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Research Article

RESEARCH ARTICLE

eDNA from roots: a robust tool for determining *Phytophthora* communities in natural ecosystems

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One sentence summary: This study provides a comparison of traditional isolation methods to metabarcoding techniques to determine *Phytophthora* species diversity.

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ABSTRACT

Proper isolation and identification of *Phytophthora* species is critical due to their broad distribution and huge impact on natural ecosystems throughout the world. In this study, five different sites were sampled and seven methods were compared to determine the *Phytophthora* community. Three traditional isolation methods were conducted (i) soil baiting, (ii) filtering of the bait water and (iii) isolation from field roots using Granny Smith apples. These were compared to four sources of eDNA used for metabarcoding using *Phytophthora*-specific primers on (i) sieved field soil, (ii) roots from field, (iii) filtered baiting water and (iv) roots from bait plants grown in the glasshouse in soil collected from these sites. Six *Phytophthora* species each were recovered by soil baiting using bait leaves and from the filtered bait water. No *Phytophthora* species were recovered from Granny Smith apples. eDNA extracted from field roots detected the highest number of *Phytophthora* species (25). These were followed by direct DNA isolation from filters (24), isolation from roots from bait plants grown in the glasshouse (19), and DNA extraction from field soil (13). Therefore, roots were determined to be the best substrate for detecting *Phytophthora* communities using eDNA.

Keywords: *Phytophthora* diversity; high-throughput sequencing; baiting; filters; bait plants; eDNA

INTRODUCTION

Phytophthora diseases cause significant losses to plants in agriculture, horticulture and natural ecosystems throughout the world. Many species, including *Phytophthora cinnamomi* one of the world's most devastating invasive species, are also known for their huge impact on natural ecosystems. *Phytophthora cinnamomi* has a massive impact on the natural ecosystems in Australia, the Cape Province of South Africa and the Iberian Peninsula in Europe (Burgess *et al*. 2017a). Interest in *Phytophthora* diseases of natural ecosystems has increased since the emergence of *Phytophthora ramorum* as the causal agent of sudden oak death in California (Hansen, Reeser and Sutton 2012). About 50 new species have been described since 2010; most of these species have been isolated from natural ecosystems, and very little is known about their distribution and impact on natural ecosystems (Burgess *et al*. 2017b). Proper detection, and identification of *Phytophthora* species, is of great importance for biosecurity and quarantine. The number of *Phytophthora* species has risen to over 150 and this number will likely increase due to extensive surveys of previously unexplored forest and river ecosystems.

Non-selective media used for isolating true fungi are not suitable for *Phytophthora* species due to antagonism and rapid growth of secondary microorganisms, and slow revival of dormant *Phytophthora* survival structures, such as oospores and chlamydospores (Tsao 1990). The genus *Phytophthora* is difficult

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to isolate from infected plants and soil, but the efficiency of isolation has greatly increased by the use of baiting techniques and selective isolation media (Tsao 1990). Eckert and Tsao (1962) reported the first medium 3P (pimaricin-penicillin-polymyxin) for selective isolation of *Phytophthora*. Different media have been used for selective isolation of *Phytophthora* since then (see Table S1, Supporting Information), most recently used include NARH (Simamora *et al*. 2017), *Phytophthora* selective medium of Burgess *et al*. (2008) and CMA-PARPBH (Pérez-Sierra *et al*. 2010).

Phytophthora species are disseminated through soil, water and aerially, (Erwin and Ribeiro 1996) and can be isolated directly from plant tissues, such as leaves, roots, stem and twigs without any surface sterilization when infection is active by plating sections of an advancing lesion margin onto a selective medium (Streito, Jarnouen de Villartay and Tabary 2002). However, the presence of *Phytophthora* is not always associated with visible symptoms and it can be recovered from symptomless plant tissues (Hüberli, Tommerup and Hardy 2000). The rate of success of isolation also depends on the pathogen's activity, which varies between seasons. Autumn was found to be the best season for isolation of alder *Phytophthora* from plant tissues (Streito, Jarnouen de Villartay and Tabary 2002). Similarly, saprophytic microorganisms and antagonistic bacteria affect the efficiency of isolation (Hüberli, Tommerup and Hardy 2000; Streito, Jarnouen de Villartay and Tabary 2002; Jung and Blaschke 2004). Therefore, surface sterilization with 70% ethanol followed by drying on paper towel prior to plating can increase chances of isolation (Martin *et al*. 2012). Finally, washing plant tissues to leach out phenolic compounds from plants like *Eucalyptus* and *Alnus* species prior to plating can also increase isolation of *Phytophthora* species (Hüberli, Tommerup and Hardy 2000; Streito, Jarnouen de Villartay and Tabary 2002).

Various traditional methods have been reported for the isolation of *Phytophthora* species from soils. Direct plating of soil onto selective media is not suitable for recovery of *Phytophthora* species, as there are few viable propagules per gram of soil and lots of contaminants (Hendrix and Kuhlman 1965). Soil baiting is more effective for isolation of *Phytophthora* species for a number of reasons. Firstly, a large amount of soil can be tested, which increases chances of isolation when species are present at a low population density (Martin *et al*. 2012). Secondly, it is more effective for isolation of homothallic species, which often survive as dormant oospores (Jeffers and Aldwinckle 1987). Species are more frequently isolated when soil is kept between 15°C and 20°C and the bait is not wounded, which discourages *Pythium* and bacterial colonization (Hwang, Oak and Jeffers ; Ghimire *et al*. 2009). Leaf tissues are more commonly used as baits than fruits, but all leaf tissues are not equally attractive to multiple *Phytophthora* species. Young and succulent leaves of *Camellia*, *Rhododendron* and *Quercus* spp. and *Pimelea ferruginea* and *Eucalyptus sieberi* cotyledons have been successfully used in recent years (Jung, Blaschke and Oßwald 2000; McDougall, Hardy and Hobbs 2002; Fichtner, Lynch and Rizzo 2007; Hwang, Oak and Jeffers).

Currently, baits and filter-based approaches are mainly used to isolate *Phytophthora* species from water. A variety of plant baits (Klotz, Wong and DeWolfe 1959; McIntosh 1966; Erwin and Ribeiro 1996; Oudemans 1999; Hüberli *et al*. 2013; Dunstan *et al*. 2016) as well as filter membranes and filtering methods (MacDonald *et al*. 1994; Von Broembsen and Wilson 1998; Hong, Richardson and Kong 2002) have been used. However, not all membranes are equally efficient for recovery of diverse *Phytophthora* species. Hong, Richardson and Kong (2002) compared nine hydrophilic membranes for isolation of Pythiaceous species in water, and found Durapore5 and Millipore5 to be more efficient than other membranes. Filtration has been found to be more efficient for isolation of *Phytophthora* in water than baiting (Hwang, Oak and Jeffers).

Fruit baits are also used for isolation of *Phytophthora*. These have included apple (Campbell 1949; Newhook 1959), lemon (Klotz and DeWolfe 1958), avocado (Zentmyer, Gilpatbick and Thorn 1960), tomato (Reis *et al*. 2003), and pear and cucurbits (Gevens *et al*. 2007). Apples are usually not very effective for isolation of *Phytophthora* species from soil because saprophytic fungi, such as *Mucor*, *Rhizopus* and *Penicillium* species produce rapid soft rots, which inhibit the growth of *Phytophthora* (Chee and Newhook 1965). Also, *Trichoderma viride* is commonly present in soil and causes a hard rot of apple. Although both may cause distinct isolatable rots in a single apple, it is more likely that *T. viride* will suppress growth of *Phytophthora* (Chee and Newhook 1965). Furthermore, Jeffers and Aldwinckle (1987) compared different baits, such as apple, pear, apple seedlings, cotyledons and seedling leaf pieces, and found that apple and pear were not suitable for isolation of *P. cactorum* from naturally infested soil. However, pears have been reported as useful baits for isolation of *P. cinnamomi* from naturally infested soil (Greenhalgh 1978).

Although these conventional methods are useful for the isolation of many *Phytophthora* species (Erwin and Ribeiro 1996; Drenth *et al*. 2006), it can be a laborious and difficult task. Additionally, it can be difficult to identify species based on morphology, especially now that so many new species have been described in the past 10 years. Some species, such as *P. mirabilils*, *P. infestans* and *P. ipomoeae* have similar sporangia (semi-papillate and caducous) and oospore characteristics, and therefore, cannot be distinguished by morphology alone (Erwin and Ribeiro 1996; Flier *et al*. 2002). Moreover, morphology of *Phytophthora* is plastic (Braiser 1992), and not all *Phytophthora* species can be cultured on agar media (Mircetich 1970). DNA-based identification is a fast and reliable method for the identification of *Phytophthora* species (Martin, Blair and Coffey 2014) and has been used in numerous studies (Ristaino *et al*. 1998; Burgess *et al*. 2009; Oh *et al*. 2013; Català, Pérez-Sierra and Abad-Campos 2015).

The term environmental DNA (eDNA) refers to DNA isolated from environmental samples (e.g. air, soil and water) without first isolating any desired organism (Taberlet *et al*. 2012). This term was first used by Ogram, Sayler and Barkay (1987) while isolating microbial DNA from a range of sediment types. It is composed of intracellular DNA from living cells and extracellular DNA from naturally lysed cells. High Throughput Sequencing (HTS) has made it possible to characterize microbial and fungal communities in eDNA without time-consuming and expensive cloning (Sogin *et al*. 2006; Coince *et al*. 2013). Environmental substrates are usually easy to sample and can be collected by non-specialists (Lear *et al*. 2018). HTS is an effective tool for epidemiological studies when description of new or rare taxa is required (Vannini *et al*. 2013). Previous studies have mainly concentrated on detection of all organisms that can be present in environmental samples by targeting barcoding genes, such as ITS and 18S (Nakayama *et al*. 2013; Weber, Vilgalys and Kuske 2013), but only a few have focused on targeting specific organisms (Bergmark *et al*. 2012; Li *et al*. 2013).

Although the metabarcoding approach has greatly improved the detection of *Phytophthora* species in environmental samples (Vannini *et al*. 2013; Català, Pérez-Sierra and Abad-Campos 2015; Prigigallo *et al*. 2015; Català *et al*. 2016), none of the studies have been specifically targeted to determine the best substrate for isolation and detection of *Phytophthora* species. The current study compared traditional isolation methods to metabarcoding using

Phytophthora specific primers on samples taken in natural environments.

MATERIALS AND METHODS

Sampling sites and sampling procedure

Rhizosphere soil including roots was collected from five different urban parks in Perth; Bold Park, Kings Park, Attadale Foreshore, Manning Park, and Bibra Lake in the late (June) autumn (Table 1). At each site, a bulked soil sample was collected (3 kg comprised of 10 × 300 g sampled from 10 different locations to a depth of 10–20 cm). Emphasis was placed on the collection of rhizosphere soil containing fine roots. The soil samples were placed into plastic bags and kept in an insulated box to protect samples from high temperature and direct sunlight and carried to the laboratory.

In the laboratory, the samples were mixed thoroughly. Some fine roots were removed from the soil, rinsed with tap water to remove soil particles and chopped into 1–2 mm segments. Chopped roots (approximately 1000 mg) were placed into three Eppendorf tubes and frozen at −20 °C until used for DNA extraction, while others were used in apple baits. A sub-sample of soil (200 g) was air dried for DNA extraction from soil, and three subsamples of soil (each approx. 400 g) were taken for traditional baiting. Finally, four subsamples of soil (approx. 1500 g) were used for growing *E. sieberi* and *Banksia attenuata* seedlings in the glasshouse as susceptible living 'baits' for *Phytophthora*.

Traditional isolation from soil using bait leaves

Soils from each sample were placed in 1.5 L rectangular polypropylene containers (167 mm × 108 mm). Each soil sample was replicated three times. Combined, the roots and soil occupied one-third of the container. The soil/root mix was then pre-moistened with distilled water overnight to stimulate the pathogens' activity. The next morning, the samples were flooded with distilled water in a 1:3 soil/water ratio and young leaves of *Quercus ilex*, *Q. suber*, *Pimelea ferruginea*, *Poplar* sp., *Scholtzia involucrata* and *Hedera helix* were floated on the surface (Fig. 1a and c). The containers were incubated at 20°C (±5°C) under ambient conditions.

The baited leaves were observed for the appearance of lesions every 1–2 days for seven days. Leaves with brownish lesions were blotted dry on paper toweling, the lesions were cut into 2 × 2 mm pieces, and plated onto NARH (Simamora *et al.* 2017). The plates were incubated at 20°C (±5°C) in the dark and examined under 10X magnification for the presence of hyphae typical for *Phytophthora*. After 1–2 days, any *Phytophthora*-like cultures were transferred onto fresh plates of NARH twice and finally transferred onto individual vegetable juice agar (V8A) plates [100 ml/L filtered vegetable juice (Campbells V8 vegetable juice; Campbell Grocery products Ltd., Norfolk, UK), 900 ml/L distilled water, 0.1 g/L $CaCO_3$, pH adjusted to 7 and 17 g Grade A Agar (Becton, Dickenson and Company, Sparks, MD, USA). After seven days, the soil was allowed to air dry and then baiting was repeated (double baiting) to increase isolation (Jeffers and Aldwinckle 1987; Davison and Tay 2005).

Isolation from filtered bait water

Filtration was performed with a filtering funnel (Nihon Millipore K.K, Tokyo-Japan) and a glass flask connected to a vacuum pump (Fig. 1d). Between samples, plastic containers (funnel, porous plate, rubber bung) and a glass flask were placed in a detergent Pyroneg (L88Z, Diversey) and then washed in a separate container containing water, sterilized with 4% sodium hypochlorite for a minute, and then finally placed in a separate container containing water for approximately 5 min before handling another bait sample.

Approximately, 250 mL of bait water was filtered from each 'bait tray' each time after finishing baiting (on day 7) through a 47 mm circular filter paper with 5 μm pore size UltraSep Polyethersulfone (GVS Life Sciences, Sanford, USA). Three filters were collected for each sample. Finally, tap water was passed through a fresh filter as a filter control. Each filter was cut into two halves. Half of each filter was placed topside down onto the surface of NARH medium. After 12 h, the filter was removed and colonies were transferred to fresh NARH plates (five to six sub-cultures per plate). *Phytophthora* like cultures were transferred onto V8A plates after 2–3 days. Soil used in each 'bait tray' was allowed to air dry and the same procedure was repeated for filtered bait water from the second round of baiting (double baiting).

Isolation using Granny Smith apple baits

Granny Smith apples were used as baits for roots from the field sites. Briefly, two holes (about 10 mm width) were made with a sterile scalpel on opposite sides of each apple; the column was taken out and chopped fine roots were placed into each hole and blocked with the removed apple column, and sealed with gladwrap (plastic film) (Fig.1e). Each apple was kept for 5–7 days in a separate zip-lock bag (Sandvik, Australia) at 20 ± 5°C and brown discolored lesions around holes were plated onto NARH.

Bait plants in glasshouse

Seed of *B. attenuata* was grown in sterilized sand for two weeks and transplanted to free-draining pots (18 × 6.5 cm L x W) containing soil collected from each site and placed into an evaporatively cooled glasshouse. *Eucalyptus sieberi* seed was then directly germinated in the same pots and watered daily (Fig. 1f). There were four replicate pots for each site. Seedlings showing disease symptoms while growing were cut into 1–2 cm segments and plated onto NARH. After 2–3 days, any *Phytophthora*-like cultures were transferred onto individual V8A plates. After nine weeks, the *E. sieberi* and *B. attenuata* seedlings were harvested by severing shoots from roots. Roots were carefully washed over a 1-mm sieve immediately after harvesting to remove soil particles and stored in collection tubes in triplicates at −20 °C for DNA extraction.

Morphological and molecular identification of isolates

Living isolates were maintained on V8A. Isolates were divided into morphotypes based on their gross colony morphology and hyphal characteristics examined at 10x magnification under a light microscope. Finally, two to three isolates from each morphotype were selected for sequence-based identification using the ITS gene region. ITS sequence data were obtained for all isolates, and their identity was confirmed by conducting BLAST search in GenBank (www.ncbi.nlm.nih.gov/genbank/).

DNA extraction

DNA was extracted in triplicate from (i) fine roots collected from field soil samples using the PowerPlant® DNA isolation kit following the manufacturer's instructions; (ii) the air-dried soil

Table 1. Site location and host plants from which *Phytophthora* isolates were obtained.

Location	Site key	GPS location	Vegetation type
Bold Park	1	31°56′47.68″S 115°46′41.96″E	Mixed *Eucalyptus gomphocephala* and *Banksia* woodlands, with mixed *Acacia rostellifera* and *Melaleuca acerosa* understory
Kings Park	2	31°57′50.08″S 115°49′19.60″E	Mixed *Eucalyptus*, *Banksia* and *Xanthorrhoea preissii* woodlands
Attadale foreshore	3	32° 1′2.05″S 115°47′52.85″E	Mixed *Eucalyptus marginata* and *Corymbia calophylla* woodlands, with *Banksia*, *Melaleuca*, and *Agonis flexuosa* midstory, *and Sporobolus virginicus* open grassland
Manning Park	4	32° 5′31.76″S 115°45′58.49″E	Mixed *Eucalyptus gomphocephala* and *E. decipiens* woodland, with *Acacia*, *Melaleuca hugelii* and *M. acerosa* understory
Bibra Lake	5	32° 5′41.96″S 115°49′14.33″E	Mixed *Eucalyptus* woodland with *Banksia attenuata*, *B. menziesii* midstory and *Melaleuca teretifolia* and/or *Astartea* aff. *fascicularis* understory

Figure 1. Techniques for isolating *Phytophthora* from soil and root samples; (**A**) a typical soil sample in a baiting tray; (**B**) fine roots collected from soil for eDNA extraction and placement into Granny Smith apples; (**C**) traditional baiting assay from a soil and root samples; (**D**) filtration apparatus for filtering bait water; (**E**) isolation from field roots using Granny Smith apples and (**F**) isolation from field soil using bait plants (*Banksia attenuata* and *Eucalyptus sieberi*) grown in the glasshouse.

sample was sieved and 100 g of this soil was crushed to a fine powder using the TissueLyser LT (Qiagen, Haan, Germany) and DNA was extracted using the Mo Bio PowerSoil® DNA isolation kit following the manufacturer's instructions except for the first step the buffer from the kit was replaced with 1 ml of saturated phosphate buffer (Na2PO 4; 0.12 M; pH 8) to the samples (500 mg) to maximize extracellular DNA isolation (Taberlet *et al.* 2012); (iii) the remaining two halves of filters from the first and second rounds of filtered bait water using PowerSoil® DNA isolation kit (filter halves obtained from the first and second rounds were bulked to reduce the cost), and (iv) fine roots recovered from glasshouse bait plants using the PowerPlant® DNA isolation kit. Extreme care was taken to avoid any possible contamination during extraction and extraction controls were also included.

Amplicon pyrosequencing and clustering

Amplicon libraries for ITS gene region were created using the *Phytophthora*-specific primers (Scibetta *et al.* 2012) and Promega GoTaq Host Start Polymerase using a nested PCR approach as optimized by (Burgess *et al.* 2017b). Negative controls were included each time a PCR reaction was setup, and carried forward to the second round in the same manner as for the samples. PCR products were cleaned twice with AMPure XP Beads (Beckman Coulter Genomics) following the Short Fragment removal protocol according to manufacturer's instructions. After purification, the PCR products were visualized on agarose gels and then pooled (based on the band intensity) to standardize each sample's DNA contribution to pooled samples. The final pooling was diluted to 1/5000 of the original concentration, and 50 μl of the dilution was again cleaned with AMPure XP Beads. DNA was quantified as described previously (Burgess *et al.* 2017b). The emulsion PCR reactions were carried out according to the Roche GS Junior emPCR Amplification Method Manual Lib-L (March 2012). The libraries were sequenced using Junior Genome Sequencer plates (454 Life Sciences/Roche Applied Biosystems, Nutley, NJ, USA). Bioinformatics was conducted in GENEIOUS version R9 (http://www.geneious.com/). Reads were then clustered into molecular operational taxonomic units (MOTUs) based on 99% sequence similarity, which allows identification of closely related species. Identities were assigned to MOTUs after phylogenetic analysis against a dataset containing verified sequences of all known *Phytophthora* species. These identities are considered phylotypes acknowledging that this is based on sequence data rather than a living isolate. Chimeras were discarded after making alignments of consensus MOTUs for each barcode. Identities were assigned to phylotypes as described by (Burgess *et al.* 2017b).

Data processing and statistical analysis

One-way analysis of variance (ANOVA) test was used to determine the differences in the number of unique *Phytophthora* species isolated and detected by the different techniques. Additionally, Welch's T-test (Welch 1951) was used to compare the number of unique *Phytophthora* species recovered by traditional isolations and metabarcoding. Assumptions of normality were assessed using the Shapiro–Wilk test and observation frequency histograms. Both Levene's and Bartlett's tests of homogeneous variance were undertaken before the analysis. Three of the six detection methods were not normally distributed. An ANOVA was performed as distribution were not similar and the test was robust enough to handle violations of normality assumption (Schmider *et al.* 2010). After performing the ANOVA, a Tukey HSD *post hoc* test was conducted when the predictive variable was significant. All analyses were performed in R (R Core Team 2015) using the 'stats', 'graphics' and 'car' (John and Sanford 2011) packages. Diversity indices were calculated for metabarcoding techniques using the R package 'vegan' (Oksanen *et al.* 2017).

It should be noted that the Granny Smith apple technique (in which no *Phytophthora* was recovered), and failed PCR runs from two sites (2 and 3) in case of field roots eDNA were excluded from the analysis because no amplifications were achieved for these sites.

RESULTS

Traditional isolation from soil using bait leaves

All baits developed brownish necrotic lesions within 2–4 days. However, *Phytophthora* species could only be recovered from sites 3, 4 and 5. These included *P. thermophila*, *P. rosacearum*, *P.* 'oreophila', *P. amnicola*, *P. multivora* and *P. inundata* (Table 2; Table S2, Supporting Information). *Pythium* species were also readily isolated from all sites.

Isolation from filtered bait water

No *Phytophthora* species were recovered from sites 1, 2 and 4. Species recovered from the other two sites included *P. amnicola, P. thermophila*, *P. multivora*, *P. rosacearum*, *P.* 'oreophila' and *P. gregata* (Table 2; Table S2, Supporting Information).

Isolation using Granny Smith apple baits

No *Phytophthora* species were recovered by using this technique. However, a few unidentified *Pythium* (data not shown) isolates were recovered.

Phytophthora species detected from eDNA

All the soil, filters and glasshouse root samples, and nine of 15 extractions from field roots from five sites yielded PCR products. Across all runs, a total of 81 324 quality reads were produced from samples that yielded PCR products. Across all sites, 30 phylotypes corresponding to 25 known species, three designated but undescribed species and two potentially new species were obtained. Some closely related species relevant to this study cannot be separated based solely on ITS1 (Fig. S1, Supporting Information): (i) *P. citrophthora* and *P. terminalis*, (ii) *P. capsici* and *P. glovera*, (iii) *P. arenaria*, *P. boodjera* and *P. alticola* and (iv) *P. versiformis, P. quercina and P. castenatorum*. With the exception of *P. arenaria* and *P. boodjera* that are both found in Australia, for the other groups only the first named species in known in Australia.

There were 25 phylotypes from field roots, 24 from filters, 19 from glasshouse bait roots, and 12 from soil (Table 2; Table S4, Supporting Information). The three most abundant phylotypes were *P. multivora* (66.73%), followed by *P. pseudocryptogea* (12.69%) and *P. amnicola* (3.38%) (see Table S3, Supporting Information). Diversity indices were calculated to determine species richness and diversity. According to all the alpha diversity indices (α, α_{SI}, α_S) (Simpson 1949), higher diversity in detections was displayed in the field roots eDNA having had the most diverse *Phytophthora* community than other substrates (Table 3). According to multiplicative beta diversity ($\beta_{\gamma/\alpha}$), higher novelty in detection was displayed in filters' eDNA than any other substrate. Finally, the Bray–Curtis dissimilarity index (Bray and Curtis 1957) showed

Table 2. Number of sites (from a total of 5) from which each *Phytophthora* species were (A) isolated using traditional techniques, or (B) detected by metabarcoding.

		(A) Isolations			(B) Metabarcoding			
Species	Clade	Baiting	Filters	Apple	Soil	Filters	Field[a] roots	Glasshouse bait roots
P. nicotianeae	1					3	3	5
P. AUS 1D	1				4	2	3	2
P. capensis	2					1	1	3
P. capsici	2					1		1
P. elongata	2					1	1	
P. frigida	2					1		1
P. multivora	2	2	1		5	5	3	5
P. pachypleura	2					1	1	
P. 'acacia'	2						1	1
P. citrophthora	2					1	2	3
P. AUS 2C	2						1	
P. arenaria	4					1	1	3
P. palmivora	4					1		
P. amnicola	6	1	1		2	5	3	5
P. fluvialis	6						1	
P. gregata	6		1			1	1	
P. inundata	6	1			2	4	2	4
P. litoralis	6						1	
P. moyootj	6					1	1	
P. 'oreophila'	6	1	1		2	5	3	5
P. rosacearum	6	2	2		2	1	2	2
P. kwongonina	6				1	5	2	3
P. thermophila	6	1	1		3	5	3	5
P. cambivora	7					1		
P. cinnamomi	7				1	2	3	5
P. melonis	7				1			
P. psuedocryptogea	8				5	5	3	5
P. 'kelmania'	8						1	
P. constricta	9				1	2	3	3
P. versiformis	11				2	1	1	1
Total no. of species		6	6	0	13	24	25	19

[a]Amplification was only achieved for three of the five sites.

Table 3. The table below displays gamma, alpha and beta diversity indices. Indices that account for species abundance (α_{SW}, α_S, β_{BC},) were calculated with the number of reads. Symbols: γ represents gamma diversity; symbol α represents species richness; α_{SI} means Shannon Index (Shannon 1948); α_S means Simpson diversity (Simpson 1949); $\beta_{\gamma/\alpha}$ means multiplicative beta diversity; β_{BC} means Bray–Curtis dissimilarity (Bray and Curtis 1957).

		Gamma	Alpha			Beta	
Metabarcoding substrate	Number of sites of sites in which *Phytophthora* species were detected	γ	α	α_{SI}	α_S	$\beta_{\gamma/\alpha}$	β_{BC}
Soil	5	13	6.20	0.50	0.24	2.10	0.50
Filters	5	24	11.2	0.81	0.38	2.14	0.53
Roots	3	25	15.67	1.71	0.72	1.59	0.75
Glasshouse roots	5	19	12.4	1.44	0.61	1.53	0.49

that *Phytophthora* species detected in field roots eDNA in different sites were more dissimilar than other substrates (Table 3).

Soil

Thirteen phylotypes were detected by metabarcoding; *Phytophthora* AUS 1D, *P. multivora*, *P. amnicola*, *P. inundata*, *P. versiformis*, *P. constricta*, *P. rosacearum*, *P.* 'oreophila', *P. kwongonina*, *P. thermophila*, *P. cinnamomi*, *P. melonis* and *P. pseudocryptogea* (Table 2). Species distribution varied across sites. *Phytophthora multivora* and *P. pseudocryptogea* were detected in all sites, *P.* AUS 1D was detected in four sites, and *P. thermophil*a were detected in three sites, *P. versiformis*, *P. amnicola*, *P.* 'oreophila', *P. rosacearum* and *P. inundata* were detected in two sites, and *P. kwongonina*, *P. cinnamomi*, *P. melonis* and *P. constricta* were detected at only one site (Table S4, Supporting Information).

Field roots

A total of 25 phylotypes were detected in field roots by metabarcoding: *P. nicotianae*, P. AUS 1D, *P. capensis, P. elongata, P. multivora, P. pachypleura*, P. 'acacia', *P. citrophthora*, P. AUS 2C, *P. amnicola, P. fluvialis, P. gregata, P. inundata, P. litoralis, P. moyootj, P. rosacearum*, P. 'oreophila', *P. kwongonina, P. thermophila, P. pseudocryptogea, P. versiformis, P. arenaria, P. cinnamomi*, P. 'kelmania' and *P. constricta* (Table 2). Species distribution was uneven across sites; *P. nicotianeae*, P. AUS 1D, *P. multivora, P. amnicola*, P. 'oreophila', *P. thermophila, P. cinnamomi, P. pseudocryptogea* and *P. constricta* were detected in three sites, *P. citrophthora, P. inundata, P. rosacearum* and *P. kwongonina* were detected in two sites, and *P. capensis, P. elongata, P. pachypleura*, P. 'acacia', P. AUS 2C, *P. arenaria, P. fluvialis, P. gregata, P. litoralis, P. moyootj*, P. 'kelmania' and *P. versiformis* were detected at only one site (Table S4, Supporting Information).

Filtered bait water

A total of 24 phylotypes were detected by metabarcoding: *P. nicotianae*, P. AUS 1D, *P. capensis, P. capsici, P. elongata, P. frigida, P. multivora, P. pachypleura, P. citrophthora, P. arenaria, P. palmivora, P. amnicola, P. gregata, P. inundata, P. moyootj*, P. 'oreophila', *P. rosacearum, P. kwongonina, P. thermophila, P. cambivora, P. cinnamomi, P. pseudocryptogea, P. constricta* and *P. versiformis* (Table 2). The occurrence of phylotypes also varied across sites; *P. multivora, P. amnicola*, P. 'oreophila', *P. kwongonina, P. thermophila* and *P. pseudocryptogea* were detected in five sites; *P. inundata* was detected in four sites; *P. nicotianae* was detected in three sites; P. AUS 1D, *P. cinnamomi*, and *P. constricta* was detected in two sites, and *P. capensis, P. capsici, P. elongata, P. frigida, P. pachypleura, P. citrophthora, P. arenaria, P. palmivora, P. gregata, P. moyootj, P. rosacearum, P. cambivora* and *P. versiformis* were detected in only one site (Table S4, Supporting Information).

Glasshouse bait roots

Nineteen phylotypes were detected by metabarcoding; *P. nicotianae*, P. AUS 1D, *P. capensis, P. multivora, P. capsici*, P. 'acacia', *P. citrophthora, P. versiformis, P. arenaria, P. amnicola, P. inundata, P. rosacearum, P. kwongonina, P. thermophila*, P. 'oreophila', *P. cinnamomi*, P. *pseudocryptogea*, *P. frigida* and *P. constricta* (Table 2). Considerable differences were observed in species distribution across sites. *P. nicotianae, P. multivora, P. amnicola*, P. 'oreophila', *P. thermophila, P. cinnamomi* and *P. pseudocryptogea* were detected in all five sites; *P. inundata* was detected in four sites; *P. capensis, P. citrophthora, P. arenaria, P. kwongonina* and *P. constricta* were detected in three sites; P. AUS 1D and *P. rosacearum* were detected in two sites, and *P. capsici, P. frigida*, P. 'acacia' and *P. versiformis* were detected in only one site (Table S4, Supporting Information).

Comparison of traditional isolations to metabarcoding

Significant differences were observed between traditional isolations and metabarcoding techniques tested for the isolation and detection of *Phytophthora* t(23.0.16) = 6. 827, P = 0.000). Significantly more *Phytophthora* were detected by molecular techniques (10. 88 average) compared with traditional (1.4 average). Differences among the analyzed substrates used for metabarcoding studies were also significant [F(5,22) = 10.34, P= 0.000) -]. Of the 30 *Phytophthora* phylotypes detected in this study, all were recovered in eDNA from a variety of sources and only seven of these were recovered by traditional methods (traditional isolation using bait leaves and filtered bait water). The highest number of *Phytophthora* species was detected in field soil roots eDNA (25); the lowest numbers were detected with traditional isolation using bait leaves and filtered bait water (six *Phytophthora* species each), while no *Phytophthora* species were detected in Granny Smith apple baits (Table 2).

Certain *Phytophthora* species were detected by all techniques tested in this study except Granny Smith apples. For example, *P. multivora, P. amnicola, P. inundata*, P. 'oreophila', *P. rosacearum* and *P. thermophila*. However, P. AUS 1D, *P. kwongonina, P. cinnamomi*, *P. pseudocryptogea, P. constricta*, and *P. versiformis* were detected from all sources of eDNA (soil, filters, roots from field, and bait plants in the glasshouse), but these were not recovered by traditional isolations (baiting, and filtered bait water and Granny Smith apple baits). Certain *Phytophthora* species were only detected with one technique (Table 2). For example, *P. meloni*s was detected from field soil eDNA; *P. palmivora* and *P. cambivora* were detected from filters' eDNA, and P. AUS 2C, *P. fluvialis, P. litoralis* and P. 'kelmania' were detected from field soil roots eDNA only (Table 2).

DISCUSSION

This study provides a comparison of traditional isolation methods to metabarcoding, and for the first time, an evaluation of different substrates used for isolation and detection of *Phytophthora* species was conducted. This research will have a great impact on *Phytophthora* diagnostics and its isolation and detection in natural ecosystems and is particularly relevant to studies of other root infecting organisms. Different techniques and substrates used for the isolation and detection of *Phytophthora* species showed variable results. Of the 30 *Phytophthora* phylotypes detected in this study, all were identified in eDNA from a variety of sources and only seven of these were recovered by traditional methods. We were also able to recognize two potentially new phylotypes, both of which had been detected in Australia previously (Burgess *et al.* 2017b).

Traditional techniques tested for isolation of *Phytophthora* species showed variable results. One interesting finding is that one additional species, *P. gregata*, was recovered from filtered bait water compared to traditional isolation from soil using bait leaves. This result further confirms the association between *Phytophthora* species and the type of bait leaves used (Erwin and Ribeiro 1996). It also supports the idea of using multiple bait leaves to avoid host preference and competition for food among *Phytophthora* species (Scibetta *et al.* 2012). *Scholtzia involucrata* (spiked scholtzia) and *Pimelea ferruginea* (rice flower) were found to be more successful in isolation of *Phytophthora* species in the current study; *P. ferruginea* has been successfully used in previous research (McDougall, Hardy and Hobbs 2002). Further work is suggested to establish the relationship between *Phytophthora* species and different baits. Moreover, inoculum level varies between seasons; therefore, baiting assays need to be conducted at different times of the year to get a good picture of *Phytophthora* species present (Balci and Halmschlager 2003). Also, the successful recovery of *Phytophthora* species in about one-third of the resampled sites by Balci *et al.* (2007) emphasizes the need for sampling throughout the year to avoid false negative results on sites. No *Phytophthora* species were isolated from field soil roots using apple baits. Although there had been some previous reports on successful isolation of *Phytophthora cinnamomi* from soil using fruit baits (Greenhalgh 1978), no *Phytophthora* species were recovered from field soil roots using apple as a bait, this was also the case for Aghighi (2013).

Although one additional *Phytophthora* species were recovered in filtered bait water (*P. gregata* that was not recovered with traditional baiting), no *Phytophthora* species were recovered from

site 4 using this technique. This was surprising because *Phytophthora* species were recovered from this site by traditional baiting and one would expect zoospores to be picked up on the filters as water was filtered from the same 'bait trays'. This result may be explained by the fact that colonies are difficult to purify in case of filtering method because colonies are concentrated in the area where membrane is placed (Hong, Richardson and Kong 2002). A single filter was used to filter bait water from each 'bait tray' in the current study. It has been shown that the density of *Phytophthora* propagules (cfu/L) from a single filter decreases with the increase in the amount of water filtered because multiple fast-growing *Phytophthora* and *Pythium* species interfere with the identification and growth of slow growing colonies (Reeser *et al.* 2011). Therefore, the amount of water to be filtered should be divided onto several filters to accurately measure *Phytophthora* diversity (Reeser *et al.* 2011).

The metabarcoding results were also variable. Among the different sources of eDNA tested for metabarcoding, the lowest number (13) of *Phytophthora* phylotypes was detected from field soil eDNA. It is believed that the lower detection of *Phytophthora* from soil eDNA can be due to the presence of humic acid inhibitors and high DNA degradation (Català, Pérez-Sierra and Abad-Campos 2015). The humic acid inhibitors' interpretation is not valid for the current study as we used the Mo Bio PowerSoil® DNA isolation kit, which efficiently removes humic acid and other inhibitors. Lear *et al.* (2018) reviewed practices for the extraction, storage and amplification of environmental samples for a wide range of taxa from 2010 to 2015 and found that Mo Bio PowerSoil and PowerMax Soil DNA isolation kits (now rebranded as DNeasy PowerSoil and DNeasy PowerMax by Qiagen, Carlsbad, USA) were used in almost all of the studies dealing with the detections from soil or sediment material and has been recommended by a number of international standards consortia following comparisons with many other methods (Gilbert, Jansson and Knight 2014). Therefore, a more likely explanation for the low detections from soil is that because *Phytophthora* species mainly live as parasites in plants, and survive as resting structures (mainly oospores and chlamydospores) and mycelium in soil. There is a higher chance of degradation in soil as these survival structures are more exposed to environmental extremes compared to roots where they are protected by thick layers of host tissues. *Phytophthora* species are known to respond differently to environmental extremes even in different parts of a single plant. For example, *P. cinnamomi* is more vulnerable to temperature extremes in bark tissues rather than in root tissues deep under soil (Marçais, Dupuis and Desprez-Loustau 1996).

Determining fungal diversity in soil eDNA has always been a challenge and several techniques have been used to improve the amount of DNA extracted with sufficient purity. For example, liquid nitrogen and phenol/chloroform in combination with powdered skim milk were used to reduce PCR inhibitors in case of *Fusarium oxysporum* f. sp *ciceris* (García-Pedrajas *et al.* 1999). Okubara *et al.* (2007) used pressure cycling technology (samples are subjected to alternate cycles of high and ambient pressure), rather than mechanical disruption for detecting *Rhizoctonia* and *Pythium* species from soil. Probably the most common approach used to reduce the amount of inhibitors is the dilution of DNA extract. Although this often results in the amplification, it is not very useful to detect low levels of pathogen inoculum (Bilodeau *et al.* 2012). Alternatively, aluminum sulphate (Dong *et al.* 2006) and Sephadex column (Tsai and Olson 1992) can be used to reduce humic substances. Humic acid substances can also be reduced by supplementing PCR mixtures with adjuvants, such as BSA (Lear *et al.* 2018). It is advisable to get an accurate estimation of consistency of results between replicates to get accurate results because inoculum is not always uniformly distributed in soil. Three to four replications for each extraction with sample tubes holding a total volume of 0.5 g of soil each has been reported to provide sufficient consistency in replicate results without increasing cost (Bilodeau *et al.* 2012). Further work is suggested to evaluate different soil DNA extraction techniques and optimization of sensitivity of detections from soil.

In comparison to field soil eDNA, 24 *Phytophthora* phylotypes were detected in eDNA from filters (extracted onto filters by filtered bait water). This result maybe explained by the fact that a large amount of soil (approx. 400 g) was used in filtered bait water, whereas only 50 mg soil was used for pyrosequencing analysis of soil eDNA.

The highest number (25) of *Phytophthora* phylotypes was detected in the eDNA of fine roots collected from field soil. These results are contrary to the hypothesis proposed by Coince *et al.* (2013) that high-throughput sequencing can rule out the niche differentiation between fine roots and soil, and can detect most oomycete and fungal MOTUs present in fine roots in soil as well. Prigigallo *et al.* (2015) detected three additional *Phytophthora* species out of nine species belonging to definite taxonomic groups in soil rather than roots. Landeweert *et al.* (2005) determined the diversity of an active ectomycorrhizal fungal community in root tips and total soil DNA, and did not detect a single fungal species in root tips that was not present in soil. However, Dickie, Xu and Koide (2002) found that mycorrhizal fungi obtained from fine roots can be different from those obtained from soil. Our research matches these latter findings as 15 additional *Phytophthora* species not found in the soil were detected in fine roots, which raise the possibility of using fine roots as an effective substitute for other substrates for metabarcoding studies. An issue with the detection directly from soil could be that the DNA extracted could be from dead organisms (Nocker, Cheung and Camper 2006). Roots act as a biological filter; if an organism is present in the roots it must have been alive to get there. However, we did only detect *Phytophthora* in three of the five root samples, which could be somehow linked to the type of roots collected and the relatively small sample size (as inoculum is not uniformly dispersed). More work needs to be done to optimize this methodology.

Nineteen *Phytophthora* phylotypes were detected in the roots of bait plants grown in the glasshouse. These results are consistent with those of McDougall, Hardy and Hobbs (2002), who detected *P. cinnamomi* in twice as many areas and five times as many samples with *in situ* baiting with *Banksia grandis* than *ex situ* soil and root baiting. A possible explanation for this might be that bait leaves used in traditional isolation from soil were not as attractive to *Phytophthora* as the roots of bait plants grown in the glasshouse. Another possible explanation for this could be that *Phytophthora* survival structures (oospores and chlamydospores) were dormant and the traditional baiting technique was unable to break their dormancy, even after double baiting (Balci *et al.* 2007). Similarly, antagonistic microorganisms could have suppressed the growth and germination of these viable propagules during traditional baiting (Balci and Halmschlager 2003), while living roots in soil stimulated their growth and germination. Furthermore, air-drying followed by remoistening and incubation up to three days might be required for recovery of some *Phytophthora* species (e.g. *P. cactorum*). In a study by Jeffers and Aldwinckle (1987), recovery occurred in 100% of replicate subsamples when subsamples were remoistened and incubated for three days before flooding, but only in 17% and 72% of

sub-samples when remoistened and incubated for 1 and 2 days, respectively before flooding.

Baiting is a complicated process that shows variable results in soils with different physiochemical and biological characteristics (Williams, Hardy and O'Brien 2009). Chemical composition of the soil can affect zoospore release and hence subsequent bait infection. It has been shown that the use of soil with high levels of N, P, K and organic matter resulted in the increased levels of zoospore production (Broadbent and Baker 1974; Shearer 2003). Messenger, Menge and Pond (2000) showed that high calcium levels were necessary for zoospore production. Duncan (1976) reported that one germinating oospore (producing a sporangium, which then produces 8–14 zoospores) of *P. fragariae* was able to cause detectable infection in a strawberry bait plant under optimum conditions, especially when zoospores were produced close to the roots and not at the soil surface. The quality of water used can also affect baiting as zoospores show sensitivity to toxic ions present in the un-purified water (Tsao 1983). Gerrettson-Cornell, quoted in Tsao (1983), found that the frequency of isolation of *P. cinnamomi* was 94%, 32% and 0%, respectively, when glass de-ionized water, deionized water and de-ionized water from a metal still was used. Lastly, the rate of positive detection of *Phytophthora* by baiting is usually very low (0.4% to 10%) in Western Australian soil (Podger 1978; Blowes 1980) compared to New South Wales and Queensland (27 to 58%) (Blowes 1980; Pryce, Edwards and Gadek 2002). It is not clear whether the reason for this difference in recovery is due to difference in soil composition or different climates (O'Brien, Williams and Hardy 2009).

In conclusion, all the techniques tested for the isolation and detection of *Phytophthora* species showed variable results. Although traditional baiting assays are important for obtaining living isolates, they do not represent the actual *Phytophthora* community present in a location. High-throughput amplicon pyrosequencing of eDNA detected the highest number of *Phytophthora*; therefore, it is a very useful tool for assessing *Phytophthora* diversity in environmental samples. The ITS region can fail to discriminate some species complexes (Català, Pérez-Sierra and Abad-Campos 2015; Burgess *et al.* 2017b). However, clustering at 99% of similarity or above may help in differentiating closely related species (clustering was done at 99% sequence similarity level in the present study). Català, Pérez-Sierra and Abad-Campos (2015) obtained 20% more differentiation of closely related species by including a control species mixture and clustering at 99% threshold. Despite these shortcomings, ITS is still very useful to differentiate known species and identify new ones. ITS is the main locus for molecular identification due its easy amplification for most species (Ristaino *et al.* 1998), availability of large sequence data deposited in GenBank, its importance in phylogenetic analysis (Grünwald *et al.* 2011), and more commonly the targeted region for fungal analysis than other gene regions (Lear *et al.* 2018). As the highest number of *Phytophthora* species were detected in field roots eDNA, it could be substituted for other substrates to assess *Phytophthora* diversity in environmental samples. Finally, certain *Phytophthora* could be only detected by one method; therefore, a combination of these techniques may be necessary to accurately assess the presence or absence of *Phytophthora* species.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

Conflict of interest. None declared.

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