpected assemblage of insect and invertebrate species. Expected native pollinators, such as *Leioproctus* spp. (Donovan, 1980) were detected and observed, as well as bees from the Halictidae family that contains native and exotic species (Donovan, 1980). The role of flies in pollination of our study species is not clear, but it is likely they play a role (Newstrom and Robertson, 2005). Aside from the likely pollinators such as native bees and some flies, other flower visitors included predatory flies who have a role in modulating other arthropod populations, and insects and other invertebrate groups that are phytophagous or detritivores, such as leaf beetles, weevils, thrips, and collembola.

Not all insects that visit flowers are pollinators (*i.e.*, do not transfer pollen) as some lack the structures or behaviours necessary to pollinate the flowers they visit (Wardhaugh, 2015; Nepi et al., 2018). Invertebrates visit flowers for a variety of reasons such as feeding on floral resources without pollinating the host plant (Nepi et al., 2018; Chapman et al., 2023), access to yeasts (de Vega and Herrera, 2012), and to predate on other flower visitors (Morse, 1986). Interestingly, we detected the DNA of gall midges (Diptera: Cecidomyiidae) (Fig. 3), which include the myrtle rust fly (*Mycodiplosis c onstricta*) (Kolesik et al., 2021). The larvae of this fly are a known natural enemy of myrtle rust as they develop by feeding on the rust itself (Kolesik et al., 2021). This suggests that these floral resources may be enhancing natural enemies of myrtle rust. Further, we found that the insects who visit the flowers of our study species are not regularly visiting other flowers (Fig. 2), suggesting that these flowers are a key direct or supplemental nutritional resource. Due to DNA extraction methods, the gut contents of the arthropods may bias some of the detected plant species or overstated which flowering plant species were visited for their nutritional requirements.

Globally, nocturnal pollination is understudied and is currently an emerging research area (Buxton et al., 2022). Although we cannot rule out self-pollination in mānuka (e.g., Bennik, 2009), we found eDNA evidence that nocturnal native moths were visiting the flowers of mānuka and *Lophomyrtus* (Fig 3.) and our seed set results suggest a role for nocturnal pollinators, supporting previous studies that have hinted at the role moths may play in mānuka pollination (Heine, 1937; Buxton et al., 2022). Due to New Zealand's lack of key pollinator groups commonly found elsewhere (e.g., large social bees), and New Zealand's relatively high moth diversity, this group of insects may play a more important role in pollination than might be expected (Newstrom and Robertson, 2005). Further detailed studies are needed to fully detangle potential self-pollination versus insect pollination of mānuka.

The study does highlight a key limitation of the metabarcoding eDNA approach. Although this study selected the COI marker to provide better taxonomic resolution compared to other gene regions, it is acknowledged that the resolution of the COI marker can be biased when compared to other commonly used arthropod markers due to the taxa it amplifies in the sample (Thomsen and Sigsgaard, 2019). The results also rely on a robust and accurate genetic reference database (Thomsen and Sigsgaard, 2019), and although the available databases are generally better for arthropods than other groups (such as fungi), they are generally not yet comprehensive enough to provide widescale accurate species identifications.

Conclusions

Through eDNA metabarcoding, insect observation and collection, and pollinator exclusion approaches, we revealed a complex web of flower-visiting invertebrates on *Lophomyrtus* spp. and mānuka. Importantly, the eDNA approach allowed for a more comprehensive overview of flower visitation than traditional observational methods. While some flower-visitor species were expected (native bees), others play a varied role in the ecosystem and are not usually thought to be associated with floral resources. Although we did not detect any obvious specialist or obligate pollinator(s) of mānuka or *Lophomyrtus* spp., or other rare or specialist arthropods, the genetic barcodes we recorded will be able to be revisited in future research on flower visitors to native plant species in New Zealand.

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Supplementary material

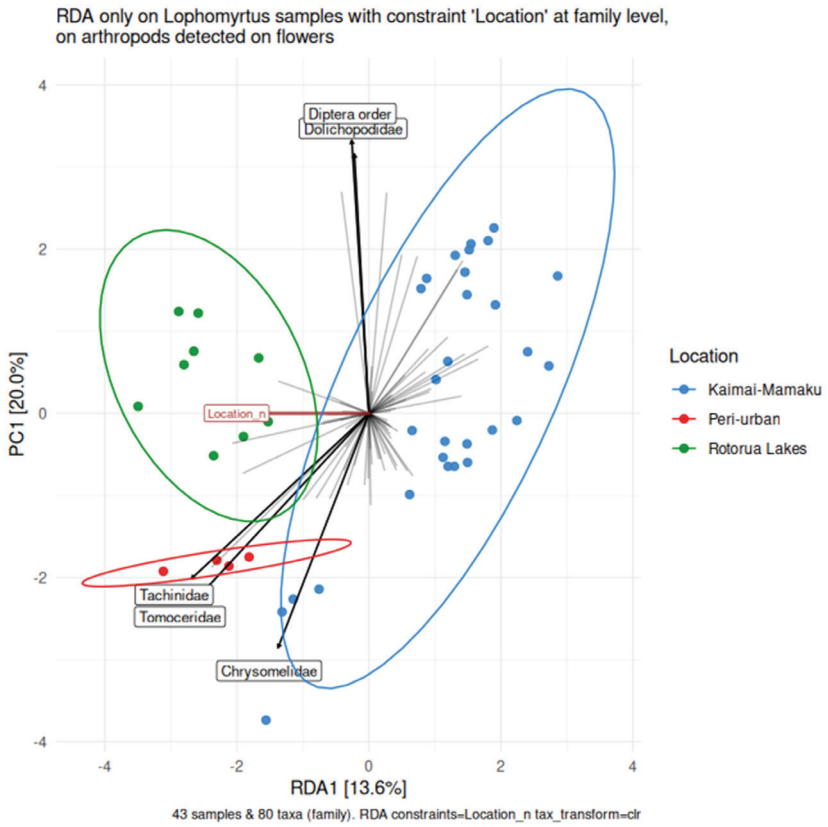

Fig. S1. The insect visitor communities on *Lophomyrtus* spp. were significantly different from each other between locations.