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ORIGINAL ARTICLE

Oomycete composition in *Proteaceae* orchards and natural stands on three continents

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Abstract

The *Proteaceae*, a diverse family of woody flowering plants in the Southern Hemisphere, contains many species known to be susceptible to *Phytophthora cinnamomi*, both in the natural environment and in cut-flower orchards. Very little is known about the prevalence of *P. cinnamomi* and other oomycetes across these landscapes. To address this knowledge gap, we used a double ITS1 and *RPS10* gene metabarcoding approach and traditional isolation protocols to investigate oomycetes in orchards and natural stands of *Proteaceae* across South Africa, South Africa (eastern and western), Australia, and Europe. The *RPS10* primers amplified more samples, including various *Pythium* species, while the ITS primers detected more *Phytophthora* phylotypes. Both datasets showed that geographic regions influenced oomycete species richness and community composition, while they did not show any variation between orchards and natural vegetation. *RPS10* metabarcoding detected the largest number of species and provided greater statistical confidence than ITS1 when considering oomycete species composition. Metabarcoding also showed that orchards had a higher abundance of *P. cinnamomi* compared to native stands, although this was not found when isolating through baiting of roots and rhizosphere soil. Direct isolation and metabarcoding are complementary, with metabarcoding serving as an early detection tool. However, it cannot distinguish living viable propagules from residual DNA of dead propagules, limiting its use for diagnostic purposes related to *Phytophthora* management and control. These results, along with those of other recent studies, show that metabarcoding offers an effective tool to describe the dynamics of soil oomycetes in different ecosystems.

Keywords High-throughput sequencing · *Phytophthora* · Proteas · Fynbos · Kwongan · Soil microorganisms · *RPS10* · ITS

Introduction

The *Proteaceae* is a large family of woody flowering plants native to the Southern Hemisphere (Weston and Barker 2006). These plants are remarkably diverse in regions with soils poor in nutrients and Mediterranean climates (Lambers et al. 2011) with approximately 1000 and 370 native species in Western Australia and South Africa, respectively (https://florabase.dpaw.wa.gov.au/science/key/proteaceae/, Crous et al. 2003; Sauquet et al. 2009). Additionally, numerous varieties and species hybrids are cultivated for cut flower production in many Mediterranean regions of Europe, Oceania, and Africa (https://www.proteaatlas.org.za/).

Despite their ecological and commercial importance, studies on the impact of oomycetes on *Proteaceae* are scarce and limited to the genus *Phytophthora*. South African literature is limited. It focuses on *Phytophthora* distribution in natural ecosystems and host response in productive plantations (Von Broembsen 1984a, b; Von Broembsen and Kruger 1985; Von Broembsen and Deacon 1997), and, with the exception of a 2023 survey on the critically endangered Fynbos *Proteaceae* species *Sorocephalus imbricatus* (Paap et al. 2023), it is not recent. In contrast, a greater number of studies have been conducted in Australia, including reports on cultivated *Proteaceae* (Hardy and Sivasithamparam 1988; Boersma et al.

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2000), endemic *Proteaceae* (Shearer and Dillon 1996; Wills 1993) and investigations on native hyper-diverse communities of local shrubland ecosystems dominated by *Banksia* and *Hakea* (Rea et al. 2011; Jung et al. 2011; Burgess et al. 2018, 2021a; Scott et al. 2009). Outside their native range, research reports limited to the diagnosis of *Phytophthora* have been conducted on *Proteaceae* imported for production purposes in Portugal (Madeira Island) and Hawaii (Rodrigues and Moura 2000; Nakao et al. 2002).

Studies on *Phytophthora* in soil samples have traditionally relied on baiting-based isolation techniques for samples from all environments and host associations. However, in the past few years, High-throughput sequencing of amplicons (metabarcoding) from environmental DNA (eDNA) has become the primary tool for studying *Phytophthora* community composition, ecology, and spread (Khaliq et al. 2019; Burgess et al. 2022). For example, large-scale surveys have explored *Phytophthora* biodiversity and distribution across Australia (Burgess et al. 2017b), a *Quercus ilex* forest in eastern Spain (Català et al. 2017), gardens and amenity woodland sites in Great Britain (Riddell et al. 2019; Green et al. 2021), as well as a rain forest in French Guiana (Legeay et al. 2020). Metabarcoding also has been successfully employed to investigate patterns of diversity and community assembly of *Phytophthora* in soil samples (Bose et al. 2018) and soil and root samples (Bose et al. 2021) of different vegetation types and across geo-climatic gradients (Redondo et al. 2018).

Most *Phytophthora* metabarcoding studies have used ITS1 *Phytophthora*-specific primers (Scibetta et al. 2012) as implemented for a metabarcoding protocol by Català et al. (2015). To extend the analysis to the whole soil-rhizosphere oomycete communities, alternative barcoding regions such as the *coxI* and *coxII* mitochondrial genes can be used (Sapp et al. 2019; Landa et al. 2021; Maciá-Vicente et al. 2020). In this regard, a new method was recently validated by Foster et al. (2022) using the mitochondrial *RPS10* gene (Martin et al. 2014). The advantages and disadvantages of the different primers used for metabarcoding with oomycetes have recently been reviewed by Burgess et al. (2022).

While implementing accurate metabarcoding protocols, oomycetes research also makes extensive use of traditional isolation techniques such as baiting (La Spada et al. 2022). Isolates are required for the diagnosis and taxonomic classification of new taxa (Abad et al. 2023; Pérez-Sierra et al. 2022). However, certain limitations may arise when using both techniques to analyze the same samples, raising the problem of simultaneous use of the two approaches in the same investigation. Metabarcoding, focused on *Phytophthora* diversity, frequently reveals additional species compared to those detected with baiting (Sarker et al. 2023a). Furthermore, in some recent works, the two techniques have provided non-comparable, discordant results (Bose et al. 2018; Khaliq et al. 2018, 2021; Landa et al. 2021).

This study aimed to compare the oomycetes community composition in the roots and rhizosphere soil of *Proteaceae* across different vegetative and geographical regions. The study included orchards in South Africa, Australia, Europe (Portugal and Italy) and natural bush areas in South Africa and Australia. This comparison was achieved using both direct isolations and ITS1 and *RPS10* metabarcoding methods. Specifically, we investigated i) whether oomycete species richness and composition differ between orchards and natural stands, ii) whether there are differences among the countries, and iii) whether soil baiting and metabarcoding produced comparable results for estimating *P. cinnamomi* distribution in *Proteaceae* orchards versus natural stands of these plants.

Materials and methods

Sites sampled

Ornamental *Proteaceae* orchards were selected in thirteen different locations across four countries. The sites were grouped in four different geographic areas, South Africa east (one in Gauteng, one in Kwa-Zulu Natal, two in Eastern Cape), South Africa west (four in Western Cape), Australia (three in Western Australia), and Europe (one in Italy and one in Portugal). Sampling was conducted between June 2016 and April 2017, during the season with local higher rain occurrence (summer in South Africa East, late spring in South Africa West, late autumn in Australia, mid-spring in Europe (Fig. 1A and Table S1).

Four sampling plots per location consisted of a 50–60 m transect with five sampling units equidistant along the transect, each consisting of 5 plants (4 sampling plots × 5 sampling units, 20 sampling units per location; Fig. 1B). Two sampling plots were surveyed and sampled within *Proteaceae* orchards, one planted with the cultivar "pink ice" (*Protea compacta* × *Protea susannae* P Matthews), and one planted with the cultivar "safari sunset" (*Leucadendron salignum* × *Leucadendron laureolum*; J Stevens and I Bell). Two additional plots were surveyed and sampled outside the cultivated orchards in natural vegetation containing *Proteaceae* vegetation. However, in Europe, where natural *Proteaceae* do not occur, sampling outside the orchards was conducted on native vegetation close to the plantation borders in Portugal. In Italy, where *Proteaceae* orchards were not present, samples were collected from a nursery located in a large area cultivated for intensive agricultural and nursery production, far from natural ecosystems. As a result, samples from native vegetation were not included in the collection from Italy.

Processing of samples

From each sampling unit, the under-canopy surface litter of each of the five plants was scrapped away on a

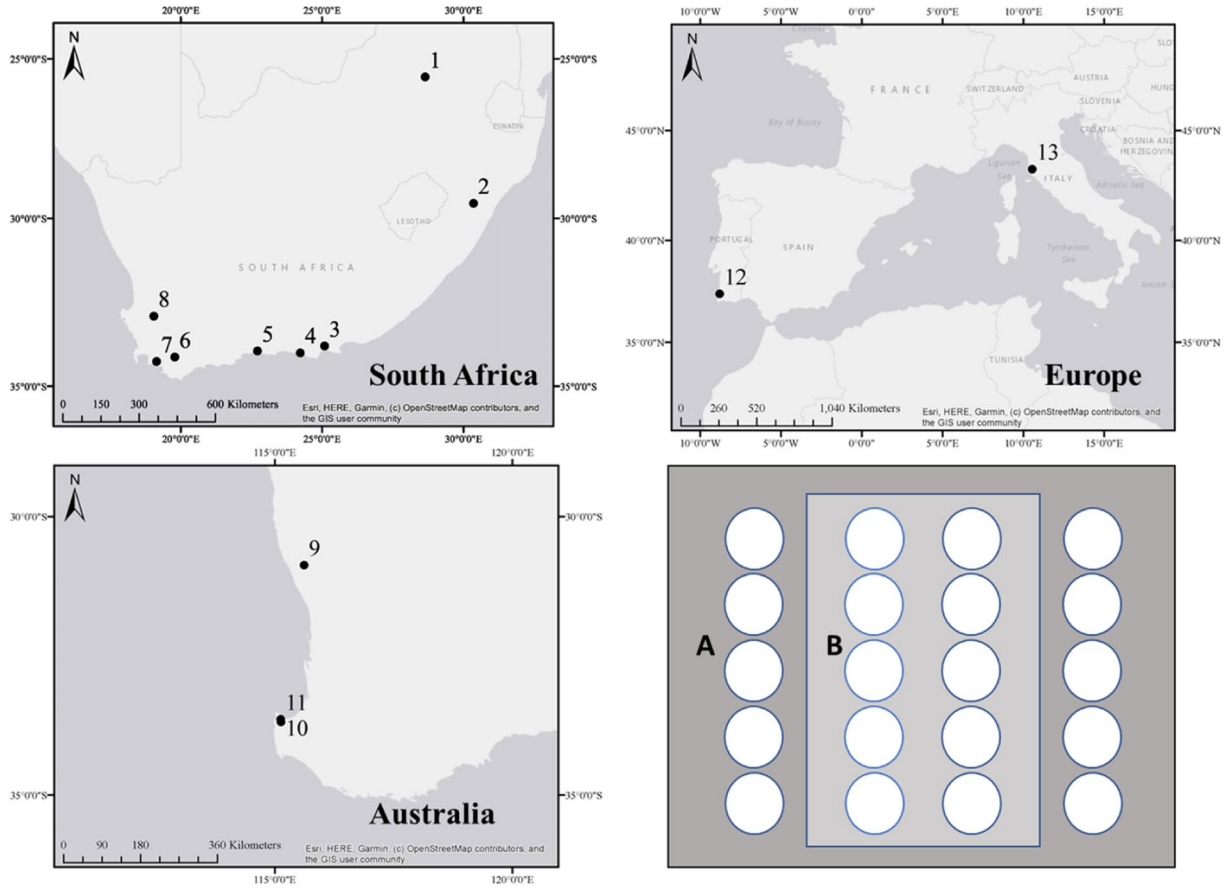

Fig. 1 Maps of the sampling locations in South Africa (east: 1 in Gauteng, 2 in Kwa-Zulu Natal, 3 and 4 in Eastern Cape; west: 5–8 in Western Cape), Australia (9–11) and Europe (12 in Portugal and 13 in Italy) and scheme of the sampling design in the left down corner of the image: four sampling plots per location consisting of a 50–60 m transect with five sampling units (the circles) equidistant along the transect for a total of twenty sampling units; two transects were realized in natural ecosystems vegetation out of the farm (**A**) and two inside the farm in planted *Proteaceae* (**B**); each sampling unit contained 5 plants

20 × 20 cm spot. At a depth of 15 cm, a soil sample of approximately 100 g including roots from the target plant was taken from each hole and mixed in a plastic bag to make a 500-g bulk sample. The bags were kept open to prevent condensation. On the same day, approximately 5 g of cluster roots was picked out of this bulk sample and allowed to dry naturally in paper bags for 7 days before being transferred to 2-mL microcentrifuge tubes (Pirouet) and stored at – 80 °C. The soil bound with the roots was not actively removed, although some fell away during the drying process. Consequently, eDNA was extracted from roots and attached to rhizosphere soil. These samples were then subjected to metabarcoding to detect the presence of oomycete species, while the remaining bulk samples were processed by baiting to isolate *Phytophthora* species. Sampling tools were cleaned between each sampling unit with a brush using water and commercial dishwashing detergent to remove all soil residues and then sprayed with 70% ethanol.

DNA extraction

Approximately 200 mg of each root sample was ground with a Qiagen TissueLyser attached to a grinding jar (Qiagen-manufactured by Retch) for 4 min at 24 Hz using two sterile 0.5-mm iron balls. Approximately 50 µg of each ground root sample was transferred into the extraction kit tubes, and the total genomic DNA was extracted using the Mo Bio Laboratories, Power Plant® Pro DNA Isolation Kit (Cat# 13400–50) following the manufacturer's protocol. A total of 273 samples were extracted, and the DNA was stored at – 20 °C.

Library preparation

Separate Illumina metabarcoding runs were conducted using two primer sets. The ITS1 gene region was amplified with *Phytophthora*-specific primers. A nested PCR

approach was used with *Phytophthora*-specific primers 18h2f and 5.8RBis (Scibetta et al. 2012) in the first round, and nested primers ITS6 and 5.8S-IR (Cooke et al. 2000; Scibetta et al. 2012) in the second round, following the methods of Scibetta et al. (2012) and Català et al. (2015). The 40S ribosomal protein S10 (*RPS10*) gene region was amplified with oomycete-specific primers (Foster et al. 2022). A nested approach was employed with PRV9-F and PRV9-R primers in the first round (Martin and Coffey 2012) and oomycete-specific primers *RPS10*-F and *RPS10*-R in the second round (http://oomycetedb.cgrb.oregonstate.edu/). In both cases, the primers used in the second round had Illumina MiSeq adapter sequences attached to the 5′ end, following standard protocols for the MiSeq platform (Illumina Demonstrated Protocols: Metagenomic Sequencing Library Preparation).

Each 25 µL PCR reaction included 12.5 µL of PCR buffer KAPA HiFi HotStart ReadyMix (KAPA Biosystems), 8 µL of PCR grade water, 1 µM of each primer (1 µL), and 2.5 µL of genomic DNA (for the first round) or 1 µL of the PCR product (for the second round). No-template negative PCR controls were included each time a PCR reaction was set up and carried forward to the second round in the same manner as for the samples. PCR cycling conditions were 94 °C for 2 min, 30 cycles of 95 °C for 20 s, 60 °C for 25 s, and 72 °C for 1 min followed by a final step at 72 °C for 7 min and holding at 4 °C. The products were discarded if a band was visualized in the negative PCR controls.

First-round PCR was conducted in duplicate and combined after the second-round PCR products based on the intensity of bands on 2% agarose gel electrophoresis. Biological replicates were assigned the same barcode and combined before the Illumina run. The PCR products were then purified with Agencourt AMPure XP magnetic beads (Beckman Coulter) and uniquely barcoded. These samples were again purified with Agencourt AMPure XP magnetic beads and pooled (separately for each gene region) based on DNA concentrations as determined using Qbit kits (Invitrogen Qubit TM ds DNA HS Assay Kit). Following the manufacturer's recommendations, the two uniquely indexed libraries were sequenced at the Murdoch University laboratory facilities on an Illumina MiSeq using 500-cycle V2 chemistry (250 bp paired-end reads).

Bioinformatic analysis

Paired-end reads were merged using USEARCH v10 (Edgar 2010) with a minimum overlap length of 50 bp and no gaps were allowed in the merged alignments. Sequence deconvolution, including quality control and clustering, was also done using USEARCH v10 (Edgar 2010). Specifically, sequences less than 200 bp and low mean quality (< 20) were removed. The remaining sequences that passed quality control were clustered into amplicon sequence variants (ASVs).

The subsequent analysis was conducted in Geneious ver. R10 (https://www.geneious.com/) to assign ASVs to a species based on phylogenetic inference. Consensus sequences were aligned using the MAFFT alignment within Geneious using the default parameters. Identities of the ASVs were initially assigned by conducting an internal BLAST search against a customized reference database. For the ITS gene region this database included sequences of type isolates as identified in IDPhy (Abad et al. 2023) and consisted of 300 *Phytophthora* species and undescribed (but designated) taxa and *Phytophthora* phylotypes recognized through metabarcoding from other previous studies (Burgess et al. 2017b, a; Bose et al. 2018). For the *RPS10* gene region, the reference database was downloaded from OomyceteDB (Foster et al. 2022) and consisted of 351 sequences of oomycetes, predominantly *Phytophthora* (144 species) and *Pythium* (133 species).

For the next step, all ASVs were then separated into phylogenetic clades, and phylogenetic analyses were conducted using Geneious tree builder and verified sequences of all known *Phytophthora* species within each clade. Several ASVs often clustered together in strongly supported terminal clades. These final identities were considered to be phylotypes. A phylotype was considered to represent a putative new species if it did not match any known species in the phylogenetic analysis. For *Phytophthora* species, these phylotypes were assigned a number representing the phylogenetic clade and a letter to distinguish between putative new phylotypes in the same clade. New phylotypes from unknown clades were allocated an "X."

Baiting and identification of isolates

Baiting *Phytophthora* spp. from soil samples was carried out at 20–24 °C using young leaves from *Hedera* helix, *Quercus ilex*, *Quercus robur*, cotyledons of *Eucalyptus sieberi*, and petals of ornamental roses following best practice protocols (Burgess et al. 2021b). After 3–10 days the portion of infected baits was first placed on NARPH medium (Hüberli et al. 2000) as described by Simamora et al. (2018) and then plated onto cornmeal agar (17 g/L water of corn meal agar) to examine for the presence of *Phytophthora* structures using a dissection microscope. Oomycete-like cultures were selected based on their morphology and transferred to carrot agar (5 g/L water of corn meal agar, 400 g/L water of fresh carrots). Isolates obtained were then grouped based on culture morphology.

DNA was extracted from the mycelium of 7-day-old aerial hyphae of one representative isolate for each morphological group using PrepManTM Ultra (Applied Biosystems by Life Technologies), following the manufacturer's protocol.

The nuclear ribosomal internal transcribed spacer (ITS) was amplified with primers ITS6 (Cooke et al. 2000) and ITS4 (White et al. 1990). Each 25 µL PCR reaction contained 12.5 µL 2 × GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA), 0.5 µL of each of forward and reverse primer, 1.5 µL of DNA, and 10 µL with PCR grade water. Amplification conditions were as follows: one cycle at 94 °C for 3 min., 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final step at 72 °C for 1 min. Five microliters of each PCR product was separated by electrophoresis in 1% agarose gels in 5% TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0). The amplified PCR fragments were purified through a Sephadex spin column (Sigma, Steinheim, Germany) and sequenced with the BigDye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Products were separated with an ABI 3730 48 capillary sequencer (Perkin-Elmer Applied Biosystems).

Identities were assigned to isolates in Geneious by conducting an internal BLAST search against the same customized reference database described above, which includes sequences of type isolates of all described *Phytophthora* species as identified in IDPhy (Abad et al. 2023). All obtained sequences were submitted to GenBank, and accession numbers are provided in Table S2.

Statistical analysis

Venn diagrams were used to visualize the number of oomycete taxa shared between (1) the vegetation types (orchards and natural stands) and (2) the different geographic areas. The "VennDiagram" package in the R software (R Core Team 2021) was used to construct the Venn diagrams. Separate Venn diagrams were constructed for data obtained from barcoding (ITS1 and *RPS10*) gene regions and baiting.

To analyze oomycete species richness, the number of taxa per sample was calculated. The effects of the vegetation type, the different geographic areas, and their interaction (V × G) on species richness were analyzed with a generalized linear model (GLM). The model was fitted assuming a Poisson error distribution and a log-link function. Additionally, the natural logarithm of the total number of reads was included as an offset to consider the variation in fungal richness triggered by sequencing depth. The "vegan" package in R software (R Core Team 2021) was employed for analyzing oomycete species richness using data obtained from ITS and *RPS10* gene regions.

For analyzing the oomycete taxa community composition in different vegetation types and geographic areas, a principal coordinate analysis (PCoA) was performed using Jaccard distance. Differences in oomycetes community composition between vegetation types and/or geographic areas were assessed using permutational multivariate variance analysis (PERMANOVA). The explanatory variables used for PERMANOVA were the vegetation type, the geographic areas, and their interaction. The natural logarithm of *Proteaceae* sample sequencing depth was included as the first effect in the PERMANOVA to consider the variation in fungal composition triggered by sequencing depth. The "vegan" package in R software (R Core Team 2021) was utilized to analyze oomycete taxa community composition using data obtained from ITS and *RPS10* gene regions.

Phytophthora cinnamomi was the only species routinely detected through baiting, and thus, the baiting and metabarcoding results for *P. cinnamomi* were compared. To determine if *P. cinnamomi* showed a different abundance between vegetation types, a GLM was fitted, assuming a Poisson error distribution and a log link function. The natural logarithm of the total number of positive identifications (baiting) or natural logarithm of sequencing depth (ITS and *RPS10*) was included as an offset to account for differences in sample size. The "vegan" package in R software (R Core Team 2021) was used to analyze *P. cinnamomi* abundance using data obtained from ITS, *RPS10* gene regions and baiting to check for differences between the detection methods.

Results

Taxa identified through metabarcoding

ITS sequencing

Of the 257 samples, 36% (92) produced an amplification product. The 26,597 reads that passed quality control were clustered into 131 ASVs, of which all were classified as oomycetes; 45% of the 59 ASVs (60% of the reads) were identified as *Phytophthora*. On average 633 reads were found for each sample. Phylogenetic identification revealed 17 phylotypes, 9 of which were *Phytophthora* taxa residing in five of the eleven clades found within the *Phytophthora* phylogeny (Table S3 and Fig. S1). The highest number of reads was observed for *P. cinnamomi*, followed by *Phytophthora* clade 12A, *P. nicotianae*, *P. multivora*, *P. pachypleura*, *P. cactorum*, *P. pseudocryptogea*, *P.* AUS XA, and *P. cryptogea*. The other 10 phylotypes accounted for 40% of total reads and were assigned to *Plasmopara halstedii*, *Pythium* aff. *heterothallicum*, *Pythium rostratifingens*, *Bremia* sp., *Lagena* sp., *Myzocytiopsis subuliformis*, *Peronospora* aff. *trivialis*, and *Peronospora effusa.*

Among the 17 phylotypes, five were shared between the two vegetation types and 10 were exclusively found in root samples collected in orchards (Fig. 2a). None of these 17 phylotypes were shared among all areas, 5 were shared between two areas, 7 were found only in Australia, 3 only in South Africa, and 2 only in Europe (Fig. 2b and Appendix S1).

RPS10 sequencing

Out of the 257 samples, 64% (164) produced an amplification product. The 139,602 reads that passed quality control were clustered into 562 ASVs, of which 8% were *Phytophthora* (30% of the reads) and 92% were other oomycetes (70% of the reads). On average 3324 reads were found for each sample. Among the 65 phylotypes detected, 5 were matched with *Phytophthora* species; *Phytophthora* clade 12A, *P. cactorum*, *P. cryptogea*, *P. sojae*, and *P. cinnamomi* (Table S3). A total of 18 *Pythium* spp. were identified to the species level (28% of the reads) (Table S3 and Fig. S2); *Pythium camurandrum*, *Py. cederbergense*, *Py. conidiophorum*, *Py. contiguanum*, *Py. cryptoirregulare*, *Py. dissotocum*, *Py. folliculosum*, *Py. irregula*re, *Py. mamillatum*, *Py. mastophorum*, *Py. myriotylum*, *Py. nunn*, *Py. oopapillium*, *Py. ornamentatum*, *Py. ornacarpon*, *Py. rostratifingens*, *Py. vanterpoolii*, and *Py. volutum*. An additional 31 *Pythium* spp. were detected, of which 28 were placed into a clade, while one *Bremia* sp. and one *Phytopythium* sp. were identified to the genus level. The remaining 9 oomycete phylotypes could not be matched with a genus.

Of the 65 phylotypes, 28 were shared in the root samples of the two vegetation types, 34 were exclusively found in samples from orchards, and only 3 were exclusively in samples from native vegetation (Fig. 2c). Moreover, of the 65 phylotypes, the majority (29) were found exclusively in Australia, 5 were exclusively found in South Africa, and 5 in Europe (Fig. 2d and Appendix S1).

Taxa identified using baiting

Phytophthora spp. were recovered from 48 (19%) sampling units of which 33 were from South Africa (21 in native vegetation and 12 in orchards), 8 in Portugal (3 in native vegetation and 5 in orchards), 4 in Italy (orchards only), and three in Australia (orchards only) (Table S4). These sampling units matched five species, three of which were shared between the two vegetation types (Fig. 2e), and one was exclusively found in South African and Australian samples (Fig. 2f). *Phytophthora cinnamomi* was the most recovered species (isolated in 40 sampling units), followed by *P. cryptogea* and *P. ornamentata* (isolated in 3 sampling units), *P. niederhauserii* (isolated in 1 sampling unit), and *P. multivora* (isolated in 1 sampling unit) (Table S4). Genbank sequence number of *Phytophthora* isolates and sampling details are reported in Table S2.

Oomycete species richness and community composition

Oomycete species richness did not vary between orchards and natural vegetation. However, it was significantly influenced by geographic areas and the interaction between vegetation types and geographic areas (Table 1). Specifically, *Proteaceae* samples from South Africa showed the lowest species richness (mean ± SE: 3.6 ± 1.1 and 1.4 ± 0.2, *RPS10* and ITS1, respectively, Fig. 3). While species richness did not vary between environment or geographic area, the PCoA plot illustrates a difference in oomycetes community composition between *Proteaceae* samples from Australia and South Africa (Fig. 3). PERMANOVA confirmed that the geographic area influenced oomycetes community composition (Table 2).

Comparison of *P. cinnamomi* detection through baiting and metabarcoding

Phytophthora cinnamomi was the only species consistently isolated with baiting: 31 positive sampling units in South Africa, of which 20 were from native vegetation and 11 in orchards; 7 in Europe of which one was native vegetation and 6 in orchards and 2 in Australian orchards (Tables S3 and S4). Data analysis did not show a significant difference in *P. cinnamomi* abundance between vegetation types using baiting (Table 3). However, *P. cinnamomi* abundance significantly differed between vegetation types according to datasets obtained with *RPS10* and ITS1 metabarcoding approaches (Fig. 4 and Table 3). Specifically, *P. cinnamomi* abundance was significantly higher in orchards compared to natural stands.

Discussion

The metabarcoding approach used in this study revealed a significant interaction between the oomycetes richness of different vegetation types and on different continents. Most detected *Phytophthora* taxa were well-known pathogens with global distributions such as *P. cinnamomi*, *P. cactorum*, *P. cryptogea*, *P. multivora*, *P. pseudocryptagea*, and *P. sojae*. The oomycete-specific *RPS10* primers amplified 164 out of 257 samples, while the *Phytopthora*-specific ITS1 primers amplified only 92 out of 257 samples. As expected, more phylotypes were detected using the *RPS10* primers, including many *Pythium* species. However, more *Phytophthora* phylotypes were detected using the ITS primers. Despite these differences, both datasets demonstrated that the geographic region influenced oomycetes community composition.

Consistent with our results, other recent research has also tested different metabarcoding techniques on the same environmental samples and mock communities, demonstrating that multiple primer pairs for the same target gene (Legeay et al. 2020) or different genes (Landa et al. 2021; Burgess et al. 2022) provide partially divergent data. When taxonomic coverage is the principal aim, sequencing multiple

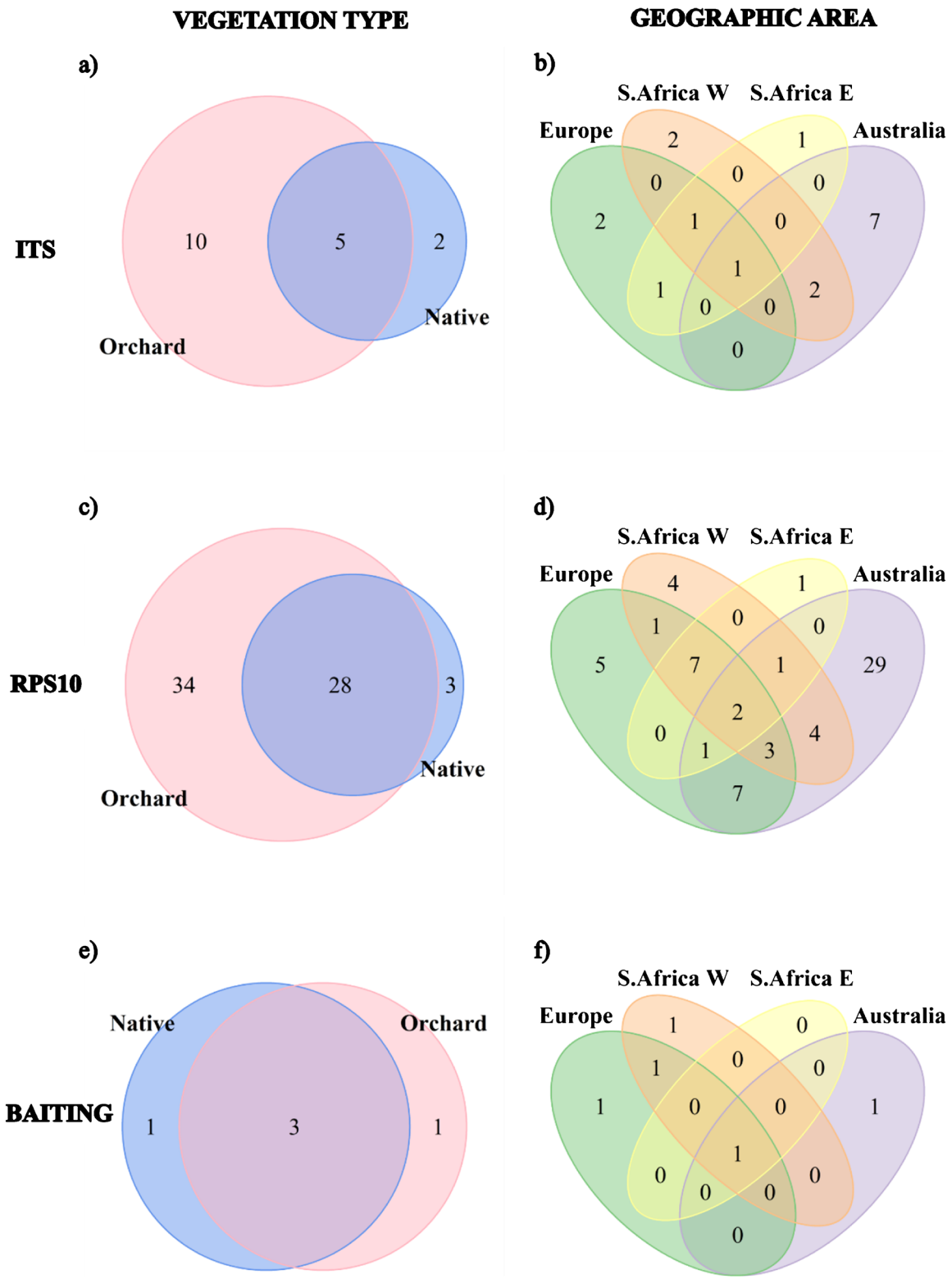

Fig. 2 Venn diagrams showing the number of oomycete species detected through ITS (**a**, **b**), *RPS10* (**c**, **d**) or baiting (**e**, **f**) in different vegetation types (**a**, **c**, and **e**) (orchards and natural stands) and geographic areas (**b**, **d**, and **f**) (South Africa E, South Africa W, Europe, and Australia)

Table 1 Results of the generalized linear model for the analysis of the effects of vegetation types (orchards and natural stands) and geographic areas (South Africa E, South Africa W, Europe, and Australia) on oomycete species richness, showing degrees of freedom (*df*), AIC, and significance levels for each variable

Gene region	Explanatory variables	*df*	AIC	*P* value
ITS1	Vegetation type (V)	1	146.99	0.9209
	Geographic area (G)	3	157.56	0.0022 *
	V × G	3	148.98	< 0.001 ***
RPS10	Vegetation type (V)	1	181.69	0.0723
	Geographic area (G)	3	183.78	0.0254 *
	V × G	3	180.46	< 0.001***

$p < 0.05$ = *; $p < 0.001$ = ***

Table 2 Results of the PERMANOVA for the analysis of the influence of the vegetation types (orchards and natural stands) and geographic areas (South Africa E, South Africa W, Europe, and Australia) on oomycete species composition identified through ITS or *RPS10* metabarcoding, showing degrees of freedom (*df*), *F* values, r^2, and significance levels

Gene region	Explanatory variable	*df*	*F* value	r^2	*P* value
ITS1	Vegetation type (V)	1	1.2400	0.0446	0.263
	Geographic area (G)	3	2.2053	0.2382	0.009 **
	V × G	3	1.1363	0.1227	0.296
	Residuals	14		0.5041	
RPS10	Vegetation type (V)	1	0.6498	0.0388	0.870
	Geographic area (G)	3	1.7640	0.3163	0.023 *
	V × G	3	0.9356	0.1678	0.588
	Residuals	7		0.4184	

$p < 0.05$ = *; $p < 0.01$ = **

regions may be advisable and provide complementary data. For example, Landa et al. (2021) found the *COI* region detected other oomycete taxa in addition to the *Phytophthora* and better differentiated several *Phytophthora* spp. from clades that were difficult to resolve using the ITS region. In our study, although the two gene regions amplified did not produce statistically significant differences in species richness between environments, the *RPS10* region gave a greater coverage, amplifying other *Peronosporaceae* in addition to *Phytophthora.*

Oomycetes association with *Proteaceae* in orchards versus natural stands

The oomycete species richness and community composition found in this study were not significantly different in cultivated orchards and natural *Proteaceae* environments. Although there is little information on this topic, recent studies across various environments and comparing various levels of disturbance have shown that less disturbed environments have fewer *Phytophthora* species (Dale et al. 2022; Redondo et al. 2018). Bose et al. (2018) found that vegetation type significantly influenced the *Phytophthora* community composition in soils. However, their study is not directly comparable to ours because they sampled different plant species from each of the different environments. By focusing solely on *Proteaceae*, we were able to demonstrate that the host had a greater impact on oomycetes diversity than the environment.

Phytophthora cinnamomi was more abundant in the sampled orchards than in the natural environment. Globally, *P. cinnamomi* is known as the most important pathogen of *Proteaceae* and other tree crops (Burgess et al. 2017a) and is often the focus of control strategies in both forestry and agriculture (Hardham and Blackman 2018), where the constant human activity contributes to its spread. We believe that the weekly use of machinery for the chemical control of pests together with a regular movement of flower pickers in *Proteaceae* orchards may have been crucial for the higher presence of this pathogen in comparison to the less disturbed natural stands sampled in this work.

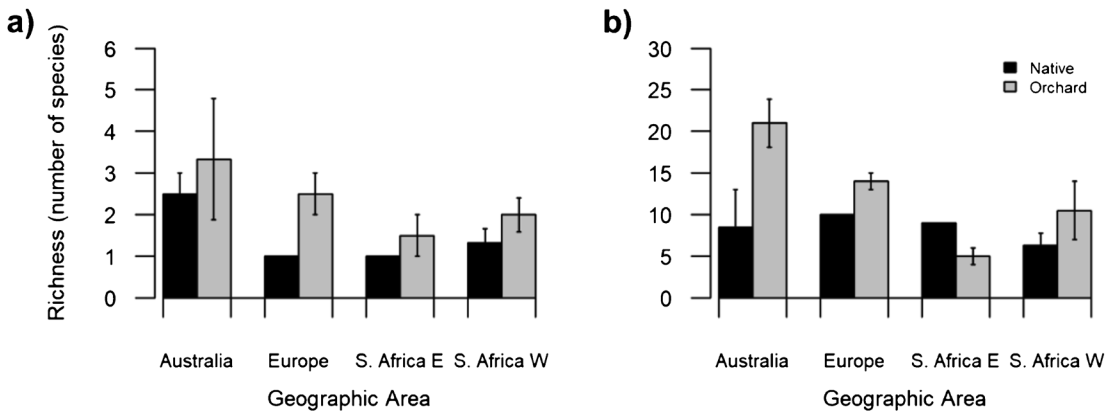

Fig. 3 Species richness was detected through ITS (**a**) or *RPS10* (**b**) in the different vegetation types (orchards and natural stands) and different geographic areas (South Africa E, South Africa W, Europe, and Australia). Data are the mean ± SE

Table 3 Results of the generalized linear model for the analysis of the effects of vegetation types (orchards and natural stands) on *P. cinnamomi* abundance, showing degrees of freedom (*df*), AIC, and significance levels for each variable

Method/protocol	Explanatory variable	df	Deviance	AIC	LRT	Pr(> chi)
Baiting			26.147	62.417		
	Vegetation type (V)	1	26.310	60.579	0.16235	0.687
ITS			16816	16879		
	Vegetation type (V)	1	17084	17145	267.75	< 0.001 ***
RPS10			64015	64078		
	Vegetation type (V)	1	78624	78685	14609	< 0.001 ***

$p < 0.001$ = ***

Oomycetes association with *Proteaceae* in different countries

The metabarcoding approach demonstrated that oomycete species richness and community composition differed in the samples collected across countries and on different continents. While numerous studies of oomycete communities have been conducted (summarized in Burgess et al. 2022), to the best of our knowledge, our work was the only one that focused on the difference of soil-borne pathogens across continents. These results represent a significant practical application for this molecular tool, which is currently underutilized in large-scale inter-country investigations of plant-associated microorganisms (Tedersoo et al. 2019). Of the few examples is the recent work by Franić et al. (2023) about the global distribution of twig fungal endophytes in trees from botanical gardens and arboreta, and two studies of fugal endophytes in twigs of ornamental plants (Migliorini et al. 2021) and on commercial seeds imported from overseas into Europe (Franić et al. 2019).

Therefore, our work advocates for the further development and implementation of the metabarcoding approach to study the global movement of plant pathogenic soil-borne oomycetes. These oomycetes are highly prevalent in the soil of potted plants traded from overseas into Europe (Eschen et al. 2015; Migliorini et al. 2015) and are responsible for causing significant ecological and economic damages (Santini et al. 2013).

Comparison of metabarcoding molecular detection methods and traditional baiting

Despite metabarcoding showing that *P. cinnamomi* was more common in orchards than in natural vegetation, isolation rates did not reflect this difference. The difference in several species detected through direct isolation (by soil/root baiting) versus metabarcoding has been a subject of

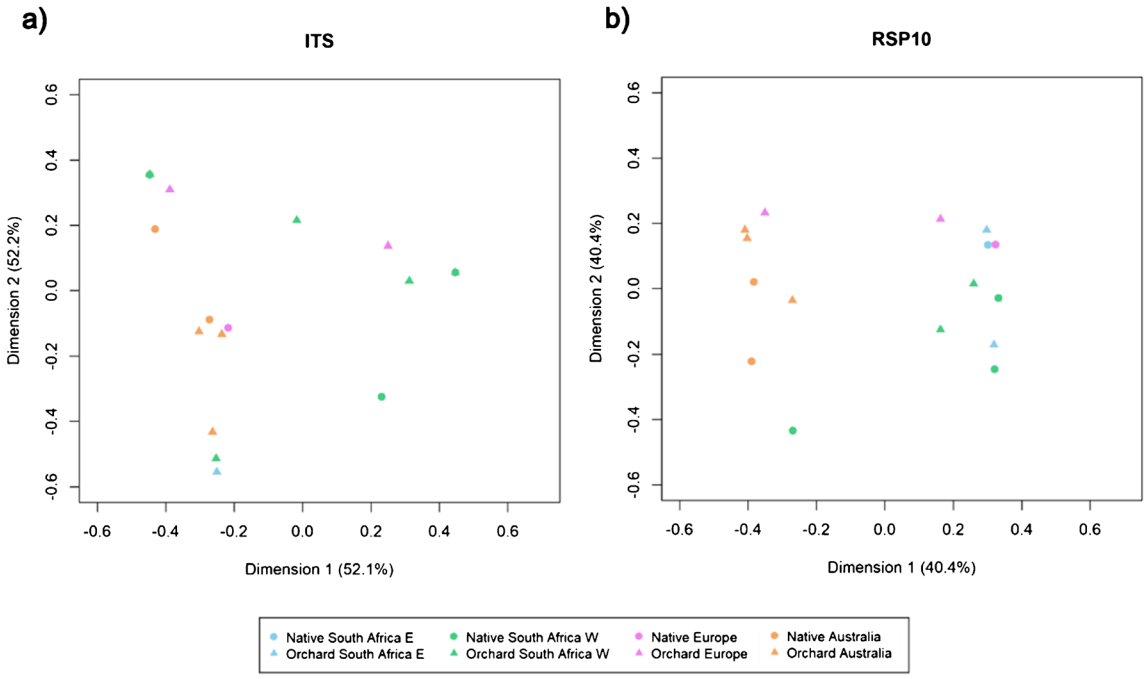

Fig. 4 Principal coordinate analysis (PCoA) of oomycete species identified through metabarcoding **a** ITS and **b** *RPS10*, associated with different vegetation types (orchards and natural stands) and geographic areas (South Africa E, South Africa W, Europe, and Australia)

long-standing debate (Sarker et al. 2023a). In soil/root baiting, the isolation outcomes depend on the presence of active *Phytophthora* propagules in the analyzed samples since the method relies on motile zoospores released from the sporangia being attracted to a live bait (Sarker et al. 2021). Baiting conditions favor fast-sporulating species, able to infect a range of bait species, achieve infection with a low number of zoospores, and grow rapidly on selective agar (Sarker et al. 2023a). For this reason, baiting success depends upon the species present and the viability of the propagules when the sample was collected (Sarker et al. 2021, 2023b). In our study, *P. cinnamomi* proved to be the most active species in our sampling areas and was therefore easily isolated.

The two methods, direct isolation and metabarcoding, when applied to the study of soil oomycetes, should be considered complementary, and results interpreted with care depending upon the study system and the aim of the investigation. Metabarcoding can be used as an early detection tool but cannot distinguish living viable propagules from residual DNA of dead propagules (false positive) (Sanzani et al. 2014; Kunadiya et al. 2021). The accurate overview of oomycetes taxonomy provided is fundamental for conducting diversity studies aimed at comparing different situations included in the sampling design, but it is unable to report the state of things at the exact moment of collection. This practical limit is acceptable for conducting diversity studies where the time range is generally not considered variable. However, metabarcoding or similar molecular methods should not be used for diagnostic purposes if the aim is the management and control of *Phytophthora*.

Conclusion

Our study provides a comprehensive analysis of the distribution and diversity of oomycete species in the soil and roots of cultivated and naturally growing *Proteaceae* across different continents. The prevalence of widely recorded *Phytophthora* species in various environments underscores the risks posed by the spread of this pathogen in both *Proteaceae* orchards and natural ecosystems.

The consistent presence of *P. cinnamomi*, especially in orchards, highlights its negative economic impact on the production sector and raises concerns about the considerable environmental risk to South African and Australian native ecosystems, in line with the recent report by Paap et al. (2023). The high isolation frequency of *P. cinnamomi* in our investigation using baiting demonstrates how propagules of this species are actively spreading in farms, emphasizing the importance for managers to establish good hygiene practices to prevent further transfer within the site and to the surrounding environment.

Furthermore, our work confirms the reliability of metabarcoding as a taxonomical diagnostic tool, marking the first-time utilization of this approach to assess oomycetes associated with *Proteaceae*. Additionally, the successful outcomes achieved using the *RPS10* gene underscore its efficiency with soil environmental DNA samples, further strengthening the applicability of this approach for ecological studies of oomycete communities.

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Author contribution All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Duccio Migliorini, Christopher Shaw, and Maria Vivas. The first draft of the manuscript was written by Duccio Migliorini and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The ITS gene dataset generated and analyzed during the current study is available in the GenBank repository, https://www.ncbi.nlm.nih.gov/genbank/. The metabarcoding dataset generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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