Hort Innovation

Final Report

Environmental DNA technologies for rapid detection and identification of avocado priority pests

Project leader:

Dr. Andrew Weeks

Report authors:

Dr. Chantelle Reid and Dr. Luke Noble

Delivery partner:

EnviroDNA

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Level 7
141 Walker Street
North Sydney NSW 2060

Telephone: (02) 8295 2300

www.horticulture.com.au

Contents

Figures

Tables

Public summary

The Australian avocado industry has a production value reaching $649 million in 2023/24—a 13% increase over three years. Production volumes have surged by 30% to nearly 151,000 tonnes, establishing avocados as a significant agricultural commodity. Australia's avocado orchards remain largely free from exotic pests and diseases that affect production in the Americas, Chile, and Asia Pacific regions. However, maintaining this requires proactive monitoring and biosecurity management to detect and respond quickly to any potential pest incursions.

Traditional methods for detecting pests and diseases often rely on visual inspection or laboratory testing of symptomatic plants. These approaches can miss early-stage infections or require expertise to identify problems before they spread. By the time visual symptoms appear, significant damage may have already occurred, making control more difficult and expensive. Environmental DNA (eDNA) offers a revolutionary approach to pest and disease monitoring. This technology uses genetic material that organisms naturally shed into their environment—whether in soil, on plant surfaces, or in the air, that can be used to identify what species are present, even when they're not easily visible. While eDNA has proven successful in water-based environments like rivers and lakes, this project focused on developing and testing these methods for land-based agricultural systems, particularly avocado orchards.

We evaluated practical and scalable sampling methods to collect eDNA from various sources relevant to pests including swabbing of plant surfaces, washing of plant/fruit surfaces to collect and concentrate eDNA, air sampling, direct collection of soil and roots/rhizosphere to detect soil-borne diseases, and insect traps.

Initial trials to validate and refine sampling methodology were done in Victoria, at a wildlife reserve and local orchard, before we conducted larger trials in Australia's major avocado-growing regions. These locations represent the majority of Australian avocado production, with Queensland contributing 53% and Western Australia 32% of national output.

1. **Initial Testing**: Controlled trials at a Victorian wildlife reserve with known species to validate air eDNA methods
2. **Local Validation**: Testing of air, plant surface, soil samples at a Mornington Peninsula avocado farm
3. **Commercial Application**: Large-scale sampling at commercial orchards in Queensland's Atherton Tablelands and Western Australia's Pemberton region

We evaluated multiple genetic markers (DNA barcodes) to detect and identify insects, animals, fungi, plants, and other microorganisms from environmental samples. We also tested an existing assay designed to detect *Phytophthora*, a harmful pathogen that causes root rot in avocado trees and developed and validated new assays to detect the causative agent of brown root rot (*Phellinus noxius* / *Pyrrhoderma noxium*).

Overall, across all production locations and sampling approaches, almost 20,000 detections of more than 1250 distinct fungi and arthropod taxa were obtained. These included many priority pathogen/pest families, with methods found to be particularly sensitive for fungi.

The methodologies developed provide the Australian avocado industry with enhanced surveillance capabilities, supporting continued growth while protecting against future biosecurity threats.

Keywords

Disease monitoring; Pest detection; Environmental DNA; Phytophthora; Soil-borne disease; Phellinus noxius; Pyrrhoderma noxium

Introduction

This research aims to enhance biosecurity monitoring by efficiently and cost-effectively identifying pest and disease incursion threats using eDNA. DNA-based tools coupled with appropriate sampling methodologies can allow rapid identification and quantification of potential pests and diseases, strengthening Australia's biosecurity preparedness and allowing avocado growers to:

- Detect pest and disease threats earlier than traditional methods
- Monitor orchard health more comprehensively
- Implement targeted management strategies before problems spread

The methodologies developed, if implemented, could provide the Australian avocado industry with enhanced surveillance capabilities, supporting continued growth while protecting against future biosecurity threats.

Methodology

1. Preliminary air eDNA sampling development and testing

An initial method validation field trial was conducted at Mt Rothwell Conservation and Research Reserve (Victoria, Australia) during summer and autumn of 2022/2023. The site was chosen as the species present have been well documented and are kept within a predator-proof fenced area.

Four Filter Master Pro (FMP) 7000 Series active sampling device were hired from Thomson Environmental Systems and deployed across the study site. Each unit incorporated a solar-powered pump system operating at a consistent air flow rate of 5 L/min, with particulate matter collected onto 0.22 µm nylon filter membranes. One active sampler was paired with a meteorological sensor that continuously recorded environmental parameters including wind speed, wind direction, temperature, relative humidity, precipitation and barometric pressure throughout the sampling period.

One hundred passive samplers, based on the Modified Wilson and Cook (MWAC) design (Wilson and Cooke 1980), were systematically distributed throughout the reserve. These samplers are designed to collect wind-transported particulate matter. Each passive sampler contained liquid preservative TE buffer to maintain DNA integrity upon collection. Weekly sampling occurred across the five sites (Figure 1) whereby the liquid preservative solution, filters and cloths were collected and replaced. Preservative solution from each of the MWAC samplers was filtered across 1.2 µm syringe filters. The 0.22 µm active air filters were collected with forceps sterilised with 10% bleach and placed into 2ml tubes. All were transported back to the lab and stored at -20°C until further processing. Three metabarcoding assays were used for broad taxonomic coverage of invertebrates (Zeale et al. 2011), vertebrates (Riaz et al. 2011) and plants (Taberlet et al. 2007) targeting the COI, 12S and trnL gene regions (Table 2).

Figure 1. Distribution of five sites across Mt Rothwell reserve

2. Field sampling Mornington Peninsula Victoria – modified sampling approaches

A second trial was conducted at a local avocado farm during Spring 2023 on the Mornington Peninsula in Victoria. We adjusted air sampling methods based on the outcomes of the Mt Rothwell trial and introduced new ones to better target invertebrates and fungal species relevant to the avocado industry including:

1. **Direct surface sampling:** Sterile swabs and wipes from leaf surfaces, branches and fruit.
2. **Plant surface washing:** Targeted water washing of plants for recovering arthropod or fungal DNA.
3. **Rhizosphere soil and roots:** Targeting the presence, diversity and distribution of fungal pathogens.
4. **A prototype active air sampler**: Development and deployment of a cost-effective air sampling device that drew air through a large volume of preservative liquid to increase DNA capture efficiency and operate under humid environments which are likely to lead to accelerated DNA degradation during extended sampling periods.

Five metabarcoding assays were assessed during this trial targeting invertebrates (Vamos, Elbrecht, and Leese 2017), fungi (Tedersoo and Lindahl 2016), general eukaryotes (Cabodevilla et al. 2023), vertebrates (Riaz et al. 2011) and *Phytophthora* (Scibetta et al. 2012) (Table 2).

3. Field sampling Atherton Tablelands North QLD

In December 2024 sampling was undertaken at a larger scale within the industry across two avocado orchards located in the Atherton Tablelands region of North Queensland. Sampling occurred across three sites on each farm with the aim to target either ‘healthy’ or ‘unhealthy’ (low foliage and fruit, potential root rot disease present) trees. At each site three trees were selected randomly within proximity to each other.

Sampling methods included:

1. **Cloth passive air samples** for collection of airborne DNA targeting invertebrates and wind dispersed fungi.
2. **Wipes** of trunks/leaves/fruit targeting visual damage to plant/fruit or fungal growth at the base of the tree trunk. Three replicates were collected from each tree at each sampling event.
3. **Rhizosphere soil and roots** targeting soil and root dispersed fungi and oomycetes. Three replicates were

collected from each tree at each sampling event.

4. **Light traps** targeting invertebrates. These were set up at the start of the week and collected at the end of the week. Batteries for the lights were replaced every day.
5. **Sticky traps** targeting invertebrates. These were suspended in the tree close to the light traps (in the trees that had these) or hung near the passive air sampler.

The collection of rhizosphere soil and roots was used to target Phytophthora dieback and brown root rot. At one orchard we were able to sample trees that were visually presenting with symptoms, neighbouring sites with no visual symptoms, and areas assumed to be disease free.

Six metabarcoding assays were used on these samples. This included previously developed and published assays targeting invertebrates (Vamos, Elbrecht, and Leese 2017; Elbrecht et al. 2019), fungi (Tedersoo and Lindahl 2016) and phytophthora (Treena I. Burgess, White, and Sapsford 2022) and two assays designed by EnviroDNA to target *Phellinus noxius / Pyrrhoderma noxium*.

4. Field sampling Pemberton Western Australia

The final field trial was conducted at one avocado orchard located in Pemberton, Western Australia in March 2025.

Four eDNA sampling methods were used.

- Wipes
- Passive cloths
- Rhizosphere soil
- Roots

Two eDNA sampling methods at packing shed

- Wipes
- Water sample (fruit wash water)

5. DNA extraction and sampling methods

For all DNA extractions a modified pre-incubation and overnight lysis protocol was developed and optimised before further processing on a QIAsymphony SP instrument using the PowerFecal Pro DNA Kit.

Table 1. Summary of eDNA samples collected over the course of the project

Sample Type	Collection dates	Location	Number of samples
MWAC - passive air	Dec 2022 - May 2023	Mt Rothwell reserve, Victoria	671
Active air – filter (Dust Master Pro 7000)	Dec 2022 - May 2023	Mt Rothwell reserve, Victoria	38
Cloth – passive air	Dec 2022 - May 2023	Mt Rothwell reserve, Victoria	44
Cloth – passive air	Sep 2023 - Dec 2023	Mornington Peninsula, Victoria	6
Active air – bubbler prototype	Sep 2023 - Dec 2023	Mornington Peninsula, Victoria	6
Wipes	Sep 2023 - Dec 2023	Mornington Peninsula, Victoria	32
Swabs	Sep 2023 - Dec 2023	Mornington Peninsula, Victoria	32
Water wash	Sep 2023 - Dec 2023	Mornington Peninsula, Victoria	4
Containers with liquid preservative – passive air	Sep 2023 - Dec 2023	Mornington Peninsula, Victoria	11

Soil	Sep 2023 - Dec 2023	Mornington Peninsula, Victoria	10
Cloth – passive air	Dec 2024	Atherton Tablelands, Queensland	11
Light trap	Dec 2024	Atherton Tablelands, Queensland	18
Sticky trap	Dec 2024	Atherton Tablelands, Queensland	11
Wipes	Dec 2024	Atherton Tablelands, Queensland	77
Soil	Dec 2024	Atherton Tablelands, Queensland	72
Roots	Dec 2024	Atherton Tablelands, Queensland	72
Cloth – passive air	March 2025	Pemberton, Western Australia	9
Wipes	March 2025	Pemberton, Western Australia	54
Soil	March 2025	Pemberton, Western Australia	54
Roots	March 2025	Pemberton, Western Australia	54
Wipes	March 2025	Donnybrook, Western Australia	9
Water (syringe filter)	March 2025	Donnybrook, Western Australia	3

Cloth passive air samples were suspended in trees and used to collect airborne DNA. These were made from blue absorbent viscose cleaning wipes cut into 20 cm x 20 cm squares, sterilised with 10% bleach, rinsed with Milli-Q water and placed into a zip lock bag prior to deployment in the field. These were initially deployed as cloths wrapped around trees (Mt Rothwell, Mornington Peninsula) or suspended with two pieces of wire, one weaved through the top and hung over the tree and the other weaved through the bottom of the cloth to weigh it down (Atherton Tablelands). A later prototype used 2 x 90 mm 45° PVC M-F elbow pipes with the cloth placed between the two to protect from UV and rain. These were collected by placing the cloth into a zip lock bag and adding 1ml of preservative buffer before transporting to the lab.

MWAC sampler prototypes were made that consisted of an array of 4 collection bottles oriented in the cardinal directions to capture any particulates transported by air. Each bottle had a 13mm inlet and outlet tube to facilitate air flow and 50 ml of liquid preservative (10x TE buffer) was added to each bottle.

Swabs for direct surface sampling were made from the above cleaning wipes cut into 10cm x 10cm squares. These were sterilised with 10% bleach, rinsed with Milli-Q water, dried and placed into zip lock bags with 600ul of 10x TE buffer added. At each tree three replicate swabs were taken, with replicates one and two used for wiping along one branch on either side of the tree, across three leaf surfaces and any fruit present on that branch. The third replicate was used for swabbing the trunk of the tree targeting any fungal growth present, focusing on the base of the tree. These were then placed back into the zip lock bags with 1ml of preservative buffer added.

Rhizosphere soil and roots, targeting soil and root dispersed fungi and oomycetes. A shovel/corer was used to dig near the base of the tree to target the rhizosphere (the narrow soil zone surrounding the roots). The roots of the avocado trees could be found within the first 10-20cm of soil. At each tree three replicates were taken, with replicates one and two collected either side of the tree at approximately 0.5 metres from the base and replicate three collected at the base of the tree. Enough soil to fill a 50ml falcon tube was collected from each spot and fresh white roots from the same area were placed into a 5ml tube. These were kept cold and refrigerated prior to being transported back to the lab.

Light traps targeting invertebrates. These were suspended close to the trunk of the tree under the canopy (when permitted, some trees had low foliage). A collection container was hung underneath of the light filled with distilled water

and two drops of dishwashing liquid. These were placed at only one of the three trees at each site. The contents of the container were collected into multiple 50ml tubes with excess water drained out. Approximately 10ml of invertebrates and water was left in each tube. 1ml of preservative buffer was added prior to transporting back to the lab. These were later pooled together per tree in the lab.

Sticky traps targeting invertebrates. These were suspended in the tree close to the light traps (in the trees that had these) or hung near the passive air sampler.

Plant surface washing involved spraying water over trees using a backpack spray unit and collecting run-off into a sterilised bucket. The water was then filtered across a 1.2 µm syringe filters (until no more water was able to be pushed across the filter) with the total volume recorded. The remaining water was expelled from the filter by drawing air into the syringe and pushing this through the filter. Liquid preservative (1 ml Qiagen ATL buffer) was then added using 3ml tubes. Pan traps containing 1L of liquid preservative (20% polyethylene glycol, 100mM TE) were also placed below trees and sampled as above after 2 weeks.

For all DNA extractions a modified pre-incubation and overnight lysis protocol was developed and optimised before further processing on a QIAsymphony SP instrument using the PowerFecal Pro DNA Kit (Qiagen).

6. Multi-species metabarcoding assays

Assessment of general surveillance metabarcoding assays

Over the course of the project, we tested a variety of metabarcoding assays for the detection of invertebrates, vertebrates, fungi, plants and eukaryotes (Table 2). We also assessed the efficacy of an existing broad-spectrum 18S assay capable of detecting oomycetes (Cabodevilla et al. 2023), more specifically Phytophthora species, from the sample types collected (Table 3).

Table 2. Metabarcoding primers used in this study

Primer reference	Target group	Gene	Forward Primer	Reverse Primer
Zeal et al. 2011	Arthropods	COI	AGATATTGGAACWTTATATTTTATTTTTGG	WACTAATCAATTWCCAAATCCTCC
Taberlet et al. 2007	Plants	trnL	CGAAATCGGTAGACGCTACG	CCATTGAGTCTCTGCACCTATC
Riaz et al. 2011	Vertebrates	12S	GATTAGATACCYCACTATGC	TAGRACAGGCTCCTCTAG
Vamos et al. 2017	Arthropods	COI	YTCHACWAAYCAYAARGAYATYGG	ARTCARTTWCCRAAHCCHCC
Cabodevilla et al. 2023	Eukaryotes	18S	GGCCGTTCTTAGTTGGTGGA	CCCGGACATCTAAGGGCATC
Tedersoo & Lindahl 2016	Fungi	ITS	GTGARTCATCRARTYTTTG	CCTSCSCTTANTDATATGC
Elbrecht et al. 2019	Invertebrates	COI	TCDGGRTGNCCRAARAAYCA	TCDGGRTGNCCRAARAAYCA
Vamos et al. 2017	Invertebrates	COI	GGDACWGGWTGAACWGTWTAYCCHCC	GTRATWGCHCCDGCTARWACWGG
Scibetta et al. 2012	Phytophthora	ITS	GAAGGTGAAGTCGTAACAAGG	GCARRGACTTTCGTCCCYRC

Once DNA was extracted, library preparation involved two rounds of PCR. The first round employed gene-specific primers designed to include Illumina overhang adapter sequences to ensure the workflow is compatible with downstream NGS sequencing technology. The second round added unique combinations p5 and p7 indexes onto each sample to enable the sequencing to be run multiplexed. Negative controls were included during library construction. Negative controls consisted of the extraction negatives as well as PCR negatives, in which nuclease-free water was used in place of DNA during both rounds of PCR. Sequencing was carried out using Illumina iSeq 100 or NextSeq 2000 platforms.

Phytophthora targeted assay

An initial test of more targeted oomycete (*Phytophthora*) specific primers (Table 3) for metabarcoding was conducted on soil eDNA samples collected from the Mornington Peninsula.

Two rounds of PCR were initially carried out with two different sets of primers (Table 3), with the first round in duplicate (10 µl volume including 2 µl of sample) and the second round pooled (5 µl of the first-round product was pooled into the duplicate plate and then 5 µl of the pooled product was added into the second-round mix for a total volume of 15 µl) using Roche KAPA3G Plant PCR Kits following manufacturer's instructions.

Both rounds of PCR were conducted following conditions used by Burgess et al. (2022) as follows, 94°C for 2 min followed by 30 cycles of 95°C for 20s, 60°C for 25s, 72°C for 1 min before a final 72°C for 7 mins. 2µl of the second PCR products were then transferred into 2µl of ExoSAP-IT *Express* PCR product cleanup reagent to inactivate excess primers and nucleotides following manufacturer's protocol. A second round of PCR incorporated unique p5 and p7 indexes for each sample using 15µl volume reactions containing Phusion DNA Polymerase HF Buffer and barcoding primers. 2µl of ExoSAP-IT product was added to the third PCR mix (for a total volume of 15µl). PCR products were visualised on a 2% agarose gel made with SYBR Safe DNA Gel Stain and all barcoded samples were pooled taking 5µl of product from each sample.

Table 3. Phytophthora specific primers

Primers are from Burgess et al. (2022) (forward and reverse Illumina adaptor sequences added to second PCR primers in bold)

Primer		Sequence
18Ph2F	PCR1	GGATAGACTGTTGCAATTTTCAGT
5.8S1R	PCR1	GCARRGACTTTCGTCCCYRC
2N-ITS6-F	PCR2	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GAAGGTGAAGTCGTAACAAGG
2N-5.8S1R	PCR2	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GCARRGACTTTCGTCCCYRC

All soil eDNA samples and eDNA sample types with reads assigned to *Oomycota* at Phylum level using the general 18S eukaryote assay were chosen to test the p4 *Phytophthora* primers. These included 2 x swab samples (swabbing trunks, branches and leaf surfaces) and 1 x bubbler sample (active solar-powered samplers that continuously collect air into a large volume of preservative liquid).

Phellinus noxius/Pyrrhoderma noxium targeted assay

To target *Phellinus noxius/Pyrrhoderma noxium* we initially considered an assay developed by Wang et al. (2016) (Table 4) targeting the ITS region. To assess primer performance, we used tissue-derived DNA extracts from samples collected in QLD. These were collected from trees that were known to be infected and presented with visual signs and growth of brown root rot. PCR was performed using Roche KAPA3G Plant PCR Kits following manufacturer's instructions and primer optimisation was conducted using gradient PCR with annealing temperatures ranging from 55.9°C to 67.2°C, with amplification products visualised on 2% agarose gels using electrophoresis to select the temperature yielding the strongest and most specific amplification.

Table 4. *Phellinus noxius/Pyrrhoderma noxium* specific primers

Forward and reverse Illumina adaptor sequences added to second PCR are in bold.

Primer name	Sequence
p-nox-g1-F	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GCCCTTTCCTCCGCTTATTG
p-nox-g1-R	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CTTGATGCTGGTGGGTCTCT

In silico primer evaluation against standard nucleotide sequences in the NCBI repository (accessed March 2025) for *Phellinus noxius* and *Pyrrhoderma noxium* identified that the reverse primer could not be located within the target gene regions, however, suggesting that it would fail to bind and amplify due to inadequate primer-template binding. Therefore,

multiple new primer pairs were designed in silico to target the ITS and 28S regions (Table 5), with the best performing versions for each locus shown below.

Table 5. Primer pairs targeting the ITS and 28s region designed by EnviroDNA.

Forward and reverse Illumina adaptor sequences added to second PCR are in bold.

Name	Sequence
p-nox-its-cr7-ngsF	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**TTGATGCTGGTGGGTCTCTG
p-nox-its-cr7-ngsR	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TCGAGCAAAAGCCCAGCTAA
p-nox-28s-cr8-ngsF	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**TAACTGCGAGTGAAGCGGG
p-nox-28s-cr8-ngsR	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ACACGTACCCGAAGATACGC

7. Extension and industry engagement

In May 2023 a webinar was delivered to QLD DAF and WA DPIRD staff. There were 51 registered attendees, the majority of which had no experience with eDNA previously. The content provided a background on eDNA and its potential application for early detection of pests and diseases in agricultural and horticultural spaces.

In May 2023 we attended a workshop to develop R&D concepts for avocado pest and disease management, meeting with researchers, industry experts and growers' representatives, which was a very useful grounding in issue facing the industry and an opportunity to establish key contacts for field trials.

In July 2024 we got in contact with Bridie Carr from QLD DAF who leads the Avocado industry development & extension project (AV17005) to seek help with raising awareness about our project and connecting with growers who would be interested in hosting us for field sampling. We put together a fact sheet summarising our project, objectives and how growers could be involved. As a result, we were able to provide awareness about the project and gather any expressions of interest through:

- An article in the Avocados Australia newsletter 'Guacamole' 9 August 2024 issue.
- A post on our company LinkedIn site.
- A fact sheet shared at the North QLD regional forum in July 2024 and WA regional forum in August 2024 hosted by Avocado industry development & extension project regional leads (Appendix D).

Through these efforts we were able to get in contact with Emily Pattison from QLD DAF who assisted us with contacting growers in the Atherton Tableland region. She is also the North QLD regional lead for the Avocado industry development & extension project.

We have also remained in contact with Mary Burton, the RD&E Coordinator for Avocados Australia, who was able to put us in contact with growers and researchers in Western Australia. She also provided an update on the project on our behalf at a regional forum hosted by Avocados Australia in March 2025.

Whilst we had anticipated presenting a summary on our diagnostic assays and field work results at the 2024/2025 Annual Diagnostics Workshop, unfortunately these events were targeted at expert speakers in the areas of invertebrate plant pests, mycology and plant bacteriology. The only webinar that would have been viable for us to present at was the invertebrate plant pests series and this was scheduled in April 2025. Due to field sampling not occurring until Dec 2024 and March 2025 in QLD and WA respectively, results and testing of assays were unable to be finalised until May/June 2025.

We expressed interest in presenting at the Avocado industry development & extension project grower event held on the 26th of March 2025 focused on the topic of pests and diseases. The regional lead Shanara Veivers from QLD DAF was contacted and provided with summary of project progress, but no response was received.

We also consulted with multiple researchers seeking advice around assay development for *Phellinus noxius/Pyrrhoderma noxium*. Associate Professor Elizabeth Dann and Dr Louisa Parkinson were contacted regarding their work with Brisbane City Council on understanding the pathogenicity and virulence nature of the pathogen. Associate Professor Andrew Geering was also contacted who is currently working with the University of Queensland on Avocado industry biosecurity capacity and capability building (AV21003a).

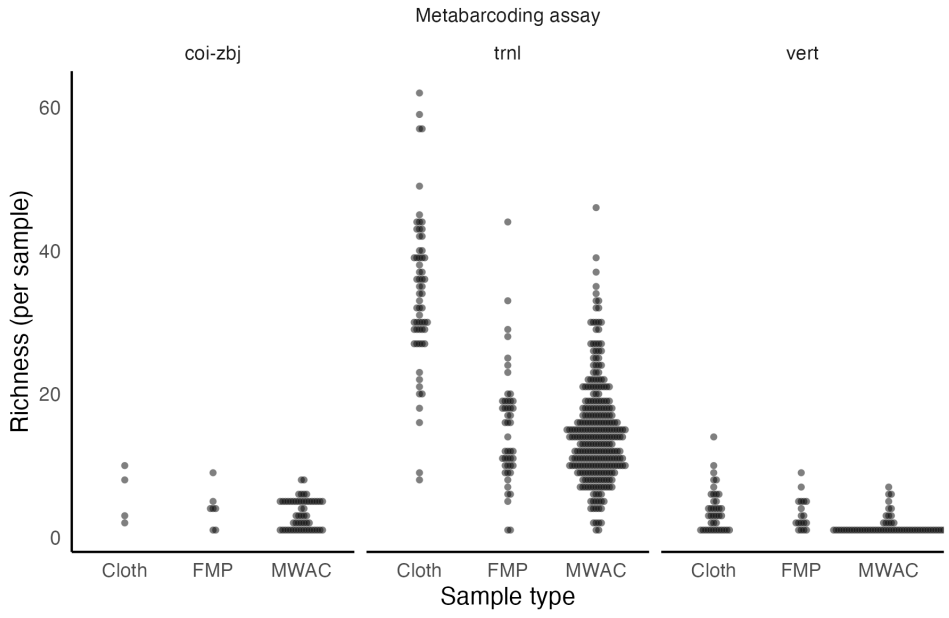
Results and discussion

1. *Initial testing of general terrestrial monitoring using airborne eDNA at Mt Rothwell*

At the time of project commencement, the prospect of using airborne eDNA as a general monitoring tool had been recently proposed (Klepke et al. 2022; Clare et al. 2021; Roger et al. 2022), building on existing research monitoring fungal spores and plant pollen (Tordoni et al. 2021; Johnson, Cox, and Barnes 2019b, 2019a; Johnson et al. 2021; Quesada et al. 2018). We undertook an initial pilot at a well-characterised reference site (Mt Rothwell conservation centre) to test a variety of terrestrial airborne eDNA techniques including solar-powered active samplers and simple, low-cost passive samplers, and the effects of distance and weather conditions on detection rates. This was an ideal location due to the existing knowledge of species that inhabit the reserve which allowed for rigorous evaluation of sampling techniques, before potential application in commercial production environments could be considered.

Across three metabarcoding assays (targeting invertebrates, plants and vertebrates), all sampling approaches resulted in the detection of species, however, passive air samplers resulted in the most robust detections and were simple to deploy. Therefore, replicated passive sampling with minimal equipment and low consumable costs may be a viable alternative to active sampling. Subsequent research has, however, demonstrated the importance of sampling volumes, and our weekly active samples of around 50 cubic metres may have been underpowered in this pilot. Detection of invertebrate taxa using these sampling approaches was relatively patchy, compared to vertebrates and plants.

Figure 2. Taxon richness per sample across amplicons and sample types.

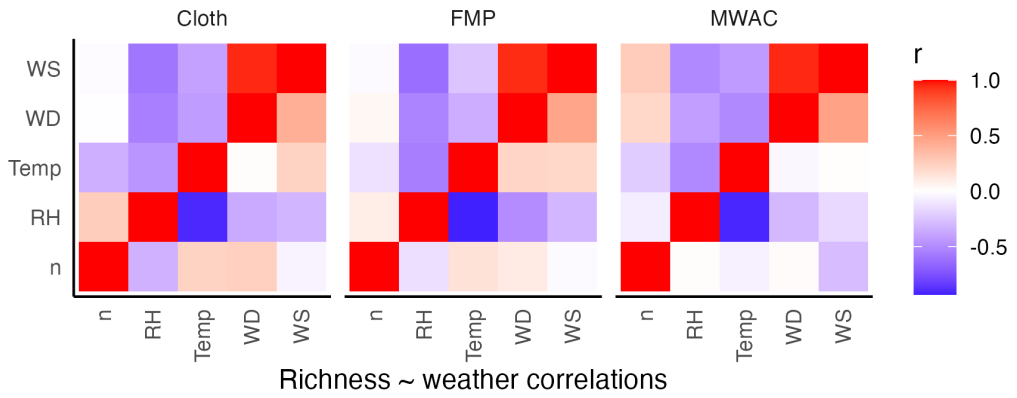


coi-zbj = arthropod assay targeting the mitochondrial COI barcode, vert – vertebrate assay targeting the mitochondrial 12S rRNA barcode, trnl = plant assay targeting the plastid trnL barcode). Sample types are passive cloth, active Filter Master Pro (FMP) and passive Modified Wilson and Cooke (MWAC).

Based on live meteorological data from one test site, we saw significant (negative) general effects of average temperature on sample richness (Poisson analysis of variance). Directional passive MWAC samplers were additionally influenced by wind speed and direction (Figure 3).

Figure 3. Meteorological effects on detection from airborne eDNA.

Each cell shows the Pearson's correlation coefficient for a pair of variables. Weather values are weekly averages (above the diagonal) or coefficients of variation (below the diagonal), taken to match sampling frequency. n = taxon richness per sample, RH = relative humidity, Temp = temperature, WD = wind direction, WS = wind speed. Sample types are passive cloth, active Filter Master Pro (FMP) and passive Modified Wilson and Cooke (MWAC).



2. *Modified sampling approaches - Avocado orchard pilot study on the Mornington Peninsula*

Following the initial validation trial at Mt Rothwell, we conducted a second trial of airborne and direct sampling approaches at an avocado orchard located on the Mornington Peninsula, Victoria. This subsequent trial applied refined methodologies based on initial findings and incorporated adjustments to enhance detection capabilities for targeting invertebrates and fungi in an agricultural setting (based on the above trial, and other relevant projects). We also trialled an alternative active air sampling method suitable for humid environments by creating our own cost-effective prototype that collects air by the Venturi effect into preservative liquid.

To better target invertebrate and fungal species relevant to avocado production, we expanded sampling methods and associated molecular assays to include:

1. **Direct Surface Sampling**: Systematic collection of biological material using sterile swabs and wipes from leaf surfaces, branches, and fruit.
2. **Plant Surface Washing**: Targeted water washing of plant tissues to recover adherent microorganisms and arthropod DNA from avocado trees.
3. **Prototype Cost-Effective Active Air Samplers**: Development and deployment of economically viable active air sampling devices specifically designed for agricultural implementation. These prototypes featured a novel collection mechanism that forced air through a large volume of preservative liquid, potentially increasing DNA capture efficiency while reducing operational costs compared to commercial alternatives.
4. **Rhizosphere soil sampling:** Collection of soil samples from the root zone to target potential microbial pathogens.

A summary of assays, sample types, and sample numbers is in Table 6.

Table 6. Overview of sampling approaches and numbers in Victoria

Victoria (Mornington Peninsula)		
environmental_sample	amplicon	n
fungi		
bubbler	fungi_gits7ngs-its4ngsuni	6
cloth	fungi_gits7ngs-its4ngsuni	6
passive air	fungi_gits7ngs-its4ngsuni	9
soil	fungi_gits7ngs-its4ngsuni	8
swab	fungi_gits7ngs-its4ngsuni	30
water wash	fungi_gits7ngs-its4ngsuni	4
wipe	fungi_gits7ngs-its4ngsuni	32
arthropods		
bubbler	fwhf1-fwhr1	6
cloth	fwhf1-fwhr1	6
passive air	fwhf1-fwhr1	10
soil	fwhf1-fwhr1	10
swab	fwhf1-fwhr1	32
water wash	fwhf1-fwhr1	4
wipe	fwhf1-fwhr1	31
eukaryotes		
bubbler	minib18s	6
cloth	minib18s	6
passive air	minib18s	10
soil	minib18s	9
swab	minib18s	32
water wash	minib18s	4
wipe	minib18s	31
phytophthora		
bubbler	phytoph2n-its6f-58sr	1
soil	phytoph2n-its6f-58sr	10
swab	phytoph2n-its6f-58sr	2
vertebrates		
bubbler	vert	6
cloth	vert	4
passive air	vert	10
soil	vert	8
swab	vert	23
water wash	vert	4
wipe	vert	29

Summaries of taxa detected for each assay (across all sampling types) are in Figures 4-6.

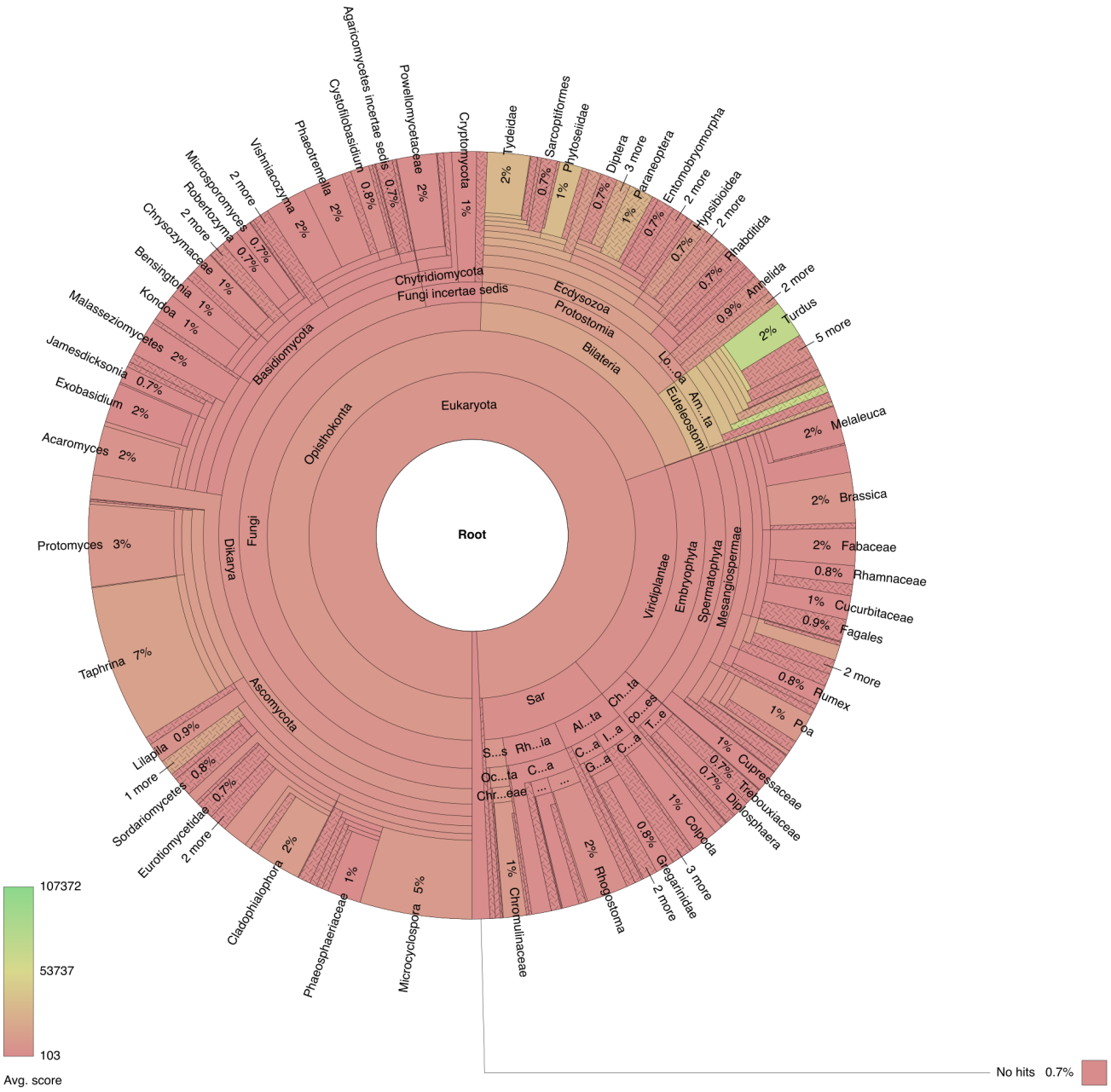


Figure 4. Mornington detections for general eukaryote 18S assay.

Plots show detections by taxonomic rank (down to family level). Colour is scaled by total read depth across all samples.

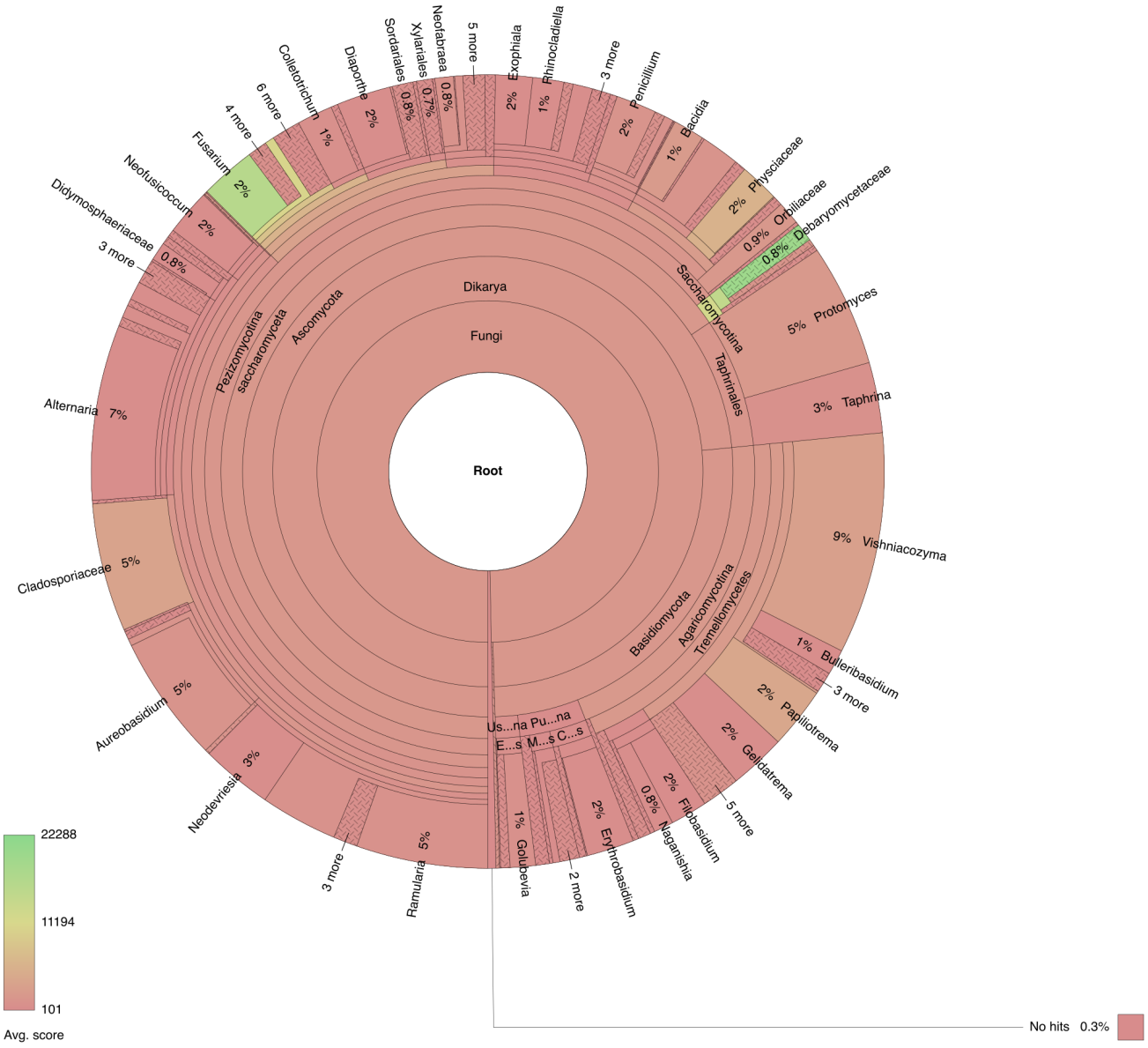


Figure 5. Mornington detection for fungi assay.

Plots show detections by taxonomic rank (down to family level). Colour is scaled by total read depth across all samples.

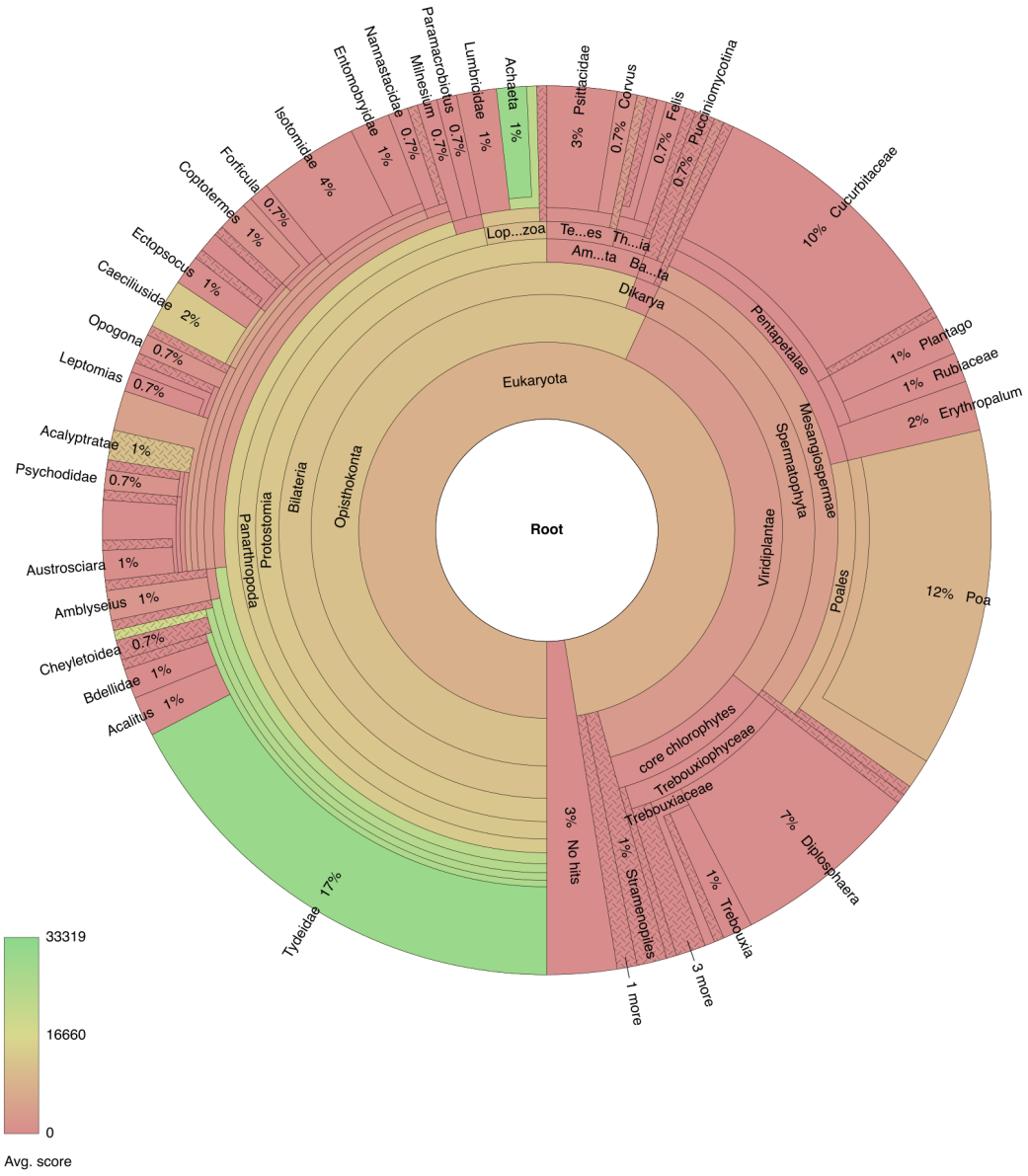


Figure 6. Mornington detections for arthropod COI assay.

Plots show detections by taxonomic rank (down to family level). Colour is scaled by total read depth across all samples.

We detected *Phytophthora* in 6/16 samples (all soil samples) and specifically *P. cinnamomi* in 3/16 samples (100% match), *P. cambivora* in 2/16 samples (99-100% match), *P. citricola/P. multivora* in 2/16 samples (97-100% match) and *P. cactorum* (most likely based on available reference data, sequence also shared with *P. pseudotsugae*) in 2/16 samples (100% match). A summary of phytophthora detections across all field sites is shown in Appendix A; sequence sharing across species at the target locus is relatively common, complicating unambiguous resolution of species (potentially a function of reference database quality and barcode evolutionary rates).

3. *Field trials – Atherton Tablelands, Queensland*

Following the implementation of sampling and analysis methodologies at the Mornington Peninsula avocado orchard, we extended field sampling to subtropical growing regions. In consultation with staff from Queensland Department of

Agriculture and Fisheries (QDAF), sampling was organised at two commercial avocado orchards located in the Atherton Tablelands region of North QLD in December 2024. This subtropical region experiences high temperatures and humidity which creates favourable conditions for the establishment and growth of pathogens such as brown root rot disease (*Phellinus noxius* / *Pyrrhoderma noxium*) and Phytophthora dieback (*Phytophthora cinnamomi*).

We introduced targeted rhizosphere root (as well as soil) sampling for detection of Phytophthora dieback and brown root rot pathogens. At one farm we were able to sample from trees displaying visual disease symptoms, adjacent asymptomatic trees, and a presumed disease-free area.

A summary of metabarcoding assays, sample types, and sample numbers is in Table 7.

Table 7. Summary of sampling approaches and numbers in Queensland

	Atherton	
environmental_sample	amplicon	n
fungi		
Unknown fungi	fungi_gits7ngs-its4ngsuni	1
roots	fungi_gits7ngs-its4ngsuni	23
soil	fungi_gits7ngs-its4ngsuni	24
swab	fungi_gits7ngs-its4ngsuni	20
invertebrates		
chux	invert-bf3-br2	11
chux	invert-fwh	11
light trap	invert-bf3-br2	18
light trap	invert-fwh	18
swab	invert-bf3-br2	68
swab	invert-fwh	67
yellow sticky trap	invert-bf3-br2	7
yellow sticky trap	invert-fwh	11
phytophthora		
roots	phytoph2n-its6f-58sr	23
soil	phytoph2n-its6f-58sr	24

Summaries of taxa detected for each assay (across all sampling types) is in Figures 7-8.

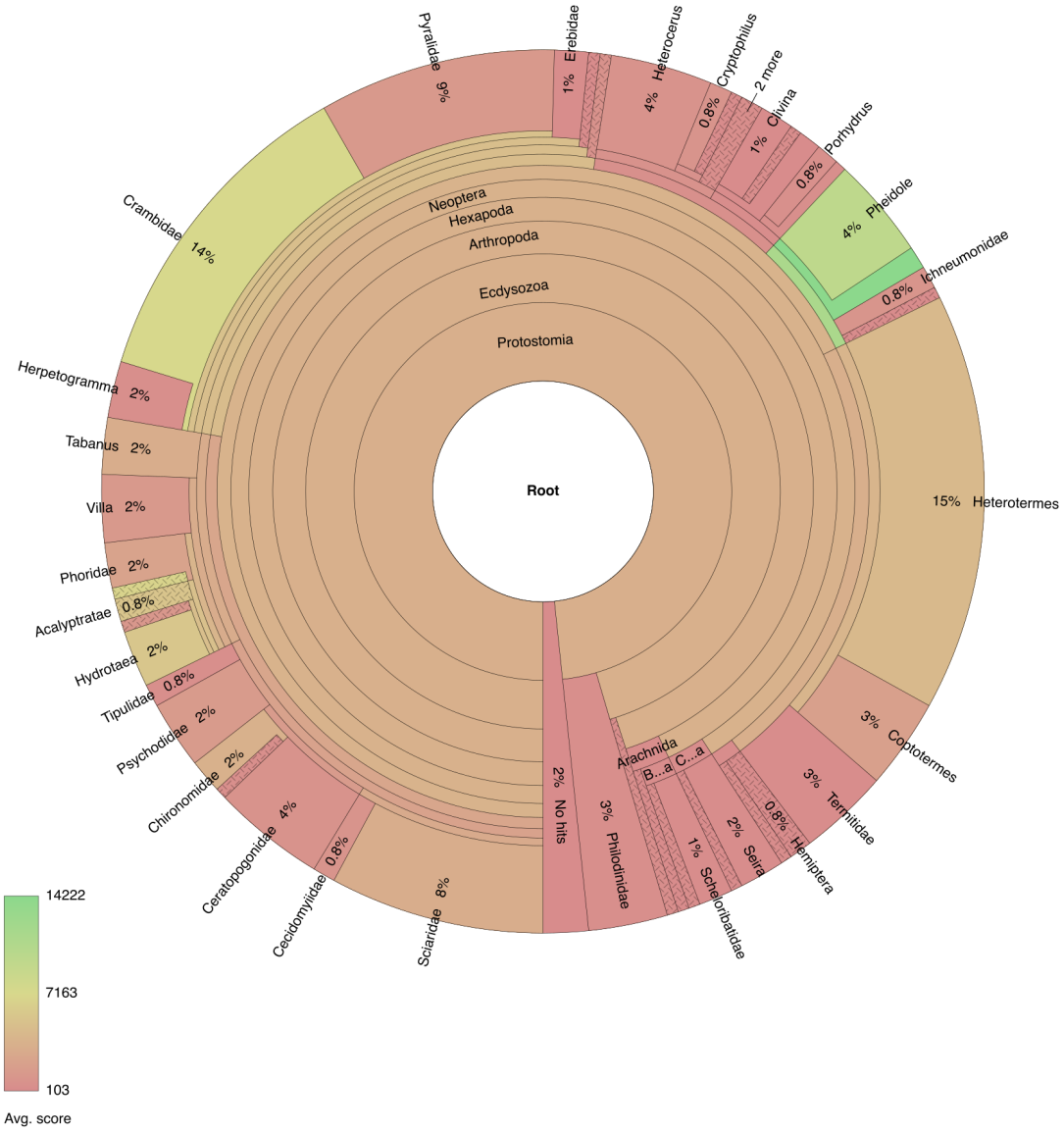


Figure 7. Atherton Tablelands invertebrate detections

Based on COI assays (invert-bf3-br2 and invert-fwh). Plots show detections by taxonomic rank (down to family level). Colour is scaled by total read depth across all samples. The nominally targeted invert-fwh assay was found to show a high rate of off-target (fungi) amplification on complex (e.g., air and swab) samples, these have been excluded for plotting.

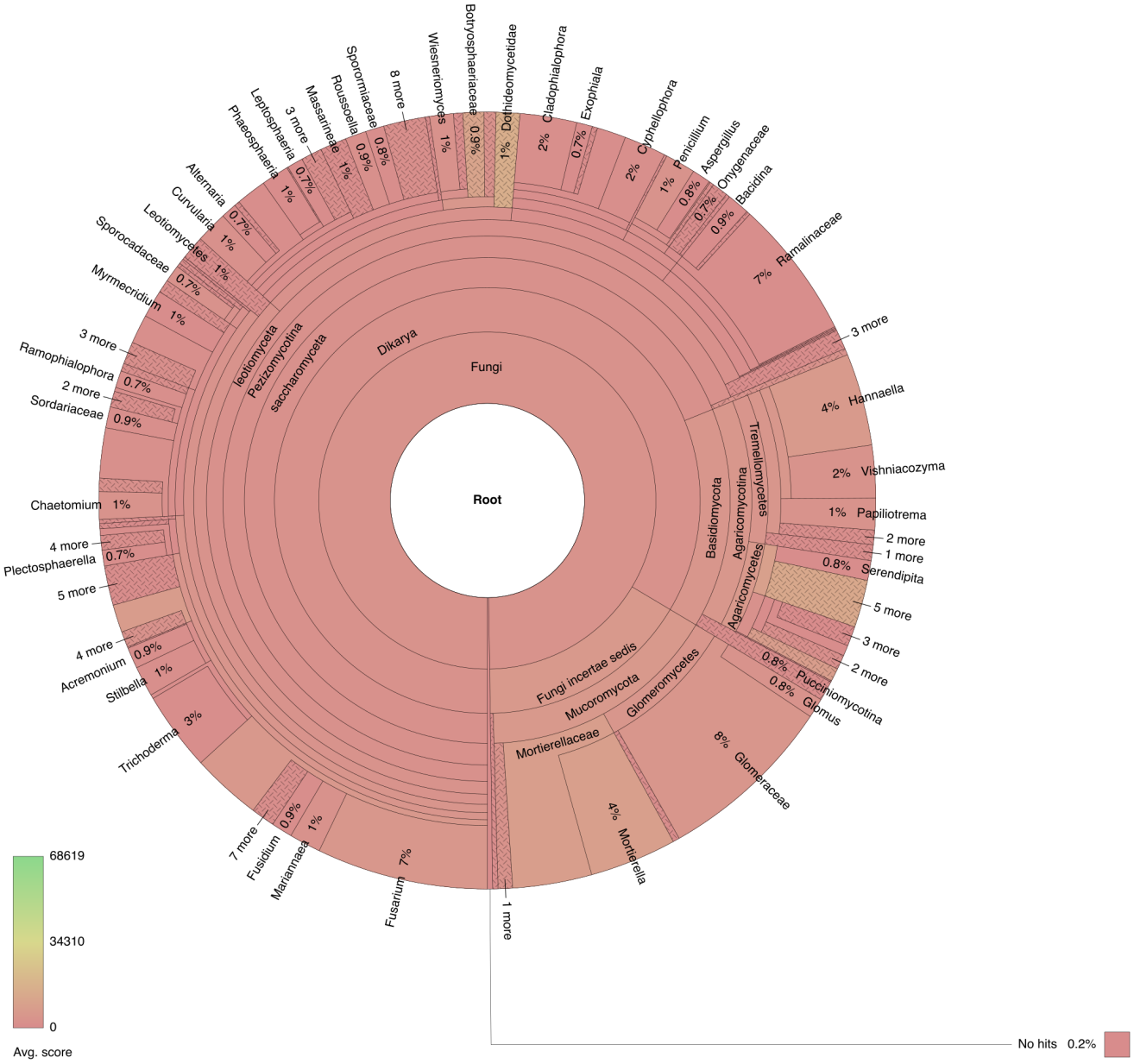


Figure 8. Atherton Tablelands fungi detections

Based on ITS2 assay. Plots show detections by taxonomic rank (down to family level). Colour is scaled by total read depth across all samples.

4. *Field trials – Pemberton and Donnybrook, Western Australia*

Four sampling methods were used in the final trial with the intention of focusing on techniques that were simple to implement, cost effective and shown to be sensitive to target species.

A summary of assays, sample types, and sample numbers is in Table 8.

Table 8. Summary of sampling approaches and numbers in WA.

environmental_sample	amplicon	n
fungi		
air	fungi_gits7ngs-its4ngsuni	9
roots	fungi_gits7ngs-its4ngsuni	47
soil	fungi_gits7ngs-its4ngsuni	18
water	fungi_gits7ngs-its4ngsuni	3
wipes	fungi_gits7ngs-its4ngsuni	58
invertebrates		
air	invert-bf3-br2	4
air	invert-fwh	9
water	invert-bf3-br2	3
water	invert-fwh	3
wipes	invert-bf3-br2	54
wipes	invert-fwh	54
phytophthora		
roots	phytoph2n-its6f-58sr	52
soil	phytoph2n-its6f-58sr	17

Phytophthora detections are summarised in Appendix A. The dominant sequences detected were most similar to an existing reference sequence for *P. citricola* (AJ854301, isolated in Italy), a wide range of *P. cinnamomi* sequences, and a more divergent sequence not closely related to any existing references. However, more distantly related sequences identified as *P. citricola* also group with *P. plurivora*, *P. citropthora*, *P. multivora* (and other poorly sampled taxa), making this identification less than certain.

Summaries of taxa detected for each assay (across all sampling types) are in Figures 9-10.

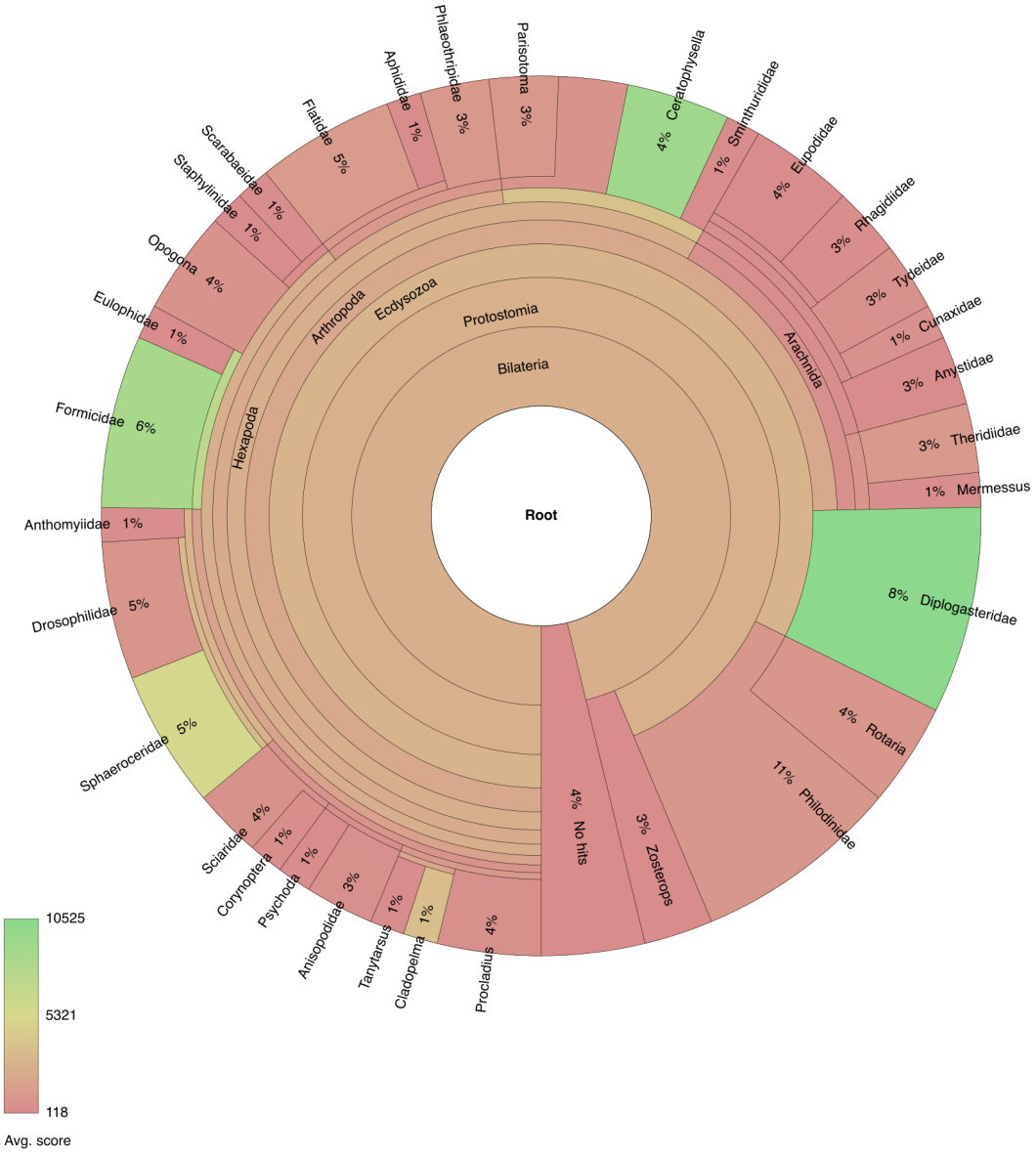


Figure 9. WA invertebrate detections

Based on COI assays (invert-fwh and invert-bf3-br2). Plots show detections by taxonomic rank (down to family level). Colour is scaled by total read depth across all samples. The nominally targeted invert-fwh assay was found to show a high rate of off-target (fungi) amplification on complex (e.g., air and swab) samples, these have been excluded for plotting.

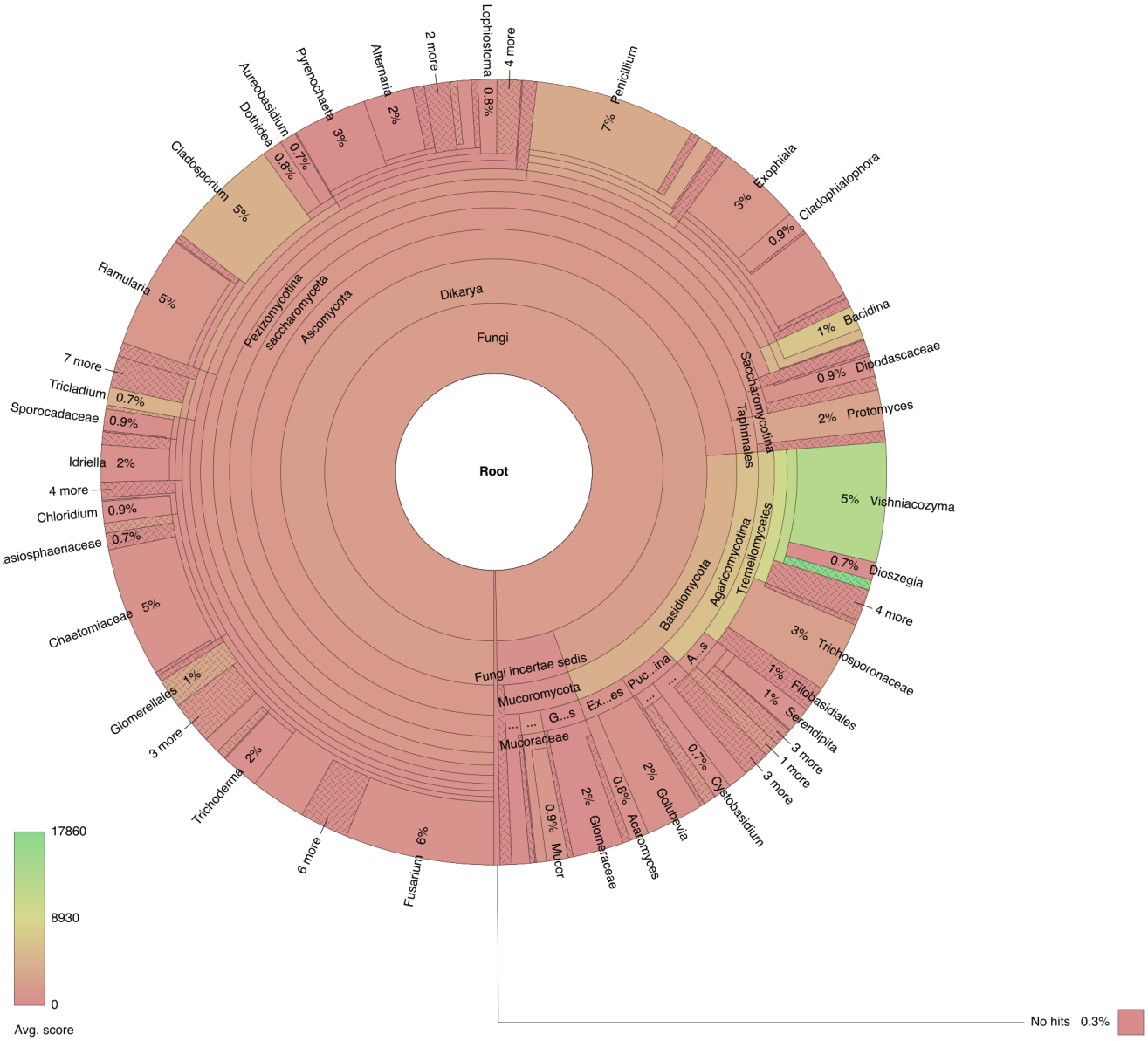


Figure 10. WA fungi detections

Based on ITS2 assay. Plots show detections by taxonomic rank (down to family level). Colour is scaled by total read depth across all samples.

5. *Metabarcoding assays targeting soil-borne pathogens*

A variety of DNA based assays were used throughout this project including general assays targeting invertebrates, fungi, plants and vertebrates. In general, the fungi ITS2 assay performs well in terms of specificity, though reference database completeness and quality, and variable evolutionary rates, mean that species-level resolution is not consistently reliable, or possible. Still, this assay should serve as a useful first line in fungal surveillance and detection programs.

In consultation with industry, we identified Brown root rot (*Phellinus noxius = Pyrrhoderma noxium*) and Phytophthora root rot (*P. cinnamomi)* as priority targets, particularly in QLD. These species can be difficult to identify visually in the early stages of infection and strategies for management and treatment differ.

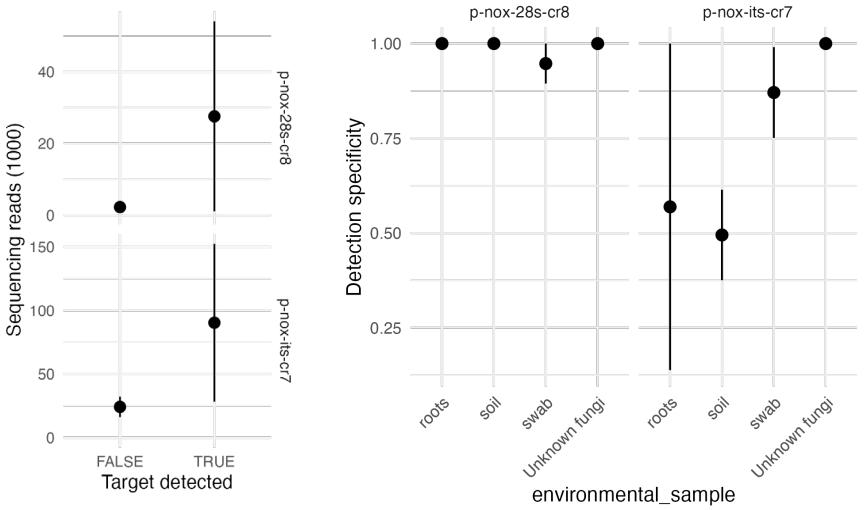


Figure 11. *P. noxius* assay specificity

Left: the sum of reads assigned to *Phellinus noxius / Pyrrhoderma noxium* (target = TRUE) versus off-target reads (target = FALSE), tested against the same set of 76 samples from Queensland. Points (error bars) are the mean (standard error of the mean). Right: average proportion of reads assigned to the target species when detected. While the ITS assay shows better efficiency (higher reads counts), the 28S assay is more specific, amplifying close to 100% of the target taxon across most sample types.

Phellinus noxius is a basidiomycete fungus that causes brown root rot disease is widespread in tropical and subtropical regions of the world (T. T. Chang 2002; T. T. Chang and Yang 1998; Kozhar et al. 2022). The fungus has a broad host range and the disease spreads between trees through root-to-root contact with infected plants showing symptoms of wilting, chlorosis, browning and defoliation (Sahashi et al. 2012). Whilst *P. noxius* DNA can be detected in both rhizosphere and non-rhizosphere soils, *P. noxius* requires the host plant tissue for viability (Wu et al. 2020). Therefore, sampling and detection of *P. noxius* in diseased host tissue (tree material or roots) is required for a true positive detection. The traditional method for identifying and isolating *P. noxius* is culturing from infected tissue using malt-extract agar, but it is not always reliable or practical (Tun Tschu Chang 1995). It can't always be isolated, and it takes time for the culture to grow which can delay timely treatment. To prevent the spread of disease, barriers are often installed to prevent root contact and complete removal of infected trees and all host tissue from the soil is required. An infected plant in early stages of disease can also be difficult to detect and most affected trees eventually die within a few months of infection (T. T. Chang and Yang 1998; Sahashi et al. 2012).

Given relatively higher rates of uncertainty in identification of many fungi (with implications for the quality and quantity of reference sequences available), we designed targeted metabarcoding assays for *P. noxius* which in theory allows both sensitive detection, where present, as well as increased confidence in the identity of detections through sequencing (versus a presence/absence result). These assays perform well when *P. noxius* is present, based on technical validation with synthetic DNA, and on inferred positive environmental samples from metabarcoding, as well as one of two suspected specimens isolated from a diseased tree confirmed by ITS metabarcoding as *P. noxius* (the second was identified as *Ganoderma australe*). The assay targeting the more slowly evolving 28S barcode shows greater specificity (Figure 11), though reference database coverage is greater for the ITS region (see Appendix B and C).

Phytophthora is a diverse genus of plant pathogens that causes devastating disease in plants worldwide. In particular, the soil-borne *Phytophthora cinnamomi* is one of the most frequently reported pathogens in Australia and poses a significant threat to forestry and agricultural industries (Cahill et al. 2008; T. I. Burgess et al. 2021). It can grow saprophytically on dead organic matter or parasitically on susceptible hosts (Hardham and Blackman 2018). It spreads within the root system causing root rotting, reduced nutrient and water uptake resulting in wilting and chlorosis of the foliage. In Australia, *Phytophthora* species impact a variety of agricultural crops including avocado, macadamia, pineapple and stone fruit, as

well as native species (T. I. Burgess et al. 2021). The disease is currently managed with regular treatment using phosphite. Whilst it is relatively well-managed within the industry, e.g. using the PEGG wheel method (Nigel Wolstenholme and Sheard 2010), monitoring its distribution will help further inform management strategies and allow for more targeted treatment.

For *Phytophthora,* primer combination p4 was chosen as this set had previously performed well when compared to 13 others in detection from environmental samples (Treena I. Burgess, White, and Sapsford 2022). Across soil and rhizosphere samples collected in Victoria, Queensland, and Western Australia, this assay showed generally good specificity under our conditions, with some cross-reactivity against other Oomycetes observed (notably in these samples were Hyaloperonospora, pathogens of Brassica crops, and Globisporangium/Pythium, pathogens of floricultural crops) depending on sample composition.

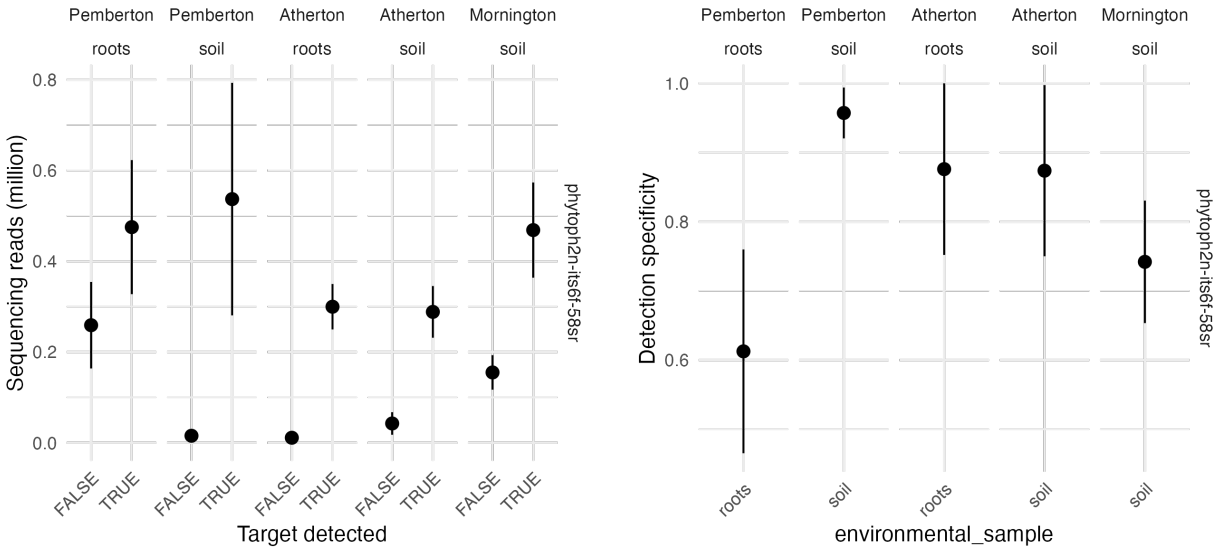


Figure 12. *Phytophthora* assay specificity across sample types.

Left: the sum of reads assigned to the *Phytophthora* genus (target = TRUE) versus off-target reads (target = FALSE) across soil and rhizosphere samples (n=72 in Pemberton, n=96 in Atherton, n=13 in Mornington). Right: average proportion of reads assigned to the target species when detected.

6. *Summary of major sampling approaches*

Sampling yields for fungi and arthropods are shown in Figure 13 For arthropods, targeted approaches are superior, as expected, though passive air sampling also performed relatively well and is far easier to scale in both field and laboratory. As expected, light traps and sticky traps targeted different arthropod communities, with light traps enriched for moths (Lepidoptera), termites (Blattodea; flying forms are strongly attracted to light), flies (Diptera) and beetles (Coleoptera), and sticky traps enriched for flies (gnats, vinegar flies, and midges), termites, moths and butterflies, and Hymenoptera (ants and wasps). Importantly, however, passive air and sticky traps targeted similar communities, with air sampling capturing greater diversity – the combination of passive air sampling / direct surface capture with light lures is worthy of exploration in future work.

For fungi, root/soil sampling yields the highest diversity, followed by air sampling and run-off from plant surfaces (water). Again, the combination of root/soil sampling and air sampling captured the full range of fungal taxa detected, with water sampling adding only replication.

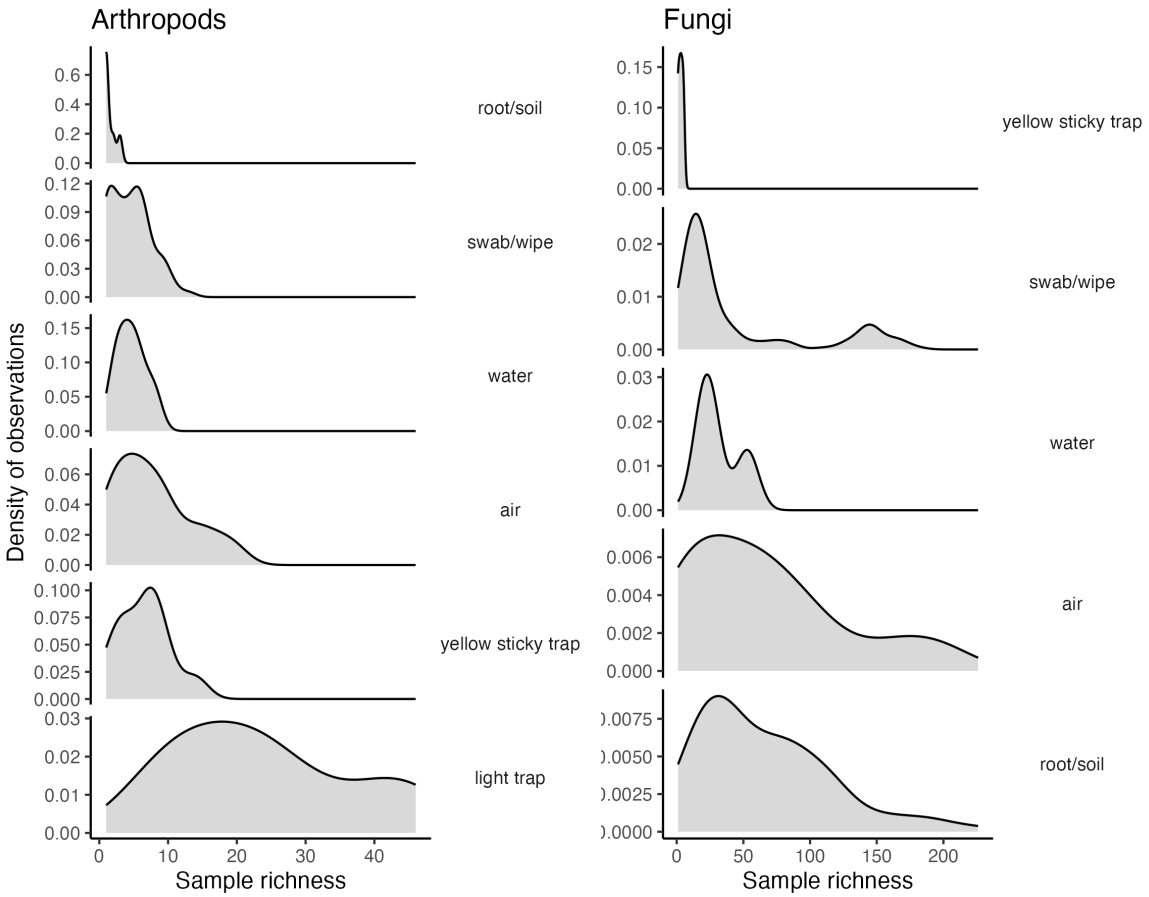


Figure 13. Yields by sample type

The density of detections per sample for artropods (left) and fungi (right) are shown for all detections at rank family or lower. Sampling types are ordered by median value.

7. Summary of pest/pathogen detections

Almost 6000 detections of fungi/water moulds from families of concern for the Avocado industry were obtained across all samples (Table 9), with around 40% of these coming from broad-spectrum metabarcoding assays (the remainder came from targeted assays for *Phytophthora*; *Phellinus/Pyrrhoderma* detections are excluded from these values as they primarily reflect assay validation effort).

The most frequently encountered of these were *Cladosporium* (associated with fruit surface damage), *Fusarium* (associated with root rot, vascular wilt, cankers), *Alternaria* (associated with fruit black spots and leaf spots), *Aspergillaceae* (associated with post-harvest rot), *Saccotheciaceae* (sooty moulds associated with fruit blemish) and *Diaporthe* cankers (associated with branch canker and dieback).

Arthropods of concern were detected far less frequently (Table 10), partly reflecting sampling effort (these are expected to be rarely detected from soil/root samples and passive air samples relative to fungi and moulds). The most frequent of these were thrips, mites, weevils and Fall Army Worm.

Table 9. Pathogenic fungi/Oomycete detections

Fungi/mould pathogens							
class	order	family	common	impact	QLD	VIC	WA
Ascomycota							
Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	Bot cankers	Canker, branch dieback, fruit rot	23	99	8
Dothideomycetes	Cladosporiales	Cladosporiaceae	Cladosporium molds	Fruit surface mold	593	2	102
Dothideomycetes	Dothideales	Saccotheciaceae	Sooty blotch	Fruit surface blemish	2	148	29
Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria rots	Fruit black spot, leaf spots	13	236	43
Eurotiomycetes	Eurotiales	Aspergillaceae	Blue/green/black molds	Post-harvest fruit rot	54	68	251
Leotiomycetes	Helotiales	Sclerotiniaceae	Brown rot, gray mold, white mold	Fruit rots, stem blights	0	5	3
Sordariomycetes	Diaporthales	Diaporthaceae	Diaporthe cankers	Branch canker, dieback	1	101	18
Sordariomycetes	Glomerellales	Glomerellaceae	Anthracnose fungi	Fruit anthracnose	13	47	21
Sordariomycetes	Hypocreales	Nectriaceae	Fusarium wilts, cankers	Root rot, vascular wilt, cankers	159	71	140
Basidiomycota							
Agaricomycetes	Cantharellales	Ceratobasidiaceae	Rhizoctonia fungi	Root rot, crown rot, thread blight	3	0	1
Agaricomycetes	Hymenochaetales	Hymenochaetaceae	Brown root rot fungi	Brown root rot	2	0	0
Agaricomycetes	Polyporales	Ganodermataceae	Bracket fungi, shelf fungi	Root/butt rot	22	0	0
Oomycota							
-	Peronosporales	Peronosporaceae	Downy mildews, Phytophthora	Root rot, canker, fruit rot	1602	35	1911
-	Pythiales	Pythiaceae	Pythium root rots	Root rot, damping-off	5	6	12

Table 10. Arthropod pest detections

Arthropod pests							
class	order	family	common	impact	QLD	VIC	WA
Arthropoda							
Arachnida	Trombidiformes	Eriophyidae	Eriophyid mites	Leaf/fruit scarring	9	5	4
Insecta	Coleoptera	Curculionidae	Weevils	Root/stem boring, fruit damage	1	4	0
Insecta	Hemiptera	Aphididae	Aphids	Honeydew, sooty mold, virus vectors	0	3	1
Insecta	Lepidoptera	Noctuidae	Cutworms, armyworms	Leaf/fruit feeding	5	0	0
Insecta	Lepidoptera	Tortricidae	Leafrollers, fruit borers	Leaf rolling, fruit boring	0	2	0
Insecta	Thysanoptera	Thripidae	Thrips	Fruit scarring, leaf damage	0	12	7

For microbial pathogens, sampling of root/soil, air, and plant surface swabbing were the most effective methods (Figure 14). For arthropods, collection of plant run-off followed by filtration yielded the most detections, however sparse detections make any conclusions tentative.

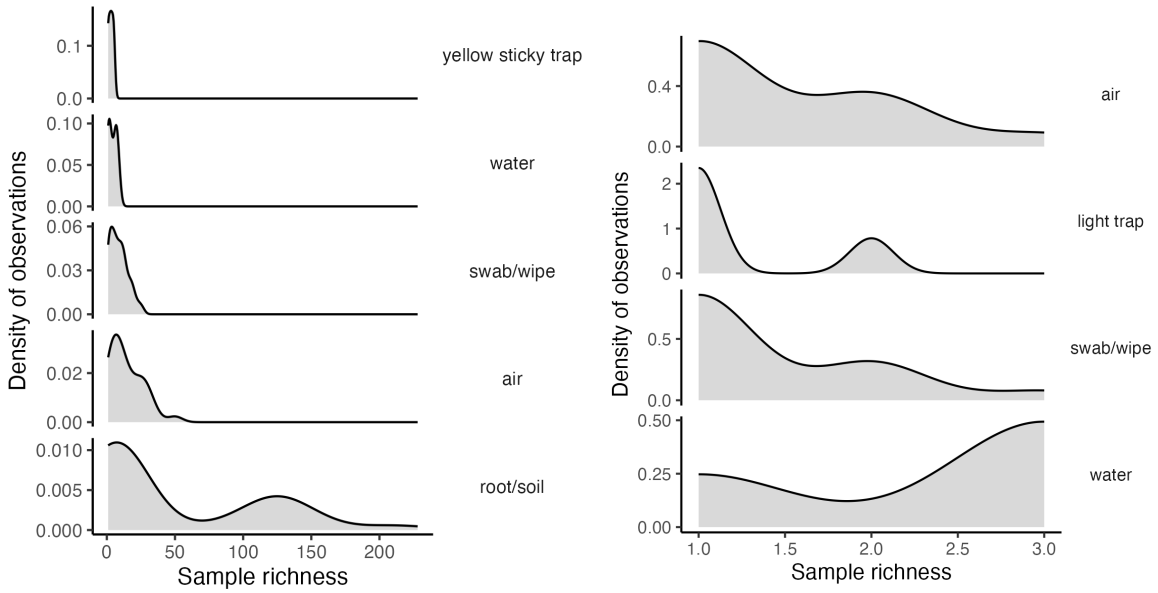


Figure 14. Pest/pathogen detections by sample type.

Per sample richness for (left) pathogenic fungi/oomycetes and (right) arthropod pests across the major sample types tested. Air = active and passive air sampling, swab/wipe = plant surface sampling, water = plant surface washing followed by water filtration.

Outputs

Table 11. Output summary

Output	Description	Detail
Webinar for WA DPIRD and QLD DAF	Webinar delivered to QLD DAF and WA DPIRD on 15th May 2023. There were 51 registered attendees.	The contents covered a background and introduction to eDNA, case studies of use in biosecurity, agriculture and horticulture, and future uses in the air eDNA and rapid testing space. Of the 51 registered attendee's majority indicated that they had no previous experience with eDNA.
Field trial and sampling with QLD DAF staff	Toured and conducted field work on two commercial avocado orchards in collaboration with two QLD DAF staff in December 2024	Through the *Avocado Industry Development and Extension project* (AV23010) we were able to connect with the North Queensland representative and arrange field work to target priority pests or pathogens in the region. They suggested that a DNA test for the soil-borne disease *Phellinus noxius* would be useful and connected us with two commercial avocado farms in the area. Two EnviroDNA staff traveled to the area in December 2024 to meet with farm staff and conduct field work in collaboration with QLD DAF staff. QDAF staff worked closely with us learning about eDNA sampling techniques and potential for use in targeted and general surveillance of pest and pathogens of concern to the industry.
Field trial and sampling in Western Australia including a packing shed	Toured and conducted field work on a commercial avocado orchard located in Pemberton WA and sampled at a fruit packing shed located in Donnybrook WA	An EnviroDNA staff member traveled to Western Australia to conduct field work in March 2025. Sampling occurred over a week and included collecting passive and active air samples and soil samples from three sites. They also traveled to Donnybrook to sample at a pack house whilst avocados were being processed collecting swabs from avocados being packed and filter water from the fruit wash prior to packing.
eDNA sampling method development	We evaluated twelve different sampling methods to collect eDNA from various sources applicable to the avocado industry	**Plant surface collection methods:** • Wipes and swabs sampling leaves, branches and fruit • Water washing plant surface area to collect and filter eDNA **Air sampling methods:** • Active solar-powered devices that filter air particles • Passive collectors that capture wind-blown material **Soil and Root Sampling:** • Collection of soil and roots to detect soil-borne diseases
Assay development and validation	We tested a variety of metabarcoding assays for the detection of invertebrates, fungi, eukaryotes, and specific pathogens (Table 2).	We assessed the efficacy of an existing targeted assay designed to amplify Phytophthora species and developed new assays to detect the causative agent of brown root rot (*Phellinus noxius* / *Pyrrhoderma noxium*). Broad spectrum metabarcoding assays for fungi and invertebrates were assessed. Existing ITS assays for fungi are specific and show generally good taxonomic resolution, subject to reference database coverage and quality. Notably, the assay

		applied is also suitable for species-level detecting exotic threats such as agents of Avocado scab *Elsinoe (Sphaceloma) perseae* and laurel wilt *Raffaelea lauricola*.
Stakeholder engagement / communication	We conducted field work at three commercial farms located in the WA and QLD regions We engaged with biosecurity staff in WA and QLD through webinars and field work We consulted with researchers from WA and QLD who are experts in soil-borne diseases	**Webinars & Presentations:** • Delivered webinar to QLD DAF and WA DPIRD staff (May 2023) - 51 registered attendees, majority with no prior eDNA experience • Provided background on eDNA applications for early pest/disease detection in agriculture/horticulture **Industry Collaboration & Outreach:** • Partnered with Bridie Carr (QLD DAF) from Avocado industry development & extension project (AV17005) for grower engagement • Created project fact sheet detailing objectives and grower involvement opportunities • Published article in Avocados Australia 'Guacamole' newsletter (August 2024) • Posted project information on company LinkedIn platform • Distributed fact sheets at North QLD (July 2024) and WA (August 2024) regional forums **Grower Network Development:** • Connected with Emily Pattison (QLD DAF, North QLD regional lead) for Atherton Tableland grower contacts • Maintained ongoing contact with Mary Burton (RD&E Coordinator, Avocados Australia) for WA grower/researcher connections • Received project update presentation at Avocados Australia regional forum (March 2025) **Research Collaboration:** • Consulted Associate Professor Elizabeth Dann and Dr Louisa Parkinson on Phellinus noxius pathogenicity research • Engaged Associate Professor Andrew Geering (University of Queensland) working on Avocado industry biosecurity project (AV21003a) **Conference Participation Attempts:** • Explored presentation opportunities at Annual Diagnostics Workshop (2024/2025) - timing constraints prevented participation • Expressed interest in March 2025 Avocado industry grower event focused on pests/diseases

Outcomes

Table 12. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
Enhanced early detection capabilities for priority avocado pathogens	Supports outcome of strengthened biosecurity preparedness and reduced crop losses through improved surveillance systems	Development and validation of sensitive eDNA detection methods for Phytophthora and *Phellinus noxius* that can identify pathogens before visual symptoms appear, enabling proactive management rather than reactive treatment	Successful detection of target pathogens across all field sites (Victoria, Queensland, WA); validated assays showing high specificity; detection of multiple Phytophthora species from soil and root samples
Cost-effective surveillance methodology suitable for commercial adoption	Aligns with industry aim of practical, scalable biosecurity tools that can be implemented by growers and extension services	Streamlined sampling protocols using passive air samplers, surface swabs, and soil collection methods that require minimal technical expertise and equipment, reducing surveillance costs compared to traditional diagnostic approaches	Field validation across 3 commercial orchards; testing of simple cloth-based passive air samplers against active samplers; successful implementation by staff with minimal training
Increased industry and biosecurity agency awareness and capacity in eDNA applications	Supports strategic objectives for knowledge transfer and capability building in emerging biosecurity technologies	Enhanced understanding among key stakeholders (growers, QLD DAF, WA DPIRD staff) of eDNA potential for agricultural surveillance, creating foundation for broader adoption	Webinar delivery to 51 biosecurity professionals (majority with no prior eDNA experience); hands-on field training with QLD DAF staff; engagement with 3 commercial operations across major growing regions
Validated broad-spectrum surveillance capability for emerging threats	Addresses outcomes for preparedness against unknown or emerging biosecurity risks beyond current priority pests	Established metabarcoding assays capable of detecting diverse arthropod, fungal, and oomycete taxa from environmental samples, providing surveillance platform adaptable to new pest threats	More than 21,000 detections of more than 1500 taxa (at rank family or below) across sampling sites using general surveillance assays; successful identification of both target pathogens and broader microbial/invertebrate communities from single samples
Geographic coverage of major production regions	Supports national biosecurity strategy requiring surveillance across diverse climatic zones and production systems	Validation of methodology effectiveness across temperate (Victoria), subtropical (Queensland), and Mediterranean (Western Australia)	Successful sampling and pathogen detection across 3 states covering different climate zones; methodology proven effective in varying

		climates representing the majority of national avocado production	humidity, temperature, and seasonal conditions
Foundation for integration with existing industry disease management programs	Aligns with industry strategy to enhance current IPM approaches with improved diagnostic capabilities	eDNA methods developed to complement existing Phytophthora management practices, providing enhanced surveillance data to optimize treatment timing and targeting	Integration with established sampling protocols at commercial orchards; compatibility with current soil sampling practices; potential for integration with existing phosphite treatment programs

Monitoring and evaluation

Table 13. Key Evaluation Questions

Key Evaluation Question	Project performance	Continuous improvement opportunities
To what extent has the project improved the ability of those undertaking surveillance activities to quickly and accurately identify pest incursion threats?	Strong foundational performance: Tested multiple sampling methods across diverse environmental conditions. Developed cost-effective active and passive sampling approaches. Successfully detected priority pathogens (*P. cinnamomi, P. noxius*) across multiple field sites with high specificity. Streamlined protocols reduce sample processing time and technical expertise requirements compared to traditional diagnostic methods.	Protocol optimization and standardization: Sampling protocols require optimization for different surveillance contexts (early detection vs population monitoring, quantification of pest pressure across different scales). Seasonal and environmental factors affecting detection sensitivity need systematic investigation. Integration pathways with existing state biosecurity surveillance programs require development. Real-time or rapid field detection capabilities for particular pests, where commercially viable, remain a future development goal.
To what extent has the project improved the knowledge of biosecurity workers in relation to eDNA sampling methods, in-field detection and the ability to train others?	Good foundational impact: Delivered comprehensive webinar to 51 biosecurity professionals across QLD DAF and WA DPIRD. Conducted hands-on field training with QLD DAF staff during commercial orchard sampling, demonstrating practical implementation challenges and solutions. Established working relationships with key regional extension staff who can facilitate future knowledge transfer. Created practical demonstration of methodology effectiveness across major production systems.	Expansion required: Practical training opportunities limited by field work coordination challenges. Need for standardized training materials and certification programs across commercial applications. Lack of follow-up assessment of knowledge retention and application. Limited train-the-trainer capacity development. More targeted technical workshops needed for different skill levels.
To what extent has the project increased the industry awareness and adoption of eDNA based methods for diagnostics and surveillance?	Mixed results: Successfully engaged 3 commercial operations across major growing regions. Demonstrated methodology across diverse production systems. Created awareness through industry newsletters and forums. Established proof-of-concept for commercial application. Farm-level engagement showed strong interest in pathogen-specific detection capabilities.	Adoption pathway development: Industry engagement proved more challenging than anticipated, with limited response to ongoing outreach efforts. Clear cost-benefit analysis for growers remains incomplete. Need for demonstration projects showing integration with existing management practices (e.g., phosphite programs, basic biosecurity control measures, packhouses). Industry communication proved less effective than direct engagement, suggesting need for different outreach strategies.
To what extent has the project met the needs of the avocado industry?	Strong technical alignment: Successfully developed targeted detection capabilities for industry-priority pathogens (*P. cinnamomi, P. noxius*) identified through stakeholder consultation.	Implementation gap closure: Economic analysis of surveillance costs vs disease management savings requires completion. Integration protocols with existing disease management systems (PEGG wheel, phosphite programs) need development.

	Validated methodology across all major production regions and climate zones. Addressed practical implementation constraints through development of low-cost, low-skill sampling approaches suitable for extension staff or trained growers. Achieved broad-spectrum surveillance capability enabling detection of emerging threats beyond current priority species (>1500 taxa detected across trials).	Scalability from research demonstration to routine operational surveillance not yet established. Regional priority variations (different pest pressures across states) may require more targeted sampling/assay portfolios.
To what extent were the avocado industry network partners included in the project?	Limited but committed engagement: Successfully partnered with three commercial operations across QLD and WA, plus one post-harvest facility, representing diverse production systems and disease pressures. Established working relationships with key extension staff (QLD DAF, Avocados Australia). Gained valuable industry-specific insight into practical implementation requirements and economic constraints.	Network expansion needed: Grower recruitment proved more challenging than anticipated, limiting opportunity for broader validation. Recommend establishing formal industry partner agreements at project initiation. Need for dedicated industry liaison resource to facilitate ongoing engagement. Consider incentive structures to support grower participation in research activities.
Have regular project updates been provided through linkage with industry communication project?	Consistent communication maintained: Regular updates provided to Hort R&D manager throughout project duration. Maintained ongoing dialogue with RD&E coordinator from Avocados Australia for industry feedback and guidance. Provided project summaries for regional forums and industry newsletters. Attempted coordination with Annual Diagnostics Workshop series, though timing constraints prevented participation.	Direct grower communication proved more effective than industry body intermediation, suggesting need for mixed communication approaches.
To what extent did the project engage with industry members and biosecurity officers through methods that were appropriate and easily understood?	Effective targeted engagement: Delivered accessible introductory materials avoiding technical jargon while maintaining scientific accuracy. In-person discussions during field work proved most effective for explaining eDNA capabilities and limitations. Provided practical demonstrations allowing stakeholders to observe sampling techniques and understand implementation requirements. Fact sheets and webinar	Broader engagement methods needed: Industry member engagement remained limited despite multiple outreach approaches. Need for more diverse engagement formats (field days, demonstration plots, case studies) to reach different learning preferences. Technical complexity requires careful balance between accessibility and accuracy. Earlier project engagement with industry extension networks could improve reach.

	presentations tailored to audiences with varying technical backgrounds received positive feedback.	
What efforts did the project make to identify areas of reduced efficiency and take steps to improve?	Systematic adaptive approach: Continuously refined sampling methods based on field trial outcomes (e.g., transition from active to passive air sampling based on cost-effectiveness analysis). Streamlined laboratory protocols to reduce processing time and costs. Adapted sampling strategies based on regional priorities and environmental conditions. Modified engagement approaches when initial industry outreach proved challenging, shifting to direct contact and personal networks.	Efficiency optimization ongoing: Grower engagement processes require significant improvement to reduce coordination time and increase participation rates. Laboratory workflow optimization could further reduce per-sample costs (e.g. by pooling at appropriate scales). Need for standardized decision frameworks to guide method selection for different surveillance objectives. Project timeline management could benefit from more flexible milestone structures accommodating field work dependencies.

Recommendations

Further Research

Immediate Priority Actions

Integration with existing Phytophthora management systems

Develop commercial eDNA surveillance service specifically targeting *Phytophthora* species differentiation (*P. cinnamomi*, *P. citricola*, *P. multivora*) to enable targeted management. Integrate eDNA detection protocols with established PEGG wheel methodology for Phytophthora management, providing quantitative pathogen load data to optimize treatment timing. Develop decision support tools linking eDNA detection thresholds to phosphite application schedules and treatment intensity recommendations. Create sampling protocols specifically designed to complement existing orchard health monitoring and nutritional management practices.

Brown vs Phytophthora root rot diagnostic service

Establish commercial diagnostic service for rapid differentiation between brown root rot (*Phellinus noxius*) and Phytophthora root rot from soil and root samples. This addresses the field-identified need where growers cannot distinguish between these diseases despite requiring completely different management approaches. Traditional culturing takes weeks and often fails, while eDNA can provides definitive identification within days (or hours if rapid field assays are developed), enabling immediate implementation of appropriate treatment strategies.

Create rapid field-deployable detection systems using LAMP, RPA, or CRISPR-based technologies for these and other high-priority pathogens. General hardware platforms such as the Nucleic Acid Barcode Identification Tool (NABIT) allow development, validation, and maintenance of custom lyophilised assays, greatly simplifying operational complexity and reducing testing costs and time-to-result.

Industry engagement enhancement

Establish formal partnerships with state biosecurity agencies to integrate eDNA capabilities into surveillance programs. Develop grower incentive programs (cost-sharing, demonstration grants) to encourage adoption of eDNA surveillance. Create industry-specific communication strategy utilizing successful grower champions rather than relying solely on industry body channels.

Medium-term Development

Optimization of sampling design, including seasonal and environmental variation

Conduct systematic multi-year study of seasonal detection patterns for priority pathogens across climatic zones. Develop region-specific sampling protocols accounting for environmental differences (monsoon patterns in QLD, Mediterranean climate in WA, temperate conditions in Victoria). Investigate eDNA persistence under varying soil moisture, temperature, and pH conditions to optimize sampling frequency and storage protocols.

The scale-dependence of airborne eDNA in particular needs to be better understood through a combination of (1) high spatial density passive sampling at local scales (2) more distributed active sampling at elevation to better capture broad-scale patterns and (3) integration of these data with meteorological data. The first generation of dedicated airborne eDNA sampling devices are now emerging, such as models from DNAir featuring high-volume, programmable, autonomous sampling and sample preservation. This will require establishment of regional eDNA surveillance networks incorporating multiple orchards to track pathogen movement and population dynamics, and associated data platforms.

Long-term Strategic Development

Predictive and precision agriculture integration

Develop machine learning models combining eDNA detection data with environmental monitoring (weather, soil sensors) to predict disease pressure and optimize intervention timing. Create precision agriculture platforms enabling site-specific treatment based on spatial pathogen distribution maps. Integrate eDNA surveillance with economic models to optimize cost-benefit ratios of preventive versus reactive management strategies.

References

Burgess, T. I., J. Edwards, A. Drenth, T. Massenbauer, J. Cunnington, R. Mostowfizadeh-Ghalamfarsa, Q. Dinh, et al. 2021. "Current Status of Phytophthora in Australia." *Persoonia* 47 (1): 151–77. https://doi.org/10.3767/persoonia.2021.47.05.

Burgess, Treena I., Diane White, and Sarah J. Sapsford. 2022. "Comparison of Primers for the Detection of Phytophthora (and Other Oomycetes) from Environmental Samples." *Journal of Fungi (Basel, Switzerland)* 8 (9): 980. https://doi.org/10.3390/jof8090980.

Cabodevilla, Xabier, Benjamín Juan Gómez-Moliner, Naiara Abad, and María José Madeira. 2023. "Simultaneous Analysis of the Intestinal Parasites and Diet through EDNA Metabarcoding." *Integrative Zoology* 18 (3): 399–413. https://doi.org/10.1111/1749-4877.12634.

Cahill, David M., James E. Rookes, Barbara A. Wilson, Lesley Gibson, and Keith L. McDougall. 2008. "Phytophthora Cinnamomi and Australia's Biodiversity: Impacts, Predictions and Progress towards Control." *Australian Journal of Botany* 56 (4): 279. https://doi.org/10.1071/bt07159.

Chang, T. T. 2002. "The Biology, Ecology and Pathology of Phellinus Noxius in Taiwan." CABI Books. February 27, 2002. https://www.cabidigitallibrary.org/doi/10.1079/9780851995427.0087.

Chang, T. T., and W. W. Yang. 1998. "Phellinus Noxius in Taiwan: Distribution, Host Plants and the PH and Texture of the Rhizosphere Soils of Infected Hosts." *Mycological Research* 102 (9): 1085–88. https://doi.org/10.1017/S0953756297005571.

Chang, Tun Tschu. 1995. "A Selective Medium for *Phellinus Noxins*." *European Journal of Forest Pathology. Journal Europeen de Pathologie Forestiere. Europaische Zeitschrift Für Forstpathologie* 25 (4): 185–90. https://doi.org/10.1111/j.1439-0329.1995.tb01002.x.

Clare, Elizabeth L., Chloe K. Economou, Frances J. Bennett, Caitlin E. Dyer, Katherine Adams, Benjamin McRobie, Rosie Drinkwater, and Joanne E. Littlefair. 2021. "Measuring Biodiversity from DNA in the Air." *Current Biology: CB*, December. https://doi.org/10.1016/j.cub.2021.11.064.

Elbrecht, Vasco, Thomas W. A. Braukmann, Natalia V. Ivanova, Sean W. J. Prosser, Mehrdad Hajibabaei, Michael Wright, Evgeny V. Zakharov, Paul D. N. Hebert, and Dirk Steinke. 2019. "Validation of COI Metabarcoding Primers for Terrestrial Arthropods." *PeerJ* 7 (October): e7745. https://doi.org/10.7717/peerj.7745.

Hardham, Adrienne R., and Leila M. Blackman. 2018. "Phytophthora Cinnamomi." *Molecular Plant Pathology* 19 (2): 260–85. https://doi.org/10.1111/mpp.12568.

Johnson, Mark D., Robert D. Cox, and Matthew A. Barnes. 2019a. "Analyzing Airborne Environmental DNA: A Comparison of Extraction Methods, Primer Type, and Trap Type on the Ability to Detect Airborne EDNA from Terrestrial Plant Communities." *Environmental DNA* 1 (2): 176–85. https://doi.org/10.1002/edn3.19.

———. 2019b. "The Detection of a Non-Anemophilous Plant Species Using Airborne EDNA." *PloS One* 14 (11): e0225262. https://doi.org/10.1371/journal.pone.0225262.

Johnson, Mark D., Mohamed Fokar, Robert D. Cox, and Matthew A. Barnes. 2021. "Airborne Environmental DNA Metabarcoding Detects More Diversity, with Less Sampling Effort, than a Traditional Plant Community Survey." *BMC Ecology and Evolution* 21 (1): 218. https://doi.org/10.1186/s12862-021-01947-x.

Klepke, Martin Johannesen, Eva Egelyng Sigsgaard, Mads Reinholdt Jensen, Kent Olsen, and Philip Francis Thomsen. 2022. "Accumulation and Diversity of Airborne, Eukaryotic Environmental DNA." *Environmental DNA (Hoboken, N.J.)* 4 (6): 1323–39. https://doi.org/10.1002/edn3.340.

Kozhar, Olga, Mee-Sook Kim, Jorge Ibarra Caballero, Ned B. Klopfenstein, Phil G. Cannon, and Jane E. Stewart. 2022. "Long Evolutionary History of an Emerging Fungal Pathogen of Diverse Tree Species in Eastern Asia, Australia and the Pacific Islands." *Molecular Ecology* 31 (7): 2013–31. https://doi.org/10.1111/mec.16384.

Nigel Wolstenholme, B., and Andrew Sheard. 2010. "INTEGRATED MANAGEMENT OF PHYTOPHTHORA ROOT ROT THE 'PEGG WHEEL' UPDATED." *South African Avocado Growers' Association Avoinfo Newsletter* 175 (October): 11–15. http://www.avocadosource.com/papers/research_articles/wolstenholmenigel2010b.pdf.

Quesada, Tania, Jennifer Hughes, Katherine Smith, Keumchul Shin, Patrick James, and Jason Smith. 2018. "A Low-Cost Spore Trap Allows Collection and Real-Time PCR Quantification of Airborne Fusarium Circinatum Spores." *Forests, Trees and Livelihoods* 9 (10): 586. https://doi.org/10.3390/f9100586.

Riaz, Tiayyba, Wasim Shehzad, Alain Viari, François Pompanon, Pierre Taberlet, and Eric Coissac. 2011. “EcoPrimers: Inference of New DNA Barcode Markers from Whole Genome Sequence Analysis.” *Nucleic Acids Research* 39 (21): e145. https://doi.org/10.1093/nar/gkr732.

Roger, Fabian, Hamid R. Ghanavi, Natalie Danielsson, Niklas Wahlberg, Jakob Löndahl, Lars B. Pettersson, Georg K. S. Andersson, Niklas Boke Olén, and Yann Clough. 2022. “Airborne Environmental DNA Metabarcoding for the Monitoring of Terrestrial Insects—A Proof of Concept from the Field.” *Environmental DNA*, March. https://doi.org/10.1002/edn3.290.

Sahashi, N., M. Akiba, M. Ishihara, Y. Ota, and N. Kanzaki. 2012. “Brown Root Rot of Trees Caused by *Phellinus Noxius* in the Ryukyu Islands, Subtropical Areas of Japan: Brown Root Rot of Trees Caused ByPhellinus Noxius.” *Forest Pathology* 42 (5): 353–61. https://doi.org/10.1111/j.1439-0329.2012.00767.x.

Scibetta, Silvia, Leonardo Schena, Antonio Chimento, Santa O. Cacciola, and David E. L. Cooke. 2012. “A Molecular Method to Assess Phytophthora Diversity in Environmental Samples.” *Journal of Microbiological Methods* 88 (3): 356–68. https://doi.org/10.1016/j.mimet.2011.12.012.

Taberlet, Pierre, Eric Coissac, François Pompanon, Ludovic Gielly, Christian Miquel, Alice Valentini, Thierry Vermat, Gérard Corthier, Christian Brochmann, and Eske Willerslev. 2007. “Power and Limitations of the Chloroplast TrnL (UAA) Intron for Plant DNA Barcoding.” *Nucleic Acids Research* 35 (3): e14. https://doi.org/10.1093/nar/gkl938.

Tedersoo, Leho, and Björn Lindahl. 2016. “Fungal Identification Biases in Microbiome Projects.” *Environmental Microbiology Reports* 8 (5): 774–79. https://doi.org/10.1111/1758-2229.12438.

Tordoni, Enrico, Claudio G. Ametrano, Elisa Banchi, Silvia Ongaro, Alberto Pallavicini, Giovanni Bacaro, and Lucia Muggia. 2021. “Integrated EDNA Metabarcoding and Morphological Analyses Assess Spatio-Temporal Patterns of Airborne Fungal Spores.” *Ecological Indicators* 121 (February): 107032. https://doi.org/10.1016/j.ecolind.2020.107032.

Vamos, Ecaterina Edith, Vasco Elbrecht, and Florian Leese. 2017. “Short COI Markers for Freshwater Macroinvertebrate Metabarcoding.” *Metabarcoding and Metagenomics* 1 (September): e14625. https://doi.org/10.3897/mbmg.1.14625.

Wilson, S. J., and R. V. Cooke. 1980. “Wind Erosion in Soil Erosion.” *John. : New York, NY*, 217–51.

Wu, Zong-Chi, Ya-Yun Chang, Qiao-Juan Lai, Heng-An Lin, Shean-Shong Tzean, Ruey-Fen Liou, Isheng J. Tsai, and Chia-Lin Chung. 2020. “Soil Is Not a Reservoir for Phellinus Noxius.” *Phytopathology* 110 (2): 362–69. https://doi.org/10.1094/PHYTO-08-19-0314-R.

Zeale, Matt R. K., Roger K. Butlin, Gary L. A. Barker, David C. Lees, and Gareth Jones. 2011. “Taxon-Specific PCR for DNA Barcoding Arthropod Prey in Bat Faeces.” *Molecular Ecology Resources* 11 (2): 236–44. https://doi.org/10.1111/j.1755-0998.2010.02920.x.

Intellectual property

No project IP or commercialisation to report.

Appendices

Appendix A: *Phytophthora* detections

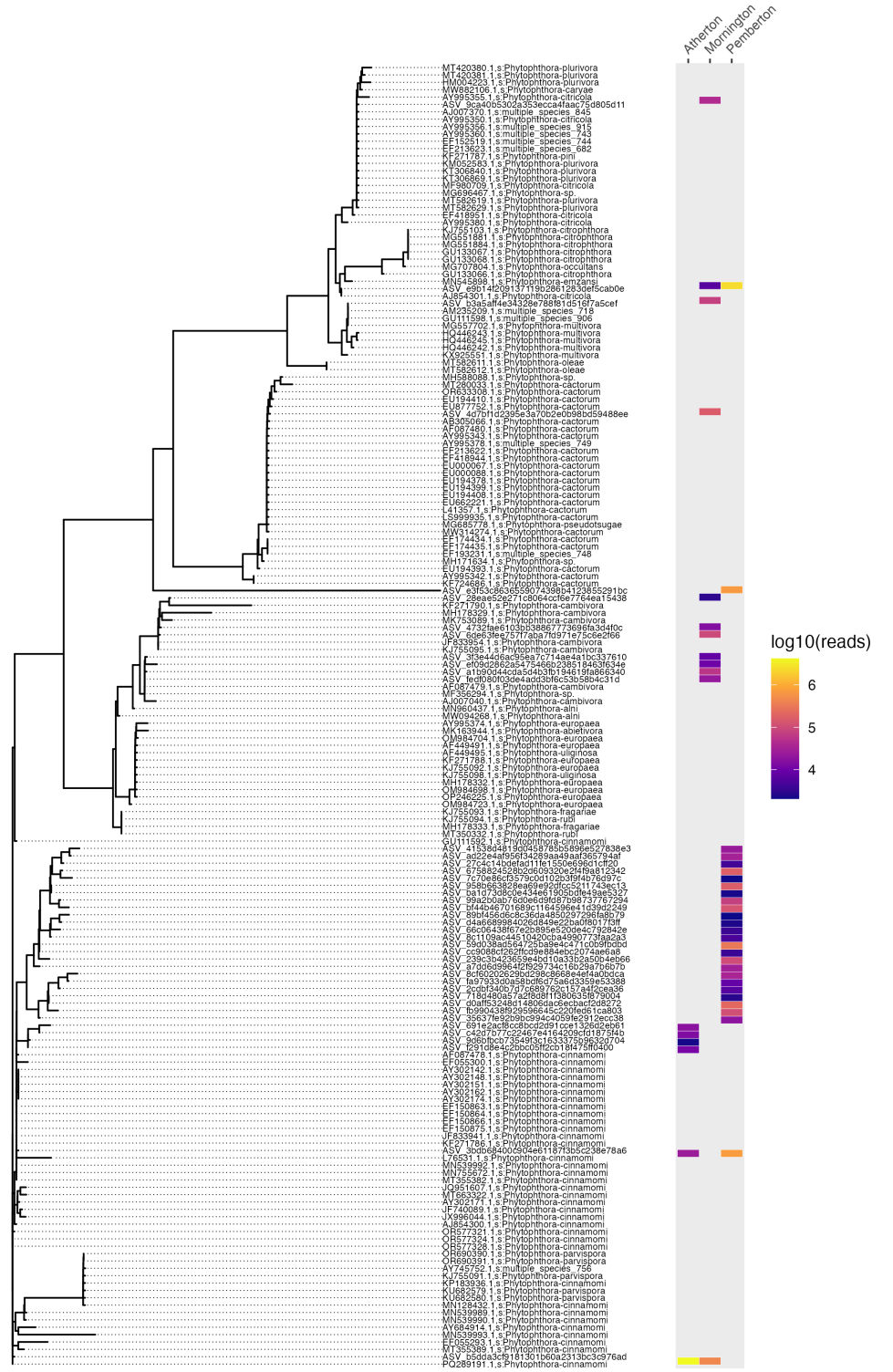


Figure 13. Phytophthora detections across all field sites.

Amplicon sequence variant (ASV) tree (bionj) for detections across Queensland, Victoria and Western Australia and similar public sequences (coverage >= 95%, identity >= 90%; NCBI nt database). Total sequencing depth across all samples within a region is shown at right.

Appendix B: *Pyrrhoderma noxium (Phellinus noxius)* detections from ITS assay

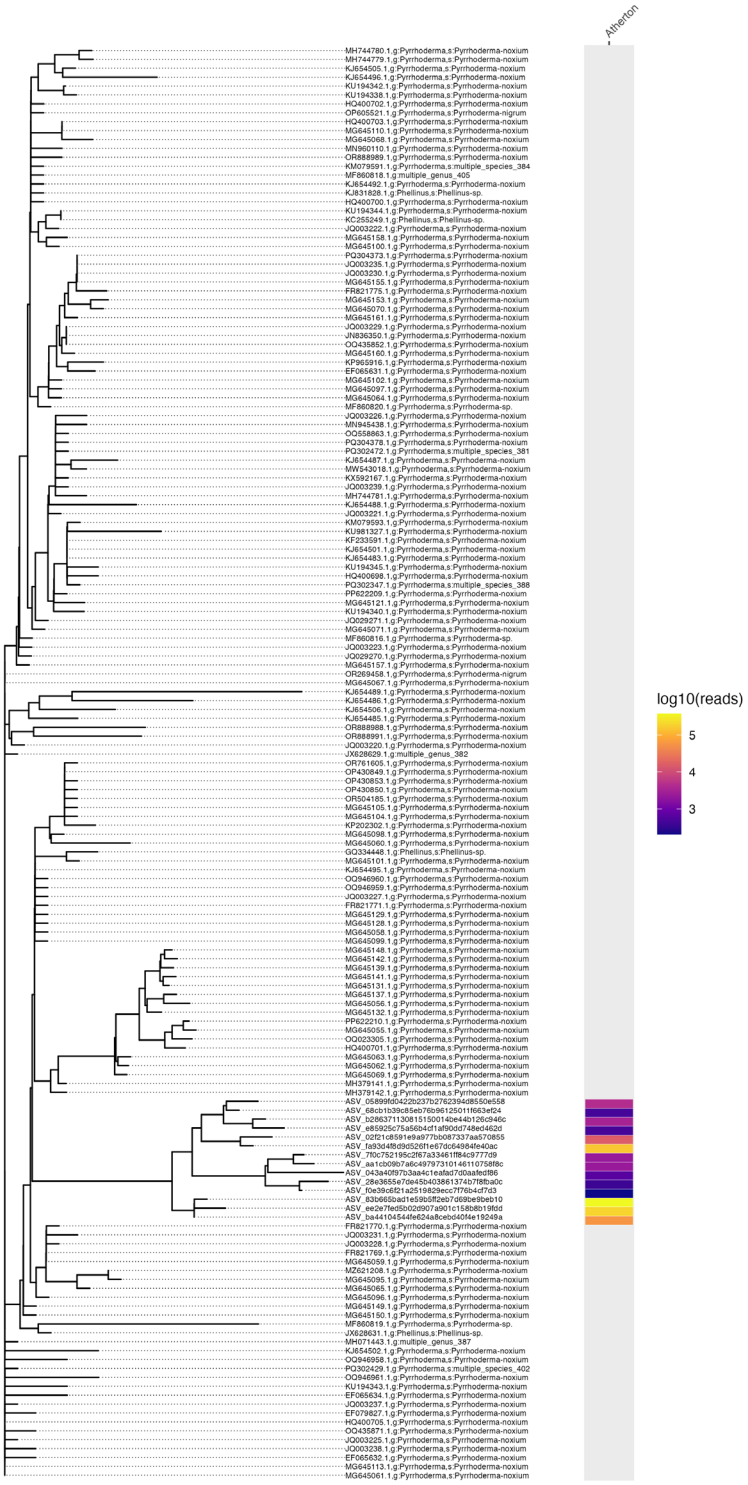


Figure 14. ITS targeted metabarcoding assay (p-nox-its-cr7).

Amplicon sequence variant (ASV) tree (bionj) for detections across Queensland and similar public sequences (coverage >= 95%, identity >= 90%; NCBI nt database).

Appendix C: *Pyrrhoderma noxium (Phellinus noxius)* detections from 28S assay

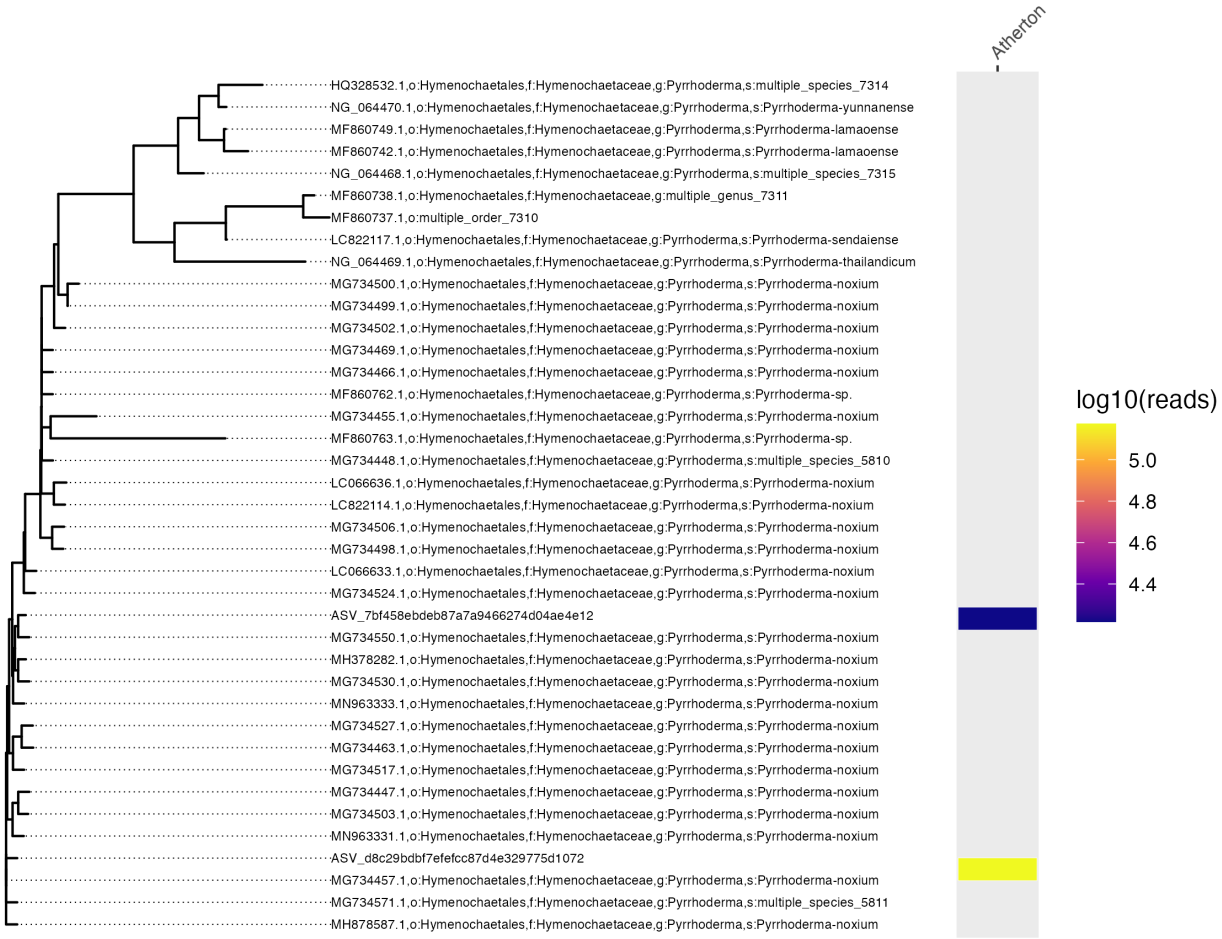


Figure 15. 28S targeted metabarcoding assay (p-nox-28s-cr8)

Amplicon sequence variant (ASV) tree (bionj) for detections across Queensland and similar public sequences (coverage >= 95%, identity >= 90%; NCBI nt database).

Appendix D: *AV21003b fact sheet*

ONGOING PROJECT – HORT INNOVATION LEVY-FUNDED PROJECT

Environmental DNA technologies for rapid detection and identification of avocado priority pests (AV21003b)

What's it all about?

This project aims to refine methods for early detection of fungal and insect pests in the Australian avocado industry using environmental DNA (eDNA) techniques. These methods use simple environmental sampling approaches to indirectly identify species that have recently been in an area, or to directly identify species that are difficult to identify by non-genetic methods.

Why is it important?

By using innovative field and lab-based testing techniques we aim to improve biosecurity monitoring and assist with undertaking surveillance activities that can accurately identify pest incursion threats in an efficient and cost-effective manner.

We need your help!

We recently completed a preliminary trial at a local Victorian avocado orchard developing some active and passive sampling techniques for eDNA monitoring of fungal and invertebrate species. We are now seeking to obtain a larger data set and collect samples in more representative regions such as QLD and WA.

We are seeking growers and/or packing sheds that are willing to let us conduct some sampling, which would involve setting up a few eDNA sampling stations or taking some water wash samples from trees/fruit/equipment. This would likely be for a few days over a one-week period. Additionally, if there is any damage or disease present from an unknown cause, we can help target our sampling to identify the source.

Please reach out and get in contact with Chantelle Reid at EnviroDNA if you're interested!

creid@envirodna.com