Decreased diversity and connectivity of endophytic fungal assemblages within cultivated European olive trees compared to their native African counterpart

Nombuso P. Ngubane [a d 1], Leanne L. Dreyer [b d], Bernard Slippers [c d], Martin Kemler [e 1], Dominik Begerow [e 1], Frederick Witfeld [e], Andreas Brachmann [f], Francois Roets [a]

[a]Conservation Ecology and Entomology, Stellenbosch University, Stellenbosch, South Africa
[b]Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa
[c]Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa
[d]DSI-NRF Centre of Excellence in Plant Health Biotechnology, University of Pretoria, Pretoria, South Africa
[e]Evolution of Plants and Fungi, Ruhr-Universität Bochum, Bochum, Germany
[f]Faculty of Biology, Ludwig-Maximilians- Universität München, München, Germany

[1] Current address: Department of Biology, Universität Hamburg, Hamburg, Germany.

*Corresponding author. Conservation Ecology and Entomology, Stellenbosch University, Stellenbosch, South Africa. Email: nombuso.ngubane@uni-hamburg.de

Highlights

- Metabarcoding was used to study fungal endophytes of *Olea europaea* in South Africa.
- Site and host identity shape fungal endophyte assemblages of *O. europaea* subspecies.
- The African olive harboured higher fungal richness than the European olive.
- More fungal endophytes were shared between hosts than were exclusive to each.

Abstract

Host identity and location help shape fungal endophyte assemblages in plants. Hosts act as uptake filters from the environment and closely related hosts in the same location may harbour similar assemblages. We assessed the influence of host identity and geographic location on endophytic fungal assemblages within the native African olive and cultivated European olive in South Africa using high throughput sequencing. As hypothesised, the two hosts were found to share many endophytic species, but alpha diversity was lower within the European olive. The two hosts had significantly dissimilar endophyte assemblages. Distance between sites positively correlated with endophyte assemblage dissimilarities, demonstrating a strong effect of the surrounding environment on endophyte assemblages. African olive individuals had highly connected endophyte assemblages, unlike those within the European olive. Microbiome sharing and disconnected assemblages may have negative impacts on the health of the cultivated host.

Keywords: *Olea europaea* subsp. cuspidate*; Olea europaea* subsp. e*uropaea; Olea Africana;* Core Cape Subregion; Mediterranean-type ecosystem; Plant health; Olive industry; Geographic location; Metabarcoding

1. Introduction

Plants are colonised by a diverse range of microorganisms that influence plant health and growth in either a positive, negative or neutral manner (Hajishengallis et al., 2012). Fungi that live within plant tissues without having any obvious effects on the plant are termed fungal endophytes (Schulz and Boyle, 2006; Hyde and Soytong, 2008). Under changing environmental or within-host conditions, the activity of some endophytes may change to either beneficial or detrimental (Saikkonen et al., 1998; Slippers and Wingfield, 2007). Fungal endophytes of closely related introduced and native hosts often expand or shift hosts to include the newly encountered taxon (Slippers et al., 2005; Glen et al., 2007; Perez et al., 2012). Despite these host shifts or expansions, exotic (often cultivated) hosts tend to harbour lower fungal endophyte diversity compared to their native relatives (Saikkonen et al., 2000; Coleman-Derr et al., 2016). In addition to taking up fungi from the new surroundings, introduced hosts can maintain associations with some endophyte species from their native range (Power et al., 2017). Therefore, although hosts lose some fungal endophytes when translocated, they retain some and take up new fungi from the surrounding flora in the introduced range.

Most plant-associated endophytic fungi are acquired from the surrounding environment (Saikkonen et al., 2000; Helander et al., 2007; Christian et al., 2016). Consequently, local conditions such as surrounding plant communities and geographic location can greatly influence endophyte assemblage composition. For example, in *Olea europaea* subsp. e*uropaea* (European olive) fungal endophyte assemblages differ markedly between different plant organs, locations and seasons (Abdelfattah et al., 2015; Martins et al., 2016; Gomes et al., 2018). Even slight differences in host identity, such as differences in olive cultivars, can have a significant influence on these assemblages (Costa et al., 2021). The differences in these endophyte assemblages can significantly impact host fitness. For example, the differences in susceptibility of European olive cultivars to diseases are thought to be driven, at least in part, by differences in their endophyte assemblages (Costa et al., 2021; Martins et al., 2021). Thus, the relationship between plants and their endophytes is complex and can be influenced by a range of biotic and abiotic factors.

The economically important European olive belongs to the *Olea europaea* L. complex, along with five other subspecies, including *O. europaea* subsp. c*uspidata* (African olive) (Besnard et al., 2002; Green, 2002). The European olive is mainly cultivated in European Mediterranean-type climates (Vossen, 2007; Therios, 2009), but it is increasingly becoming important to the agroeconomy of regions with similar climate (Abdelfattah et al., 2015). In South Africa, it is often cultivated in areas where the African olive naturally occurs, as this close relative was used as a proxy for ideal cultivation environments (Costa, 1998). The African olive is also an important plant for its wide range of uses. It is used for agricultural purposes (as root stocks for the European olive), as a garden plant, and for medicinal purposes, shade provision and as a source of firewood (Costa, 1998; Besnard et al., 2007; Amabeoku and Bamuamba, 2010; Aumeeruddy-Thomas et al., 2017). The close taxonomic relationship between the European and African olive, combined with their frequent contact due to their proximity, increase the chances of microbial exchange between them in South Africa. Yet, research has focused mainly on pest and pathogen identification and management, and on improving cultivation practices (Agricultural Research Council, 2010; Costa,

2018; van Dyk et al., 2021a, 2021b). Endophytic fungal associations have yet to receive much attention.

Given the close taxonomic relationship and the vast distances between the native habitats of the African and European olives, the ecological ramifications of sharing their endophytes, or loss of endophytic diversity are unclear. The limited available research in South Africa focuses on pathogenic fungi, mostly of the European olive, but occasionally include the African olive. In the study of Spies et al. (2020), 99 fungal taxa were isolated, an overwhelming majority of which have not previously been recorded in olives. Van Dyk et al. (2021a) investigated olive trunk pathogens in South Africa and identified *Neofusicoccum australe, Pleurostoma richardsiae*, *Phaeoacremonium parasiticum* and *Pseudophaeomoniella globosa* as culprits. Other South African research efforts have focused on economically important fungal groups, such as the Diatrypaceae and *Cytospora* (Valsaceae) occurring on different plants including the cultivated and native olives (Adams et al., 2006; Moyo et al., 2019). In addition, during screenings for causal agents of olive scab and olive anthracnose, respectively, *Venturia oleagina* and *Colletotrichum gloeosporioides*, were identified as causal agents of disease (Gorter, 1956, 1962). The origins of most of the pathogenic fungi of European olives in South Africa are unknown but may include those that expanded their host ranges from the African olive where they may occur as benign endophytes. Consequences of these expansion have yet to be investigated.

The purpose of this study was firstly to characterise the endophyte fungal mycobiome associated with twigs of both the native African olive and the closely related cultivated European olive in South Africa. Due to their close taxonomic affiliations, it was hypothesised that they would share many fungal species, especially when growing in close proximity (Bufford et al., 2016). Since introduced taxa can retain some native associates (Saikkonen et al., 2000; Martín-García et al., 2012), it was expected that some taxa known to be associated with the European olive in other olive growing regions globally would be present in this cultivated host in South Africa. Due to the long co-evolutionary history between native fungi with native hosts and reduction in endophyte diversity observed in some introduced hosts (Saikkonen et al., 2000), it was hypothesised that the native host would harbour more diverse endophytic fungal assemblages than the cultivated host. As geographic location plays a central role in endophyte assemblages of various plants (Collado et al., 1999; Gore and Bucak, 2007), it was hypothesised that endophyte assemblages of both hosts would be strongly influenced by sampling location and the distances between them. The results of this study elucidate important factors that influence the fungal mycobiome of tree species that are of both economic and ecological significance in a biodiversity hotspot, the Core Cape Subregion (Manning and Goldblatt, 2012), and may point towards ways to best conserve both.

2. Materials and methods

2.1. Site selection and sampling design

Six sites corresponding to the main olive production areas were sampled in the Core Cape Subregion (Table S1, Fig. 1). The region has a Mediterranean climate with dry summers and wet winters (Rebelo et al., 2006). *Olea europaea* subsp. e*uropaea* was sampled from olive orchards and residential gardens. *Olea europaea* subsp. c*uspidata*

was collected from the nearest natural or semi-natural areas to the sampled cultivated olives. The sampled locations of the native host covered a wide range of Fynbos Biome vegetation types (Mucina and Rutherford, 2006; Table S1).

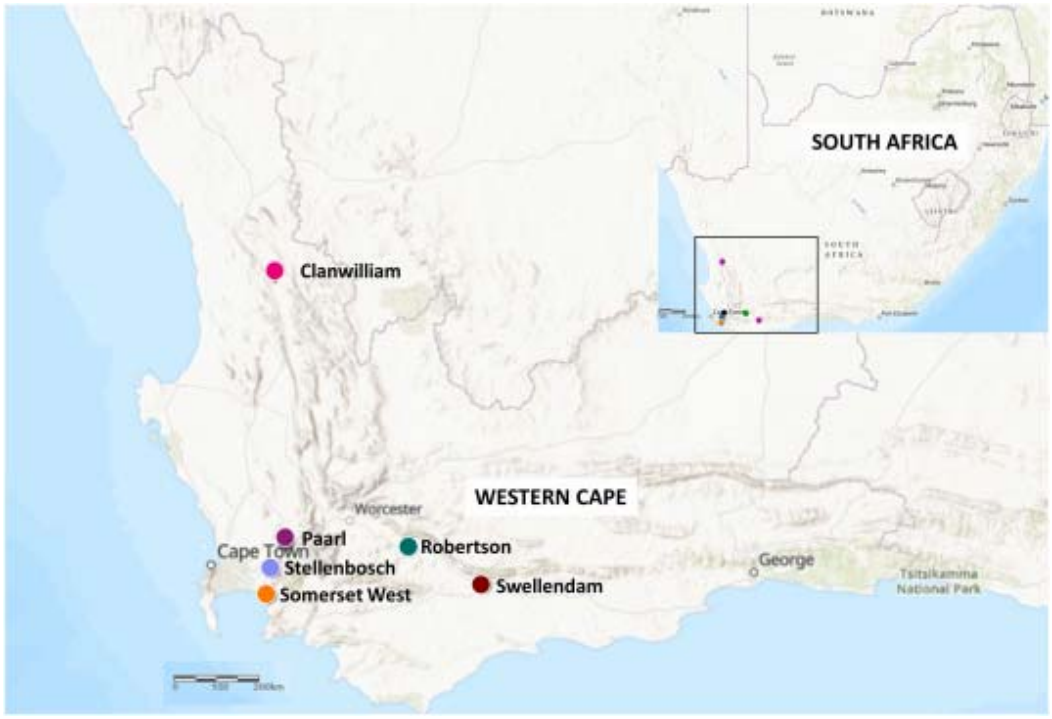

Fig. 1. Map of the Western Cape Province with the six sampled sites highlighted. Site colours correspond with the colour scheme in later figures. Insert: Map of South Africa.

Sampling was conducted between late-May and early-July 2018 to minimise possible seasonal influence. Focal trees were randomly selected and were at least 50 m apart. Four asymptomatic, 5 cm long and 3–5 mm diameter twigs were collected per tree and combined into a single sample. Samples were taken from all four sides of an individual tree to minimise possible effect of differences in canopy orientation on endophyte assemblages. At each site, twigs were collected from ten trees per host. All twigs were from the previous season's growth to minimise possible successional influences on fungal endophytes properties. Twigs from each plant were kept in a re-sealable plastic bag and stored at −80 °C awaiting further processing.

Twigs were surface sterilised through a sequential wash: 70% ethanol for 45 s, 3% sodium hypochlorite bleach for 1 min, 95% ethanol for 30 s and then double distilled water for 30 s (Slippers and Wingfield, 2007; Moral et al., 2010). A 2 cm section was cut from the centre of each sterile piece collected from each of the four sides of the tree, ground into fine powder with mortar and pestle, and combined into one tube. The mortar and pestle were cleaned using 70% ethanol, household bleach and autoclaved double distilled water between samples. Tubes were stored at −80 °C before DNA extraction.

2.2. DNA extraction

DNA extraction followed the protocol developed by Doyle and Doyle (1990) with modifications detailed in Ngubane (2022). The quantity and quality of the resulting DNA was evaluated using a photometer (Eppendorf Vertrieb Deutschland GmbH, Germany).

2.3. Library preparation

Multiplex amplification of the fungal species was carried out in two consecutive PCR reactions. The first PCR (PCR1) was conducted using the ITS primers augmented with tags that carried a binding site for the indexing primers. During PCR1, the fungal ITS region was amplified from total DNA extracted from the olive twigs using the forward primer ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA- 3′, Gardes and Bruns, 1993) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC- 3′, White et al., 1990) modified for multiplex barcoding by Metabion (Planegg/Steinkirchen, Germany). Reaction volumes and thermal conditions were identical to those in Ngubane (2022). The presence of ITS amplicons was confirmed through a 0.8% agarose gel (Bio-budget Technologies GmbH, Germany) by electrophoresis supplemented with 1 µl ethidium bromide. To remove excess primers, homodimers, heterodimers and DNA, the PCR products were purified following the ExoSap protocol (New England BioLabs Inc., USA). PCR2 was conducted to add Illumina adapters and indices to PCR1 products. PCR2 volumes and thermal cycle conditions mirrored those detailed in Ngubane (2022). The presence of amplicons was confirmed on an agarose gel using electrophoresis.

2.4. Sample pooling, purification, and library sequencing

The PCR amplicons carrying ITS primers and adaptors were sequentially combined into pools such that at each pooling step there were fewer tubes until a single pool was prepared for sequencing. Equimolar pools were prepared by quantifying band intensities using ImageJ version 1.52a (Ferreira and Rasband, 2012). After each pooling round, the resulting pools were visualised on an agarose gel using electrophoresis and photographed in a UV chamber. These images were also examined on ImageJ and the band intensities were quantified. Pools with similar intensities were further pooled. Pools were subsequently purified using the CleanPCR® Kit (CleanNA, GC biotech B.V.). After the sequential pooling, the final pool was sent to the sequencing facility at the Genetics Department, Ludwig Maximilian University of Munich. At the facility 2 × 250 bp paired-end sequencing was conducted using an Illumina MiSeq® sequencer (Illumina Inc., USA).

2.5. Sequence cleaning, identification, and quantification

Quality control and demultiplexing of the sequencing product were performed using the QIIME 1.9.1 pipeline (Caporaso et al., 2010). Sequence reads were extracted using the forward and reverse barcodes as identifiers. Forward reads were used for subsequent analyses. Tag-index barcodes were used to assign sequences to sample of origin, after which they were trimmed using the FASTX-Toolkit (v. 0.0.13, http://hannonlab.Cshl.edu/fastx_toolkit/). The trimmed sequences were clustered into operational taxonomic units (OTU) based on sequence similarities using CD-HIT-OTU

(http://weizhongli-lab.org/cd-hit-otu/; Li et al., 2012). Clusters were formed based on a 97% similarity threshold (Stackebrandt and Goebel, 1994). Representative sequences were extracted and used for taxonomic assignment using QIIME and the UNITE v. 7.2 database (Kõljalg et al., 2013).

3. Analyses of fungal endophytes within the African and European olives

3.1. Alpha-diversity

Endophytic fungal OTU richness within twigs per tree species and per site was estimated using the non-parametric Jackknife2 (Burnham and Overton, 1978; Palmer, 1991) and Chao2 (Chao and Lee, 1992) diversity measures in Primer6 (Anderson et al., 2008). Fungal endophyte OTU richness and abundance were compared between the different sites and hosts using modelling procedures in R v. 3.1.2 (R Development Core Team, 2015). Abundance measures were based on 1) total number of sequence reads per sample and 2) relative abundance defined as the total percentage of tree individuals per species per site that contained a particular OTU (White and Bennetts, 1996). Species richness was based on 1) the total number of OTUs per sample and 2) richness rarefied to samples containing the least abundant OTU (total number of reads) using "*rarefy*" in *Vegan* v. 2.5–6 (Oksanen et al., 2008; Weiss et al., 2017). Stack plots of dominant families within the two hosts at the different sites were calculated and created using the *Phyloseq* v. 1.28.0 package (McMurdie and Holmes, 2013) in R.

OTU abundance, richness and rarefied richness were compared between host taxa and sites using generalised linear models (GLM) fitted with a Laplace approximation and a Poisson family distribution in the *lme4* v. 1.1–31 package in R (Bates and Sarkar, 2007). Relative abundance data were over dispersed; thus, the negative binomial family distribution was used. To improve the model fit and account for possible spatial autocorrelation, region was used as a random variable (Cape Town = Somerset West, Boland = Paarl and Stellenbosch, Olifants River Valley = Clanwilliam, Breede River Valley = Robertson and Swellendam). Where needed, significant main effects were further separated using conservative Tukey *post hoc* tests using the *multcomp* v. 1.4-10 package (Hothorn et al., 2008) in R. The same alpha diversity analyses were also performed on a subset of the mycobiome containing only the core microbial taxa, defined as those fungal endophytes that appeared in at least 75% of the samples.

3.2. Beta-diversity

We considered two main types of beta-diversity to differentiate between beta-diversity between factors [β1: between different sites or between different host taxa] and within factors [β2: within individual sites or within individual host taxa] (Anderson, 2006). β1 and β2 were calculated following permutational multivariate analyses of variance (PERMANOVA) and permutational multivariate analyses of dispersion (PERMDISP) procedures in Primer6, respectively. These diversity measures are sensitive to differences in sample size (Anderson, 2006). Since a portion of the samples collected for barcoding failed to amplify, a random number generator (https://www.random.org/) was used to balance the sample sizes within site and host by lowering the number of individuals available per species per site to the smallest sample size available per host per site (n = 5; cumulative n = 60) prior to beta-diversity analyses.

Responses of fungal endophyte assemblages to host identity, site and their interaction were investigated based on the two datasets (abundance and richness) using PERMANOVA. For the abundance-based dataset, the data was square-root transformed before performing 999 permutations on a Bray-Curtis dissimilarity matrix (Anderson, 2001). For the incident-based dataset, 999 permutations were performed on a Jaccard's dissimilarity matrix (Magurran, 2004). Where main effects were significant *post hoc* comparisons for significant effects were conducted using pair-wise testing procedures in Primer6. PERMDISP analyses were conducted on the incident data matrix using 999 permutations of a Jaccard's distance matrix (Anderson, 2001). The above-mentioned beta-diversity analyses were also performed on a subset of the data containing only the core assemblages. Where significant differences were detected in β1, Canonical Analysis of Principal Coordinates (CAP) procedures in Primer6 were used for visualisation. To adequately visualise the significant differences between hosts, an unconstrained ordination procedure (non-metric multi-dimensional scaling, nMDS) was used to visualise the grouping differences using Primer6.

3.3. Effect of distance between sites on fungal endophyte assemblages within olives

A Euclidean distance-based geographic distance matrix (Gauch, 1973) was computed from the GPS co-ordinates of the six sites using the *Vegan* package in R. Assemblage-based distance matrices were computed from the OTU abundance and richness tables. For the abundance-based distance matrix, the assemblage matrix distance was based on a Bray-Curtis dissimilarity matrix (Bray and Curtis, 1957) constructed from square-root transformed data of the balanced dataset. The richness-based distance matrix was constructed from the presence/absence dataset to compute a Jaccard's dissimilarity matrix. These matrixes were constructed for 1) both hosts combined per site and for the 2) two hosts separate per site. The geographic distance matrix was then compared to each assemblage-based dissimilarity matrix using Mantel tests (based on a Pearson's correlation coefficient) with 999 random permutations in R using the *Vegan* package. These analyses were repeated on a subset of the data containing only the core OTUs.

3.4. Fungal endophyte co-occurrence networks

Significant fungal co-occurrences were calculated using the package *Hmisc* (Harrell, 2014) in R and visualised as networks using Cytoscape v3.7.2 (Cline et al., 2007). Significant co-occurrences ($p < 0.05$) were calculated based on Spearman's correlation coefficients ($p > 0.5$) (Spearman, 1904). Type II errors known to accumulate during multiple testing were corrected for using a Benjamini-Hochberg standard false discovery rate correction (Benjamini and Hochberg, 1995). Interactions were visualised using Cytoscape v. 3.7.2 (Cline et al., 2007). UNITE-based identifications were used to label nodes. OTUs that returned no BLAST hits based on the UNITE database were queried using the Basic Local Alignment Search Tool (BLAST) in GenBank within NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To identify fungi that could not be placed using the UNITE database, a 95% sequence similarity cut-off was applied to find the closest matches using BLAST. A collection of network description measures (such as node degree, total number of nodes, average path length, diameter, clustering coefficient, betweenness co-efficient and modularity) were calculated and reported (Freeman 1977; Newman, 2003; Assenov et al., 2008).

4. Results

A total of 1 035 012 sequences were recovered from 102 samples. These sequences resolved to 448 unique OTUs belonging to two phyla, 61 families, and 98 genera. Of these, 104 were Ascomycota, 35 Basidiomycota, 25 unidentified taxa and 194 OTUs without any BLAST hits. Out of the 448 taxa, 265 were recovered from both hosts, while 119 were found exclusively within the African olive and 64 were restricted to the European olive. Thirty-six OTUs were found in all six sites. One hundred and fifty-seven African olive OTUs, and 97 European olive OTUs had no BLAST hits. More OTUs could not be placed at genus level within the African olive (95) than in the European olive (69). The most common families within the two hosts included Corticiaceae, Elsinoaceae, Pleosporaceae, Sporocadaceae and Teratosphaeriaceae (Fig. 2). Although found within both hosts, members of the Caliciaceae were predominantly associated with the European olive. Members in the Diaporthaceae, Phaeosphaeriaceae, and Sporocadaceae were the most dominant within this host. The African olive was dominated by taxa in the Corticiaceae and Teratosphaeriaceae families.

4.1. Fungal endophyte α-diversity

The African olive harboured significantly higher fungal endophyte richness than the European olive (Table 1, Table 2, Fig. S1). The core fungal assemblages within the African olive were also richer than those in the European olive (Table 1, Table 2). Once data were rarefied, species richness was still significantly affected by host identity. Similarly, host identity also significantly affected the relative abundance of fungi within the twigs. However, host identity did not significantly influence species richness of the core taxa when rarefied richness or when relative abundance of the core taxa were considered (Table 2). Overall and core species richness were also significantly different among sampling localities (Table 2), but not all sites were significantly different from one another (Tables 1, 2 and S2). Rarefied species richness was not significantly affected by site (Table 2). Despite the significant role of site in core species richness, none of the pairwise comparisons were significant (Table S2). The interaction between host and site often significantly affected species richness (Table 2). Generally, sites that were closer to one another and had similar vegetation types tended to have more similar species richness (Table S1, S2 and S4).

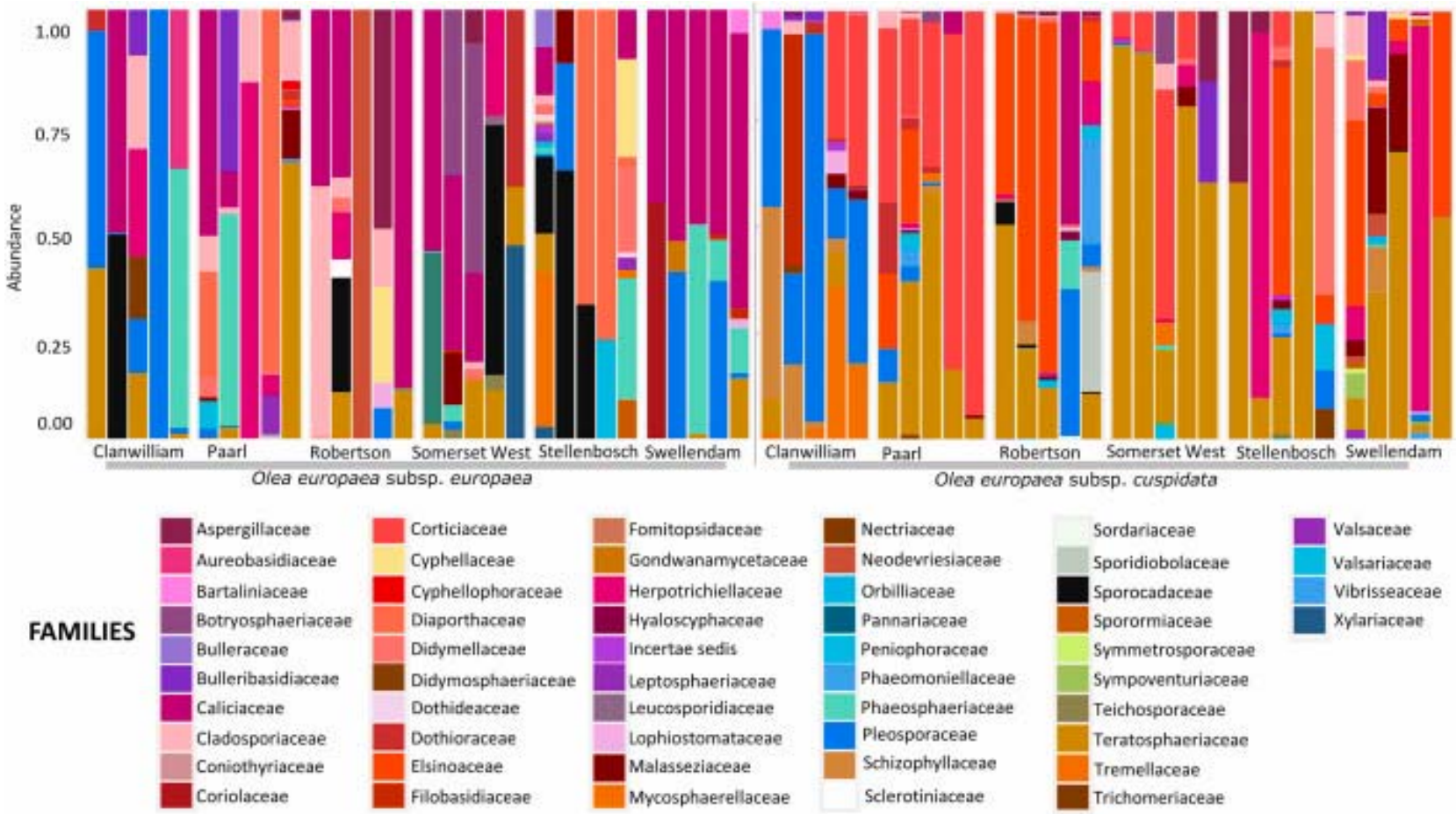

Fig. 2. Relative abundance (100% stack plots) of fungal families found within olive twigs (*Olea europaea* subsp. *cuspidata* and *Olea europaea* subsp. *europaea*) from the six sites (unbalanced dataset) based on 589 518 reads, excluding taxa that could not be placed at family level.

Table 1. Species richness estimators (Chao2 and Jackknife2) within olive twigs from both hosts (*Olea europaea* subsp. *cuspidata* and *Olea europaea* subsp. *europaea*) and from all six sites based on the full mycobiome (left) and the core mycobiome (right). Sample size, n = 102 (full and unbalanced dataset).

Factors		**Whole**			**Core**		
Site	**Host**	**Chao2**	**SD**	**Jackknife2**	**Chao2**	**SD**	**Jackknife2**
All sites	*O. europaea* subsp. *cuspidata*	531.450	41.163	565.480	17.000	0.000	17.000
	O. europaea subsp. *europaea*	471.430	67.500	445.250	10.000	0.000	10.000
Clanwilliam	Both	247.000	42.682	231.940	17.000	2.646	19.262
	O. europaea subsp. *cuspidata*	230.750	86.291	160.130	19.500	17.139	13.867
	Olea europaea subsp. *europaea*	436.170	238.930	144.290	9.000	1.323	8.967
Paarl	Both	360.020	50.066	342.370	27.125	6.080	30.056
	O. europaea subsp. *cuspidata*	337.140	84.690	262.350	23.125	9.018	22.083
	Olea europaea subsp. *europaea*	243.530	50.442	201.360	10.000	0.000	12.000
Robertson	Both	294.430	27.354	320.790	37.250	20.187	30.639
	O. europaea subsp. *cuspidata*	236.070	21.580	256.070	17.800	2.070	19.000
	Olea europaea subsp. *europaea*	152.060	46.385	115.230	10.250	3.396	11.300
Somerset	Both	239.180	28.026	260.840	22.125	3.658	24.611
West	*O. europaea* subsp. *cuspidata*	213.000	34.500	207.340	17.125	6.078	18.333
	Olea europaea subsp. *europaea*	164.180	41.730	131.130	10.600	2.162	11.000
Stellenbosch	Both	348.560	50.994	303.700	23.900	1.464	24.462
	O. europaea subsp. *cuspidata*	267.170	46.101	241.970	18.000	1.871	18.978
	Olea europaea subsp. *europaea*	174.110	28.679	171.930	11.333	1.846	12.000
Swellendam	Both	465.850	118.910	325.350	28.100	9.727	21.500
	O. europaea subsp. *cuspidata*	257.880	42.138	269.320	28.100	9.727	21.500
	Olea europaea subsp. *europaea*	230.570	79.314	134.270	10.000	3.742	10.167

∗SD = Chao2 Standard Deviation.

Table 2. Generalised linear model results based on total and core fungal endophytes of *Olea europaea*. Linear model designs considered total abundance, richness and rarefied richness of endophytes and for those of the core fungal endophytes for comparisons of the effect of host (*O. europaea* subsp. c*uspidata* and *O. europaea* subsp. *europaea*) and location across six sites (n = 102). Linear models with all fixed effects were also considered for the whole and core fungal richness and abundance, and core and whole rarefied and relative abundance. Results are significant when $p < 0.05$ (denoted by ∗). *Post hoc* results for the significant main tests are presented in Table S2.

	Richness			**Richness (core)**			**Abundance**			**Abundance (core)**		
Factor	**Chi-sq**	**Chi df**	**p-value**	**Chi-sq**	**Chi df**	**p-value**	**Chi-sq**	**Chi df**	**p-value**	**Chi-sq**	**Chi df**	**p-value**
Site	20.554	10	<0.05∗	45.929	10	<0.05∗	29,407	10	<0.05∗	27,548	10	<0.05∗
Host	301.32	6	<0.05∗	58.735	6	<0.05∗	16,607	6	<0.05∗	23,945	6	<0.05∗
Interaction	153.68	5	<0.05∗	27.332	5	<0.05∗	10,495	5	<0.05∗	23,565	5	<0.05∗
	Rarefied richness			Rarefied richness (core)			Relative abundance			Relative abundance (core)		
Factor	Chi-sq	Chi df	p-value	Chi-sq	Chi df	p-value	SS	F-value	p-value	SE	F-value	p-value
Site	65.14	0.879	0.502	9.575	2.101	0.033*						
Host	205.01	13.827	<0.05∗	1.996	2.908	0.803	9.727	5.678	<0.05∗	2.605	3.747	0.0817
Interaction	5.066	0.342	0.885	2.124	0.606	0.695						

Fungal abundance was significantly lower in the African olive than in the European olive irrespective of the measure used (Table 2 and S3, Fig. S1). Total fungal abundance was also significantly different between all sites (Tables 2 and S2). Although the abundance of core fungi was also significantly affected by site, some of the between-site comparisons were not significantly different (Table S3). The interaction between site and host identity had a significant effect on total abundance and core fungal abundance within olive twigs (Table 2 and S5).

4.2. Beta-diversity

Host played an important role to full and core fungal assemblage composition within olive twigs irrespective of whether fungal endophyte abundances or just the presence/absence of taxa were considered (Table 3, Fig. 3 and S2). The European olive samples clustered closer together than the African olive samples (Fig. 3 and S2). Full and core fungal assemblages were also significantly influenced by site, regardless of whether OTU abundances or presence/absence were considered (Table 3, Fig. 3 and S2). Accordingly, hosts from most sites harboured fungal assemblages different from each other, except when sites were close to each other and had similar vegetation types such as the Breede Shale Renosterveld vegetation in Robertson and Swellendam (Tables S1 and S6, Fig. 3).

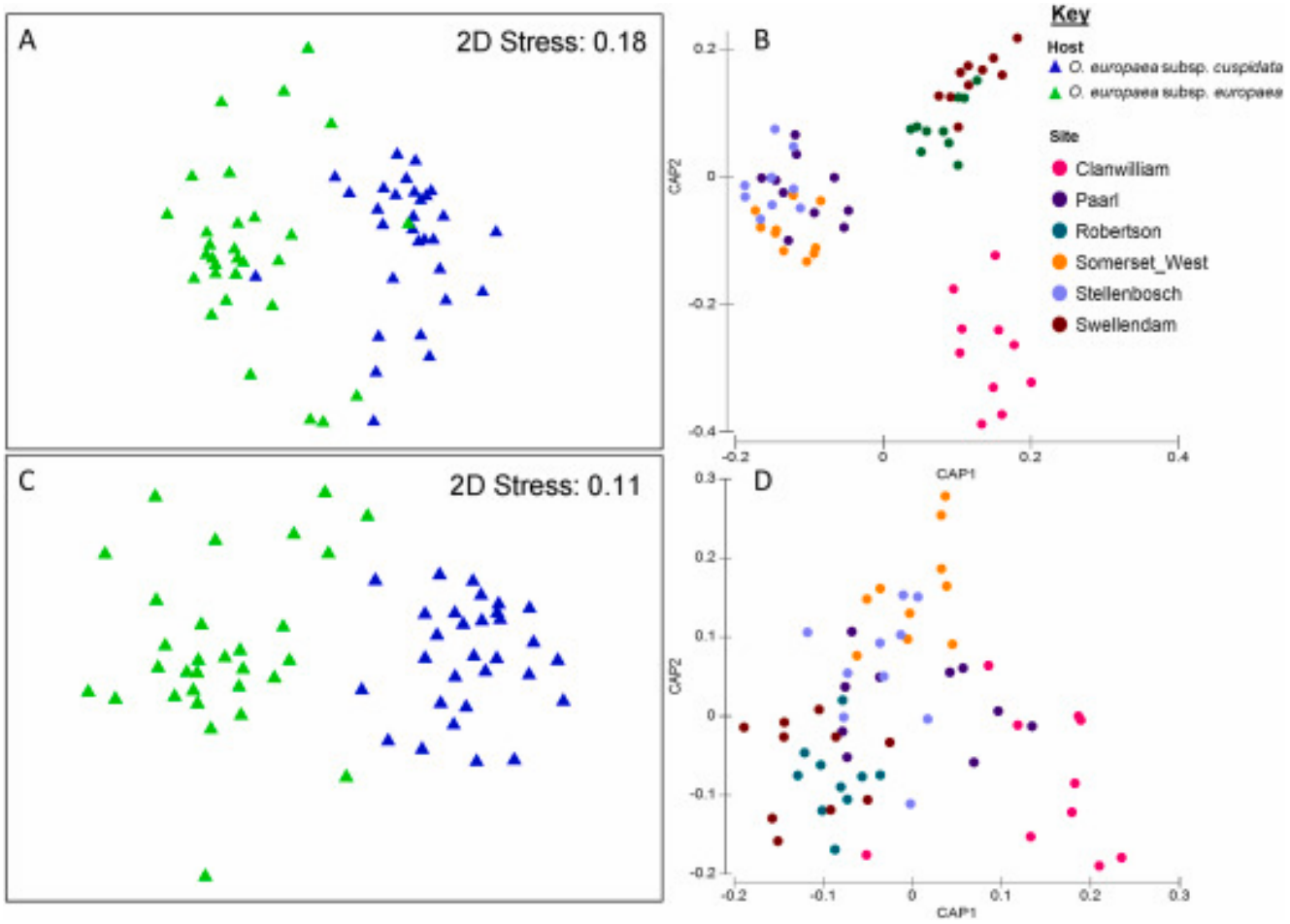

Fig. 3. Canonical analysis of principal coordinates (CAP) and non-metric multidimensional scaling (nMDS) plots based on abundance data (Bray-Curtis resemblance), for fungal endophyte assemblages sequenced from *Olea europaea* subsp. *cuspidata* and *O. europaea* subsp. *europaea* from six different sites (Fig. 1). Plots reflect the grouping of all endophytes (top) and only that of the core (bottom) within olive twigs based on host (nMDS, left) and site (CAP, right). Plots were constructed based on the reduced and balanced sample size, n = 60.

Table 3. Influence of host and site on fungal endophyte community assemblage composition (β1). Global PERMANOVA results on the overall influence of site and host and their interaction (n = 60) on fungal assemblages. Results with asterisk (∗) indicate comparisons significant at $p < 0.05$.

			Jaccard distance (incident)			Bray-Curtis distance (abundance)		
	Source	**df**	**SS**	**Pseudo-F**	**p (perm)**	**SS**	**Pseudo-F**	**p (perm)**
Whole assemblages	site	5	34,915	1.948	0.001∗	38,009	2.445	0.001∗
	host	1	19,256	5.372	0.001∗	29,662	9.541	0.001∗
	interaction	5	28,186	1.573	0.001∗	27,906	1.795	0.001∗
Core assemblages	host	1	63,193	29.141	0.001∗	58,105	23.489	0.001∗
	site	5	28,932	2.668	0.001∗	2.90 E+04	2.348	0.001∗
	interaction	5	26,594	2.453	0.001∗	2.75 E+04	2.227	0.001∗

Host identity did not influence the β2 diversity (endophyte species turnover) of the full fungal assemblages (Table 4). However, host identity was important to core fungal assemblage species turnover, with that of the introduced host higher than the native host (Table S4). β2 diversity of the full endophyte assemblages differed between sites (Table 4). Specifically, β2 diversity from samples collected in Swellendam were significantly lower than those within olive twigs collected from Clanwilliam, Paal, Somerset West and Stellenbosch (Table S7). This effect disappeared when only core endophyte assemblages were assessed. The interaction between site and host had a significant effect on β2 diversity of core fungal assemblages (Table 4, Fig. S3). Pairwise comparisons revealed that this significance was attributed to Swellendam, which had the lowest β2 diversity in both hosts, with the native host from this site having the lowest β2 diversity of all sites (Table S7).

Table 4. Homogeneity of within-group multivariate dispersions (β2) of whole endophyte assemblages, or of only core assemblages from *O. europaea* subsp. c*uspidata* and *O. europaea* subsp. e*uropaea* collected at six sites in South Africa. Results are significant at $p < 0.05$ (∗); their *post hoc* results are presented in Appendix A: Table S6.

	Source	**df**	**F**	**p-value**
Whole microbial assemblage	site	5	2.620	0.033∗
	host	1	1.624	0.211
	interaction	11	2.583	0.070
Core microbial assemblage	site	5	1.653	0.151
	host	1	5.247	0.050∗
	interaction	11	5.319	0.007∗

4.3. Effect of geographic distance between sites on endophyte community assemblage composition

Geographic distance between sites played a significant role in differentiating the full and the core fungal assemblages within olive twigs, except in core fungal endophyte assemblages of the European olive based on Bray-Curtis (Fig. 4 and S4). This pattern persisted when samples were pooled within sites regardless of host and when hosts were considered separately. The more distant sites, Clanwilliam and Swellendam,

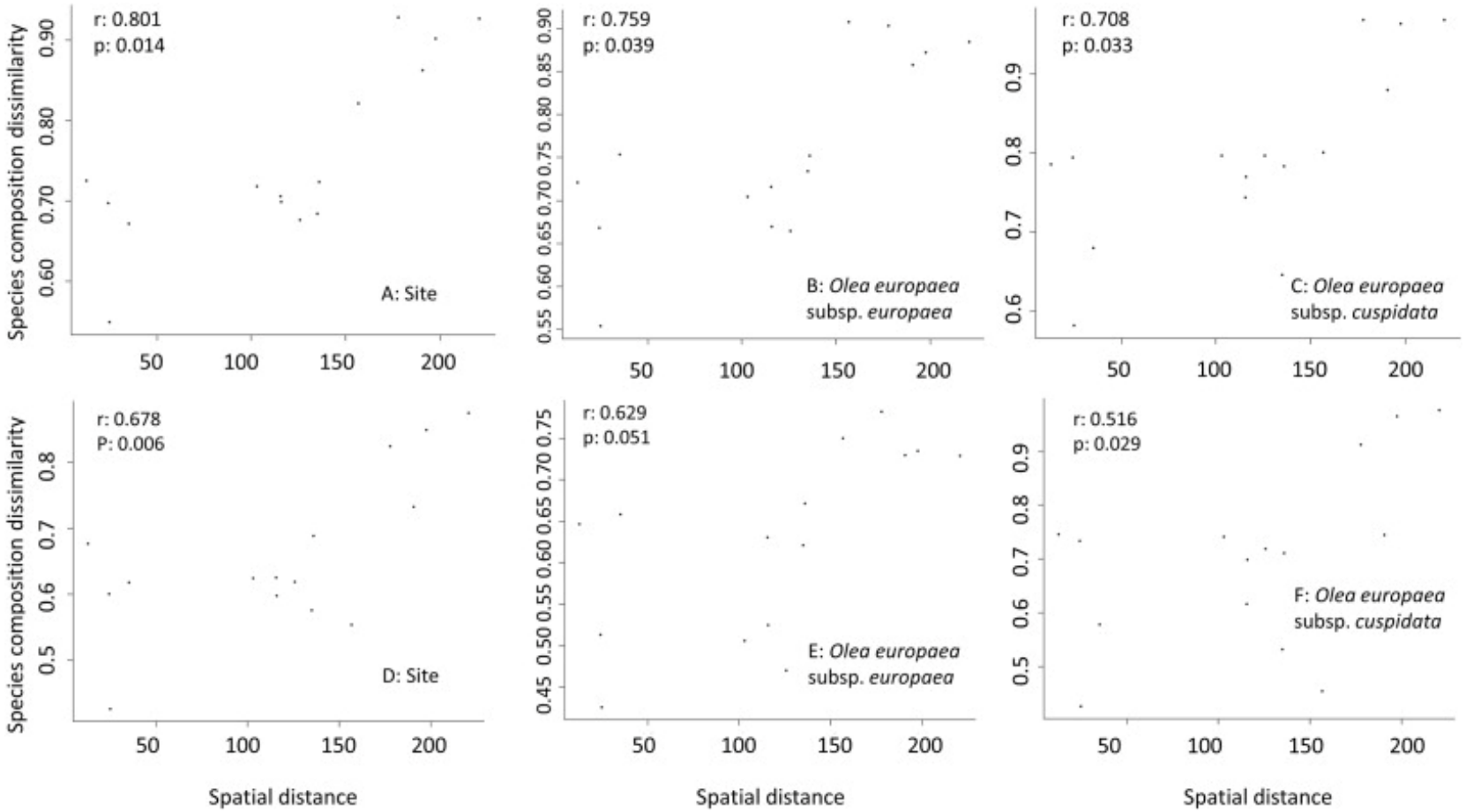

Fig. 4. Correlations between geographic distance (matrix based on Euclidean distances) and fungal endophyte assemblage compositional differences measured using Bray-Curtis dissimilarity matrices (based on abundance data). Plots A, B and C are based on full fungal assemblages, while D, E and F only consider core fungal assemblages. Correlations are meaningful at $r > 0.5$ and significant when $p < 0.05$.

consistently harboured different assemblages compared to those that were more proximal to each other.

4.4. Endophytic fungal co-occurrences

Co-occurrence networks of fungal endophytes from the two hosts were strikingly different from one another. European olive twigs had fewer significant fungal co-occurrences compared to the African olive (Table 5, Fig. 5, Fig. 6). Co-occurrences between fungal taxa within the cultivated host were largely disconnected, indicating that most of the taxa encountered here likely had no biologically meaningful relationships with each other (Fig. 6, Table 5). Only 37 of the 189 OTUs in the European olives were present in the network suggesting that many of the taxa encountered within this host were likely chance encounters rather than preferential associations. Economically important fungi often formed significant co-occurrences with other taxa in respective hosts, e.g., *Cladosporium* species, *Phoma* species and *Alternaria* species. The African olive network had a higher degree of connectivity with 41 nodes having node degrees higher than the average node degree (ad = 4.44, Table 5). In contrast, the European olive had 23 nodes with a node degree higher than the average node degree and an average node degree lower than that of the native olive (ad = 1.94, Table 5). The African olive network showed a higher degree of connectivity compared to the European olive, which had a largely disconnected network with the number of edges almost the same as the number of nodes.

Table 5. Summary network statistics for both networks (Fig. 5, Fig. 6) based on the full fungal composition within the olive twigs from the balanced sample design, n = 60 (30 per host). Cultivated olive = Olea europaea subsp. Europaea, native olive = Olea europaea subsp. Cuspidata.

	Native olive	**Cultivated olive**
No. of nodes (v)	100	37
No. of edges (e)	222	36
Average node degree (ad)	4.440	1.940
clustering coefficient (cc)	0.3513	0.638
graph density (gd)	0.045	0.054
modularity (md)	0.534	0.874
network diameter (nd)	11	4
short path length (spl)	3.917	1.436

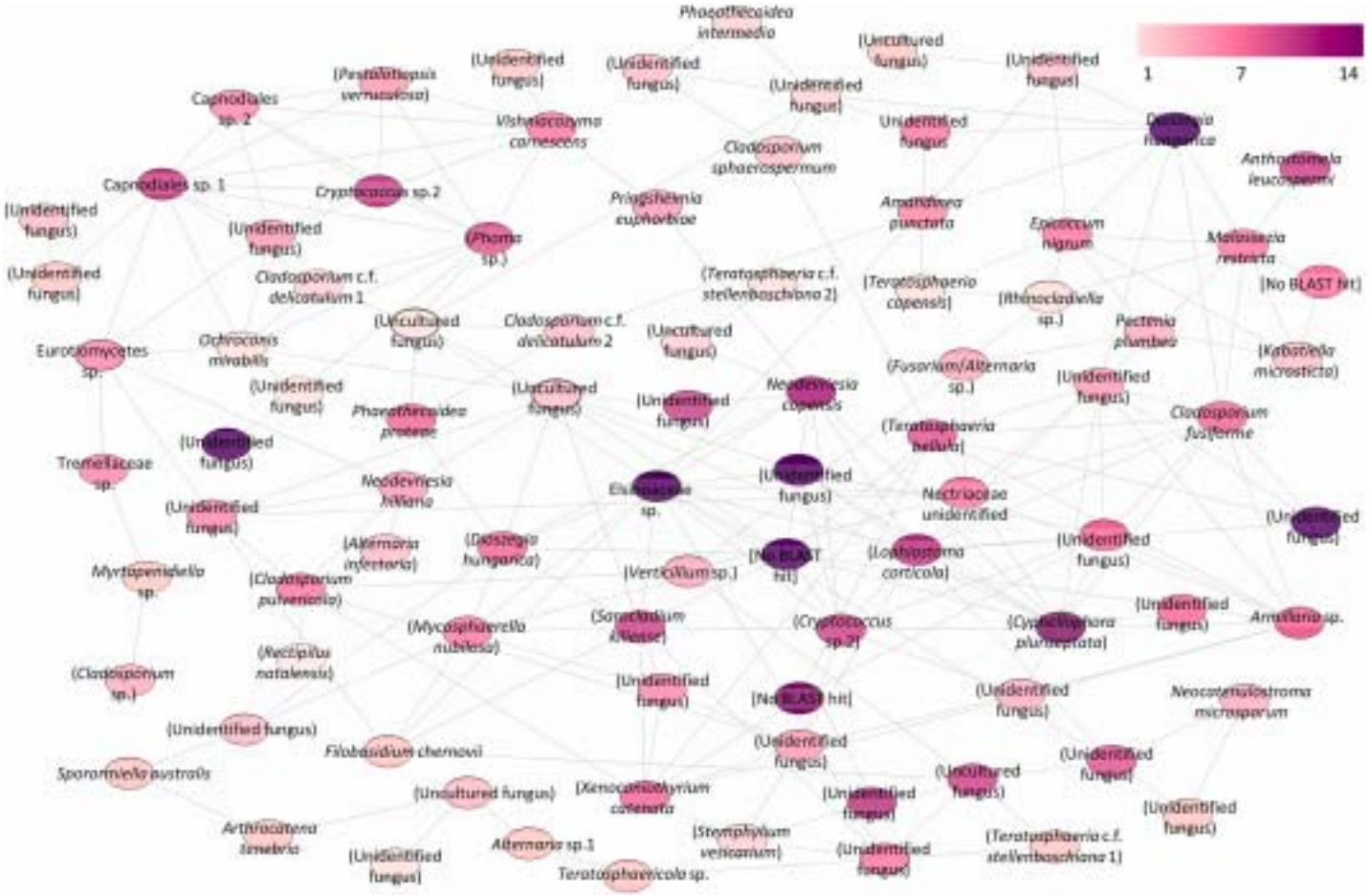

Fig. 5. An ecological co-occurrence network of fungal endophytes in *Olea europaea* subsp. *cuspidata* twigs. The colour gradient of nodes is indicative of node degree, where the darker the node the more connections it has. The thickness of the node outline indicates node betweenness centrality, the thicker the line the more important it is as a mediator that holds together node clusters on either side of it. The darker nodes with thick outlines have a higher probability of being hub taxa. Labels in rounded brackets come from GenBank (NCBI), those without are from the UNITE database and those with square brackets are taxa with no hits in either database.

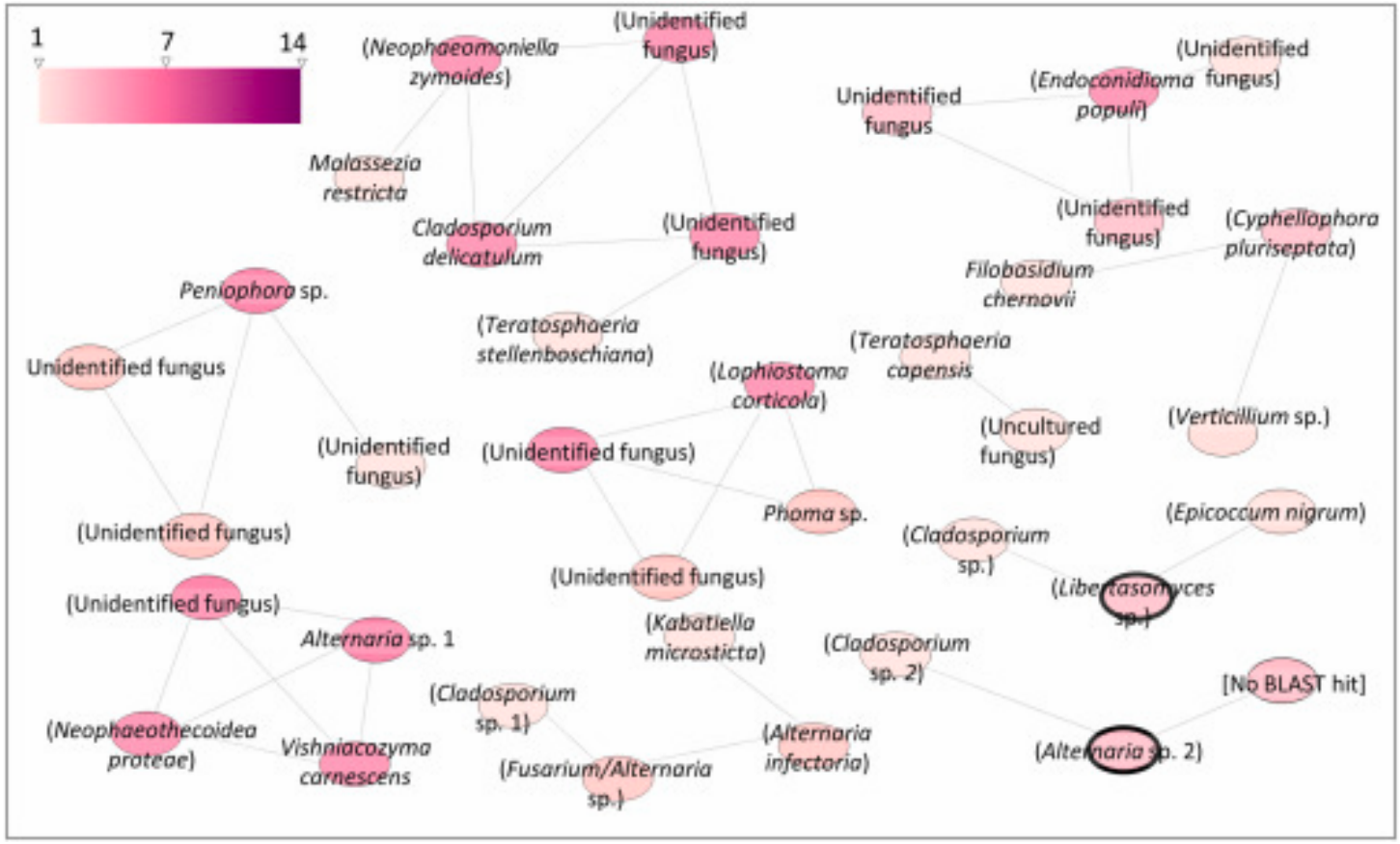

Fig. 6. An ecological co-occurrence network of fungal endophyte in *Olea europaea* subsp. *europaea*. The colour gradient of nodes is indicative of node degree, where the darker the node the more connections it makes and, conversely, the lighter it is the fewer the connections it has. The thickness of the node outline indicates node betweenness centrality (strategically placed nodes that hold together different parts of the network), the thicker the line the more important it is as a mediator that holds together node clusters on either side of it. Darker nodes with thick outlines are more likely to be hub taxa. Labels in rounded brackets originate from GenBank (NCBI), those without brackets are from the UNITE database while those with square brackets had no hits in either database.

5. Discussion

We present results of the first study using next generation sequencing to investigate fungal endophyte assemblages within two *Olea europaea* subspecies in South Africa. We show that these two host taxa share numerous fungal endophytes, which is to be expected when two closely related hosts also grow in close proximity. Many of the taxa encountered may be latent pathogens, that may have expanded their host ranges to include the newly encountered hosts. As expected, fungal endophyte alpha-diversity was higher within the native African olive than in the cultivated European olive, and their respective endophyte assemblage composition differed. In addition, fungal assemblages within the native host were much more interconnected compared to those of the cultivated olive, consistent with our hypothesis of lower endophyte richness in introduced hosts compared to native hosts. Sampling location played a significant role in the association between endophytes and the olive hosts, supporting our hypotheses that host environment plays a large role in the uptake or differentiation of fungal endophyte assemblages.

More endophyte taxa were shared between the native and non-native congeneric olive subspecies than were unique to each. This is not surprising considering studies on other hosts elsewhere that indicates that many fungal endophytes can easily colonise different hosts (Rodriguez et al., 2009; Bufford et al., 2016; Castañeda et al., 2018), but closely related native and introduced hosts generally tend to be more likely to exchange their fungi (Coleman-Derr et al., 2016; Crous et al., 2017). In the current study the two *O. europaea* subspecies were sampled where they co-occurred, thus increasing opportunity of fungal endophyte exchange. Despite these shared taxa, the European olive had significantly lower endophyte richness, and significantly different assemblage composition to the African host. The European olive also harboured fewer exclusive OTUs than the African olive. This was expected since some fungal associates may not have been co-introduced into the new range by chance or due to phytosanitary measures (e.g. fertiliser and pesticide application) and inconsistencies between the native and the introduced environments (Zhou et al., 2014; Hayward et al., 2015; Castañeda et al., 2018; Fernández-González et al., 2020b). The similarities in the core assemblages of the European olive from different sites may indicate that these endophytic taxa have been co-introduced with their hosts. Additionally, the high incident of shared taxa between the hosts suggests that the introduced host may have accrued much of its fungal endophyte diversity from the surrounding environments (Colautti et al., 2004; Martín-García et al., 2012).

Co-occurrence networks revealed a dense and highly connected network of endophytes within the African olive, while fungal endophytes within the European olive were very disconnected. This suggests that although the European olive takes up fungal endophytes from its surroundings, these acquisitions are infrequent and inconsistent from one individual to the next. In contrast, the highly connected co-occurrence network of the fungal endophytes of the African olive suggests that many of the taxa likely co-occur deliberately, thus may be important to the persistence of both the fungi and the host. Complex and highly connected networks have been associated with the resilience of microbial assemblages to changes in their environment (Rybakova et al., 2017; Santolini and Barabási, 2018; Fernández-González et al., 2020a). In this regard, the assemblages associated with the African olive may be more adapted to changes in their environment (both within the host and in the surrounding environment) than the

cultivated olive. Characterising the relationship between fungal endophytes, their hosts and the environment will greatly improve our understanding of the consequences of changes in ecosystems structures in the anthropogenic era and may prove useful for the commercial production of olives.

Most of the taxa encountered in the European olive network also appeared in the network of the native host. However, the consequences of their occurrence within the cultivated olive may be of concern. For example, a single *Verticillium* species was amongst the species that appeared in the disconnected network of the cultivated olive. Although this taxon was presented in the network of the native host, species in this genus (especially *Verticillium dahlia*) are known pathogens of the cultivated olive in other olive cultivating regions such as Spain, Italy and California (Chliyeh et al., 2014). While the consequences of the presence of this fungus are well known in the cultivated olive, the nature of its relationship with the native African olive is unclear but may remain neutral. Indeed, a notorious pathogen (*Hymenoscyphus fraxineus*) of *Fraxinus excelsior* is also found in other *Fraxinus* species where it is either a weak pathogen or a harmless endophyte (Kosawang et al., 2018, 2019). Additionally, the recruitment of some taxa may result from coinfections aided by the actions of other species (such as pathogens) in the interaction. To this effect, the presence of pathogens has been linked to a significant change in fungal endophyte assemblages in plants, including *O. europaea* (Gomes et al., 2019; Varanda et al., 2019).

Geographic location played a significant role in shaping endophytic fungal assemblages within twigs from both olive hosts. These patterns were like those found in fungal assemblages in olives in Portugal (Martins et al., 2016), olives in Brazil (de Oliveira et al., 2022), *Quercus ilex* in Spain (Collado et al., 1999), and in native and non-native *Phragmites* species from Michigan (Bickford et al., 2018), amongst others. The role of locality becomes more evident when considering sites located closest to each other, such as Somerset West and Stellenbosch, and Robertson and Swellendam. These sites harboured more similar assemblages within both hosts than sites further apart, a pattern mirrored in other studies in other systems (Slabbert et al., 2010; Martins et al., 2016). There is therefore an overwhelming effect of surrounding environment on the endophyte assemblages within olive hosts. Factors that may influence this are numerous and include differences in surrounding plant communities (Ngubane, 2022) and pollution levels (Lumibao et al., 2018). In the present study, similarities in the surrounding vegetation types at sites near each other (e.g. Swartland Shale and Granite Renosterveld in Stellenbosch and Paarl) are likely responsible for the similarities in fungal assemblages. Similar vegetation types not only experience similar climatic conditions, but also have similar plant communities from which many of the fungal endophyte taxa are sourced (Saikkonen et al., 2000; Helander et al., 2007; Christian et al., 2016).

Different plant species often share endophytic fungal taxa as exemplified by the known occurrence of some of the fungal taxa encountered in the present study on other hosts in South Africa (e.g. Crous et al., 2009; van Niekerk et al., 2004) and in olives elsewhere (e.g. Úrbez-Torres et al., 2013). For example, *Neofusicoccum australe* identified from both olive hosts here has also previously been recorded in grapevines, olive orchards and olive nurseries in South Africa (de Wet et al., 2008; Spies et al., 2020; van Dyk et al., 2021b) and in olives in Uruguay (Hernández-Rodríguez et al., 2022). *Dothiorella iberica* was identified from both olive hosts in the present study and

has been implicated as a causal agent of olive diseases in California (Úrbez-Torres et al., 2013). *Teratospharia stellenboschiana* recorded within the cultivated olive is known from leaves of *Eucalyptus* species in Stellenbosch and Pretoria (Crous et al., 2009). Although typically species level identification using metabarcoding ITS sequences is not recommended, the appearance of fungal taxa already identified in the area and/or in the same host stratifies the identity of the fungal endophytes identified in the present study. Nevertheless, the commonalities between fungal endophytes in our olives and fungal endophytes in olives elsewhere and other hosts in South Africa, some of which are associated with disease symptoms, raise plant health concerns that warrant further investigations.

Some endophytic fungi present conflicting lifestyles upon exiting the endophytic phase such that they can either become beneficial or harmful. Endophytic fungi that can become beneficial to their host often reside in genera such as *Epicoccum*, *Cladosporium* and *Penicillium* (Khan et al., 2016; Dzoyem et al., 2017; Gomes et al., 2019). A single *Epicoccum* species (*Epicoccum nigrum*) was identified from both hosts. *Epicoccum nigrum* has been encountered from members of the Restionaceae in South Africa (Lee et al., 2004) and in many hosts globally, including *Fraxinus* species (Kosawang et al., 2018) and olives (Gomes et al., 2019). A harmless (with potential to become beneficial) nature of the European olive – *E. nigrum* association was hinted at by the consistent discovery of *E. nigrum* in asymptomatic olive twigs and its absence from symptomatic twigs (Gomes et al., 2019). However, *Epicoccum nigrum* has also been associated with olive fruit rot in Iran (Torbati et al., 2014). This may suggest that this fungus is capable of a wide range of lifestyles depending on the prevailing conditions and the identity of other microbes in the microhabitat. Five *Cladosporium* taxa (including two undescribed taxa), found within the native olive, also formed significant co-occurrences within the cultivated olive twigs. Although beneficial to other hosts, *Cladosporium* species such as *Cladosporium herbarum* and an undescribed *Cladosporium* species have also been implicated as causal agents of olive fruit rots and olive leaf mould in other countries including Australia and Iran (Arnold et al., 2003; Faiza et al., 2011). The seemingly conflicting effects of these endophytic fungi to their associated hosts highlight the need to improve our understanding of endophytic fungi-host associations and the factors triggering lifestyle switches.

This study contributes to the growing knowledge of fungi associated with agricultural crops cultivated in the Core Cape Subregion of South Africa. Studying fungal endophyte assemblages within crops and native hosts can shed light on fungal movement and host expansion. Knowledge on fungal endophytes present in crop plants is especially critical for those that are known to shift to become pathogens that affect crop productivity. In addition, as microbiome manipulation to improve productivity is gaining attention, it is important to understand fungal assemblages within agricultural crops and their closest native relatives. Due to the conservative nature of the ITS region used in metabarcoding studies, further barcoding and confirmation activities are required, especially for the taxa we identified that are also of biotechnological interests. The prevalence of undescribed taxa indicates a trove of potentially important taxa that remains to be discovered and described in a biodiversity hotspot.

Data statement

Our data is not publicly available but may be made available upon request.

Declaration of competing interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nombuso Portia Ngubane reports financial support was provided by University of Pretoria Centre Of Excellence In Tree Health Biotechnology.

Acknowledgments

We thank the DSI-NRF Centre of Excellence in Plant Health Biotechnology for funding this research. Thank you to private landowners, protected areas managers, olive orchard managers and olive orchard owners for allowing us to sites for sample collection. Thanks to Courtney Morris and Kyla Janse van Rensburg for their assistance with field and laboratory work. Thank you to Tanja Rollnik for providing guidance during metabarcoding library preparation. We also thank Drs Palesa Mothapo and Maria Vivas for their assistance with data analyses.

References

Abdelfattah, A., Li Destri Nicosia, M.G., Cacciola, S.O., Droby, S., Schena, L., 2015. Metabarcoding analysis of fungal diversity in the phyllosphere and carposphere of olive (Olea europaea). PLoS One 10, 1–19. https://doi.org/10.1371/journal.pone.0131069.

Adams, G.C., Roux, J., Wingfield, M.J., 2006. *Cytospora* species (Ascomycota, diaporthales, valsaceae): introduced and native pathogens of trees in South Africa. Australas. Plant Pathol. 35, 521–548. https://doi.org/10.1071/AP06058.

Agricultural Research Council, 2010. Directorate Plant Production. Olives — Production guideline, Pretoria, South Africa, pp. 1–32.

Amabeoku, G.J., Bamuamba, K., 2010. Evaluation of the effects of *Olea europaea* L. subsp. africana (Mill.) P.S. Green (Oleaceae) leaf methanol extract against castor oil-induced diarrhoea in mice. J. Pharm. Pharmacol. 62, 368–373. https://doi.org/10.1211/jpp/62.03.0012.

Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. PRIMER-E, Plymouth, pp. 1–218.

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

Anderson, M.J., 2006. Distance-based tests for homogeneity of multivariate dispersions. Biometrics 62, 245–253. https://doi.org/10.1111/j.1541-0420.2005.00440.x.

Arnold, A.E., Mejía, L.C., Kyllo, D., Rojas, E.I., Maynard, Z., Robbins, N., Herre, E.A., 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proc. Natl. Acad. Sci. U. S. A 100, 15649–15654. https://doi.org/10.1073/pnas.2533483100.

Assenov, Y., Ramírez, F., Schelhorn, S.E.S.E., Lengauer, T., Albrecht, M., 2008. Computing topological parameters of biological networks. Bioinformatics 24, 282–284. https://doi.org/10.1093/bioinformatics/btm554.

Aumeeruddy-Thomas, Y., Moukhli, A., Haouane, H., Khadari, B., 2017. Ongoing domestication and diversification in grafted olive – oleaster agroecosystems in Northern Morocco. Reg. Environ. Change 17, 1315–1328. https://doi.org/10.1007/s10113-017-1143-3.

Bates, D.M., Sarkar, D., 2007. Lme4: linear mixed-effects models using S4 classes. In: R Package, vols. 1–29. https://cran.r-project.org/src/contrib/Archive/lme4/.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and
powerful approach to multiple testing. J. Roy. Stat. Soc. B 57, 289–300. https://doi.org/10.2307/2346101.

Besnard, G., Henry, P., Wille, L., Cooke, D., Chapuis, E., 2007. On the origin of the invasive olives (*Olea europaea* L., Oleaceae). Heredity 99, 608–619. https://doi.org/10.1038/sj.hdy.6801037.

Besnard, G., Khadari, B., Baradat, P., Bervillé, A., 2002. *Olea europaea* (Oleaceae) phylogeography based on chloroplast DNA polymorphism. Theor. Appl. Genet. 104, 1353–1361. https://doi.org/10.1007/s00122-001-0832-x.

Bickford, W.A., Goldberg, D.E., Kowalski, K.P., Zak, D.R., 2018. Root endophytes and invasiveness: no difference between native and non-native Phragmites in the Great
Lakes Region. Ecosphere 9, 1–14. https://doi.org/10.1002/ecs2.2526.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. Ecol. Monogr. 27, 325–349.

Bufford, J.L., Hulme, P.E., Sikes, B.A., Cooper, J.A., Johnston, P.R., Duncan, R.P., 2016. Taxonomic similarity, more than contact opportunity, explains novel plant–pathogen associations between native and alien taxa. New Phytol. 212, 657–667. https://doi.org/10.1111/nph.14077.

Burnham, K.P., Overton, W.S., 1978. Estimation of the size of a closed population when capture probabilities vary among animals. Biometrika 65, 625–633.

Caporaso, J., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F., Costello, E., Fierer, N., Peña, A., Goodrich, J., Gordon, J., Huttley, G., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 415–425. https://doi.org/10.1038/nmeth.f.303.QIIME.

Castañeda, L.E., Miura, T., Sánchez, R., Barbosa, O., 2018. Effects of agricultural management on phyllosphere fungal diversity in vineyards and the association with adjacent native forests. PeerJ 6, e5715. https://doi.org/10.7717/peerj.5715.

Chao, A., Lee, S.-M., 1992. Estimating the number of classes via sample coverage. J. Am. Stat. Assoc. 87, 210–217.

Chliyeh, M., Touati, J., Selmaoui, K., Touhami, A.O., Filali-Maltouf, A., El Modafar, C., Moukhli, A., Benkirane, R., Douira, A., 2014. Bibliographic inventory of the olive tree (*Olea europaea* L.) fungal diseases in the world. Int. J. Pure App. Biosci. 2, 46–79.

Christian, N., Sullivan, C., Visser, N.D., Clay, K., 2016. Plant host and geographic location drive endophyte community composition in the face of perturbation. Microb. Ecol. 72, 621–632. https://doi.org/10.1007/s00248-016-0804-y.

Cline, M., Smoot, M., Cerami, E., Kuchinsky, A., Landys, N., Workman, C., Christmas, R., Avila-Campilo, I., Creech, M., Gross, B., Hanspers, K., Isserlin, R., Kelley, R., Killcoyne, S., Lotia, S., Maere, S., Morris, J., Ono, K., Pavlovic, V., Pico, A.R., Vailaya, A., Wang, P.-L., Adler, A., Conklin, B.R., Hood, L., Kuiper, M., Warner, G.J., Ideker, T., Bader, G.D., 2007. Integration of biological networks and gene expression data using Cytoscape. Nat. Protoc. 2, 2366–2382. https://doi.org/10.1038/nprot.2007.324.Integration.

Colautti, R.I., Ricciardi, A., Grigorovich, I.A., MacIsaac, H.J., 2004. Is invasion success explained by the enemy release hypothesis? Ecol. Lett. 7, 721–733. https://doi.org/10.1111/j.1461-0248.2004.00616.x.

Coleman-Derr, D., Desgarennes, D., Fonseca-garcia, C., Gross, S., Clingenpeel, S., Woyke, T., North, G., Visel, A., Partida-Martinez, L.P., Tringe, S.G., 2016. Plant compartment and biogeography affect microbiome composition in cultivated and native *Agave* species. New Phytol. 209, 798–811.

Collado, J., Platas, G., Gonzalez, I., Pelaez, F., 1999. Geographical and seasonal influences on the distribution of fungal endophytes in Quercus ilex. New Phytol. 144, 525–532. https://doi.org/10.1111/j.1439-0329.2007.00509.x.

Costa, C., 1998. Olive Production in South Africa: A Handbook for Olive Growers. Agricultural Research Council, Pretoria, pp. 1–124.

Costa, C., 2018. Advances and opportunities in olive production: why research and technology development is required for success? Met. Ind. News 48–49.

Costa, D., Fernandes, T., Martins, F., Pereira, J.A., Tavares, R.M., Santos, P.M., Baptista, P., Lino-Neto, T., 2021. Illuminating *Olea europaea* L. endophyte fungal community. Microbiol. Res. 245, 1–10. https://doi.org/10.1016/j.micres.2020.126693.

Crous, C.J., Burgess, T.I., Le Roux, J.J., Richardson, D.M., Slippers, B., Wingfield, M.J., 2017. Ecological disequilibrium drives insect pest and pathogen accumulation in non-native trees. AoB Plants 9, 1–16. https://doi.org/10.1093/aobpla/plw081.

Crous, P.W., Summerell, B.A., Carnegie, A.J., Wingfield, M.J., Hunter, G.C., Burgess, T.I., Andjic, V., Barber, P.A., Groenewald, J.Z., 2009. Unravelling *Mycosphaerella*: do you believe in genera? Persoonia 23, 99–118. https://doi.org/10.3767/003158509X479487.

De Oliveira, A.A.D., Ramalho, M.D.O., Moreau, C.S., Campos, A.E.D.C., Harakava, R., Bueno, O.C., 2022. Exploring the diversity and potential interactions of bacterial and fungal endophytes associated with different cultivars of olive (*Olea europaea*) in Brazil. Microbiol. Res. 263, 1–17. https://doi.org/10.1016/j.micres.2022.127128.

de Wet, J., Slippers, B., Preisig, O., 2008. Phylogeny of the Botryosphaeriaceae reveals
patterns of host association. Mol. Phylogenet. Evol. 46, 116–126. https://doi.org/10.1016/j.ympev.2007.08.016.

Doyle, J.J., Doyle, J.L., 1990. A rapid total DNA preparation procedure for fresh plant tissue. Focus 12, 13–15.
Dzoyem, J.P., Melong, R., Tsamo, A.T., Maffo, T., Kapche, D.G.W.F., Ngadjui, B.T., McGaw, L.J., Eloff, J.N., 2017. Cytotoxicity, antioxidant and antibacterial activity of four compounds produced by an endophytic fungus *Epicoccum nigrum* associated with *Entada abyssinica*. Brazilian J. Pharmacogn. 27, 251–253. https://doi.org/10.1016/j.bjp.2016.08.011.

Faiza, I., Wahiba, K., Nassira, G., Chahrazed, B., Atik, B.F., 2011. Antibacterial and antifungal activities of olive (*Olea europaea* L.) from Algeria. J. Microbiol. Biotechnol. Res. 1, 69–73.

Fernández-González, A.J., Cardoni, M., Gómez-Lama Cabanás, C., Valverde-Corredor, A., Villadas, P.J., Fernández-López, M., Mercado-Blanco, J., 2020a. Linking belowground microbial network changes to different tolerance level towards Verticillium wilt of olive. Microbiome 8, 1–19. https://doi.org/10.1186/s40168-020-0787-2.

Fernández-González, A.J., Wentzien, N.M., Villadas, P.J., Valverde-Corredor, A., Lasa, A. V., *Cytospora*, C.G.L., Mercado-Blanco, J., Fernández-López, M., 2020b. Comparative study of neighboring Holm oak and olive trees-belowground microbial communities subjected to different soil management. PLoS One 15, 1–21. https://doi.org/10.1371/journal.pone.0236796.

Ferreira, T., Rasband, W., 2012. ImageJ user guide. ImageJ/Fiji 1, 155–161.

Freeman, L.C., 1977. A set of measures of centrality based on betweenness. Sociometry 40, 35–41.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes, application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–118, 10.1111/J.1365-294x.1993.Tb00005.

Gauch Jr., H.G., 1973. A quantitative evaluation of the Bray-Curtis ordination. Ecology 54, 829–836.

Glen, M., Alfenas, A.C., Zauza, E.A.V., Wingfield, M.J., Mohammed, C., 2007. *Puccinia psidii*: a threat to the Australian environment and economy - a review. Australas. Plant Pathol. 36, 1–16. https://doi.org/10.1071/AP06088.

Gomes, T., Pereira, J.A., Benhadi, J., Lino-neto, T., Baptista, P., 2018. Endophytic and epiphytic phyllosphere fungal communities are shaped by different environmental factors in a Mediterranean ecosystem. Microb. Ecol. 76, 668–679.

Gomes, T., Pereira, J.A., Lino-Neto, T., Bennett, A.E., Baptista, P., 2019. Bacterial disease induced changes in fungal communities of olive tree twigs depend on host genotype. Sci. Rep. 9, 1–10. https://doi.org/10.1038/s41598-019-42391-8.

Gore, M.E., Bucak, C., 2007. Geographical and seasonal influences on the distribution of fungal endophytes in Laurus nobilis. For. Pathol. 37, 281–288.

Gorter, G.J.M.A., 1956. Anthracnose fungi of olives. Nature 178, 1129–1130.

Gorter, G.J.M.A., 1962. The identity of the fungus causing anthracnose of olives in South Africa. Bothalia 7, 769–778.

Green, P.S., 2002. A revision of Olea L. (Oleaceae). Kew Bull. 57, 91–140.

Hajishengallis, G., Darveau, R.P., Curtis, M.A., 2012. The keystone-pathogen hypothesis. Nat. Rev. Microbiol. 10, 717–725. https://doi.org/10.1038/nrmicro2873.

Harrell Jr., F.E., 2014. Package 'Hmisc'. R Package, vols. 1–389. http://biostat.mc. vanderbilt.edu/Hmisc. https://github.com/harrelfe/Hmisc.

Hayward, J., Horton, T.R., Pauchard, A., Nunez, M.A., 2015. A single ecto-mycorrhizal fungal species can enable a Pinus invasion. Ecology 96, 1438–1444.

Helander, M., Ahlholm, J., Sieber, T.N., Hinneri, S., Saikkonen, K., 2007. Fragmented environment affects birch leaf endophytes. New Phytol. 175, 547–553. https://doi.org/10.1111/j.1469-8137.2007.02110.x.

Hernández- Rodríguez, L., Mondino-Hintz, P., Alaniz-Ferro, S., 2022. Diversity of Botryosphaeriaceae species causing stem canker and fruit rot in olive trees in Uruguay. J. Phytopathol. 170, 264–277.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50, 346–363. https://doi.org/10.1002/bimj.200810425.

Hyde, K.D., Soytong, K., 2008. [Commentary] the fungal endophyte dilemma. Fungal Divers. 33, 163–173.

Khan, M.I.H., Sohrab, M.H., Rony, S.R., Tareq, F.S., Hasan, C.M., Mazid, M.A., 2016. Cytotoxic and antibacterial naphthoquinones from an endophytic fungus. Cladosporium sp. Toxicol. Reports 3, 861–865. https://doi.org/10.1016/j.toxrep.2016.10.005.

Kõljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F., Bahram, M., Bates, S. T., Bruns, T.D., Bengtsson-Palme, J., Callaghan, T.M., Douglas, B., Drenkhan, T., Eberhardt, R., Dueñass, M., Grebenc, T., Griffith, G.W., Hartmann, M., Kirk, P.M., Kohout, P., Larsson, E., Lindahl, B.D., Lücking, R., Martín, M.P., Matheny, P.B., Nguyen, N.H., Niskanen, T., Oja, J., Peay, K.G., Peintner, U., Peterson, M., Põldmaa, K., Saag, L., Saar, I., Schüßler, A., Scott, J.A., Sen´es, C., Smith, M.E., Suija, A., Taylor, D.L., Telleria, M.T., Weiß, M., Larsson, K.-H., 2013. Towards a unified paradigm for sequence-based identification of Fungi. Mol. Ecol. 22, 5271–5277. https://doi.org/10.1111/mec.12481.

Kosawang, C., Amby, D.B., Bussaban, B., McKinney, L.V., Xu, J., Kjær, E.D., Collinge, D.B., Nielsen, L.R., 2018. Fungal communities associated with species of *Fraxinus* tolerant to ash dieback, and their potential for biological control. Fungal Biol 122, 110–120. https://doi.org/10.1016/j.funbio.2017.11.002.

Kosawang, C., Sørensen, H., Kjær, E.D., Dilokpimol, A., McKinney, L.V., Collinge, D.B., Nielsen, L.R., 2019. Defining the twig fungal communities of *Fraxinus* species and *Fraxinus* excelsior genotypes with differences in susceptibility to ash dieback. Fungal Ecol 42, 100859. https://doi.org/10.1016/j.funeco.2019.08.003.

Lee, S., Mel'nik, V., Taylor, J.E., Crous, P.W., 2004. Diversity of saprobic hyphomycetes on proteaceae and Restionaceae from South Africa. Fungal Divers. 17, 91–114.

Li, W., Fu, L., Niu, B., Wu, S., Wooley, J., 2012. Ultrafast clustering algorithms for metagenomic sequence analysis. Briefings Bioinf. 13, 656–668. https://doi.org/10.1093/bib/bbs035.

Lumibao, C.Y., Formel, S., Elango, V., Pardue, J.H., Blum, M., Van Bael, S.A., 2018. Persisting responses of salt marsh fungal communities to the Deepwater Horizon oil spill. Sci. Total Environ. 642, 904–913. https://doi.org/10.1016/j.scitotenv.2018.06.077.

Magurran, A.E., 2004. An index of diversity. In: Magurran, A.E. (Ed.), Measuring Biological Diversity. Blackwell Publishing Ltd, Oxford, pp. 100–130.

Manning, J., Goldblatt, P., 2012. Plants of the Greater Cape Floristic Region 1: the Core Cape Flora, Strelitzia 29. South African National Biodiversity Institute, Pretoria, pp. 1–870.

Martín-García, J., Müller, M.M., Diez, J.J., 2012. ITS-based comparison of endophytic mycota in twigs of native *Populus nigra* and cultivated *P. x euramericana* (cv. I-214) stands in Northern Spain. Ann. For. Sci. 69, 49–57. https://doi.org/10.1007/s13595-011-0129-4.

Martins, F., Pereira, J.A., Bota, P., Bento, A., Baptista, P., 2016. Fungal endophyte communities in above- and belowground olive tree organs and the effect of season and geographic location on their structures. Fungal Ecol 20, 193–201. https://doi.org/10.1016/j.funeco.2016.01.005.

Martins, F., Cameirão, C., Mina, D., Benhadi-Marín, J., Pereira, J.A., Baptista, P., 2021. Endophytic fungal community succession in reproductive organs of two olive tree cultivars with contrasting anthracnose susceptibilities. Fungal Ecol 49, 1–11. https://doi.org/10.1016/j.funeco.2020.101003.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, 1–12. https://doi.org/10.1371/journal.pone.0061217.

Moral, J., Muñoz-Díez, C., Gonz´alez, N., Trapero, A., Michailides, T.J., 2010. Characterization and pathogenicity of Botryosphaeriaceae species collected from olive and other hosts in Spain and California. Ecol. Epidemiol. 100, 1340–1351.

Moyo, P., Mostert, L., Halleen, F., 2019. Diatrypaceae species overlap between vineyards and natural ecosystems in South Africa. Fungal Ecol 39, 142–151. https://doi.org/10.1016/j.funeco.2018.11.015.

Mucina, L., Rutherford, M.C., 2006. The Vegetation of South Africa, Lesotho and Swaziland, Strelitzia 19. South African National Biodiversity Institute, Pretoria. Newman, M.E.J., 2003. Mixing patterns in networks. Phys. Rev. 67, 1–13. https://doi.org/10.1103/PhysRevE.67.026126.

Ngubane, N.P., 2022. Fungal Endophyte Assemblages Associated with Twigs of Olives in the Core Cape Subregion, South Africa. Stellenbosch University, PhD Thesis.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Simpson, G.L., Solymos, P.M., Stevens, M.H.H., Wagner, H., 2008. The vegan package. Community ecology package. R Package 1–191.

Palmer, M.W., 1991. Estimating species richness: the Second-order Jackknife reconsidered. Ecology 72, 1512–1513.

Perez, C.A., Wingfield, M.J., Altier, N., Blanchette, R.A., 2012. Species of *Epicoccum nigrum* ceae and Teratosphaeriaceae on native Myrtaceae in Uruguay: evidence of fungal host jumps. Fungal Biol 117, 94–102. https://doi.org/10.1016/j.funbio.2012.12.002.

Power, M.W.P., Hopkins, A.J.M., Chen, J., Bengtsson, S.B.K., Vasaitis, R., Cleary, M.R., 2017. European *Fraxinus* species introduced into New Zealand retain many of their native endophytic fungi. Balt. For. 23, 74–81.

R Development Core Team, 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna.

Rebelo, A.G., Boucher, C., Helme, N., Mucina, L., Rutherford, M.C., 2006. Fynbos Biome. In: Mucina, L., Rutherford, M.C. (Eds.), The Vegetation of South Africa, Lesotho and Swaziland. South African National Biodiversity Institute, Pretoria, pp. 53–219.

Rodriguez, R.J., White, J.F., Arnold, A.E., Redman, R.S., 2009. Fungal endophytes: diversity and functional roles. New Phytol. 182, 314–330.

Rybakova, D., Mancinelli, R., Wikström, M., Birch-Jensen, A.S., Postma, J., Ehlers, R.U., Goertz, S., Berg, G., 2017. The structure of the *Brassica napus* seed microbiome is cultivar-dependent and affects the interactions of symbionts and pathogens. Microbiome 5, 1–16. https://doi.org/10.1186/s40168-017-0310-6.

Saikkonen, K., Ahlholm, J., Helander, M., Lehtimäki, S., Niemeläinen, O., 2000. Endophytic fungi in wild and cultivated grasses in Finland. Ecography 23, 360–366. https://doi.org/10.1111/j.1600-0587.2000.tb00292.x.

Saikkonen, K., Faeth, S.H., Helander, M., Sullivan, T.J., 1998. Fungal endophytes: a continuum of interactions with host plants. Annu. Rev. Ecol. Systemat. 29, 319–343. https://doi.org/10.1146/annurev.ecolsys.29.1.319.

Santolini, M., Barab´asi, A.-L., 2018. Predicting perturbation patterns from the topology of biological networks. Proc. Natl. Acad. Sci. USA 115, E6375–E6383. https://doi.org/10.1073/pnas.1720589115.

Schulz, B., Boyle, C., 2006. Microbial root endophytes. Soil Biol. 9, 1–14. https://doi.org/10.1007/3-540-33526-9.

Slabbert, E., Kongor, R.Y., Esler, K.J., Jacobs, K., Slabbert, E., 2010. Microbial diversity and community structure in Fynbos soil. Mol. Ecol. 19, 1031–1041. https://doi.org/10.1111/j.1365-294X.2009.04517.x.

Slippers, B., Stenlid, J., Wingfield, M.J., 2005. Emerging pathogens: fungal host jumps following anthropogenic introduction. Trends Ecol. Evol. 20, 420–421. https://doi.org/10.1016/j.tree.2005.04.024.

Slippers, B., Wingfield, M.J., 2007. Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. Fungal Biol. Rev. 21, 90–106. https://doi.org/10.1016/j.fbr.2007.06.002.

Spearman, C., 1904. Proof and measurement of association between two things. Am. J. Psychol. 15, 72–101.

Spies, C.F.J., Mostert, L., Carlucci, A., Moyo, P., van Jaarsveld, W.J., du Plessis, I.L., van Dyk, M., Halleen, F., 2020. Dieback and decline pathogens of olive trees in South Africa. Persoonia Mol. Phylogeny Evol. Fungi 45, 196–220. https://doi.org/10.3767/persoonia.2020.45.08.

Stackebrandt, E., Goebel, B.M., 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 168, 846–849. https://doi.org/10.1016/S0140-6736(01)43317-4.

Therios, I., 2009. History of olive growing. In: Therios, I. (Ed.), Olives. CABI Publishing, London, United Kingdom, pp. 1–7.

Torbati, M., Arzanlou, M., Azadmard-Damirchi, S., Babai-Ahari, A., Alijani, S., 2014. Effect of fungal species involved in the olive fruit rot on the qualitative properties of olive oil. Arch. Phytopathol. Plant Protect. 47, 292–297. https://doi.org/10.1080/03235408.2013.809183.

Úrbez-Torres, J., Peduto, F., Vossen, P., Krueger, W., Gubler, W., 2013. Olive twig and branch dieback: etiology, incidence, and distribution in California. Plant Dis. 97, 231–244.

Van Dyk, M., Spies, C.F.J., Mostert, L., van der Rijst, M., du Plessis, I.L., Moyo, P., van Jaarsveld, W., Hallen, F., 2021a. Pathogenicity testing of fungal isolates associated with olive trunk diseases in South Africa. Plant Dis. 105, 4060–4073.

Van Dyk, M., Spies, C.F.J., Mostert, L., Halleen, F., 2021b. Survey of trunk pathogens in South African olive nurseries. Plant Dis. 105, 1–10. https://doi.org/10.1094/PDIS-04-20-0798-RE.

Van Niekerk, J.M., Crous, P.W., Groenewald, J.Z., Fourie, P.H., Halleen, F., 2004. DNA phylogeny, morphology and pathogenicity of Botryosphaeria species on grapevines. Mycologia 96, 781–798. https://doi.org/10.1080/15572536.2005.11832926.

Varanda, C.M.R., Materatski, P., Landum, M., Campos, M.D., F´elix, M.D.R., 2019. Fungal communities associated with peacock and cercospora leaf spots in olive. Plants 8, 1–14. https://doi.org/10.3390/plants8060169.

Vossen, P., 2007. Olive oil: history, production, and characteristics of the world's classic oils. Hortscience 42, 1093–1100.

Weiss, S., Xu, Z.Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., Lozupone, C., Zaneveld, J.R., V´azquez-Baeza, Y., Birmingham, A., Hyde, E.R., Knight, R., 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5, 1–18. https://doi.org/10.1186/s40168-017-0237-y.

White, G.C., Bennetts, R.E., 1996. Analysis of frequency count data using the negative binomial distribution. Ecology 77, 2549–2557.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols a Guide to Methods and Applications. Academic Press, New York, pp. 315–322.

Zhou, Z., Zhang, C., Zhou, W., Li, W., Chu, L., Yan, J., Li, H., 2014. Diversity and plant growth-promoting ability of endophytic fungi from the five flower plant species collected from Yunnan, Southwest China. J. Plant Interact. 9, 585–591. https://doi.org/10.1080/17429145.2013.873959.