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Community composition and distribution of *Phytophthora* species across adjacent native and non-native forests of South Africa

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ABSTRACT

The diversity of *Phytophthora* species associated with various ecological niches is poorly understood. In this study, the community composition and distribution of *Phytophthora* species associated with non-native plantation trees, *Eucalyptus grandis* and *Acacia mearnsii,* was compared with adjacent natural forests in South Africa using soil baiting and metabarcoding approaches. Through soil baiting, 85 *Phytophthora* isolates were recovered representing five taxa: *P. alticola*, *P. cinnamomi, P. frigida, P. multivora* and *P. pseudocryptogea*. Metabarcoding revealed molecular operational taxonomic units corresponding to 32 *Phytophthora* taxa. Among these, 14 were new reports from South Africa, including seven undescribed taxa. The community composition of *Phytophthora* species clustered according to vegetation type. Most species in plantations were present in the natural forest sites, but few species were exclusively associated with the non-native plantations. Overall, the results revealed a substantial diversity of *Phytophthora* species that includes both described and novel phylotypes previously unknown from South Africa.

1. Introduction

Phytophthora species are amongst the most destructive plant pathogens (Haas et al., 2009; Ribeiro, 2013) and yet relatively little is known regarding their global diversity (Scott et al., 2013). Surveys for, and discoveries of, new *Phytophthora* species have been significantly facilitated by the application of baiting techniques (Drenth and Sendall, 2001) and the utilization of selective media (Erwin and Ribeiro, 1996). In recent years, molecular techniques have also contributed strongly to the discovery, detection and identification of cryptic species as well as to diversity studies. For example, the *P. citricola* (Jung and Burgess, 2009; Scott et al., 2009; Bezuidenhout et al., 2010) and *P. cryptogea* (Safaiefarahani et al., 2015) complexes have been resolved using these techniques.

The application of high throughput sequencing technology to environmental samples has the capacity to expand our knowledge of species diversity and distribution, especially as it offers the potential to detect rare or unculturable organisms missed in traditional studies. However, there are some weaknesses with these approaches such as: (i) the inability to generate pure cultures, crucial for taxonomic and genomics studies, and (ii) the amplification of relatively short fragments (~500-600bp) of DNA making it challenging to discriminate between closely related species (Huse et al., 2007; Burgess et al. 2017a). Nonetheless, the genus specific primers available for *Phytophthora* (Scibetta et al., 2012), based on the ITS1 gene region have provided sufficient data to be able to conduct phylogenetic analyses for identification purposes (Català et al. 2015, 2017; Burgess et al., 2017a). As such, metabarcoding is an efficient tool to investigate the diversity of *Phytophthora* species in natural forests, nurseries and agricultural ecosystems.

Although *Phytophthora* species have a worldwide distribution, relatively few species have been reported from Africa, and the majority of these have been reported from South Africa (Nagel et al., 2013; Scott et al., 2013). In South Africa, *Phytophthora* species affect agricultural crops, native forests, plantations and orchards of non-native species (Nagel et al., 2013). The severity of *Phytophthora* diseases varies greatly within South Africa, depending on the area and crop being planted. For example, the root rot of avocado caused

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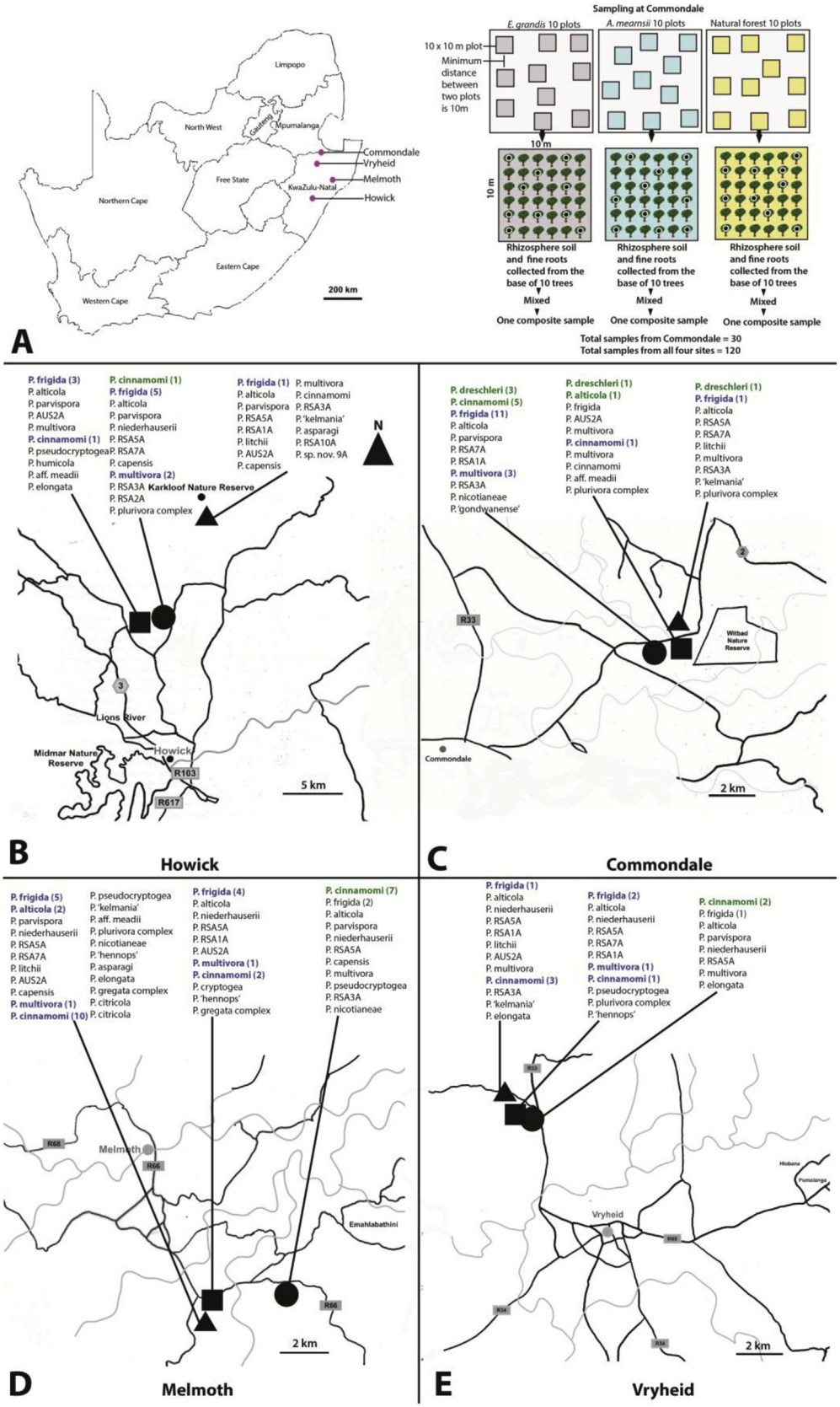
A
Limpopo
North West
Gauteng
Mpumalanga
Free State
KwaZulu-Natal
Northern Cape
Eastern Cape
Western Cape
Commondale
Vryheid
Melmoth
Howick
200 km
Sampling at Commondale
E. grandis 10 plots
A. mearnsii 10 plots
Natural forest 10 plots
10 x 10 m plot
Minimum distance between two plots is 10m
10 m
Rhizosphere soil and fine roots collected from the base of 10 trees
Mixed
One composite sample
Total samples from Commondale = 30
Total samples from all four sites = 120
B
P. frigida (3)
P. alticola
P. parvispora
P. AUS2A
P. multivora
P. cinnamomi (1)
P. pseudocryptogea
P. humicola
P. aff. meadii
P. elongata
P. cinnamomi (1)
P. frigida (5)
P. alticola
P. parvispora
P. niederhauserii
P. RSA5A
P. RSA7A
P. capensis
P. multivora (2)
P. RSA3A
P. RSA2A
P. plurivora complex
P. frigida (1)
P. alticola
P. parvispora
P. RSA5A
P. RSA1A
P. litchii
P. AUS2A
P. capensis
P. multivora
P. cinnamomi
P. RSA3A
P. 'kelmania'
P. asparagi
P. RSA10A
P. sp. nov. 9A
Karkloof Nature Reserve
N
Lions River
Howick
Midmar Nature Reserve
R103
R617
5 km
Howick
C
P. dreschleri (3)
P. cinnamomi (5)
P. frigida (11)
P. alticola
P. parvispora
P. RSA7A
P. RSA1A
P. multivora (3)
P. RSA3A
P. nicotianeae
P. 'gondwanense'
P. dreschleri (1)
P. alticola (1)
P. frigida
P. AUS2A
P. multivora
P. cinnamomi (1)
P. multivora
P. cinnamomi
P. aff. meadii
P. plurivora complex
P. dreschleri (1)
P. frigida (1)
P. alticola
P. RSA5A
P. RSA7A
P. litchii
P. multivora
P. RSA3A
P. 'kelmania'
P. plurivora complex
R33
Witbad Nature Reserve
Commondale
2 km
Commondale
D
P. frigida (5)
P. alticola (2)
P. parvispora
P. niederhauserii
P. RSA5A
P. RSA7A
P. litchii
P. AUS2A
P. capensis
P. multivora (1)
P. cinnamomi (10)
P. pseudocryptogea
P. 'kelmania'
P. aff. meadii
P. plurivora complex
P. nicotianeae
P. 'hennops'
P. asparagi
P. elongata
P. gregata complex
P. citricola
P. citricola
P. frigida (4)
P. alticola
P. niederhauserii
P. RSA5A
P. RSA1A
P. AUS2A
P. multivora (1)
P. cinnamomi (2)
P. cryptogea
P. 'hennops'
P. gregata complex
P. cinnamomi (7)
P. frigida (2)
P. alticola
P. parvispora
P. niederhauserii
P. RSA5A
P. capensis
P. multivora
P. pseudocryptogea
P. RSA3A
P. nicotianeae
R68
Melmoth
R66
Emahlabathini
R66
2 km
Melmoth
E
P. frigida (1)
P. alticola
P. niederhauserii
P. RSA5A
P. RSA1A
P. litchii
P. AUS2A
P. multivora
P. cinnamomi (3)
P. RSA3A
P. 'kelmania'
P. elongata
P. frigida (2)
P. alticola
P. niederhauserii
P. RSA5A
P. RSA7A
P. RSA1A
P. multivora (1)
P. cinnamomi (1)
P. pseudocryptogea
P. plurivora complex
P. 'hennops'
P. cinnamomi (2)
P. frigida (1)
P. alticola
P. parvispora
P. niederhauserii
P. RSA5A
P. multivora
P. elongata
R34
Vryheid
R65
R34
R34
Hlobane
Pumalanga
2 km
Vryheid

by *P. cinnamomi* (Milne et al. 1974, 1975; Kotze et al., 1987) and of citrus species caused by *P. citrophthora* and *P. citricola* (Doidge, 1925; Von Maltitz and Von Broembsen, 1985) has a severe economic impact. There are also some reports of *Phytophthora* diseases in natural ecosystems in South Africa, the best-known being those caused by *P. cinnamomi* in the Cape Floristic Region (CFR) in the Western Cape province. The CFR has received the most attention (Von Broembsen, 1984; Bezuidenhout et al., 2010) due to its extraordinary floral diversity as well as the high levels of susceptibility of the Proteaceae in this region to *Phytophthora* infections (Van Wyk, 1973).

In South Africa, *Phytophthora* species cause diseases of various species of the non-native plantation tree genera *Pinus*, *Eucalyptus* and *Acacia mearnsii*. *Phytophthora cinnamomi* causes root and collar-rot of both *Pinus* and *Eucalyptus* species (Linde et al., 1994), and until the early 1990's *P. cinnamomi* was the only species known to cause disease on these trees. Later studies reported *P. alticola*, *P. boehmeriae*, *P. frigida, P. meadii* and *P. nicotianae* as pathogens of *A. mearnsii* and *Eucalyptus* species (Zeiljemaker, 1967; Zeijlemaker and Margot, 1970; Zeijlemaker, 1971; Linde et al., 1994; Roux and Wingfield, 1997; Maseko et al., 2007).

While a few studies have focused on *Phytophthora* diseases of non-native plantation trees, no studies have considered natural forests as a source of the *Phytophthora* species found in plantations of non-native trees in South Africa. Consequently, this study sought to determine the community composition of *Phytophthora* species associated with plantations of non-native *Eucalyptus grandis* and *Acacia mearnsii* and adjacent natural forests. In addition, it aimed to determine whether this community composition varies between these three very different environments. Soil baiting complemented with metabarcoding using a pyrosequencing platform was used to address the following hypotheses: (1) Community composition of *Phytophthora* species differs between the three vegetation types; (2) community composition of *Phytophthora* species differs between sites; and (3) the *Phytophthora* community is less diverse in monocultures than in the natural forests.

2. Materials and methods

2.1. Collection of soil samples

Soil samples were collected from four locations in southeastern Mpumalanga and KwaZulu-Natal Provinces of South Africa in November 2014 and 2015. The four collection sites were near Howick, Melmoth, Vryheid and Commondale (Fig. 1A). Howick and Commondale were sampled in 2014 and Melmoth and Vryheid in 2015. These sites were chosen where plantations of non-native *E. grandis* and *A. mearnsii* trees and native natural forests were located in close proximity (Fig. 1C–D). The age of the plantations was between 10 and 15 y for *E. grandis* and 8–10 y for *A. mearnsii*. The natural forests were healthy protected remnants with high plant species diversity typical of the region. Some common native trees included *Allophylus natalensis, Bequaertiodendron natalense, Celtis africana, Combretum krausii, Curtisia dentate, Cussonia spicata, Ekebergia capensis, Euclea natalensis, Heteropyxis natalensis, Ilex mitis, Kiggelaria africana, Millettia grandis, Ocotea bullata, Podocarpus latifolius, Prunus africana, Sideroxylon inerme, Vepris undulate* along with various species of *Eugenia* and *Syzygium*.

A total of 1200 soil samples were collected from these four sites (4 sites × 3 vegetation types × 10 plots × 10 trees). Ten plots within each plantation as well as adjacent natural forest were selected arbitrarily (Fig. 1A). Soil samples along with fine roots were arbitrarily collected from the rhizosphere of 10 trees within each 10 × 10 m plot after removing the plant debris and 4–5 cm of topsoil. These 10 soil samples from each plot were merged together thereafter 2 kg of this composite soil mix served as one sample (Fig. 1A). A portion of the 120 composite soil samples (4 sites × 3 vegetation types × 10 plots) was used for soil baiting, while the remaining were air-dried at room temperature (22–25 °C) for metabarcoding.

2.2. Isolation of Phytophthora using soil baiting

All 120 composite soil samples were baited in a controlled environment where the temperature was kept between 22 and 25 °C and the humidity between 70 and 75%. Each of the soil samples was baited separately in a 24 × 14 × 6 cm plastic trough using 300 g of soil following the protocol of the Centre for *Phytophthora* Science and Management (CPSM), Murdoch University. Soils were mixed thoroughly and pre-moistened overnight before flooding with water to a depth, twice that of the soil. After removing the floating debris, two leaves each of *Duranta repens, Hedera helix*, *Hibiscus rosa-sinensis, Rhododendron indicum*, white rose petals and cotyledonous leaves of *Eucalyptus sieberi* were added and served as baits. The baits were monitored regularly for 10 d for signs of infection. Lesions from infected baits were plated onto *Phytophthora*-selective medium, NARPH (Masago et al., 1977), followed by the establishment of pure cultures. Pure cultures were maintained on 10% clarified V8-Agar (10 ml clarified V8 juice, Campbell Soup Company USA; 15 g Difco™ Agar, Becton, Dickinson and Company, Sparks, USA) as well as half-strength Potato Dextrose Agar (PDA; Becton, Dickinson and Company, Sparks, USA, 19.5 g PDA powder, 7.5 g of agar and 1L of distilled water) and also as agar plugs in glass vials with sterile deionized water. Where the initial baiting did not show any signs of infection on the baits, the same soil was re-baited after drying at room temperature (22–25 °C).

2.3. Identification of Phytophthora isolates recovered through baiting

Phytophthora isolates were grown on half strength PDA in Petri dishes at 20 °C for 10 d. Mycelium was harvested from each isolate by scraping this from the agar surface. Thereafter, genomic DNA was extracted using ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research, USA) following the manufacturer's protocol. The region spanning the internal transcribed spacer region (ITS1-5.8S-ITS2) of ribosomal DNA was amplified using the primers ITS6 (Cooke et al., 2000) and ITS4 (White et al., 1990). Individual PCRs were performed using 5 × GoTaq Flexi Buffer (Promega, MI) – 5 μl, 25 mM $MgCl_2$ (Promega, MI) - 2.5 μl, 0.1 mM dNTPs (Promega, MI) - 1.5 μl, BSA (Amresco, OH) – 1 μl, 1U GoTaq Hot Start Polymerase (Promega, MI), 0.5 μl of each primer and the final volume was made up to 25 μl with PCR grade water. The PCRs were carried out with initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min and final elongation at 72 °C for 5 min. The PCR products were sequenced with an ABI PRISM BigDye® Terminator Cycle Sequencing Kit 3.1 (Life Technologies-Applied Biosystems, Foster City, CA). Electrophoresis was

Fig. 1. (A) Soil sampling sites and strategy across Mpumalanga and KwaZulu-Natal provinces of South Africa. Sampling strategy at Commondale is illustrated here as an example. List of *Phytophthora* species detected at each environment is indicated in close-up maps for each site (C) Howick, (D) Commondale, (E) Melmoth, and (F) Vryheid. In C-F, taxa names in blue bold font were recovered through both soil baiting and metabarcoding. Taxa names in green bold font were recovered only through soil baiting. Numbers within parenthesis indicates the number of isolates recovered through soil baiting. The three vegetation types are indicated as ■ = *Acacia mearnsii*, ● = *Eucalyptus grandis* and ▲ = Natural forests.

performed by the DNA Sequencing Facility of the University of Pretoria. Geneious R8 (Kearse et al., 2012) was used for assembling the amplicons. All the *Phytophthora* species were identified using BLAST available via NCBI GenBank through 100% sequence similarity. All the complete ITS sequences of the isolates obtained in this study were deposited in GenBank and cultures are maintained in the microbial culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table S1).

2.4. Metabarcoding and analysis of data

About 50 g of each of the 120 composite soil samples were pulverized using a Retsch ® grinding jar attached to a Qiagen® TissueLyser II. DNA was extracted from 500 mg of each soil sample in duplicate using the Mo-Bio PowerSoil® DNA Isolation Kit (Carlsbad, CA). Environmental DNA (eDNA) amplifications and amplicon library generation were carried out using a nested PCR approach following Scibetta et al. (2012) and Català et al. (2015). Autoclaved fine sand served as controls. For each pyrosequencing run there were two sets of controls. These included (1) grinding controls where sterile sand was ground during the pulverization process to serve as a sample and (2) eDNA extraction controls where for each set of eDNA extractions, 0.5 g of autoclaved sand served as a control sample. Grinding and eDNA extraction controls were assigned Multiplex Identifiers (MIDs) and processed with the same protocol as soil samples, although no product could be visualized on the gel during electrophoresis.

PCR products were visualized using 1% agarose gel electrophoresis and then pooled based on band intensity into groups of 5–6 (total volume 30 μl). Each group was cleaned twice with Agencourt AMPure XP PCR purification beads (Beckman Coulter Genomics, USA) following the manufacturer's instructions. After cleaning, the PCR products were again visualized on an agarose gel. The samples were further pooled into a single unit based on the band intensity to standardize the DNA contribution for each sample. 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. The amplicons were sequenced at the Western Australian State Agricultural Biotechnology Centre (SABC), Murdoch University following the Roche GS Junior Sequencing Method Manual (March 2012) using GS Junior Titanium Chemistry and GS Junior Pico Titre Plates (454 Life Sciences/Roche Applied Biosystems, Nutley, NJ, USA). The reads were analyzed and clustered into molecular operational taxonomic units (MOTUs) based on 99% similarity using Geneious R8. Metabarcoding data is available at the NCBI under the accession numbers SRX3228069 and SRX3228070.

Initial species identification was carried out based on sequence similarity against a reference database containing ITS1 sequences from 192 *Phytophthora* taxa including 169 identified species and 23 designated, but as yet undescribed, *Phytophthora* species made available by the CPSM (see Burgess et al., 2017a). For the purpose of phylogenetic identification of the MOTUs, the database was divided into five groups: (1) clades 1 and 2, (2) clades 3 and 4, (3) clades 5 and 6, (4) clades 7 and 8 and (5) clade 9 and 10 in order to increase resolution within a clade. All the datasets were aligned using MAFFT (Katoh et al., 2002) available via Geneious R8. Phylogenetic analyses using maximum likelihood (ML) approach were performed using RAxML v8 (Stamatakis, 2014). The general time reversible model along with gamma distribution (GTR GAMMA) was selected using jModelTest 2.1 (Guindon and Gascuel, 2003; Darriba et al., 2012). Fifty replicated likelihood searches were executed for each dataset followed by 1000 bootstrap replicates. The resultant trees were rooted and modified using FigTree v1.4 and Adobe Illustrator CS6.

2.5. Statistical analyses

For isolates recovered by soil baiting, a Chi-square test was conducted to determine whether the total number of *Phytophthora* species differed between the sites and the vegetation types.

Phylotypes of *Phytophthora* species recovered through metabarcoding were analyzed after consolidating the data for each vegetation type (4 sites × 3 vegetation types). Presence/absence data was used rather than abundance data because of sequencing bias, which has been highlighted as a problem (Catalá et al., 2015). To visualize variation in *Phytophthora* species community composition among the soil samples, a non-metric multidimensional scaling (NMDS) of *Phytophthora* species was conducted using Jaccard distance (k = 3) and the "metaMDS" function in the vegan package in R (Oksanen et al., 2015). Moreover, the "ordiellipse" function available in the vegan package (R core Team, 2018) was used to generate confidence ellipses (conf = 0.95) to cluster points based on the vegetation type. To asses differences among the four sampling sites (Commondale, Howick, Melmoth and Vryheid) and the three vegetation types (plantations of *E. grandis* and *A. mearnsii* and natural forests) on community composition, a permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001) was performed using the "adonis" function and the Jaccard's dissimilarity index in the vegan package (R core Team, 2018).

To analyze oomycete diversity, *Phytophthora* species richness was calculated for each of the 12 samples. The effects of the sampling site and the vegetation type were analyzed with a generalized linear model, where the dependent variable richness fitted a Poisson distribution (R Core Team, 2018). Finally, *Phytophthora* species that were shared between vegetation types were visualized using a Venn diagram constructed in R with the VennDiagram package (R Core Team, 2018).

3. Results

3.1. Phytophthora isolates recovered through baiting

In total, 85 isolates of *Phytophthora* were recovered using baiting (Fig. 1C–F; Table S1). Based on the sequence similarity search using BLAST (Altschul et al., 1990), the isolates represented five taxa: *P. alticola, P. cinnamomi, P. frigida, P. multivora* and *P. pseudocryptogea*. Most isolates were identified as *P. frigida* (33) and *P. cinnamomi* (32) (Table S1). Among the baits used white rose petal was the most efficient followed by *D. repens*, *E. sieberi*, *R. indicum*, *H. helix* and *H. rosa-sinensis* (Table S1).

The total number of isolates of each species differed significantly ($P < 0.05$) across vegetation types (plantations of *E. grandis*, *A. mearnsii* and natural forest). Most of the isolates were recovered from plantations of non-native *A. mearnsii*, followed by natural forest and lastly plantations of non-native *E. grandis*. When the five *Phytophthora* species were taken into consideration separately there was no significant difference across the vegetation types.

3.2. Phytophthora species detected from soil eDNA

The two pyrosequencing runs collectively generated 123,459 reads (approximately 71.3% of the wells gave good quality reads), which corresponded to 314 MOTUs. The average read length was 306 bp. Approximately 98.4% of the reads corresponded to *Phytophthora* and about 0.5–1% of these reads were chimeras. Chimeras were discarded after making alignments of consensus MOTUs for each barcode. The MOTUs were initially identified using BLAST against a reference database with ITS1 sequences of 192 *Phytophthora* species and undescribed (but designated) taxa. After phylogenetic analysis each MOTU was assigned an identity (Fig. 2).

Some closely related species relevant to this study could not be separated exclusively based on ITS1 sequences: (1) *P. plurivora, P. acerina* and *P. pini* and (2) *P. gregata*, *P. gibbosa* and *P.* taxon raspberry (Fig. 2). In order not to complicate results, these are hitherto referred to as either *P. plurivora* complex or *P. gregata* complex (Fig. 2).

Clustering of the MOTUs and phylogenetic identification revealed 32 distinct *Phytophthora* phylotypes (Table S2, Fig. 2). These mostly corresponded to well-defined taxa; two represented informally described species, while six were identified as putatively new phylotypes (one each from Clade 1, 2, 3, 5, 7 and 10, Fig. 2). Of the 32 *Phytophthora* species detected by metabarcoding, the

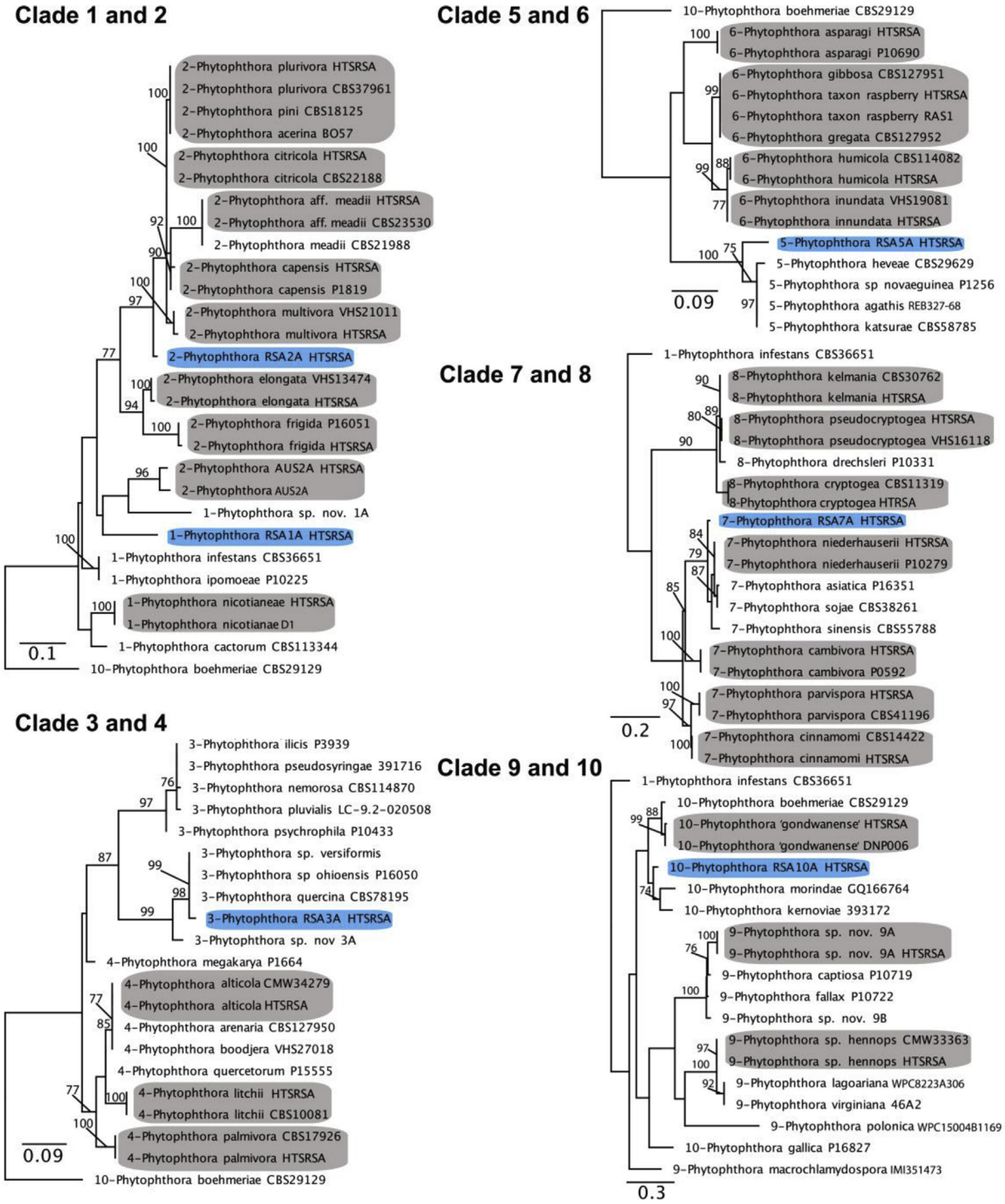

Fig. 2. Internal Transcribed Spacer 1 (ITS1) phylogenies of *Phytophthora* species recovered through metabarcoding. MOTUs that were designated as new phylotypes are highlighted in blue. MOTUs that clustered with well-defined *Phytophthora* species are highlighted in grey. Suffix HTRSA indicates MOTUs recovered through high throughput sequencing from South Africa. Although ITS1 is highly variable still some species could not be separated based on it alone. Hence, these species are grouped within the same coloured block and have been referred to as a complex throughout the article.

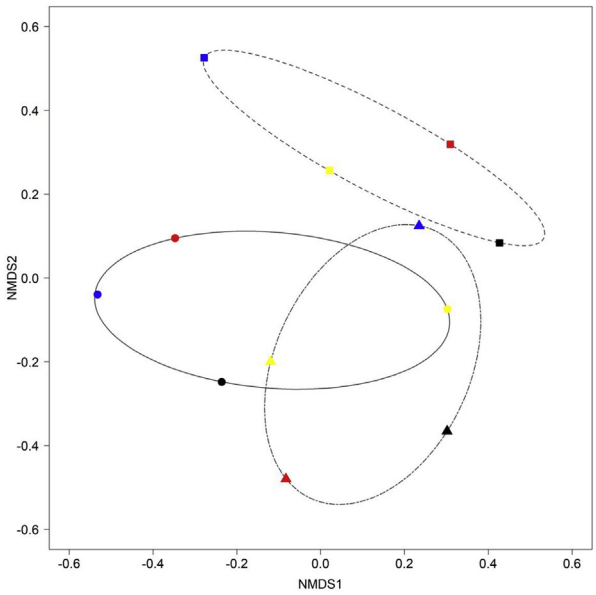

Fig. 3. Non-metric multidimensional scaling (nMDS) of *Phytophthora* species identified through metabarcoding (presence-absence data), among the four sites (red = Howick, black = Melmoth, yellow = Vryheid and blue = Commondale) and the three vegetation types (■ = *Acacia mearnsii*, ● = *Eucalyptus grandis*, ▲ = Natural forests). Confidence ellipses (conf = 0.95) were drawn to cluster the data points based on vegetation types.

greatest numbers of MOTUs were recovered for *P. frigida, P. alticola, P. parvispora, P. niederhauserii,* and *Phytophthora* RSA5A (Table S2). Twelve species were new reports from South Africa and these included *P. parvispora, P. lichii, P. pseudocryptogea, P. 'kelmania', P. humicola, P.* aff. *meadii, P. gondwanense, P. asparagi, P. elongata, P. gregata* complex, *P. inundata* and *P. cambivora.* Two undescribed Australian species (Burgess et al., 2017a) were also identified as *Phytophthora* AUS2A and *Phytophthora* AUS9A (Fig. 1C–F; Table S2).

The community composition of the *Phytophthora* species from the soil samples was different between the vegetation types, but not between the sampling sites. The NMDS plot supported the difference in *Phytophthora* species between the three vegetation types (Fig. 3). PERMANOVA confirmed that vegetation type was the only factor significantly explaining the variation in *Phytophthora* species ($r^2 = 0.309$, $P < 0.01$). Moreover, *Phytophthora* species richness was influence by site not by vegetation type ($P < 0.05$ and $P > 0.05$, respectively). The greatest species richness was recorded from the native natural forests at Melmoth where 27 species were detected. The Venn diagram shows that, of the 32 *Phytophthora* species detected, 13 were recorded from all three vegetation types (Fig. 4).

4. Discussion

Metabarcoding using *Phytophthora* specific primers to amplify eDNA extracted from forest and plantation soils in South Africa

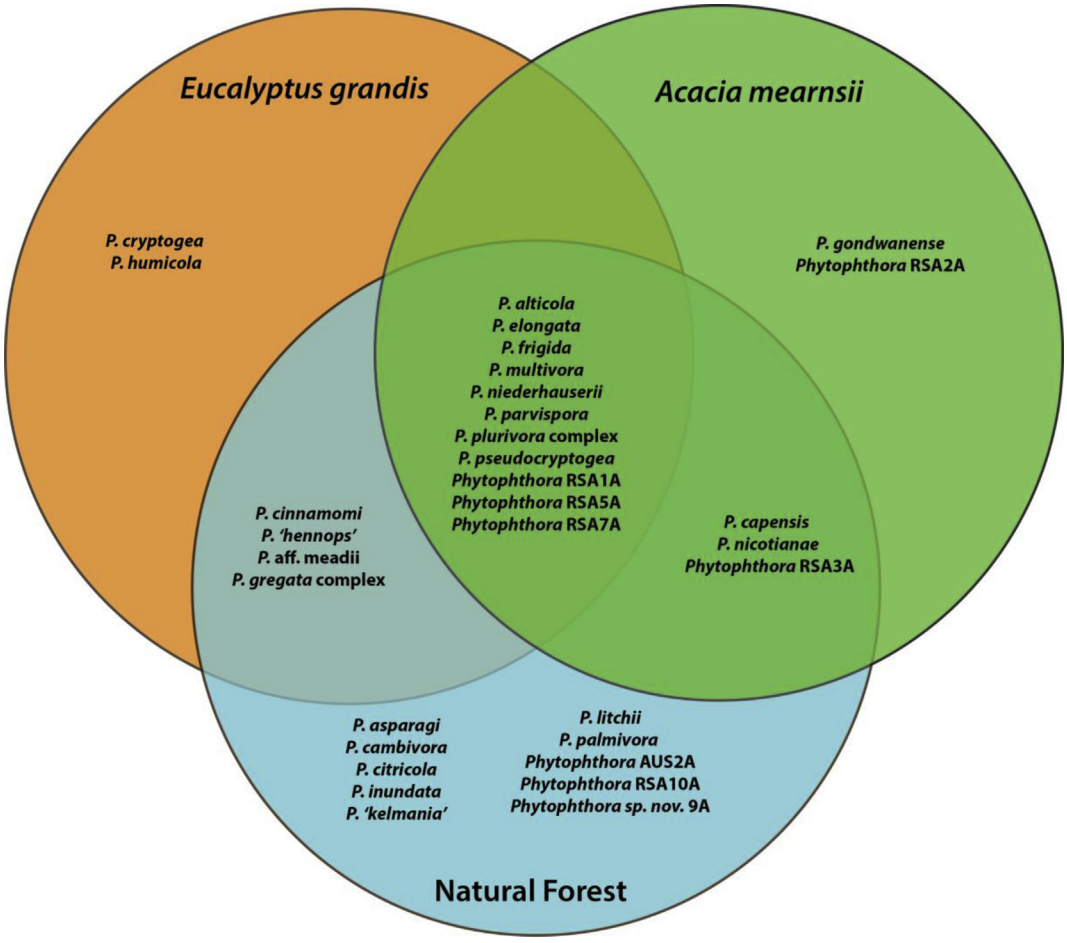

Fig. 4. Venn diagram showing the distribution of *Phytophthora* species identified through metabarcoding among the three vegetation types.

detected 32 *Phytophthora* species across 10 clades recognized within the complete *Phytophthora* phylogeny. These included seven undescribed phylotypes and 14 new records for South Africa. The majority of the *Phytophthora* species from natural forests were also recovered from the adjacent plantations of non-native *E. grandis* and *A. mearnsii.* Both plantations of non-native trees and natural forests had exclusive *Phytophthora* species. However, the natural forests had greater numbers of exclusive *Phytophthora* species than the plantations. *Phytophthora* species composition was influenced by vegetation type, while *Phytophthora* species richness was influenced by site.

The number of *Phytophthora* species detected in the current study was comparable to similar investigations (Vannini et al., 2013; Català et al. 2015, 2017; Prigigallo et al., 2016; Burgess et al., 2017a). Of those studies, Burgess et al. (2017a) detected the greatest number of species (68) in a survey of over 500 sites across Australia. The remaining studies (Vannini et al., 2013 (15), Català et al., 2015 (36), Prigigallo et al., 2016 (15), and Català et al., 2017 (14)) were comparable in size and scope to the current study and detected a similar number of species.

Two previous studies (Català et al., 2015; Burgess et al., 2017a), and the present investigation, examined natural ecosystems with diverse habitat types. Natural ecosystems have consistently yielded the greatest number of *Phytophthora* species (Català et al., 2015; Burgess et al., 2017a). However, our findings suggest that *Phytophthora* species richness is linked to sites. This could be due to either a variation in silviculture practices or the local climate. Plantations at all sites were owned by different forestry companies. Hence, post-harvest soil treatments, sourcing of saplings as well as post-planting silviculture practices would vary between the forestry companies. These silviculture practices would most likely affect the soil microbial community. The sampling sites also stretched across three different climatic zones that would certainly have influenced the *Phytophthora* species richness at different sites.

Community composition of *Phytophthora* species in the present study differed among vegetation types. In particular, the community composition in the *A. mearnsii* plantations was significantly different from the natural forest and *E. grandis*. The sampled plantations were 10–15 y old and had been established on areas of cleared native vegetation. The *Phytophthora* community composition was most likely the same in both the planted forest environments (*A. mearnsii* or *E. grandis*) originally, but would have altered over time due to differences in host plants. The differences in *Phytophthora* species found in the *A. mearnsii* plantations and in the adjacent native forest could have been due *Phytophthora* species introduced into the plantations from nurseries during the establishment phase, as commonly occurs in Europe (Jung et al., 2016).

The rare or new *Phytophthora* species detected in the present study were not isolated using soil-baiting, a finding echoed in other studies considering both direct baiting and metabarcoding (Vannini et al., 2013; Khaliq et al., 2018). The discrepancy between isolation success and molecular detection could be due to several factors. Metabarcoding would detect a species even if it was dead. Some species, especially those unknown in culture, could be obligate biotrophs and not culturable, as has been found for the related genus *Peronospora* (Cooke et al., 2002). Efficacy of baits can also influence the variety of *Phytophthora* species recovered (Cooke et al., 2007; O'Brien et al., 2009), and this might have been a factor in the present study. However, Reeser et al. (2011) concluded that the type of bait was not important, but rather how it was handled. Likewise, antibiotics used in the selective media, low inoculum levels and dormant propagules could also have affected isolation success (Jeffers and Martin, 1986; Drenth and Sendall, 2001).

Metabarcoding allows identification of several *Phytophthora* species without isolation into culture, but it also has various limitations (Huse et al., 2007). The ITS1 gene region is highly variable in *Phytophthora* but it cannot separate some closely related species (Català et al., 2015) including, for example, *P. plurivora, P. acerina* and *P. pini* in the present study. The key limitation here is the lack of living cultures to allow the inclusion of data for other gene regions. Additionally, the 454-platform has sequencing bias using these *Phytophthora* specific primers (Català et al. 2015) and thus interpretation of results such as those in the present study must be predominantly qualitative.

The known *Phytophthora* diversity in South Africa, including those revealed in this study, most likely includes both native and introduced species. This has been shown for many countries where biodiversity studies have used traditional isolation methods, including Europe and North America (Hansen et al., 2012), Argentina (Greslebin et al., 2005) and South Africa (Oh et al., 2013). It is also true for investigations including the present study, applying high-throughput sequencing platforms (Vannini et al., 2013; Català et al. 2015, 2017; Prigigallo et al., 2016; Burgess et al., 2017a). Among the 32 *Phytophthora* species detected in the present study, *P. frigida, P. capensis, P.* 'hennops', and *P. alticola* and the newly identified species *Phytophthora* RSA1A, RSA2A, RSA3A, RSA5A, RSA7A and RSA10A are known only from South Africa (Maseko et al., 2002; Oh et al., 2013; Bose et al., 2017), and they could be native to the country. *Phytophthora* AUS2A, *P. elongata, P. gondwanense* and *P.* 'kelmania' have been reported from at least one other country apart from South Africa.

In South Africa, *Phytophthora* species infect and impact both *Eucalyptus* and *Acacia mearnsii* plantations. Among the 32 *Phytophthora* species detected in the present study, 20 were either new reports or new phylotypes; their pathogenicity toward *E. grandis* and *A. mearnsii* is unknown. Both *P. nicotianae* and *P. capensis* were detected from natural forests and *A. mearnsii* plantations. The former species is a pathogen of *A. mearnsii,* while the later species infects *Curtisia dentata* (Bezuidenhout et al., 2010), a species commonly observed in the natural forests surveyed in the present study. *Phytophthora cinnamomi* was detected from *E. grandis* and natural forest and has been previously reported to infect *Eucalyptus* and native trees in South Africa (Nagel et al., 2013). Among the species shared between all three environments, *P. alticola* and *P. frigida* are known pathogens of various *Eucalyptus* species grown in South Africa (Maseko et al., 2007).

Several *Phytophthora* species detected in the present study were previously unknown in South Africa, but are known as pathogens elsewhere in the world. The global dispersal of *Phytophthora,* especially species known in agriculture, would have been very common in the past and continues today through the live plant trade (Eschen et al., 2015). This has been clearly documented for well-known pathogens such as *P. cinnamomi* (Burgess et al., 2017b). Thus, the *Phytophthora* species newly detected in the present study, but already known from other parts of the globe, most likely entered South Africa through trade of live plant materials and agricultural commodities as has been demonstrated for the root-rot pathogen *Armillaria mellea* (Coetzee et al., 2001).

In conclusion, and in response to the proposed hypotheses, community composition of *Phytophthora* species differed significantly between the three vegetation types but not across sites. High-throughput sequencing platforms have positively influenced studies focused on species discovery and distribution of *Phytophthora* species globally. Results of the present study contribute to our knowledge of the community composition of *Phytophthora* species in South Africa. Future surveys should include many other areas of the country, such as the Cape Floristic Region in the Western Cape province, the Garden Route National Park in the Eastern Cape province and the Soutpansberg Afromontane region in the

Limpopo province, where some of the world's most diverse flora occur.

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Supplementary data

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26, 32–46.

Bezuidenhout, C.M., Denman, S., Kirk, S.A., Botha, W.J., Mostert, L., McLeod, A., 2010. *Phytophthora* taxa associated with cultivated *Agathosma*, with emphasis on the *P. citricola* complex and *P. capensis sp. nov*. Persoonia 25, 32–49.

Bose, T., Burgess, T.I., Roux, J., Wingfield, M.J., 2017. *Phytophthora alticola*; revised description based on new collections and a neotype. Sydowia 69, 161–170.

Burgess, T., White, D., McDougall, K.M., Garnas, J., Dunstan, W.A., Català, S., Carnegie, A.J., Worboys, S., Cahill, D., Vettraino, A.M., Stukely, M.J.C., Liew, E.C.Y., Paap, T., Bose, T., Migliorini, D., Williams, B., Brigg, F., Crane, C., Rudman, T., Hardy, GEStJ., 2017a. *Phytophthora* distribution and diversity across Australia. Pac. Conserv. Biol. 23, 1–13.

Burgess, T.I., Scott, J.K., Mcdougall, K.L., Stukely, M.J.C., Crane, C., Dunstan, W.A., Brigg, F., Andjic, V., White, D., Rudman, T., Arentz, F., 2017b. Current and projected global distribution of *Phytophthora cinnamomi*, one of the world's worst plant pathogens. Global Change Biol. 23, 1661–1674.

Català, S., Berbegal, M., Pérez-Sierra, A., Abad-Campos, P., 2017. Metabarcoding and development of new real-time specific assays reveal *Phytophthora* species diversity in holm oak forests in eastern Spain. Plant Pathol. 66, 115–123.

Català, S., Pérez-Sierra, A., Abad-Campos, P., 2015. The use of genus-specific amplicon pyrosequencing to assess *Phytophthora* species diversity using eDNA from soil and water in northern Spain. PLoS One 10 e0119311.

Coetzee, M.P., Wingfield, B.D., Harrington, T.C., Steimel, J., Coutinho, T.A., Wingfield, M.J., 2001. The root rot fungus *Armillaria mellea* introduced into South Africa by early Dutch settlers. Mol. Ecol. 10, 387–396.

Cooke, D.E., Drenth, A., Duncan, J.M., Wagels, G., Brasier, C.M., 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. Fungal Genet. Biol. 30, 17–32.

Cooke, D.E., Schena, L., Cacciola, S.O., 2007. Tools to detect, identify and monitor *Phytophthora* species in natural ecosystems. J. Plant Pathol. 89, 13–28.

Cooke, D.E., Williams, N.A., Williamson, B., Duncan, J.M., 2002. An ITS-based phylogenetic analysis of the relationships between *Peronospora* and *Phytophthora*. In: Spencer-Phillips, P.T.N., Gisi, U., Lebeda, A. (Eds.), Advances in Downy Mildew Research. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 161–165.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772-772.

Doidge, E.M., 1925. Brown rot in citrus fruits (*Phythiacystis citrophthora* (R. and E. Sm.). J. Dep. Agric. (Repub. Irel.) 1920 (10), 499–503. Union of South Africa.

Drenth, A., Sendall, B., 2001. Practical Guide to Detection and Identification of *Phytophthora*, Version 1.0. CRC for Tropical Plant Protection, Brisbane, pp. 1–39.

Erwin, D.C., Ribeiro, O.K., 1996. *Phytophthora* Diseases Worldwide. American Phytopathological Society, St. Paul, Minnesota.

Eschen, R., Britton, K., Brockerhoff, E., Burgess, T.I., Dalley, V., Epanchin-Niell, R.S., Gupta, K., Hardy, GEStJ., Huang, Y., Kenis, M., Kimani, E., 2015. International variation in phytosanitary legislation and regulations governing importation of plants for planting. Environ. Sci. Pol. 51, 228–237.

Greslebin, A.G., Hansen, E.M., Winton, L.M., Rajchenberg, M., 2005. *Phytophthora* species from declining *Austrocedrus chilensis* forests in Patagonia, Argentina. Mycologia 97, 218–228.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Haas, B.J., Kamoun, S., Zody, M.C., Jiang, R.H., Handsaker, R.E., Cano, L.M., Grabherr, M., Kodira, C.D., Raffaele, S., Torto-Alalibo, T., Bozkurt, T.O., 2009. Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. Nature 461, 393–398.

Hansen, E.M., Reeser, P.W., Sutton, W., 2012. *Phytophthora* beyond agriculture. Annu. Rev. Phytopathol. 50, 359–378.

Huse, S.M., Huber, J.A., Morrison, H.G., Sogin, M.L., Welch, D.M., 2007. Accuracy and quality of massively parallel DNA pyrosequencing. Genome Biol. 8, R143.

Jeffers, S.N., Martin, S.B., 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Dis. 70, 1038–1043.

Jung, T., Burgess, T.I., 2009. Re-evaluation of *Phytophthora citricola* isolates from multiple woody hosts in Europe and North America reveals a new species, *Phytophthora plurivora sp. nov*. Persoonia 22, 95.

Jung, T., Orlikowski, L., Henricot, B., Abad-Campos, P., Aday, A.G., Aguín Casal, O., Bakonyi, J., Cacciola, S.O., Cech, T., Chavarriaga, D., Corcobado, T., 2016. Widespread *Phytophthora* infestations in European nurseries put forest, semi-natural and horticultural ecosystems at high risk of *Phytophthora* diseases. For. Pathol. 46, 134–163.

Katoh, K., Misawa, K., Kuma, K.I., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059–3066.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649.

Khaliq, I., Hardy, GEStJ., White, D., Burgess, T.I., 2018. eDNA from roots: a robust tool for determining *Phytophthora* communities in natural ecosystems. FEMS Microbial Ecology 94 fiy048.

Kotze, J.M., Moll, J.N., Darvas, J.M., 1987. Root rot control in South Africa: past, present and future. South Afr. Avocado.Grow. Assoc.Yearbk. 11, 89–91.

Linde, C., Kemp, G.H., Wingfield, M.J., 1994. *Pythium* and *Phytophthora* species associated with eucalypts and pines in South Africa. Eur. J. For. Pathol. 24, 345–356.

Masago, H., Yoshikawa, M., Fukada, M., Nakanishi, N., 1977. Selective inhibition of *Pythium* spp. on a medium for direct isolation of *Phytophthora* spp. from soils and plants. Phytopathology 67, 425–428.

Maseko, B.O., Burgess, T.I., Coutinho, T.A., Wingfield, M.J., 2007. Two new *Phytophthora* species from South african *Eucalyptus* plantations. Mycol. Res. 111, 1321–1338.

Maseko, B.O., Coutinho, T.A., van Staden, J., 2002. Pathogenicity of *Phytophthora* and *Pythium* species associated with citrus root rot in South Africa. South Afr. J. Bot. 68, 327–332.

Milne, D.L., Brodrick, H.T., Hughes, J.P., 1975. Progress in solving the *Phytophthora* problem when replanting avocados. Citrus Subtrop. Fruit J. 502, 22–24.

Milne, D.L., Hughes, J.P., Brodrick, H.T., de Villiers, E.A., Frean, R.T., 1974. Attempts to control *Phytophthora* root rot on established avocado trees. Fruit World Mark. Grow. 478, 5–7.

Nagel, J.H., Gryzenhout, M., Slippers, B., Wingfield, M.J., 2013. The occurrence and impact of *Phytophthora* on the African continent. In: Lamour, K. (Ed.), *Phytophthora*: a Global Perspective. CABI International, United Kingdom, pp. 204–214.

O'Brien, P.A., Williams, N., Hardy, GEStJ., 2009. Detecting *Phytophthora*. Crit. Rev. Microbiol. 35, 169–181.

Oh, E., Gryzenhout, M., Wingfield, B.D., Wingfield, M.J., Burgess, T.I., 2013. Surveys of soil and water reveal a goldmine of *Phytophthora* diversity in South African natural ecosystems. IMA Fungus 4, 123–131.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H., Wagner, H., 2015. Vegan: Community Ecology Package. R Package Version 3.2, vol. 3, pp. 117–118.

Prigigallo, M.I., Abdelfattah, A., Cacciola, S.O., Faedda, R., Sanzani, S.M., Cooke, D.E., Schena, L., 2016. Metabarcoding analysis of *Phytophthora* diversity using genus-specific primers and 454 pyrosequencing. Phytopathology 106, 305–313.

R Core Team, 2018. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL. https://www.R-project.org/.

Reeser, P.W., Sutton, W., Hansen, E.M., Remigi, P., Adams, G.C., 2011. *Phytophthora* species in forest streams in Oregon and Alaska. Mycologia 103, 22–35.

Ribeiro, O.K., 2013. A historical perspective of *Phytophthora*. In: Lamour, K. (Ed.), *Phytophthora*: a Global Perspective, pp. 1–10.

Roux, J., Wingfield, M.J., 1997. Survey and virulence of fungi occurring on diseased *Acacia mearnsii* in South Africa. For. Ecol. Manag. 99, 327–336.

Safaiefarahani, B., Mostowfizadeh-Ghalamfarsa, R., Hardy, GEStJ., Burgess, T.I., 2015. Re-evaluation of the *Phytophthora cryptogea* species complex and the description of a new species, *Phytophthora pseudocryptogea sp. nov*. Mycol. Prog. 14, 108.

Scibetta, S., Schena, L., Chimento, A., Cacciola, S.O., Cooke, D.E., 2012. A molecular method to assess *Phytophthora* diversity in environmental samples. J. Microbiol. Meth. 88, 356–368.
Scott, P., Burgess, T.I., Hardy, GEStJ., 2013. Globalization and *Phytophthora*. In: Lamour, K. (Ed.), *Phytophthora*: a Global Perspective. CABI International, United Kingdom, pp. 226–232.
Scott, P.M., Burgess, T.I., Barber, P.A., Shearer, B.L., Stukely, M.J., Hardy, GEStJ., Jung, T., 2009. *Phytophthora multivora sp. nov.*, a new species recovered from declining *Eucalyptus, Banksia, Agonis* and other plant species in Western Australia. Persoonia 22, 1–13.
Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313.
Van Wyk, P.S., 1973. Root and crown rot of silver trees. J. S. Afr. Bot. 39, 255–260.
Vannini, A., Bruni, N., Tomassini, A., Franceschini, S., Vettraino, A.M., 2013. Pyrosequencing of environmental soil samples reveals biodiversity of the *Phytophthora* resident community in chestnut forests. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 85, 433–442.
Von Broembsen, S., 1984. Occurrence of *Phytophthora cinnamomi* on indigenous and exotic hosts in South Africa, with special reference to the South-Western Cape Province. Phytophylactica 16, 221–225.
Von Maltitz, P.M., Von Broembsen, S., 1985. Lemon shoot tip blight caused by *Phytophthora citricola* in propagation tunnel. Phytophylactica 17, 47–48.
White, T.J., Bruns, T., Lee, S.J., Taylor, J.L., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18, 315–322.
Zeijlemaker, F.C.J., 1971. Black-butt disease of black wattle caused by *Phytophthora nicotianae* var. *parasitica*. Phytopathology 61, 144–145.
Zeijlemaker, F.C.J., Margot, P., 1970. Black-butt Disease of Black Wattle. Report, vol. 1971. Wattle Research Institute, University of Natal, pp. 49–50.
Zeiljemaker, F.C.J., 1967. The Gummosis of Black Wattle: a Complex of Disease. in: Wattle Research Institute (Pietermaritzburg, South Africa) Report, pp. 40–43.