Fungal endophyte assemblages associated with twigs of olives in the Core Cape Subregion, South Africa

by

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Declaration

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Abstract

Fungal endophytes are increasingly gaining recognition for their role in plant health. In the face of global change and unprecedented biodiversity loss, it has become an urgent concern to understand these valuable microbes. The main objectives of the work presented here were two-fold, 1) to gain better understanding of the fungal endophytes in a threatened biodiversity hotspot and 2) to improve our understanding of fungal endophyte assemblages associated with ecologically and agriculturally important *Olea* species. The Core Cape Subregion provides a rare and important study area since it is one of the few areas of olive cultivation with close native relatives, including *O. europaea* subsp. *cuspidata*.

Many olive (*O. europaea* subsp. *europaea*) orchards in the Core Cape Subregion are near natural *O. europaea* subsp. *cuspidata* populations. In Chapter 2, I investigated the role of host identity and geographic distance on fungal endophyte assemblages associated with the two *Olea europaea* subspecies in South Africa. Although many taxa were shared between these hosts, the native host harboured significantly higher alpha diversity. The beta diversity of fungal endophytes also differed significantly between hosts. Geographic distances played a significant role in shaping fungal endophyte assemblages of both hosts, more so in the native host.

The native *O. europaea* subsp. *cuspidata* is a widely distributed plant growing across a variety of habitats that is also a favoured shade plant, planted in gardens, parks and roadsides. In Chapter 3, the response of fungal endophytes to different levels of disturbance (habitat context) and to differences in surrounding vegetation types (vegetation contrast) were assessed. Endophyte species richness was influenced by habitat context and vegetation contrast. However, fungal endophyte assemblage composition was only affected by habitat context. This suggests that although the host can tolerate different habitat context levels, its fungal endophytes are particularly sensitive to even the mildest of disturbances found in the semi-natural habitat context.

In the Core Cape Subregion, two additional *Olea* species (*O. capensis* and *O. exasperata*) are native to South Africa. This made it possible to assess the impact of host identity and relatedness on fungal endophyte assemblages of native hosts (Chapter 4). Fungal endophytes were documented in five native hosts (three *Olea* and two non-*Olea* hosts) in the Kogelberg Biosphere. Although fungal endophyte assemblages were significantly different between hosts, this was not correlated to host relatedness (phylogeny). Other factors, other than host phylogeny, were more important to fungal endophytes in this area.

The lack of a phylogenetic signal reflected in fungal endophyte assemblages of native Oleaceae hosts suggests that the differences in fungal endophyte assemblages between *O. europaea* susp. *europaea* and *O. europaea* susp. *cuspidata* are likely due to differences in their histories. The differences in planted African olive trees versus those in the natural context, and the differences between the cultivated and the native olives demonstrate the importance of habitat context. The dynamic nature and diversity of fungal endophytes within the investigated hosts highlights the need to improve our understanding of fungal endophytes in South Africa, especially in native hosts.

Opsomming

Endofitiese fungi ontvang meer en erkenning vir hulle rol in plantgesondheid. In die lig van globale verandering en ongekende verlies aan biodiversiteit, het dit ‘n dringende kommer geraak om hierdie waardevolle mikrobe te verstaan. Die hoof doelwitte van die werk wat hier aangebied word is tweeledig, 1) om ‘n beter begrip te verkry van die endofitiese fungi in ‘n bedreigde biodiversiteit brandpunt en 2) om ons begrip van die endofitiese fungi versamelings wat met die ekologies en landboukundig belangrike *Olea* species geassosieerd is. Die Kern Kaapse Substreek verskaf ‘n skaars en belangrike studie area aangesien dit een van baie min areas van olyfproduksie is waar naby natuurlike naverwantes, insluitend *O. europaea* subsp. *cuspidata*.

Baie olyfboorde (*O. europaea* subsp. *europaea*) in die Kern Kaapse Substreek kom naby natuurlike *O. europaea* subsp. *cuspidate* populasies voor. In Hoofstuk 2 het ek die rol van gasheer identiteit en geografiese afstand op fungus versamelings wat met die twee *Olea europaea* subspesies in South Africa geassosieer word ondersoek. Alhoewel baie taksa deur hierdie gashere gedeel is, het die natuurlike gasheer ‘n beduidend hoër alfa-diversiteit. Die beta-diversiteit van endofitiese fungi het ook beduidend verskil tussen die twee gashere. Geografies afstand het ‘n beduidende rol gespeel in die vorming van endofitiese fungi gemeenskappe in beide gashere, meer so in die natuurlike gasheer.

Die natuurlike *O. europaea* subsp. *cuspidata* is ‘n wydverspreide plant wat in verskeidenheid van habitatte en is ook ‘n gunsteling skadu plant, wat in tuine, parke langs paaie aangeplant is. In Hoofstuk 3 word die reaksie van endofitiese fungi tot verskillende vlakke van versteuring (habitat konteks) en tot verskillende omringende vegetasie tipes (vegetasie konteks) vergelyk. Endofitiese spesiesrykheid was beinvloed deur habitat konteks en plantegroei kontras. Dit suggereer dat, alhoewel die gasheer verskillende habitat konteks vlakke kan verdra, is sy endofitiese funge besonder sensitief tot selfs die geringste versteurings aanwesig in die semi-natuurlike konteks.

Die Kern Kaapse Substreek sluit twee addisionele natuurlike Olea species (*O. capensis* en *O. exasperata*) in. Dit het dit moontlik gemaak om die impak van gasheer identiteit en verwantskap op die endofitiese fungi versamelings te assesseer (Hoofstuk 4). Die endofitiese fungi van vyf natuurlike gashere (3 *Olea* en twee nie-*Olea*) in die Kogelberg Biosfeer is gedokumenteer. Alhoewel endofitiese fungi samestellings beduidend verskil het tussen gashere, was dit die gekorreleer met gasheer verwantskap (filogenie) nie. Ander faktore, benewens gasheer filogenie, was meer belangrik vir endofitiese fungi in hierdie area.

Die gebrek aan filogenetiese sein wat gereflekteere word in die endofitiese fungi samestellings van natuurlike Oleaceae gashere stel voor dat die verskille in endofitiese fungi samestellings tussen *O. europaea* susp. *europaea* en *O. europaea* susp. *Cuspidate* waarskynlik toegeskryf kan word aan verskille in hulle geskiedenis. Die verskille in aangeplante Afrika olyfbome teenoor dié in die natuurlike konteks, en die verskille tussen gekultiveerde en natuurlike olywe demonstreer die belangrikheid van habitat konteks. Die dinamiese aard en diversiteit van endofitiese fungi binne die gashere wat ondersoek is, beklemtoon die behoefte om ons begrip van endofitiese fungi in Suid-Afrika te verbeter, veral in natuurlike gashere.

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"*In the end we will conserve only what we love. We will love only what we understand. We will understand only what we are taught*." *– Baba Dioum, 1968. International Union for Conservation of Nature*

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CHAPTER 1: Literature Review

Ecology of plant-associated fungal endophytes and their role in adaptation and plant health, with a special focus on the endophytes of Oleaceae

1.1 The microbiome

Plants associate with a wide range of organisms, the most common and diverse of which are microbes. These microbes can be found in the phyllosphere (in and around above-ground organs) and rhizosphere (in and on below-ground organs) (Compant et al., 2010; Singh and Mondal, 2018), and are often grouped based on their habitat/niche requirements relative to their host. For example, microbes on the surface of above-ground plant organs are referred to as epiphytes, while those that exist internally within plant tissues are referred to as endophytes (Singh and Mondal, 2018). The nature of the relationship between plants and their symbiotic microbes can be beneficial (mutualism), neutral (commensalism) or harmful (pathogen) (Hajishengallis et al., 2012; Herre et al., 2007; Rodriguez et al., 2009).

Plant-associated microbes play a critical role in plant health, ecosystems function and in sustainable agriculture (Barge et al., 2019; Leach et al., 2017; Singh and Mondal, 2018; Sivakumar et al., 2020). In ecosystem function, microbes aid in ecosystems restoration, enhancing resilience of plant communities, and contribute to adaptive strategies (Barea et al., 2002; Singh and Mondal, 2018). In addition, microbes play a critical role in physiological functioning, defence, and immunity of their hosts (Archie and Tung, 2015; Bahrndorff et al., 2016; McFall-Ngai et al., 2013). A testament to the importance of microbes to plant health can be seen in disease symptoms and fecundity of hosts with microbial assemblages that deviate from their preferred assemblage composition (Bettenfeld et al., 2020; Denman et al., 2018; Sapp et al., 2016). For example, shifts in microbial assemblage composition have been linked with disease symptoms in many woody plants such as the Acute Oak Decline syndrome in *Quercus* L. species and *Verticillium* wilt in *Olea europaea* subsp. *europaea* L. (Denman et al., 2018; Fernández-González et al., 2020).

Microbes have received considerable attention since the 17th century, both as a scourge and a benefit to society and the environment (Berg et al., 2021). Over time, research on microbes has evolved from a focus on single organisms to a more encompassing multi-organism (within and across taxonomic groups) approach, including their reciprocal influence on each other and the environment. With the increasingly evolving interests and scope of research on microbes, the need to define the collective

has become critically important. To this end, the term 'microbiome' was introduced and continues to be used and redefined. The most basic definition of a microbiome is as a collective of micro-organisms that co-exist and interact with each other in a defined environment (Berg et al., 2021). The earliest definition of the microbiome viewed it as the characteristic microbial community in a reasonably well-defined habitat with well-defined physio-chemical properties within which the community performs its activities (Whipps et al., 1988). The earlier definition, only focused on fungi and bacteria, but was later expanded to include viruses and protists too (Berg et al., 2021; Marchesi and Ravel, 2015). Other definitions have expanded to include genome- or method-driven perspectives. For example, the microbiome can also be defined as the collection of genes and genomes from the members of the microbiota, where the microbiota encompasses all the living organisms within a defined environment (Marchesi and Ravel, 2015). However, the resolution of what constitute members of the microbiota has been controversial (e.g. with reference to virus-like organisms and relic DNA) (Berg et al., 2021). In this PhD I focus on a subset of the plant microbiome, namely organisms from the kingdom Fungi that live inside asymptomatic above-ground parts of plants. The collection of fungi associated with a particular niche or organism is often referred to as the mycobiome.

1.2 Endophytes

Microbial organisms found within plant tissue without causing any visible harm are called endophytes (Hyde and Soytong, 2008; Schulz and Boyle, 2006). The endophytic nature of a microbe is labile and may change to either become beneficial or harmful to the host should the environment or state of the host change (Slippers and Wingfield, 2007; Smith et al., 1996). Endophytes may therefore include those micro-organisms that can also become saprophytes or that are latent pathogens (Peršoh, 2013; Stone, 1987). Thus far, endophytes have been encountered in all major plant groups examined, and are believed to inhabit all living plants (David et al., 2016; Huang et al., 2008; Rodriguez et al., 2009; Strobel and Daisy, 2003; van der Heijden and Hartmann, 2016).The term endophyte covers a hyper diverse community of microbes within plant tissues that include bacteria, fungi and protists (Arnold et al., 2000; Hardoim et al., 2015; Porras-Alfaro and Bayman, 2011). These organisms, although unseen, comprise of a considerable proportion of the total microbial diversity on earth (Blackwell, 2011; Rodriguez et al., 2009). Microbe colonisation of healthy plant tissues may confer protection from pathogens and offer novel adaptive traits to the host (Baldrian, 2017; Muller et al., 2016).

1.3 Fungal endophytes

Fungal endophytes include a diverse and polyphyletic group of species (mainly ascomycetes) that live within asymptomatic plant tissues of all known plant taxa, in all natural and anthropogenically altered

habitats (Bazzicalupo et al., 2013; Matsumura and Fukuda, 2013). Symbiotic relationships between plants and their endophytic fungi have a long history that has been dated back to over 400 Mya (Krings et al., 2006; Redecker et al., 2000; Rodriguez and Redman, 2008). Endophytic fungi are divided into two general groups, clavicipitaceous (Class 1 fungal endophytes) and nonclavicipitaceous (Class 2 – 4) (Kuldau and Bacon, 2008; Naik, 2019; Rodriguez et al., 2009). Clavicipitaceous endophytes are ascomycetes in the family Clavicipitaceae that associate with above-ground structures of grasses and some sedges (Kuldau and Bacon, 2008). The rest of fungal endophytes outside Clavicipitaceae are nonclavicipitaceous endophytes and they occur in asymptomatic tissues of angiosperms, conifers, ferns and allies and nonvascular plants (mosses) (Kuldau and Bacon, 2008; Rodriguez et al., 2009). Fungal endophytes are further divided into different classes based on their mode of transfer between host generations combined with their host preference:

- **Class 1** (clavicipitaceous) endophytic fungi are vertically transferred between plant host generations, with a mutualistic association with the rhizospheres, endorhizae, and aerial tissues of their grass hosts (Saikkonen et al., 2010; Vijayabharathi et al., 2016).
- **Class 2** endophytic fungi, in contrast, are horizontally transferred (Rodriguez et al., 2009). Members of the subkingdom Dikarya (mostly Phylum Ascomycota) are well-known class 2 endophytes (Kumar and Radhakrishnan, 2020). Class 2 endophytic fungi differ from other endophytes in that they inhabit plant organs such as roots, stems and leaves and extend deep inside host organs (Meena and Siddhardha, 2019). These endophytes are capable of forming beneficial associations by conferring enhanced fitness of the host, while they gain nutrition (for growth and reproduction) and shelter from external stresses (Meena and Siddhardha, 2019; Rodriguez et al., 2009).
- **Class 3** endophytic fungi also include members of Dikarya (mostly Ascomycota), however, unlike Class 2 they reproduce through hyphal propagules and reproduce sexually or asexually on aerial tissues of host plants once the host dies (Meena and Siddhardha, 2019). These endophytes are horizontally-transferred and are restricted to above-ground structures of non-vascular plants, seedless vascular plants, conifers and angiosperms (Giauque and Hawkes, 2016; Meena and Siddhardha, 2019).
- **Class 4** endophytes (also called dark septate endophytes, DSE) are diagnosed by their darkly melanised septa (Liu et al., 2017; Naik, 2019; Rodriguez et al., 2009). They are predominantly Ascomycota associating with root tissues of angiosperms often in harsh, nutrient poor arid and semiarid areas (Meena and Siddhardha, 2019).

Endophytic fungi of different classes can occur together within hosts, depending on the needs of the host. The relationships between fungal endophytes and their plant hosts have significant implications for both host and endophyte. For example, their association can confer fitness advantages for both

plant host and associated fungal endophytes, impacting on plant community structuring, plant ecology and plant evolution, and can exert strong influences on other associated organisms such as bacteria (Meena and Siddhardha, 2019; Rodriguez et al., 2009). As an example, in fungal endophytes that have the potential to protect their hosts from toxins in polluted habitats, genome mapping has revealed a suite of genes that are involved in the breakdown of toxins (Ijaz et al., 2016). Fungal endophytes that possess these genes aid their hosts to thrive in polluted systems by promoting growth and fitness through phytoremediation (Feng et al., 2017; Liu et al., 2017; Mohd et al., 2017). This is just one of many examples of fitness advantages that endophytes confer on their hosts.

Fungal endophytes can increase plant vigour, drought resistance and pathogen resistance (Arnold et al., 2003; Bae et al., 2009; Saikkonen, 2007; Sieber, 2007). As a result, they hold promise for crop manipulation for agricultural, forestry and pharmaceutical purposes. To this end, numerous bioprospecting efforts have led to the identification of fungal sources of biological compounds with biotechnological and pharmaceutical applications (Abdalla and McGaw, 2018; Suryanarayanan et al., 2009). Fungal endophytes are capable of producing compounds such a vitamins and phytohormones also produced by the host plant, thus providing alternative sources of compounds extracted from plants that are over exploited (Meena and Siddhardha, 2019; Wani et al., 2015). Efforts are also focusing on screening fungal endophytes from wild plants (*e.g.* wild grass, *Celtica gigantea* (Link) F.M.Vázquez & Barkworth) for fungi that may enhance health and growth in agricultural crops (*e.g.* cereal, *Tritordeum* hybrid) (Vázquez de Aldana et al., 2021).

Fungal endophytes contain a suite of genes that encode an extensive variety of novel secondary metabolites that aid in adaptation (Lugtenberg et al., 2016). Peramine, a secondary metabolite produced by *Epichloë* (Fr.) Tul. & C. Tul endophytes, deters insect feeding (Clay and Schardl, 2002), and as such can be explored as biological control option. For example, it holds potential for managing certain root diseases in important crops such as wheat and maize (Lugtenberg et al., 2016). Sugarcane-associated *Epicoccum nigrum* Link is a popular biological control agent against pathogens such as *Pythium* Pringsheim species and *Monilinia* Honey species that plague cotton and peaches, respectively (De Cal et al., 2009; Hashem and Ali, 2004; Larena and Melgarejo, 2009). Phomopsichalasin from a fungal endophyte, *Phomopsis* sp., has antibacterial properties against notorious pathogens such as *Bacillus subtilis* Cohn and *Staphylococcus aureus* Rosenbach (Horn et al., 1995; Strobel et al., 2004). In *Artemisia mongolica* (Fisch. Ex Besser) Nakai, *Colletotrichum gloeosporioides* (Stoneman) Spauld. & H. Schrenk produces colletotric acid which has antibacterial and antifungal activities against *Helminthsporium sativum* Pammel, C.M. King & Bakke (Zou et al., 2000). Endophytes thus offer a very diverse group of fungi to explore for secondary metabolites for use in medicine and agriculture.

1.4 Variation in fungal endophyte assemblages

Fungal endophytes may show spatial and temporal variations at different scales (Martins et al., 2016; Wu et al., 2013). The nature and strength of the relationship between fungal endophytes and plants can depend on host (above and below species level) characteristics and environmental conditions (e.g., habitat quality, location, moisture, pH and elevation) (Faeth and Hamilton, 2006; Zimmerman and Vitousek, 2012; Coleman-Derr et al., 2016; Stone et al., 2018; Wemheuer et al., 2019). Fungal endophyte assemblages can also differ between natural and altered habitats. In some instances, these changes may be adaptive and aid in host persistence, while in other cases fungal endophytes may be lost due to intolerable conditions that lead to their demise.

1.4.1 Spatial and host related influences on fungal endophytes

Fungal endophyte assemblages are influenced by spatial factors (e.g. geography and geographic features), and host-related properties (e.g. host identity, genotype, cultivar and phylogeny) (Harrison and Griffin, 2020; Matsumura and Fukuda, 2013; Rajamani et al., 2018; Sun et al., 2012; Wearn et al., 2012). Geographic features such as elevation have been found to significantly affects fungal endophytes in a wide range of hosts (Zimmerman and Vitousek, 2012), for example, *Acer saccharum* Marshall (Wallace et al., 2018), *Erica dominans* Killick (Kohout and Tedersoo, 2017), *Fagus sylvatica* L. (Cordier et al., 2012) and *Mussaenda shikokiana* Makino (Qian et al., 2018). Differences in geographic distance and host identity influence foliar fungal endophyte assemblages in environments such as the tropics (Arnold et al., 2000). In Canada, fungal endophytes from three plant lineages differed based on host phylogenetic relationships and geography (Higgins et al., 2007). Moreover, in three *Agave* L. species, fungal endophyte assemblages differed between the cultivated and native hosts and between the three collection sites in the USA and Mexico (Coleman-Derr et al., 2016). The influence of host- and geography-related factors on fungal endophyte assemblages are widely observed across a variety of hosts and locations.

Plants experience different habitat conditions and react to these either directly or indirectly through their associated microbiome. Foliar fungal endophytes within *Populus trichocarpa* (Torr. & A.Gray ex Hook.) Brayshaw appear to be dictated by environmental context (e.g. moisture availability) rather than dispersal capabilities (Barge et al., 2019). In the event of stress, plant-associated microbes can intervene to mediate environmental pressures on behalf of their host (Rodriguez et al., 2009). As such, associated microbes such as fungal endophytes can differ markedly within the same plant host across different environmental conditions. Fungi with special capabilities that enable the plants to thrive in otherwise unfavourable habitats do so by way of secondary metabolites and genes that enable them to perform tasks such as the breakdown of toxins (Cherif-Silini et al., 2019). Additional factors such as

moisture availability, salinity, heat and soil chemistry also directly and indirectly structure fungal endophyte assemblages (Rodriguez et al., 2005; Hawkes et al., 2020).

Within the same host, the spatial distribution of fungi throughout the plant can be partitioned between organs. For example, *Olea europaea* subsp. *europaea* L. often harbour very distinct fungal assemblages in above- and below-ground organs (Martins et al., 2016). Similarly, the fungal endophyte assemblages within *Alnus incana* (L.) Moench and *Corylus avellana* L. were significantly influenced by tree organ, specifically, trunk assemblages were distinct from those in the leaves and branches (Küngas et al., 2020). In the United Kingdom, fungal endophyte assemblages of grassland forbs differed remarkably between leaves and roots (Wearn et al., 2012). The outcomes of these studies suggest that some fungal endophytes may prefer organ-specific microhabitats and/or cater to organ-specific needs of their host.

1.4.2 Temporal variation of fungal endophyte assemblages

When the host is affected by external conditions such as seasonal fluctuation, moisture availability and others, the fungal endophytes are also directly and indirectly affected. For example, during dry periods some fungal propagules may desiccate, thus fluctuating as seasons change (Hoekstra, 2002). In *Laurus nobilis* L. and *Quercus ilex* L. sampled in spring and autumn, fungal endophyte assemblages were shaped by season, with samples collected in spring harbouring higher fungal endophyte diversity than those sampled in autumn (Collado et al., 1999; Gore and Bucak, 2007). Fungal endophyte assemblages in cruciferous crops were also influenced by season, with fungal diversity highest in autumn (Chen et al., 2020). In *Quercus macrocarpa* Michx. Buds, high fungal endophyte numbers was noted during the bud opening period, and they continued to accumulate during the following months (Jumpponen and Jones, 2010). The fungal accumulations and fluctuations in plants are also thought to be linked to fungal life cycle and environmental tolerance such as rain and wind patterns (Jumpponen and Jones, 2010; Moricca and Ragazzi, 2008).

1.4.3 Fungal endophytes in extreme environmental conditions

Many plants are adapted to thrive in extreme conditions such as deserts. In these habitats the associated fungal endophytes can differ markedly between plant species, organs and different environmental conditions (Li et al., 2019; Moghaddam et al., 2021; Xie et al., 2017). For example, fungal endophyte assemblages within plant hosts in a desert in China were heavily reliant on nutrient availability and host nutrient enzymatic activity (Xie et al., 2017). In a manipulation study, dark septate endophytes (such as *Paraphoma* sp. Morgan-Jones & J.F. White, *Embellisia chlamydospora* (Hoes, G.W. Bruehl & C.G. Shaw) E.G. Simmons, and *Cladosporium oxysporum* Berk. & M.A. Curtis) isolated from the desert shrub, *Hedysarum scoparium* Fisch. & C.A. Mey., were shown to aid hosts in drought tolerance by influencing root formation (Li et al., 2019). Yet, in the Namib desert, foliar fungi associated

with *Welwitschia mirabilis* Hook.f. were dominated by generalist fungi rather than specialist fungi that can be advantageous in extreme conditions (Kemler et al., 2021). At four sites in the central deserts of Iran, fungal endophytes from eight hosts were dominated by a few fungal endophytes including *Neocamarosporium chichastianum* Berk. & M.A. Curtis, which when inoculated into barley plants, showed enhanced salinity and drought tolerance (Moghaddam et al., 2021). These authors also found that host species, sampling site, season and interactions between soil and organ types significantly influenced endophytic fungal community composition, although host species was the main driving factor. Fungal endophytes appear to play a role in the survival of desert plants, but the exact mechanisms through which this is achieved varies greatly between plants, deserts, and associated microbes.

1.5 Fungal endophytes in anthropogenically altered habitats

Anthropogenic activities such as agricultural activities, urbanisation, pollution, and plantations can have adverse consequences for fungal endophyte assemblages associated with vegetation dependent on the habitats (e.g. Jumpponen and Jones, 2010; Lumibao et al., 2018). In response, fungal endophytes can dramatically shift in the face of disturbance and some species can perform bioremediation activities for their plant hosts, thus allowing the plants to thrive under otherwise intolerable conditions (Kandalepas et al., 2015).

1.5.1 Fungal endophytes in contaminated habitats

In polluted systems, the presence of fungal endophytes with bioremedial properties can aid in plant tolerance and survival. The persistence of dark septate fungi in the roots of *Arrhenatherum elatius* L. growing in heavy metal contaminated soils suggests that the presence of these fungi may be important to the persistence of these plants in this environment (Deram et al., 2011). Experimental evidence supports the bioremedial activities of some fungal endophytes, for example, the fungal endophyte Lindgomycetaceae P87 and the combination of Lindgomycetaceae P87 and *Aspergillus* sp. A31 were shown to successfully aid *Aeschynomene fluminensis* Vell. Plants in mercury contaminated sites (Pietro-Souza et al., 2020). Studies investigating the influence of the Deepwater Horizon oil spill in the Gulf of Mexico on fungal assemblages associated with salt marshes and salt marsh plants consistently differed significantly between contaminated and control sites (Bik et al., 2012; Kandalepas et al., 2015; Lumibao et al., 2018). The persistence of a *Phaeosphaeria* sp., a primary decomposer in salt marshes, in *Spartina alterniflora* Loisel. was also consistent with the known oil contamination bioremediation capabilities of fungi in this genus (Kandalepas et al., 2015; Viswanathan et al., 2014). This highlights a complex influence of contamination on fungal endophytes and their hosts and feedback responses of fungal endophytes to environmental changes through mediation activities.

1.5.2 Fungal endophytes in agricultural systems

Agricultural activities have an important influence on plant communities and their associated fungal endophyte assemblages. Due to their sensitivity to environmental changes, fungal endophytes represent a useful indicator of effects of agricultural activities and their associated management regimes (Kandalepas et al., 2015; Lumibao et al., 2018). For example, five Cacao plantation regions under different agricultural management systems in Cameroon near varying sizes of natural forest lands harboured significantly different fungal endophyte assemblages (Wemheuer et al., 2020). Particularly, dominant fungal endophyte orders (Botryosphaeriales, Eurotiales and Hypocreales) were also significantly different between sites. In addition, management of silver birch (*Betula pendula* Roth.) forests had a significant effect on fungal endophyte assemblages, leading to leaf assemblages that differ between silviculture and natural forests (Helander et al., 2006). The dark septate endophytic fungus, *Sclerobasidium humicola* G.L. Barron & L.V. Busch, found in natural and agricultural conditions improved plant growth in tomatoes when grown in agricultural conditions supplemented in organic nitrogen (Mahmoud and Narisawa, 2013). In a forest-agricultural landscape, spore deposition was significantly more strongly influenced by the vegetation type than weather conditions and distance from the source (Redondo et al., 2020). In this landscape, the composition of fungi and the dominant taxa were different between deciduous forests and agricultural wheat fields. The effects of land-use activities on fungal endophytes can be long-lasting, failing to return to the composition reflecting that of undisturbed remnants even when the vegetation has been restored (Gooden et al., 2020). These examples demonstrate the importance of understanding how agricultural systems influence other organisms associated with the vegetation in the area. In turn, this will allow for better and more informed conservation and orchard management decisions.

1.5.3 Fungal endophytes in fragmented and urban habitats

Fungal assemblages in fragmented natural forest that result from land use and urbanisation activities can significantly differ from assemblages in the natural habitats (Boeraeve et al., 2019; Grilli et al., 2012, 2017; Newbound et al., 2010). In eastern Japan, fungal endophyte alpha and beta diversity showed a significant decrease in urban areas compared to rural forests (Matsumura and Fukuda, 2013). In urban environments fungal endophytes associated with *Platanus acerifolia* (Aiton) Willd. Were mainly affected by levels of urban disturbance (Robles et al., 2015). A general trend of fungal endophytes differing between urban and rural assemblages has been reported in other systems in Europe (Jumpponen and Jones, 2009; 2010).

1.6 Disease induction by latent pathogenic fungal endophytes

Pathogenic fungi have the potential to either bypass or overpower host plant resistance (Gilbert, 2002; McDonald and Linde, 2002). Genome sequences of fungal endophytes with the capacity to become pathogenic revealed a well-developed set of genes associated with pathogenicity and virulence factors that can disarm hosts and cause serious diseases (Blanco-Ulate et al., 2013; Marsberg et al., 2017; Morales-Cruz et al., 2015). When the respective needs of the host and endophyte conflict, the needs of the endophyte may inflict harm on the host, thus leading to a transition from harmless endophyte to pathogen (Moricca and Ragazzi, 2008). However, the nature and extent of effect of the endophytic fungus on their host also depends on the host and host-associated properties (Rajamani et al., 2018; Sun et al., 2012; Wearn et al., 2012). Thus, the same fungal endophyte may be capable of inducing disease in one host, but not in another. When plants are stressed, some fungi may exit the endophytic lifestyle to enter a pathogenic one (Desprez-Lousteau et al., 2007; Wargo, 1996).

1.7 High-throughput sequencing as a tool for studying fungal assemblages

While classical techniques of isolating and identifying microfungi remain important to characterise fungi, and to obtain isolates for further study, it is also widely recognised that they are limited in their ability to characterise overall fungal assemblages. There is a high cost in growing thousands of fungal isolates and sequencing these individually. It is also challenging to monitor and study microfungi due to their inconspicuous nature (Mueller et al., 2004; Orgiazzi et al., 2012). Additionally, some fungi fail to grow on artificial media (Blackwell, 2011; Sieber, 2007). Factors that induce the alteration between the asexual and the sexual phases of many fungi are poorly understood and therefore reproductive structures needed for identification are often absent in culture (Mueller et al., 2004).

High-throughput sequencing techniques such as metabarcoding practices have become popular to characterise microbial assemblages, including fungi (Kemler et al., 2013; Purty and Chatterjee, 2016; Siddique and Unterseher, 2016). This technique is culture-independent (i.e. species that do not grow in culture can also be identified), and provides a fast and more effective way for characterisation of all microbial diversity in a sample (Abdelfattah et al., 2015; Tringe and Rubin, 2005). At the rate we are going, if we continued to rely on using traditional tools it would take over a thousand years to uncover the rest of unknown fungal species (Hibbett and Taylor, 2013). Metabarcoding techniques provide efficient tools to uncover the diversity of microbial organisms in a given sample through multiplexed PCRs, thus bypassing the time consuming constraints of culture-based methods (Tringe and Rubin, 2005; Hajibabaei et al., 2011). Metabarcoding techniques are increasingly used in various fields focused on microbial assemblages. For example, in the Microbiome project of the National Institutes

of Health (NIH) metagenomic sequencing was used to test whether changes in microbiome assemblages can be linked to certain disorders and medical conditions (The NIH Working Group, 2009).

Metabarcoding techniques need to target loci that can be amplified in most species and for which extensive databases are available. For fungal diversity, this method predominantly uses the different fungal primers targeting the Internal Transcribed Spacer (ITS) region (Bellemain et al., 2010; Hibbett et al., 2011; Schoch et al., 2012; Hibbett and Taylor, 2013). However, biases have been noted in the ability of some ITS targeting primers; some primer pairs display biases towards ascomycetes, while others are biased towards basidiomycetes (Bellemain et al., 2010; Tedersoo et al., 2015). In addition, while the ITS is a popular barcoding tool, it has been criticised for its inability to separate closely related species because of the degree of conservation in this region (Abdelfattah et al., 2015). The ITS-based high-throughput sequencing of environmental samples may therefore underestimate total biodiversity in a sample. The ITS region is also known to occur in multiple copies per individual (Lindner et al., 2013) and evolve at different rates between lineages (Nilsson et al., 2008).

Efforts are continuously being made to further optimise the ITS markers to address the effects of associated biases (Alberdi et al., 2018; Balint et al., 2016; Collins et al., 2018; Ihrmark et al., 2012). Despite these problems, ITS markers remain the best available markers of choice due to the ease of use and the amount of barcoding data available that can be used for taxonomic assignment (Kõljalg et al., 2013). The use of ITS primers in metabarcoding is advantageous due to the availability of an extensive marker database (such as UNITE and GenBank) and the consistent ease with which this region can be amplified in fungi (Kõljalg et al., 2013; Nilsson et al., 2019). Due to the poor resolution at species level, it has been advised that interpretation of data from this marker be limited to genus level taxonomic resolution (Abdelfattah et al., 2015; Callewaert et al., 2018). Despite shortcomings and problems discussed above, ITS metabarcoding has proven to be very successful, as demonstrated in numerous studies exploring fungal diversity and ecology (e.g. Jumpponen and Jones, 2009; Arnold and Lutzoni, 2007; Arnold et al., 2000).

High-throughput sequencing has been remarkably successful in the detection of both described and undescribed fungal taxa (Abdelfattah et al., 2015). The ease of use and access to high-throughput sequencing data has made it possible to study the ecology of microbes at a scale previously unimaginable (Nilsson et al., 2019; Siddique and Unterseher, 2016). Techniques are being streamlined and many guides for users are easily accessible (e.g. Callewaert et al., 2018; Lindahl et al., 2013). Powerful bioinformatics tools and pipelines have been developed, many of which are open access and intuitive to use (e.g. Bengtsson-Palme et al., 2013; Caporaso et al., 2010; Kõljalg et al., 2013).

1.8 Oleaceae hosts and their associated fungal endophytes as focal study organisms

The plant family Oleaceae is of global importance. It accommodates the economically important timber source, *Fraxinus* L. species, and the source of olives and olive oil, *Olea europaea* subsp. *europaea* L. (the commercial olive) (Dobrowolska et al., 2011; Sebastiani and Busconi, 2017; Wallander and Albert, 2000). The olive family comprises of at least 20 genera, including the prominent genus *Olea* L. (Besnard et al., 2002). *Olea* includes approximately 35 species, including *Olea europaea* (Besnard et al., 2002; Green, 2002; Green and Kupicha, 1979). The cultivated olive, *O. europaea* subsp. *europaea*, belongs in the *O. europaea* L. complex along with five other subspecies, *O. europaea* subsp. *cuspidata* (Wall. & G.Don) Cif. (*syn*.= *O. europaea* subsp. *africana* (Mill.) P.S. Green), *O. europaea* subsp. *maroccana* (Greuter & Burdet) P.Vargas & al., *O. europaea* subsp. *guanchica* P.Vargas & al., *O. europaea* subsp. *laperrinei* (Batt. & Trab.) Cif. (Batt. & Trab.) Cif. and *O. europaea* subsp. *cerasiformis* G.Kunkel & Sunding (Besnard et al., 2002; Green, 2002; Vargas et al., 2000). *Olea europaea* subsp. *cuspidata* has a wide distribution range across a variety of environmental conditions (Coates-Palgrave, 1977). It accommodates species previously described as *Olea cuspidata* Wall. Ex G. Don (demarcated from Iran to China), *Olea chrysophylla* Lam. (from East Africa to Arabia), and *Olea africana* Mill. (from East to South Africa) that are now considered synonyms (Besnard et al., 2002; Cuneo and Leishman, 2006).

Olea europaea subsp. *europaea* is a very long lived evergreen shrub/tree with one of the oldest living trees (olive of Vouves, in Crete) aged between 2000 to 4000 years old (Maravelakis et al., 2012). It is an iconic tree of the Mediterranean region with a >6000 years history of domestication (Besnard et al., 2001; Liphschitz et al., 1991; Terral et al., 2004). Olive planting and hand harvesting date back to the Minoan civilisation on the island of Crete in 3500 BC (Therios, 2009). Olive cultivation expanded westwards into France, Spain, Italy, Algeria, Tunisia, and Morocco (Vossen, 2007). Since its domestication, the spread of the European olive has been human mediated (Besnard et al., 2007). Most of the world's olive production occurs in Europe, centred mainly in Spain, Italy and Greece (Therios, 2009; Vossen, 2007). Olive cultivation has now also expanded to other countries with Mediterranean Type Ecosystems such as Australia, New Zealand, Chile, Argentina, and South Africa (Vossen, 2005; Besnard et al., 2007; Barranco et al., 2010). The olive tree, like its African olive relative, is a drought tolerant plant (Connor, 2005; Guerfel et al., 2009; Coates-Palgrave, 1977) and is therefore well suited to the arid conditions of the Core Cape Subregion of South Africa.

Fungal endophytes associated with economically important Oleaceae species have received some attention in the literature (Costa et al., 2021; Kosawang et al., 2018; 2019; Nicoletti et al., 2020). Specifically, the importance of fungal endophytes of the European natives, *Fraxinus* sp. (European Ash)

and *Olea europaea* subsp. *europaea* L. (the European olive) have been well established. Fungal endophyte assemblages were found to differ significantly between different species of *Fraxinus* (Kosawang et al., 2019). In the same study, fungal endophyte assemblages were found to be significantly different between different *Fraxinus excelsior* L. genotypes. When the European *Fraxinus* species was introduced into New Zealand, this host retained many of the endophytes from their native range despite being present since the 1800s (Power et al., 2017). This suggests that although hosts can take up fungi from the surrounding flora, they still retain endophytes from their native range despite being away from it for over 100 years. In *Fraxinus* species, Pleosporales and Hypocreales were particularly common, represented by taxa such as *Boeremia exigua* (Desm.) Aveskamp, Gruyter & Verkley, *Diaporthe* Fuckel sp., *Epicoccum nigrum* Link, and *Fusarium* Link sp. (Kosawang et al., 2018). *Fraxinus excelsior* and *O. europaea* subsp. *europaea* share the latent pathogen *Biscogniauxia nummularia* (Bull.) Kuntze (Oražem et al., 2016; Scholtysik et al., 2013). These hosts also share the latent pathogen *Neofusicoccum mediterraneum* Crous, M.J. Wingf. & A.J.L. Phillips in California where they are both introduced (Moral et al., 2010). In East Asia, foliage and buds of Manchurian ash (*Fraxinus mandschurica* Rupr.) harboured a weak pathogen *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (Zhao et al., 2012), which threatens *F. excelsior* in Europe (Kosawang et al., 2018, 2019; Kowalski et al., 2016). Fungal endophytes associated with *Fraxinus ornus* L. were found to be vastly different between the north and south facing sides of the Alps (Ibrahim et al., 2017). These examples highlight the dynamic nature of the plant-fungal endophyte relationships.

The economically important *Olea europaea* subsp. *europaea* has been the subject of many studies investigating the community ecology of their fungal endophytes (Abdelfattah et al., 2015; Martins et al., 2016). These studies reveal a complex relationship between the commercial olive and its endophytic fungi. For example, fungal endophytes of *O. europaea* subsp. *europaea* differed markedly between cultivars (Costa et al., 2021). Metabarcoding analysis of fungal composition and diversity (195 unique OTUs) in Portuguese olives revealed assemblage differentiation between organs and phenological stages (Abdelfattah et al., 2015). In Italian olive orchards, fungal endophyte assemblages also varied greatly between organs and season (Martins et al., 2016). In this study, an underestimation of total diversity was noted and proposed to be linked to the isolation-based technique used. In olive cultivars (Cobrançosa and Madural) with different levels of susceptibility to olive anthracnose, fungal endophyte assemblages were significantly different (Martins et al., 2021). In this study, phenology also significantly influenced fungal endophyte assemblages. It was suggested that the fungal endophyte differences between cultivars may be associated with their susceptibility to the olive anthracnose. These studies highlight the sensitivity of olive-associated endophyte fungal assemblages to factors such as host organ, phenological stages and geographic distances between orchards.

In areas of *O. europaea* subsp. *europaea* cultivation, latent pathogens have been recorded (Carlucci et al., 2013; Ivic et al., 2010; Jiménez-Díaz et al., 2012; Sergeeva et al., 2009). In Spain, three species were identified that cause die-back of branches and decay of ripe fruits, namely, *Diplodia seriata* De Not. and *Neofusicoccum mediterraneum* Crous, M.J. Wingf. and A.J.L. Phillips in the former and *Botryosphaeria dothidea* (Moug.) Ces. & De Not. in the latter (Moral et al., 2010). In Australia, leaf necrosis and fruit rot in olives was shown to be caused by *Neofusicoccum luteum* (Pennycook and Samuels) Crous, Slippers and A.J.L. Phillips (Sergeeva et al., 2009). In southern Italy, *Pleurostomophora richardsiae* (Nannf.) L. Mostert, W. Gams & Crous, *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai and *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips were associated with a serious decline of olive trees (Carlucci et al., 2013). In California, an extensive survey was conducted in order to identify fungal pathogens associated with olive twig and branch dieback (Úrbez-Torres et al., 2013). Many pathogens were isolated from this study, including *Diaporthe viticola* Nitschke, *Diatrype stigma* (Hoffm.) Fr., *Diplodia mutila* (Fr.) Mont., *Dothiorella iberica* A.J.L. Phillips, J. Luque & A. Alves, *Phomopsis* Sacc. & Roum. Species, *Neofusicoccum vitifusiforme* (van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips and *P. aleophilum.* Many of these species appear within the European olive both in and outside of its native range and may also be present in the South African orchards.

1.8.1 A brief history of the olive industry in South Africa

Compared to major olive producers such as Spain and Portugal, South Africa is relatively new to this market (Breton et al., 2009; Costa, 1998; Maravelakis et al., 2012). Along with South Africa, New World olive growers also include Australia, Argentina, Chile, and the United States of America (Barranco et al., 2000; Johnson, 2008). The first record of the commercial olive trees successfully growing in South Africa was found in the diary of Jan van Riebeek, referring to two olive trees in Boscheuvel in 1661 (Costa, 1998). Simon van der Stel established the first olive farm in the Cape, known as Constantia (Callaghan, 2009). Late in the 19th century, Mr J. Minnaar planted a few olive trees in Paarl in what is now referred to as De Hoop Farm (Costa, 1998). In 1903, Ferdinando Costa (The father of the olive industry in South Africa) started the South African olive industry by grafting the European olive onto rootstocks of the African olive (Agricultural Research Council and Directorate Plant Production, 2010; Costa, 1998). However, it was not until 1925 that the South African olive cultivation industry was established when Costa setup an oil mill at the Nervi Farm in Paarl (Costa, 1998). Now, many cultivars are successfully grown in the country, including the Mission, Kalamata, Manzanilla, Barouni and Frantoio cultivars (Agro processing Business Unit, 2013). In 2013, olive orchards were estimated to cover at least 3000 – 4 000 ha in South Africa (Agro processing Business Unit, 2013), these estimates are expected to have grown substantially since then.

In South Africa, the native African (*O. europaea* subsp. *cuspidata*) and introduced European olives are often grown in proximity. This is because in the early days of cultivation, the location of olive orchards was selected based on where the African olive grew well (Costa, 1998). One of the modes of olive planting has been through grafting onto favourable roots such as those of the African olive, *O. europaea* subsp. *cuspidata* (Agricultural Research Council and Directorate Plant Production, 2010; Costa, 1998; Vossen, 2007). The taxonomic similarity and the physical proximity of these olives present the opportunity for them to also share their fungal endophyte associates. should fungal endophytes be exchanged upon contact, this would expose naïve hosts to novel fungal endophytes with unknown consequences. Questions relating to fungal endophytes exchange between related cultivated and native plants have been extensively explored in many other countries, but not yet in olives of South Africa. Host jumps, hybridisations and changes in virulence play an important role in the ability of fungal pathogens to invade and thrive in a novel range (Desprez-Loustau et al., 2007). In addition, there is new evidence that species of *Lasiodiplodia* Ellis & Everh. (Botryosphaeriaceae) are able to form hybrids (Cruywagen et al., 2017; Rodriguez-Galvez et al., 2017), which may also increase the chances of survival and adaptive potential of this taxon. The current and future implication of fungal exchange and possible hybridisation in olives and other agricultural crops remains unknown. Added to this, it is unclear how common hybridisation between closely related host species are, especially in countries where olive cultivation forms an important part of the agricultural sectors.

1.9 Dissertation focus

The overarching focus of this dissertation is to characterise fungal endophyte assemblages associated with *Olea europaea* in South Africa. Specifically, I assess how fungal endophyte assemblages compare across different locations in the native *O. europaea* subsp. *cuspidata* and the cultivated *O. europaea* subsp. *europaea* (Chapter 2). Given the wide distribution and the favour of *O. europaea* subsp. *cuspidata* as shade and ornamental plant, I investigated the influence of an anthropogenic activity gradient and surrounding plant forms on fungal endophyte assemblages within this host (Chapter 3). Additionally, *O. europaea* subsp. *cuspidata* has three close relatives found within the Core Cape Subregion of South Africa (Green, 2002). This provided an opportunity to assess whether similarities in fungal endophyte assemblage composition within host species are correlated to host taxonomic similarities (Chapter 4).

1.9.1 CHAPTER 2: Diversity of endophytic fungi within native African and cultivated European olive trees in the Mediterranean climatic zone of South Africa

Fungal assemblages within native hosts can be influenced by introduced hosts just as those in the introduced hosts can be influenced by the assemblages in the native hosts. The ability of fungi to migrate among host plants can facilitate infections of novel hosts and threaten the sustainability of both commercially important plants and native ecosystems (Desprez-Loustau et al., 2007; Fisher et al., 2012). In South Africa, many studies have shown that Botryosphaeriaceae species can jump hosts between cultivated stands and native surrounding species (e.g. Mehl et al., 2016; Pillay et al., 2013). For example, the native *Sclerocarya birrea* subsp. *caffra* (Sond.) Kokwaro (Anacardiaceae) and cultivated *Mangifera indica* L. (Anacardiaceae) share 11 Botryosphaeriaceae with *N. parvum* as the dominant shared latent pathogen (Mehl et al., 2017). These authors also found shared genotypes between *N. parvum* from *M. indica* and the adjacent *S. birrea* subsp. *caffra*, providing further evidence that these hosts do indeed exchange their Botryosphaeriaceae associates. Pillay et al., (2013) documented four overlapping species of *Neofusicoccum* (including *N. parvum*) and *L. pseudotheobromae* between two Myrtaceae species (*Eucalyptus grandis* W. Hill and *Syzygium cordatum* Hochst. Ex Krauss). It is unclear whether these fungi are directly transferred between these hosts or if other nearby plants act as bridges.

The influence of geographic location and the correlation of geographic distances to community distances between fungal endophyte assemblages has been studied in many hosts across different geographic scales. Endophytic fungal assemblages within leaves, twigs and barks of *L. nobilis* and *Q. ilex* are shaped by geographic influences rather that season of sampling (Collado et al., 1999; Gore and Bucak, 2007). Similarly, *Cephalotaxus harringtonii* (Forbes) K. Koch from Japan and France hosted fungal endophyte assemblages that differed in response to geographic location (Langenfeld et al., 2013). Interestingly, in symptomatic twigs and barks of *F. excelsior* fungal endophyte assemblages were similar between sites, but differed significantly between early necrosis and late necrotic stages (Kowalski et al., 2016). Thus, although geographic distances play a critical role in shaping fungal assemblages, it appears that in the face of infection, plant responses to the infection determine fungal community composition rather than geographic distances.

In this chapter, I assess how host identity and geographic distances affect fungal assemblages in twigs of the native *O. europaea* subsp. *cuspidata* and the cultivated *O. europaea* subsp. *europaea*. Host range expansion of fungal endophytes (some of which are latent pathogens) has been recorded between native and related introduced hosts (Crous et al., 2017; Gioia et al., 2020; Mehl et al., 2016; Slippers et al., 2005). Considering this, I hypothesise that, given the taxonomic similarities between these hosts,

they will harbour fungal endophyte assemblages that overlap significantly. However, given the different histories (native vs. introduced) of these hosts the native host is expected to harbour a higher diversity and higher number of fungal taxa not yet known to science. Additionally, both host taxonomy and geographic distances between sites will play an important role in shaping assemblages of these hosts (Collado et al., 1999; Costa et al., 2021; Kosawang et al., 2019). It is also expected that the cultivated host will harbour more taxa known from economical important crops.

1.9.2 CHAPTER 3: Fungal endophyte assemblages within African wild olive from areas that differ in habitat quality and in contrast with the surrounding vegetation

Most endophytic fungi in woody hosts are horizontally transmitted (Helander et al., 2007; Saikkonen et al., 2000; Whitaker et al., 2020). Consequently, local conditions such as surrounding plant communities and geographic location can greatly influence fungal endophyte community composition. Given the limited dispersal capabilities of fungal endophytes, hosts that occur near each other are expected to harbour similar fungi (David et al., 2016; Seabloom et al., 2019). Additionally, the type of vegetation in the vicinity is expected to influence fungal assemblages in the area (similar to Redondo et al., 2020), as they serve as the immediate propagule source. More similar hosts are expected to exchange their fungal associates more readily, since the microbial organisms are likely to be familiar with the habitat within the plants.

The importance of fungal endophytes in ecosystems is becoming increasingly apparent (Desprez-Loustau et al., 2007; Peay et al., 2013) especially in the face of perturbation (Christian et al., 2016). In disturbed environments, fungal assemblages have been known to shift with some fungi struggling and perishing, while others accumulate and dominate (Beyer et al., 2016; Lumibao et al., 2018). The shift in community composition is expected to become more apparent with increasing disturbance (Zhang et al., 2011). In addition, hosts in transformed habitats may be exposed to a different suite of fungal endophytes than those that grow in natural contexts. In disturbed environments, cosmopolitan, opportunistic and pioneer microbial species tend to be abundant, thus exposing hosts to altered microbial assemblages that are underrepresented in native hosts (Jumpponen and Jones, 2010; Matsumura and Fukuda, 2013; Newbound et al., 2010).

The first aim of this chapter was to characterise and compare fungal endophyte assemblages found in twigs of the African olive growing in urban settings (planted), seminatural green belts (semi-natural) and in the protected natural pockets (natural). I expected that the fungal assemblages within trees in the natural habitats would be more structured compared to those in the other two habitat types. I also expected that fungal assemblages within the natural habitat context would be significantly different

from the planted context. The African olives in the planted context were expected to largely harbour cosmopolitan and pioneer endophytic fungi while twigs from the natural context were expected to largely harbour fungal endophytes new to science.

The second aim of this chapter was to assess how fungal endophytes are affected by the differences between the host of focus (*O. europaea* subsp. *cuspidata*) and the contrast level with the surrounding vegetation. I expected that, similar to Redondo et al., (2020) the surrounding vegetation would be important to fungal endophyte assemblages in the African olive. I expected that fungal endophyte richness would be highest when the sampled olive tree grew amongst other trees/shrubs that are not olives. This is because, it is expected that the higher diversity of trees/shrubs in the area would have provided the African olive an opportunity to encounter a larger suite of fungal endophytes capable of infecting it.

1.9.3 CHAPTER 4: Complex interactions between host identity and surrounding environmental conditions dictate fungal endophyte assemblages within trees in a global biodiversity hotspot

Host taxonomy and phylogeny can have major implications for fungal endophyte assemblages within plants. In New Zealand, foliar fungal endophyte assemblages associated with three *Nothofagus* Blume species displayed patterns of phylogenetically tethered degrees of similarity, where the species in the subgenus *Fuscopora* (*N. solandri* (Hook.f.) Oerst. And *N. fusca* (Hook.f.) Oerst.) harboured more similar assemblages compared to *N. menziesii* (Hook.f.) Oerst. (subgenus *Lophozonia*) (Johnston et al., 2012). Similarly, endophytic fungi of three *Nicotiana* L. species native to Australia revealed a strong phylogenetic signal, where related species resembled each other in their fungal assemblages more than hosts would if drawn at random (Dastogeer et al., 2018; Münkemüller et al., 2012). In addition, endophytic fungi of these *Nicotiana* hosts were not influenced by plant organ or host location (Dastogeer et al., 2018). Fungal endophyte assemblages within different genotypes of *Arabidopsis thaliana* (L.) Heynh. showed different levels of tolerance towards an infection with *Albugo* (Pers.) Roussel *sp.* (Agler et al., 2016). Furthermore, novel plant-pathogen associations between native and introduced taxa were found to be governed by taxonomic similarity rather than chance encounter (Bufford et al., 2016). Given the strong dependence of fungal endophytes on the host environment, it is reasonable to expect that hosts with similar internal environments (often related) would harbour more compositionally and taxonomically similar fungal endophytes.

In Chapter 4, the aim was to characterise fungal endophyte assemblages of three *Olea* and two non-*Olea* species in and around the Harold Porter National Botanical Garden (HPNBG), Betty's Bay, South Africa. The chosen *Olea capensis* subsp. *capensis* Verdoorn grows within the HPNBG, while *O. europaea*

subsp. *cuspidata* occurs just outside the HPNBG, and *Olea exasperata* Jacq. Is found in the dunes immediately outside the HPNBG. To ensure a broad and varying degrees of host relatedness, twigs of *Halleria lucida* L. (Order: Lamiales) and *Olinia ventosa* (L.) Cufod. (Order: Myrtales) were also sampled from HPNBG. *Olea capensis* subsp. *capensis* and *Olea exasperata* both reside in section *Ligustroides* making them the most closely related hosts sampled, while *O. europaea* subsp. *cuspidata* belongs to the section *Olea* of the genus *Olea*, and *H. lucida* resides in the order Lamiales along with all these *Olea* species (Besnard et al., 2002; Coates-Palgrave, 1977; Green, 2002). *Olinia ventosa* represented the basal host, as it was the only host outside Lamiales instead residing in the order Myrtales (Oxelman et al., 2005; Sebola and Balkwill, 2013). I expected that degrees of similarities in fungal endophyte assemblages would mirror the relationships between the sampled hosts, where fungal assemblages associated with *Olea* plants would be distinct from the non-*Olea* hosts sampled, with *O. ventosa* harbouring the most distinct assemblages.

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CHAPTER 2: Diversity of endophytic fungi within native African and cultivated European olive trees in the Mediterranean climatic zone of South Africa

2.1 Abstract

A growing body of literature points towards a strong influence of host identity and geography on fungal endophyte assemblages. Most endophytes are transmitted horizontally, therefore hosts act as uptake filters of fungi from the immediate environment. Closely related hosts in the same geographical area should therefore harbour similar endophyte assemblages with the level of assemblage similarities decreasing with an increase in geographic distance and taxonomical dissimilarities. In this study, I set out to determine the influence of host identity and geographic location on endophytic fungal assemblages associated with closely related native *Olea europaea* subsp. *cuspidata* and cultivated *Olea europaea* subsp. *europaea* using metabarcoding techniques. To test this, both hosts (as close to each other as possible) were collected from six sites across the Core Cape Subregion in the Western Cape Province. I found that both host identity and geographic location significantly affected fungal endophyte alpha- and beta diversity. Although many taxa were shared between the two hosts. Geographic distance significantly affected the degree of similarities between assemblages from different sites, demonstrating the strong effect of the surrounding environment on plant endophyte assemblages. Significant co-occurrences between fungal endophytes were more prominent and more connected in the native host than in the cultivated one. Results suggest that although native and commercial olives in South Africa currently share many endophytic species, many of these fungal endophytes occurred in lower frequencies within the cultivated hosts. Native olives have diverse and highly connected endophyte assemblages, unlike the cultivated olives. The dynamics of the fungal endophyte assemblages in these olives have unknown consequences for the fitness of both hosts.

2.2 Introduction

Plants are colonised by a diverse range of microorganisms that can influence plant health and growth in a positive, negative or neutral manner (Hajishengallis et al., 2012). Benefits towards their hosts include increased plant vigour, drought resistance and pathogen resistance (Arnold et al., 2003; Bae et al., 2009). For example, fungi such as *Epicoccum* Link, *Cladosporium* Link and *Penicillium* Link species, often encountered within healthy plant tissues, can protect their hosts from pathogenic fungi (Gomes et al., 2019; Khan et al., 2016). Fungi, such as these three, that live within plant tissues with no obvious

effects are termed endophytes (Hyde and Soytong, 2008; Schulz and Boyle, 2006). Under changing environmental or within-host conditions, the activity of some endophytes may change to become either beneficial or detrimental (Saikkonen et al., 1998; Slippers and Wingfield, 2007). For example, fungal species in the Botryosphaeriaceae and Teratosphaeriaceae may turn from harmless endophytes into pathogens when plants are stressed (Abdollahzadeh et al., 2013; Andjic et al., 2010; Carlucci et al., 2013; Moral et al., 2010). The effects of changing conditions on plant mycobiomes in commercially important plants are complex, but are increasingly documented (Caffarra et al., 2012; Vujanovic et al., 2019).

Host biology and ecology can influence endophytic fungi, dictate their presence or absence, and the nature of their interaction under different conditions faced by the host. Fungal endophyte associates of related cultivated and native hosts can expand or shift hosts (Glen et al., 2007; Perez et al., 2012; Slippers et al., 2005). Despite these host shifts or expansions, cultivated hosts tend to harbour lower fungal diversity compared to their native relatives (Hoffman and Arnold, 2018). A study on grasses in Finland showed that cultivated grasses harboured much lower fungal diversity than native grasses, supporting the notion that many endophytes can be lost during cultivation (Saikkonen et al., 2000). Similarly, fungal endophyte assemblages within cultivated *Agave tequilana* Weber in Mexico were much diminished compared to those in the native *A. salmiana* Otto ex Salm-Dyck (Coleman-Derr et al., 2016). In addition to taking up fungi in the new surroundings, hosts can continue to be associated with the endophyte assemblages from their native range, such as is the case in European *Fraxinus* L. species introduced into New Zealand (Power et al., 2017). This suggests that although hosts lose some fungal endophytes when translocated, they retain some and take up new fungi from the surrounding flora in the novel range. This helps explain why the fungal assembly in related native and cultivated alien hosts can differ considerably.

Most plant-associated endophytic fungi are horizontally transmitted (Christian et al., 2016; Helander et al., 2007; Saikkonen et al., 2000). Consequently, local conditions such as different plant organs, surrounding plant communities and geographic location can greatly influence endophyte assemblage composition. For example, in *Olea europaea* subsp. *europaea* L. (Oleaceae) plants from nine different Portuguese groves, fungal endophyte assemblages differed markedly between different plant organs, locations and seasons (Martins et al., 2016). Specifically, in late spring and autumn, fungal assemblages in different organs of these plants consistently differed from each other, although assemblages from above-ground organs (leaves and twigs) were more similar to each other than to those from the roots. In the northeast of Portugal, fungal endophyte abundance, richness and diversity of olives were found to be higher in twigs than in leaves, but fungal community composition was very similar between the two organs with approximately 43 % species overlap (Gomes et al., 2018). These authors also identified rainfall and temperature as major drivers of fungal endophyte assemblages in olives.

The economically important *O. europaea* subsp. *europaea* (the cultivated European olive) belongs to the *Olea europaea* L. complex, along with five other subspecies, including *O. europaea* subsp. *cuspidata* (Wall. & G. Don) Cif. (the African olive) (Besnard et al., 2002; Breton et al., 2008; Green, 2002). *Olea europaea* subsp. *europaea* is an important agricultural crop that is mainly cultivated in Mediterranean type climates (Therios, 2009; Vossen, 2007). Although olive product consumption can be dated back to biblical times in the Mediterranean basin, grafting experiments of *O. europaea* subsp. *europaea* onto *O. europaea* subsp. *cuspidata* root stocks for cultivation purposes only started in 1903 in South Africa (Breton et al., 2009; Costa, 1998; Maravelakis et al., 2012; Vossen, 2007). In South Africa, olive orchards were conservatively estimated to cover an approximate 3 000 – 4 000 ha in 2013 (Agro processing Business Unit, 2013). As the awareness of the health benefits of olive products increased, their consumption has also increased significantly (Abdelfattah et al., 2015). As of 2013, the demand for olive products far exceeded the supply in South Africa (Agro processing Business Unit, 2013). Due to these facts, it is reasonable to expect that the olive industry will expand and to become an even more important part of the South African economy.

Olea europaea subsp. *europaea* is a drought tolerant plant (Connor, 2005) and is therefore well suited to the arid conditions of the Core Cape Subregion. The closely related *O. europaea* subsp. *cuspidata* is also an important plant for its wide range of uses in South Africa. It was used for agricultural purposes (as root stocks for *Olea europaea* subsp. *europaea*) and is used for medicinal purposes, shade provision and as a source of firewood (Amabeoku and Bamuamba, 2010; Aumeeruddy-Thomas et al., 2017; Besnard et al., 2007; Costa, 1998). In the early days of olive cultivation, the location of olive orchards was selected based on where African olive grew very well (Costa, 1998). In addition, many of the *O. europaea* subsp. *europaea* trees in the country were planted through grafting onto favourable roots such as those of the African olive (Agricultural Research Council and Directorate Plant Production, 2010; Costa, 1998; Vossen, 2007). With the close taxonomic relationship between these hosts combined with their frequent contact due to their proximity (Powell et al., 2019), there is an increased chance of microbial exchange (including fungal endophytes) between them. Yet, research in the country has focused mainly on pest and pathogen identification and management, and on improving cultivation practices of the cultivated olive (Agricultural Research Council and Directorate Plant Production, 2010; Costa, 2018; van Dyk et al., 2020). Endophytic associations have yet to receive attention.

Olea europaea subsp. *cuspidata* has a wide distribution range and is found across a wide variety of environmental conditions. It accommodates taxa previously known as *Olea africana* Mill., *Olea chrysophylla* Lam. And *Olea cuspidata* Wall. with the distribution ranges from South Africa to East Africa, East Africa to Arabia, and Iran to China, respectively (Besnard et al., 2002; Green and Kupicha, 1979). It is one of the most widespread species in the botanically diverse Core Cape Subregion

(Goldblatt and Manning, 2012; Coates-Palgrave, 1977). This species grows either as a bush, shrub or tree in a diverse range of habitats such as forests, rocky slopes, fynbos thickets, fynbos, and renosterveld (Green, 2002; Coates-Palgrave, 1977; Palmer, 1977). The Core Cape Subregion in the Western Cape is also the main area of olive (*O. europaea* subsp*. europaea*) cultivation in South Africa (Agricultural Research Council and Directorate Plant Production, 2010; Costa, 1998). Here, olive production started in *ca*. 1925 and contributes significantly to the local economy (Agro processing Business Unit, 2013). Production is, however, threatened by pests and diseases, some of which are of possible African origin such as the parasitoid wasps (e.g. *Utetes africanus* Szépligeti) and olive fruit fly (*Bactrocera oleae* Rossi) (Caleca et al., 2019; Giacalone et al., 2015; Powell et al., 2019). Olive anthracnose and likely many as yet undescribed pathogenic fungi may threaten the olive industry and the native *Olea* species as the climate continues to change in the region (Agricultural Research Council and Directorate Plant Production, 2010; Cacciola et al., 2012; Costa, 2018).

Given the close taxonomic relationship and the vast distances between the native habitat of the South African *O. europaea* subsp. *cuspidata* and *O. europaea* subsp. *europaea*, the ecological ramifications of their endophytes coming into contact are unclear. The lack of general fungal endophyte diversity information within these hosts hinders our ability to adequately determine whether plant histories (cultivated vs native) or taxonomy shapes fungal assemblages in *Olea europaea* in South Africa. The limited available research focuses on pathogenic fungi, mostly of *O. europaea* subsp. *europaea*, but occasionally include *O. europaea* subsp. *cuspidata.* An investigation into the causal agents of olive tree die-back and decline was recently focused mainly on symptomatic trees of *O. europaea* subsp. *europaea* (145 trees) and some symptomatic *O. europaea* subsp. *cuspidata* (40 trees) (Spies et al., 2020). In this study, 99 fungal taxa were isolated, however, an overwhelming majority of these taxa have not previously been recorded in olives (Spies et al., 2020). Most of the identified fungi resided in the Basidiomycota, Botryosphaeriaceae, *Cytospora* Ehrenb, *Diaporthe*, Diatrypaceae, *Phaeoacremonium*, Phaeomoniellales, and *Pleurostoma* (Spies et al., 2020). Van Dyk et al., (2021) conducted a comprehensive screening focused on olive trunk pathogens from two major nurseries in the Western Cape (South Africa). They identified pathogens already known to cause trunk diseases and other potential pathogens in woody plants. Amongst the already known olive trunk pathogens were *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, *Pleurostoma richardsiae* (Nannf.) L. Mostert, W. Gams & Crous and *Phaeoacremonium parasiticum* (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf. (Spies et al., 2020).

Other South African research efforts have mainly focused on economically important fungal groups, such as Diatrypaceae and *Cytospora* (Valsaceae) (Adams et al., 2006; Moyo et al., 2019). Moyo et al., (2019) found cultivated grapes to house a higher documented fungal diversity than *Olea europaea* subsp. *europaea*, although they only focused on fungi with pathogenic effects. In addition, during

screenings for causal agents of olive scab and olive anthracnose, respectively, *Cycloconium oleaginum* Castagne and *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., were identified as causal agents of disease (Gorter, 1956; 1962). Although largely independently screened, the pathogenic agents identified within these hosts are highly alarming as they pose a significant pathogenicity threat to *Olea europaea* in South Africa.

During the screening of pathogenic *Cytospora* species by Adams et al., (2006), the isolates from South Africa (representing at least 14 species) were predominantly isolated from introduced hosts. All the isolates from *Olea europaea* subsp. *cuspidata* were placed in the *Cytospora pruinosa* (Fr.) Sacc complex. Although they were distinct from the *C. pruinosa* from *Fraxinus* and *Syringa* L. in North America and in Europe, respectively, suggesting they might represent new species potentially native to this host. To my knowledge, no *Cytospora* species have been reported in the cultivated olive within South Africa. However, there have been numerous reports of these species (mainly *Cytospora oleina* Berl.) as pathogens of cultivated olives in Greece, Spain, and Italy (Carlucci et al., 2013; Fisher et al., 1992; Moral et al., 2017; Rumbos, 1988). While these *Cytospora* species predominantly kept to the introduced hosts, *C. pruinosa* is a widespread and generalist cosmopolitan endophyte found in hosts belonging in genera such as *Fraxinus* and *Olea* (Adams et al., 2006) therefore may poses an ongoing potential threat to native hosts in these genera.

It is important to Identify the microbes associated with crops, especially in the light of increasing environmental pressures on these crops through climate change. Amongst other things, this information can be used to conduct risk assessments based on the conditions known to favour pathogenic taxa and disease development. The lack of information of the microbial associates of *O. europaea* subsp. *europaea* and its close relative, *O. europaea* subsp. *cuspidata*, hampers such predictive and monitoring work. The purpose of this study was firstly to characterise the endophyte fungal mycobiome associated with the younger stage above-ground plant organs (twigs) of both the native *O. europaea* subsp. *cuspidata* and cultivated *O. europaea* subsp. *europaea* trees. Due to their close taxonomic similarities, I hypothesised that they will share many endophyte species, especially when found in close proximity and under similar environmental conditions (Bufford et al., 2016). Since introduced taxa can retain some native associates (Martín-García et al., 2012; Saikkonen et al., 2000), I expected that some taxa known to be associated with the cultivated taxa in other olive growing regions globally will be present in the cultivated host in South Africa. Due to the long co-evolutionary history between native fungi with native hosts and reduction in endophyte diversity in some introduced hosts, I hypothesise that the native host will harbour more diverse endophytic fungal diversity than the cultivated host (Saikkonen et al., 2000). As geographic location plays a central role in endophyte assemblages of various plants (Collado et al., 1999; Gore and Bucak, 2007), I hypothesise that endophyte assemblages of both hosts will be strongly influenced by distances between sampled

sites. Results of this study will elucidate important factors influencing the 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.

2.3 Materials and Methods

2.3.1 Site selection and sampling design

Six sites were selected for sampling in the winter rainfall region of the Core Cape Subregion corresponding to the main olive production areas (from Clanwilliam to Stellenbosch to Swellendam) (Table 1, Figure 1). The region has a Mediterranean climate with dry summers and wet winters (Rebelo et al., 2006). *Olea europaea* subsp. *europaea* was sampled from olive orchards and residential gardens, while *Olea europaea* subsp. *cuspidata* was collected from the nearest natural trees. To capture as much as possible of the fungal endophyte diversity, the sampled cultivated trees covered as many of the different cultivars in the orchards as possible. The sampled locations of the native host covered a wide range of Fynbos Biome vegetation types (Mucina and Rutherford, 2006) (Table 1).

Table 1: Summary of the properties of the sites from which the host plant material was collected. Habitat and host characteristics that were recorded were altitude (m), mean annual precipitation (MAP) and prevailing vegetation types surrounding the sampled olive trees. Sampling was conducted during the autumn/winter months of May, June and July of 2018. When sites allowed, sampling was spread out to cover as much of the location as possible to allow maximum coverage of the olive endophytes in the area. Sampling was also spread out to cover all the cultivars on the farms sampled.

Location		Host	Cultivars	Altitude (m)	MAP (mm)	Vegetation
Clanwilliam*	Alpha Excelsior Farm Cederberg Mountain	*Olea europaea* subsp. *europaea* *Olea europaea* subsp. *cuspidata*	Mission, Frantoio	500	180–600	Cederberg Sandstone Fynbos
Paarl*	De Hoop Farm & residential gardens Paarl Mountain Reserve	*Olea europaea* subsp. *europaea* *Olea europaea* subsp. *cuspidata*	Mission, Leccino, Frantoio	200 – 300	270–2 220	Boland Granite Fynbos Swartland Shale Renosterveld Swartland Granite Renosterveld
Stellenbosch[%]	Residential gardens	*Olea europaea* subsp. *europaea*	Mission	50 – 240	610–2 220	Boland Granite Fynbos Swartland Shale Renosterveld
	Coetzenburg Mountain, Jan Marais Nature Reserve & Onderpapegaaiberg	*Olea europaea* subsp. *cuspidata*				
Somerset West[Δ]	Residential gardens	*Olea europaea* subsp. *europaea*	Mission	100 – 150	610–2 220	Boland Granite Fynbos
	Helderberg Nature Reserve	*Olea europaea* subsp. *cuspidata*		150		
Robertson[Δ]	Marbrin Olive Growers	*Olea europaea* subsp. *europaea*	Frantoio, Coratina, Mission	240	210–610	Breede Shale Renosterveld
	Langeberg mountain foothills	*Olea europaea* subsp. *cuspidata*		330		
Swellendam*	Mardouw Olive Estate	*Olea europaea* subsp. *europaea*	Frantoio, Leccino, Coratina	160	300–1 300	Breede Shale Fynbos
	Neighbouring foothills of Langeberg mountain	*Olea europaea* subsp. *cuspidata*				

*Hosts sampled were in adjacent plots, [Δ]Hosts sampled were distant from each other (separated by 5 to 10 km), [%]Samples of both hosts were spread out and intermingled with each other

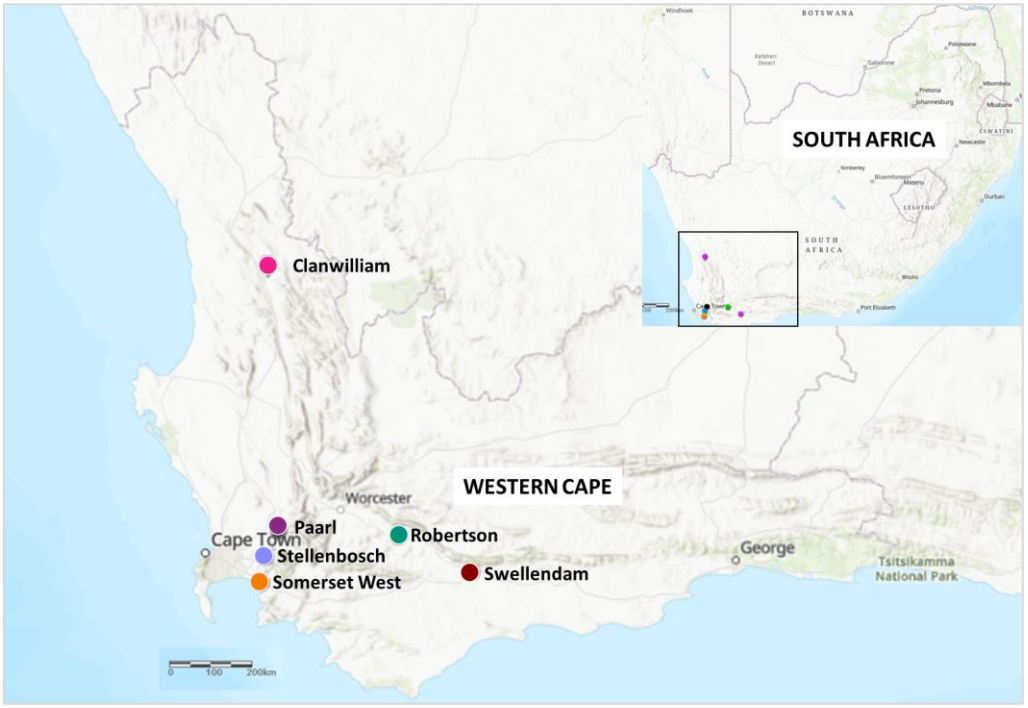

Figure 1: Map of the Western Cape Province with the six sampled sites highlighted. Colours correspond with the colour scheme in later figures. Insert: Map of South Africa.

Sampling was conducted between late-May and early-July 2018 to minimise possible seasonal effects on endophyte assemblages. Focal trees were randomly selected and were at least 50 meters apart. Four asymptomatic, 5 cm long and 3-5 mm wide twigs (one each from the north, south, east, and west side of the focal tree,) were collected per tree and combined in a single sample. Samples were taken from all four sides to minimise possible effect of differences in canopy orientation on endophyte assemblages (Gomes et al., 2018). At each site twigs were collected from ten trees per host. All twigs were from the previous season's growth to minimise possible successional effects in properties (e.g. exposure duration, physiology and nutrients available) that may have affected fungal endophyte assemblages (Canham and Marks, 1985; Spaeth et al., 2002). Twigs from each plant were sealed 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 rinsed with autoclaved double distilled water for 30 s (Moral et al., 2010; Slippers and Wingfield, 2007). A 2 cm section was cut from the centre of each sterile piece collected from each of the four sides of the focal tree, ground into fine powder with mortar and pestle, and combined into one tube. The mortar and pestle were cleaned by subsequent washings using 70 % ethanol, household bleach and autoclaved double distilled water between samples. Tubes were stored at -80°C before DNA extraction.

2.3.2 DNA extraction

DNA extraction followed a modified version of the protocol developed by Doyle and Doyle (1990). Adjustments made included using 2 µl mercaptoethanol (instead of 1 µl) with 500 µl 2x CTAB buffer (supplemented with 5 µl RNAse A (120 U/mg) and 7 µl Proteinase K (2.5 U/mg)). An extra wash step was included: 100 µl double distilled water, 75 µl of 5 M Kac and gently mixed followed by an addition of 700 µl of ice cold 70% ethanol. The quantity and quality of the resulting DNA was evaluated using a photometer (Eppendorf, BioPhotometer).

2.3.3 Library preparation

Multiplex amplification of the fungal species was carried out in two PCR reactions. The first PCR (PCR1) was conducted using the ITS primers augmented with tags that carried a binding site for the indexing primers. The second PCR (PCR2) was conducted to add indexing primers onto the binding site carried by PCR1 primers. Indexing primers carried proprietary Illumina adaptors that were to be used during the sequencing process. Amplicons from the same tree carried a unique tag and adaptor combination that was used to assign sequencing products to their respective samples.

In 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 and synthesised for multiplex barcoding by metabion (Planegg/Steinkirchen, Germany). PCR volumes of 12.5 µl per sample contained 6.25 µl GoTaq® G2 Hot Start Colorless Master Mix (Promega, USA), 0.25 µl of 0.1 µM of each primer, 5.25 µl ddH_2O and 0.5 µl of the 1:10 diluted template DNA. PCR reactions were conducted using a BIO-RAD (DNA Engine®) thermocycler under the following conditions: an initial denaturation step at 95 ^oC for 3 min, followed by 32 cycles (denaturation at 94 ^oC for 27 s, annealing at 57 ^oC for 1 min, and elongation at 72 ^oC for 90 s) and then termination with a final elongation step at 72 ^oC for 7 min. To confirm the presence of ITS amplicons the products were visualised on an 0.8 % agarose gel by electrophoresis (Bio-budget Technologies GmbH, Germany) supplemented by 1 µl Ethidium Bromide.

To remove unused primers, homodimers, heterodimers and excess DNA, the PCR products were purified following the ExoSap protocol (New England BioLabs Inc., USA). The Exonuclease 1 (*E. coli*) and Shrimp Alkaline Phosphatase (rSAP) mix was prepared by adding 0.2 µl Exo and 0.2 µl Sap to 1.6 µl sterile ddH_2O. ExoSap (2 µl) was added to 5 µl PCR product and then incubated at 37 °C in the thermocycler for 45 min.

PCR2 was conducted to add Illumina adapters (P5 and P7) and indices to PCR1 products. PCR2 volumes were 25 µl per sample, containing 12.5 µl GoTaq, 0.5 µl of 0.1 µM of each primer (containing sequences complimentary to PCR1 primers), 6.5 µl ddH_2O and 5 µl cleaned PCR1 product. PCR reaction conditions were identical to those for PCR1, except that only 5 cycles were conducted instead of 32 cycles. Similar to PCR1, the presence of amplicons was confirmed on an agarose gel using electrophoresis. Samples were tagged and indexed such that each sample contained a unique combination of labels (tag and index).

2.3.4 Sample pooling, purification and library sequencing

The PCR amplicons carrying ITS primers and adaptors were then 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). Band intensities were measured as proxies for product molarity with the ladder intensities used to standardise between gels. At each pooling step, samples with similar intensities were combined.

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. The resulting pools were purified using the CleanPCR® Kit (CleanNA, GC biotech B.V.). To determine the success of the cleaning step another agarose gel electrophoresis was run, and the results visualised and photographed. The cleaning procedure was repeated until only one band was visible per well. The cleaned pools were further combined until all the pools were pooled into one superpool. The superpool was sent to the sequencing facility at the Genetics Department, Ludwig Maximilian University in Munich for 2 × 250 bp paired-end sequencing on an Illumina MiSeq® sequencer (Illumina Inc., San Diego, CA, USA) using the MiSeq Reagent Kit v3 Chemistry.

2.3.5 Sequence cleaning, identification and quantification

The sequencing product from the sequencing facility was received as a batch of sequences carrying indexes and tags. Quality control and demultiplexing were performed using the QIIME 1.9.1 pipeline (Caporaso et al., 2010). Demultiplexing of samples was performed by using the tag-index combination (introduced during PCR1 and PCR2) to assign sequences to their samples of origin. Following demultiplexing, only forward reads were used for subsequent analyses; to avoid loss of taxa data that would have resulted from merging of forward and reverse reads. Quality control was conducted to identify, and discard reads with low quality base calls, reads that were too short, and chimeras (Abdelfattah et al., 2018). Chimeras are sequences that contain pieces from different sequences that

are combined to appear as if they are from the same amplicons. Forward and reverse barcodes were used to extract sequence reads. Sequence reads were extracted using the forward and reverse barcodes as identifiers. Sequences with acceptable scores (Phred quality threshold $\geq$ 0.35) were retained. The tag-index combinations together with the reference mapping file were used to assign sequence reads to their corresponding sample IDs. After this, the tag-index sequences were trimmed such that only the ITS sequences remained. Trimming was performed using the FASTX-Toolkit (v. 0.0.13, http://hannonlab. Cshl.edu/fastx_toolkit/). The resulting ITS sequences were checked for possible chimeras using an abundance-based method in the USEARCH platform (Edgar, 2010). The remaining trimmed ITS1 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). An OTU table was created and the frequency of each OTU per sample was used to infer abundance.

2.4 Analyses of fungal endophytes within the African and European olives

2.4.1 Alpha diversity

Endophytic fungal OTU richness within asymptomatic twigs per focal 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), as samples contained many rare species. Fungal endophyte OTU richness and abundance were compared between the different sites and hosts using linear 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 each 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 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 modelling (GLM) fitted with a Laplace approximation and a Poisson family distribution in the *lme4* package in R (Bates and Sarkar, 2007). This model was selected after the data were identified as non-normally distributed with a Poisson distribution using the Shapiro-Wilks test in

nortest and histogram plots, respectively (Gross and Ligges, 2015). Abundance data showed signs of over dispersion when tested using the *lme4* package, thus the Poisson family was replaced with a negative binomial family distribution. Models contained host identity, site, and their interaction as fixed effects. To improve the model fit and account for spatial autocorrelation, region was used as a random variable (Cape Town = Somerset West, Boland = Paarl and Stellenbosch, Olifants River Valley = Clanwilliam, Brede River Valley = Robertson and Swellendam). Models for relative abundance data could only include host taxon as fixed effect and was fitted to a binomial distribution as it was based on percentage data (White and Bennetts, 1996). Where needed, significant main effects were further separated using a conservative Tukey *post hoc* tests using the *multcomp* package (Hothorn et al., 2008) in R, which allows for multiple comparisons between medians and that generates p-values for these comparisons.

The same alpha diversity analyses were also performed on a subset of the mycobiome containing only the core microbial taxa. Less than 10 fungal taxa made up the top 25 percentile, thus core taxa were redefined as those OTUs that made up the top 50 % percentile calculated based on the number of samples the OTU appeared in. The core mycobiomes were calculated separately for each host at each of the six sites.

2.4.2 Beta diversity

Numerous forms of beta diversity have been described (Tuomisto, 2010). Here I considered two types of beta diversity to differentiate between beta diversity between factors [β1: between different sites or between different host taxa] and beta diversity within factors [β2: within individual sites or within individual host taxa] (Anderson, 2006). B1 and β2 were calculated following permutational multivariate analyses of variance (PERMANOVA) and permutational multivariate analyses of dispersion (PERMDISP) procedures in Primer v6, respectively. These diversity measures are particularly sensitive to differences in sample size (Anderson, 2006). A portion of the samples collected and barcoded failed to amplify, therefore, to balance the design, I randomly chose individuals (using the random numbers generator, https://www.random.org/) to the lowest number of individuals available per species per site (i.e. the site containing the lowest number of individuals of a host taxon from which I was able to obtain sequence data; n =5 (cumulative n = 60)) prior to analyses. Analyses containing full, unbalanced datasets (n = 102) were also conducted and are presented in the appendix.

Responses of fungal endophyte assemblage composition to host identity, site and their interaction were investigated based on the two datasets (abundance and richness) using PERMANOVA. In the first dataset I considered abundances of fungal endophyte (total number of reads) as input matrix (full dataset). However, given primer affinity towards certain taxa in metagenomics analyses (Agler et al.,

2016). I also reduced this matrix to only consider presence/absence of OTUs (richness) in each sample (simplified dataset). For the abundance-based dataset the data was first square-root transformed to reduce the effect of common OTUs, 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). *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 abovementioned beta diversity analyses were also performed on a subset of the data containing only the core assemblages calculated as previously mentioned. Where significant differences were detected in β1, the Canonical Analysis of Principal Coordinates (CAP) procedures in Primer6 was used to find axes that best visualised these differences. 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 in Primer6. Differences in assemblage composition based on species richness data were visualised in nMDS with the Jaccard distance matrix, while differences in assemblage composition based on fungal abundance data was visualised using the Bray–Curtis distance matrix.

2.4.3 Effect of distance between sites on fungal endophyte assemblages within olives

Distance matrices were computed using the Vegan package in R. A Euclidean distance-based geographic distance matrix (Gauch, 1973) was computed from the GPS co-ordinates of the six sampled sites using the Vegan package in R. GPS co-ordinates were taken from the centre of the sampling radius per region. Assemblage distance matrices were computed from the abundance and richness OTU 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 (reduced) 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 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 assemblages.

2.4.4 Fungal endophyte co-occurrence networks

Ecologically meaningful fungal associations and interactions were identified by calculating significant fungal co-occurrences and visualising these in networks. Significant fungal co-occurrences were

calculated using the package *Hmisc* 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 ($_{\rho}>0.5$) (Spearman, 1904). Benjamini-Hochberg standard false discovery rate correction was used to correct for type II errors that occur during multiple testing (Benjamini and Hochberg, 1995). The resulting graph was visualised in Cytoscape v. 3.7.2. UNITE OTU identifications were used to label nodes. The OTUs that returned no BLAST hits based on the UNITE database were queried using the Basic Local Alignment Tool (BLAST) located in GenBank within NCBI. In an attempt to place fungi that could not be placed using the UNITE database, a 95 % sequence similarity cut-off was applied to identify the closest matches using BLAST. Although it was rare, in cases where an OTU sequence was equally similar to more than one taxon, the option that either originated from the same host, same geographic origin as one of the two hosts or taxa that have been previously recorded within olives or Oleaceae were selected as the most likely taxon. In cases where multiple taxa were equally likely to be correct and met the same number of ecological criteria, all likely matches were reflected on the network.

A collection of measures (such as node degree, total number of nodes, average path length, diameter, clustering coefficient, betweenness co-efficient and modularity) were calculated and reported to describe the networks (Assenov et al., 2008; Newman, 2010). Node Degree shows the number of nodes connected to an individual node (Newman, 2010). The degree of a node is defined as the number of nodes the node of interest is connected to and the degree distribution is the probability distribution of these degrees over the whole network (Assenov et al., 2008; Newman, 2010). When a node has a higher than the average node degree, this node has the potential to be a hub (Agler et al., 2016). Hubs are nodes/species that play a bigger role in keeping the network as connected as it appears (Delmas et al., 2019). The number of nodes or vertices (v) signifies the number of species (or OTUs) in a network (Newman, 2003). The connections between these nodes are measured by the number of edges it takes to connect them to each other. The shortest distance (fewest number of edges) it takes to achieve the connections (direct and indirect) between all the nodes is called the short path length (spl) (Newman, 2010). Network diameter (nd) is measured as the longest path connecting any pair of nodes that are directly or indirectly connected (Newman, 2010). Some nodes may be more connected to each other than they are to the rest of the network, and thus create a subnetwork within the bigger network. This tendency is referred to as modularity (Newman, 2003). The clustering coefficient (cc) provides an indication of cliquishness of nodes in a network, as it measures the likelihood of connected nodes being part of a subnetwork connected to the large network (Watts and Strogatz, 1998). The clustering coefficient (cc) measures how densely connected the nodes are, thus the higher the cc is the more cliquish the nodes are.

In a network some nodes will be more influential than others, which is referred to as centrality (Delmas et al., 2019). The proximity of a node to all the other nodes in a network is called closeness centrality (C_C; Freeman, 1978). The more central (closeness centrality) a node is, the closer it is to all other nodes. Higher closeness centrality reflects the tendency of a node to be a hub. Some nodes play a large part in connecting different components of the network, called betweenness centrality (C_B; Freeman, 1977). Betweenness centrality reflects which nodes are strategically placed and serve as a bridge/mediator through which many paths pass to connect the node clusters on either side of the mediator.

2.5 Results

A total of 1 035 012 sequences were recovered from the 102 samples. These sequences resolved to 448 unique OTUs. OTUs belonging to two phyla, 61 families, and 98 genera were identified within olive twigs. Of these, 104 were Ascomycota, 35 Basidiomycota, 25 unidentified taxa and 194 OTUs without any BLAST hits. Out of the 448 taxa, 265 were shared between the two hosts, while 119 were found exclusively within *O. europaea* subsp. *cuspidata* and 64 were restricted to *O. europaea* subsp. *europaea.* Thirty-six OTUs were found in all sites sampled. One hundred and fifty-seven *O. europaea* subsp. *cuspidata* OTUs, and 97 *O. europaea* subsp. *europaea* OTUs had no BLAST hits. *Olea europaea* subsp. *cuspidata* had 95 OTUs that could not be placed at the genus level, while *O. europaea* subsp. *europaea* had 69.

The most common families within the two hosts included Corticiaceae, Elsinoaceae, Pleosporaceae, Sporocadaceae and Teratosphaeriaceae (Figure 2; Appendix A: Figure S1). Although found within both hosts, members of the Caliciaceae were predominantly associated with *O. europaea* subsp. *europaea.* In addition to Caliciaceae, members in the Diaporthaceae, Phaeosphariaceae, and Sporocadaceae were the most dominant within *O. europaea* subsp. *europaea. Olea europaea* subsp. *cuspidata* was dominated by taxa in the Corticiaceae and Teratosphaeriaceae.

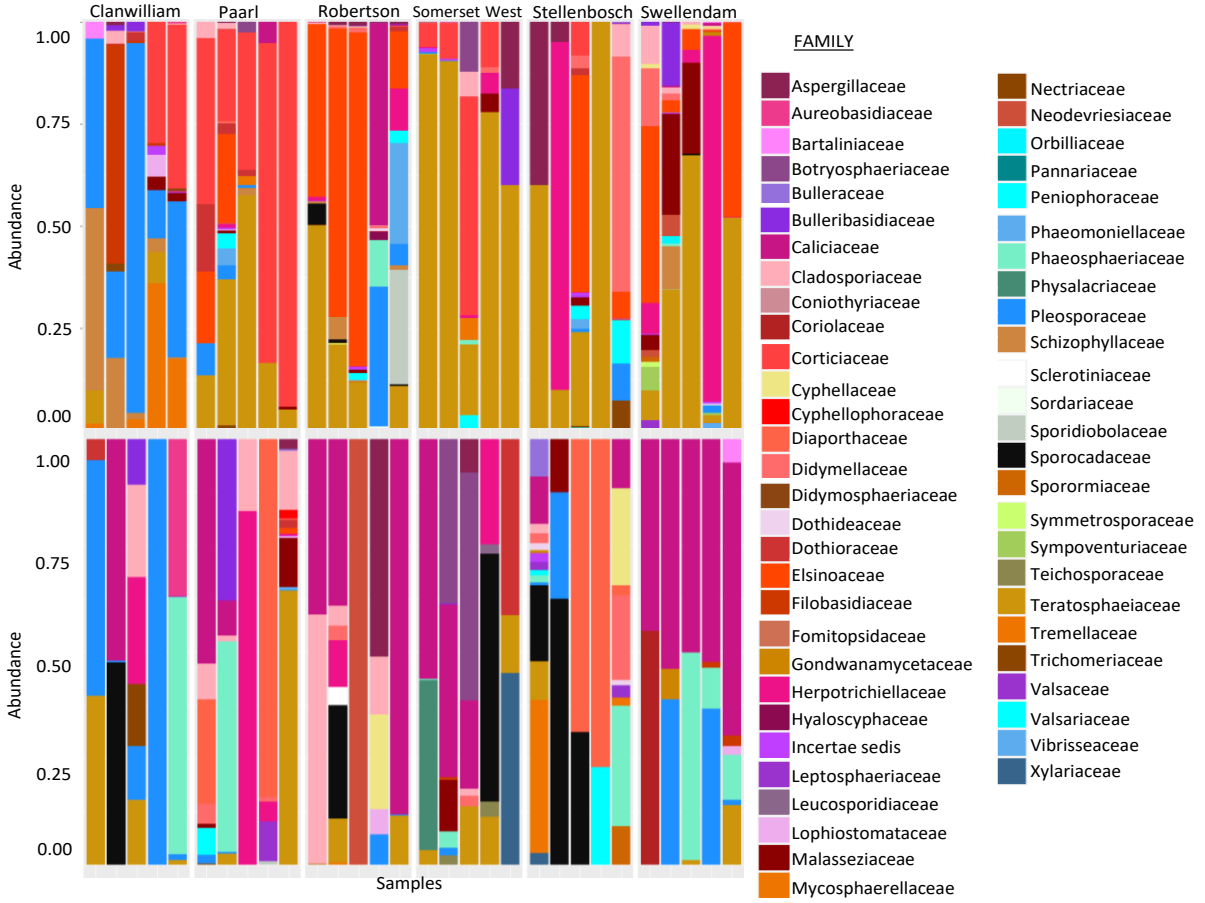

Figure 2: Relative abundance (100 % stack plots) of fungal families found within olive twigs (*Olea europaea* subsp. *cuspidata* (above) and *Olea europaea* subsp. *europaea* (below)) from the six sites (unbalanced dataset) based on 589 518 reads, excluding taxa that could not be placed at family level. Stack plots including operational taxonomic units with no blast hits or family placement are presented in Appendix A, Figure S1.

2.5.1 Fungal endophyte richness and abundance between sites and hosts (α-diversity)

Species richness was significantly affected by host identity (Table 2). *Olea europaea* subsp. *cuspidata* harboured significantly higher fungal endophyte richness than *O. europaea* subsp. *europaea* (Table 3, Appendix A: Figure S2). The core fungal assemblages within the African olive were also more diverse than those in the cultivated olive (Table 3). Once rarefied, species richness was still significantly affected by host identity. Similarly, host identity also significantly affected the relative abundance of fungi within the olive twigs. However, host identity did not significantly influence species richness of the core taxa when rarefied or when relative abundance of the core taxa was analysed (Table 3).

Both overall species richness and core species richness were significantly affected by sampling locality (Table 2). *Post hoc* analyses revealed that this significance was driven by the significant differences between species richness from all sites, except for species richness between Somerset West and Clanwilliam, Stellenbosch and Paarl, and Swellendam and Robertson (Table 3, Appendix A: Table S1). In contrast, the significance in core species richness was driven by Clanwilliam, which was significantly

different from Stellenbosch, Swellendam and Robertson, and the significant differences between Swellendam and Paarl, and between Swellendam and Somerset West (Table 2, Table 3). Species richness was not significantly affected by site when data were rarefied, but when only the core taxa was considered from rarefied data, host played a significant role in species richness (Table 3). Despite the significant role of site in core species richness, none of the pairwise comparisons between sites were significant (Appendix A: Table S1).

Total fungal abundance was significantly affected by site (Table 2). This significance was driven by significant differences between all pairwise comparisons (Appendix A: Table S2). However, the box and whisker plots revealed mostly overlapping spread between sites (Appendix A: Figure S2). Although the abundance of fungi within the OTUs that make up the core was also significantly affected by site, some of the between-site comparisons were not significantly different. For example, Clanwilliam was significantly different from Robertson and Swellendam, but not from Stellenbosch and Paarl.

Fungal richness and core fungal richness were significantly affected by the interaction between host and site (Table 2). Species richness within *O. europaea* subsp. *cuspidata* twigs within all sites were significantly different from each other (Appendix A: Table S3). Species richness within *O. europaea* subsp. *europaea* was largely significantly different between all sites, except between Somerset West and Clanwilliam, and between Stellenbosch and Robertson. Core species richness within *O. europaea* subsp. *cuspidata* was significantly different from Robertson, Stellenbosch and Swellendam (Table 2; Appendix A: Table S3). The sites closest to each other, Stellenbosch, Somerset West and Paarl, had similar species richness. In contrast, none of the pairwise comparisons of core fungal richness within *O. europaea* subsp. *europaea* were significantly different from each other.

Fungal abundance and the core fungal abundance within olive twigs was significantly affected by the interaction between site and host identity (Table 2). Fungal abundance within *O. europaea* subsp. *cuspidata* from Clanwilliam was significantly different from Robertson and Swellendam (Appendix: Table S2, Figure S2). Fungal abundance within twigs from the African olive in Robertson was significantly different from those originating from Paarl, Somerset West, Stellenbosch and Swellendam. Despite their proximity (hosts adjacent to each other), fungal abundance between the African and European olives from Swellendam were significantly different from each other. European olive fungal abundance in Paarl was significantly different from those originating from Clanwilliam, Robertson and Somerset West. In Robertson, fungal abundance within the twigs of the African and European olive were also significantly different from each other. In contrast, fungal abundance from both hosts within Clanwilliam, Paarl, Somerset West and Stellenbosch were not significantly different from each other. Fungal abundance of the core mycobiome was predominantly different in pairwise comparisons.

Table 2: Generalised linear model results based on total and core fungal endophytes and reduced to balance host sample sizes. 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. *cuspidata* and *O. europaea* subsp. *europaea*) and location across 6 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 Appendix A: Table S1.

	Richness			Richness (core)			Abundance			Abundance (core)		
	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*	29407	10	<0.05*	27548	10	<0.05*
Host	301.32	6	<0.05*	58.735	6	<0.05*	16607	6	<0.05*	23945	6	<0.05*
Interaction	153.68	5	<0.05*	27.332	5	<0.05*	10495	5	<0.05*	23565	5	<0.05*
	Rarefied richness			Rarefied richness (core)			Relative abundance			Relative abundance (core)		
							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						

Table 3: Species richness estimators (Chao2 and Jackknife2) within olive twigs from both hosts and from all six sites based on the full mycobiome (left) and the core mycobiome (right). Sample size, n = 102 (full and unbalanced dataset).

		Whole			Core		
Site	Host	Chao2	Chao2 (SD)	Jackknife2	Chao2	Chao2 (SD)	Jackknife2
All sites	*Olea europaea subsp. cuspidata*	531.450	41.163	565.480	17.000	0.000	17.000
	Olea 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
	Olea 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
	Olea 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
	Olea 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	*Olea 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
	Olea 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
	Olea 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

2.5.2 Endophyte assemblage composition between hosts and sites (β1)

Full and core fungal assemblage composition within olive twigs were significantly influenced by host identity irrespective of whether fungal endophyte abundances or only their presence-absence were considered (Table 4). The core fungal assemblages within hosts formed separate host clusters, but this was especially evident when only considering the core fungal assemblages (Figures 3 and 4). Clustering of samples from *O. europaea* subsp. *europaea* based on the core assemblages was much tighter than clustering of fungi from the native host (Figures 3 and 4). Interestingly, the patterns uncovered with the reduced and balanced sample design were consistent with those from the larger, but unbalanced, sample design (Appendix B: Table S7). Here fungal assemblages were also significantly influenced by host.

Full and core fungal assemblages were also significantly influenced by site, irrespective of whether including abundance or only richness data (Table 4, Figures 3 and 4). Accordingly, hosts from most sites harboured fungal assemblages different from each other, except between Paarl and Stellenbosch, and Robertson and Swellendam (Appendix A, Table S5). These sites were also

geographically close to each other (Figure 1). Sites distant from Swellendam (Clanwilliam, Paarl, Somerset West, and Stellenbosch) harboured significantly different full fungal assemblages from this site (Appendix A: Table S6). Sites far from each other tended to have core assemblages distinct from each other, while closer sites harboured more similar core assemblages. However, fewer pairwise comparisons were significantly different in the core fungal assemblages (Appendix A: Table S5). Also, when considering only the core fungal assemblages, the influence of site was seemingly reduced compared to the full endophyte assemblages as samples from different sites overlapped in CAP analyses (i.e. core assemblages were more similar between sites than when plotting full assemblages) (Figures 3 and 4). As with host identity, patterns uncovered with the balanced sample designs were largely consistent with those from the unbalanced sample designs (Appendix B: Table S7).

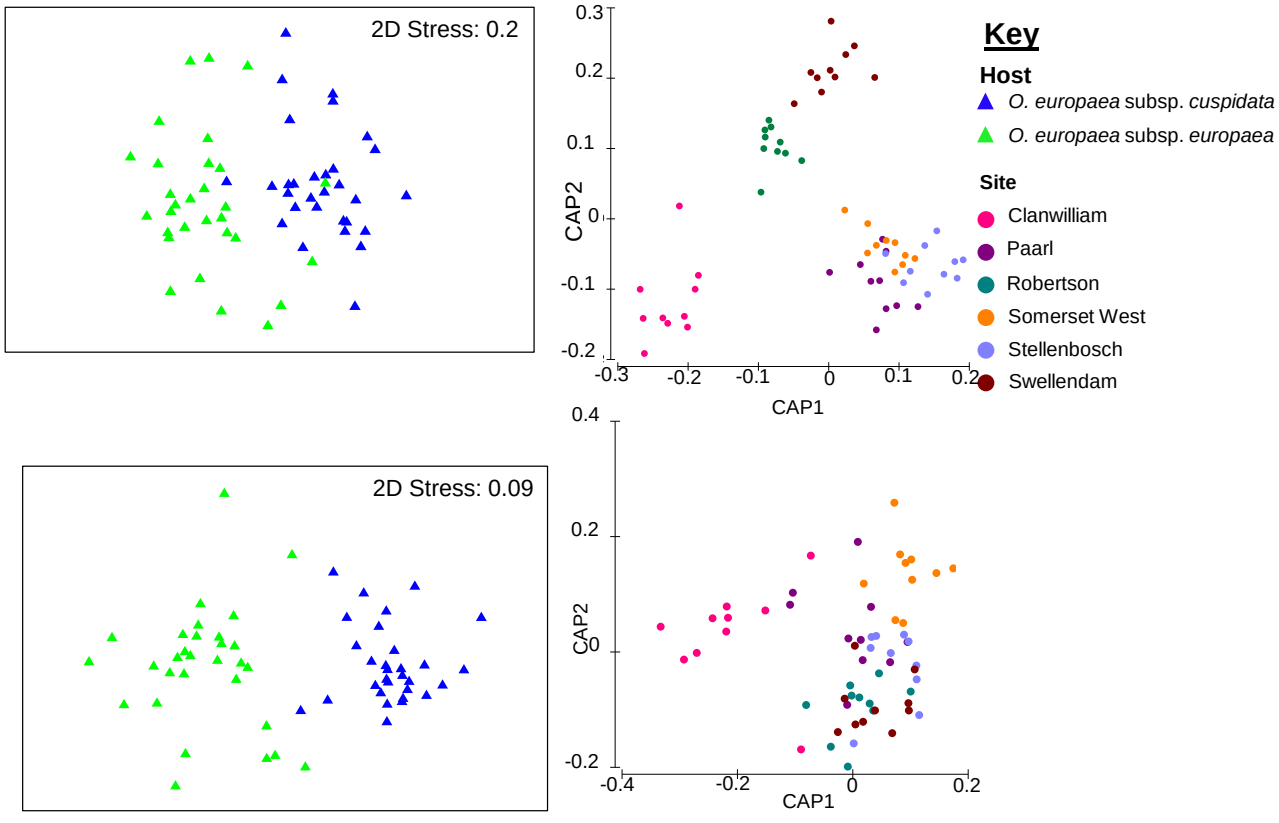

Figure 3: Canonical analysis of principal coordinates (CAP) and non-metric multidimensional scaling (nMDS) plots based on incident data (Jaccard resemblance) for fungal endophyte assemblages sequenced from *O. europaea* subsp. *cuspidata* and *O. europaea* subsp. *europaea* from six different sites. 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 4: 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 communities. 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 microbial community	site	5	34915	1.948	0.001*	38009	2.445	0.001*
	host	1	19256	5.372	0.001*	29662	9.541	0.001*
	interaction	5	28186	1.573	0.001*	27906	1.795	0.001*
Core microbial community	host	1	63193	29.141	0.001*	58105	23.489	0.001*
	site	5	28932	2.668	0.001*	2.90E+04	2.348	0.001*
	interaction	5	26594	2.453	0.001*	2.75E+04	2.227	0.001*

2.5.3 Endophyte assemblage composition within hosts and sites (β2)

Dispersion relative to the centroid of the full fungal assemblage was not significantly influenced by host identity (Table 5). However, the influence of host identity on the sample dispersion around the centroid based on the core fungal assemblages was significant, with that of the introduced host higher than the native host (Appendix A; Table S6).

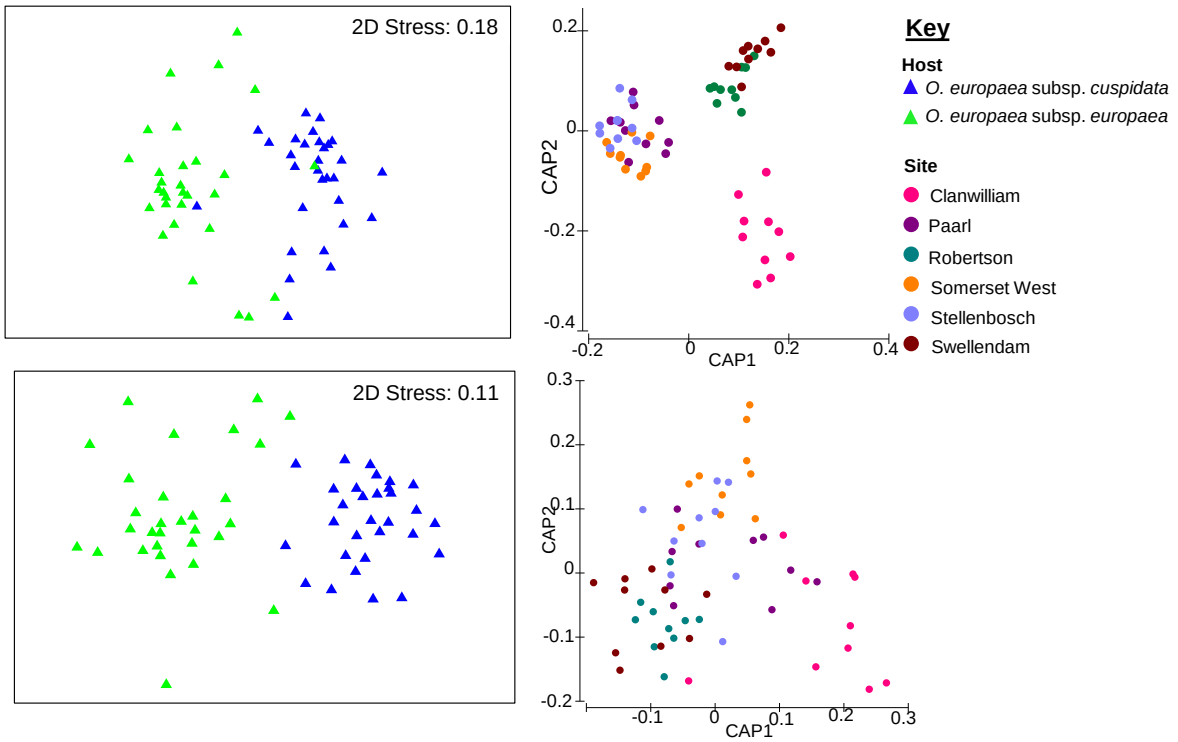

Figure 4: Canonical analysis of principal coordinates (CAP) and non-metric multidimensional scaling (nMDS) plots based on abundance data (Bray-Curtis resemblance), for fungal endophyte assemblages sequenced from *Olea europaea* subsp. *cuspidata* and *O. europaea* subsp. *europaea* from six different sites (Figure 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

Dispersion around the centroid of the full endophyte assemblages differed between sites (Table 5). Specifically, β2 diversity from samples collected in Swellendam were significantly lower than those within olive twigs collected from Clanwilliam, Paal, Somerset West and Stellenbosch (Appendix A: Table S6). This effect disappeared when only considering the core endophyte assemblages.

The interaction between site and host had a significant effect on species turnover of core fungal assemblages (Table 5). Pairwise comparisons revealed that this significance was attributed to Swellendam, which had the lowest species turnover for both hosts, with the native host from this site having the lowest species turnover of the two hosts (Appendix A: Table S6).

Table 5: Homogeneity of within-group multivariate dispersions (β2) of whole endophyte assemblages, or of only core assemblages from *O. europaea* subsp*. cuspidata* and *O. europaea* subsp*. europaea* 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
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*

2.5.4 Effect of geographic distance on endophyte community assemblage composition between sites

The Mantel tests revealed that geographic distance played a significant role in differentiating the full and the core fungal assemblages within olive twigs whether considering presence-absence data (Figure 5) or whether also considering the abundance of OTUs (Figure 6). This pattern was reflected both when hosts were combined and when hosts were considered separately (Figures 5 and 6). This accounted for the earlier results that showed that the more distant sites, Clanwilliam and Swellendam, consistently harboured different assemblages compared to those that were physically closer to each other.

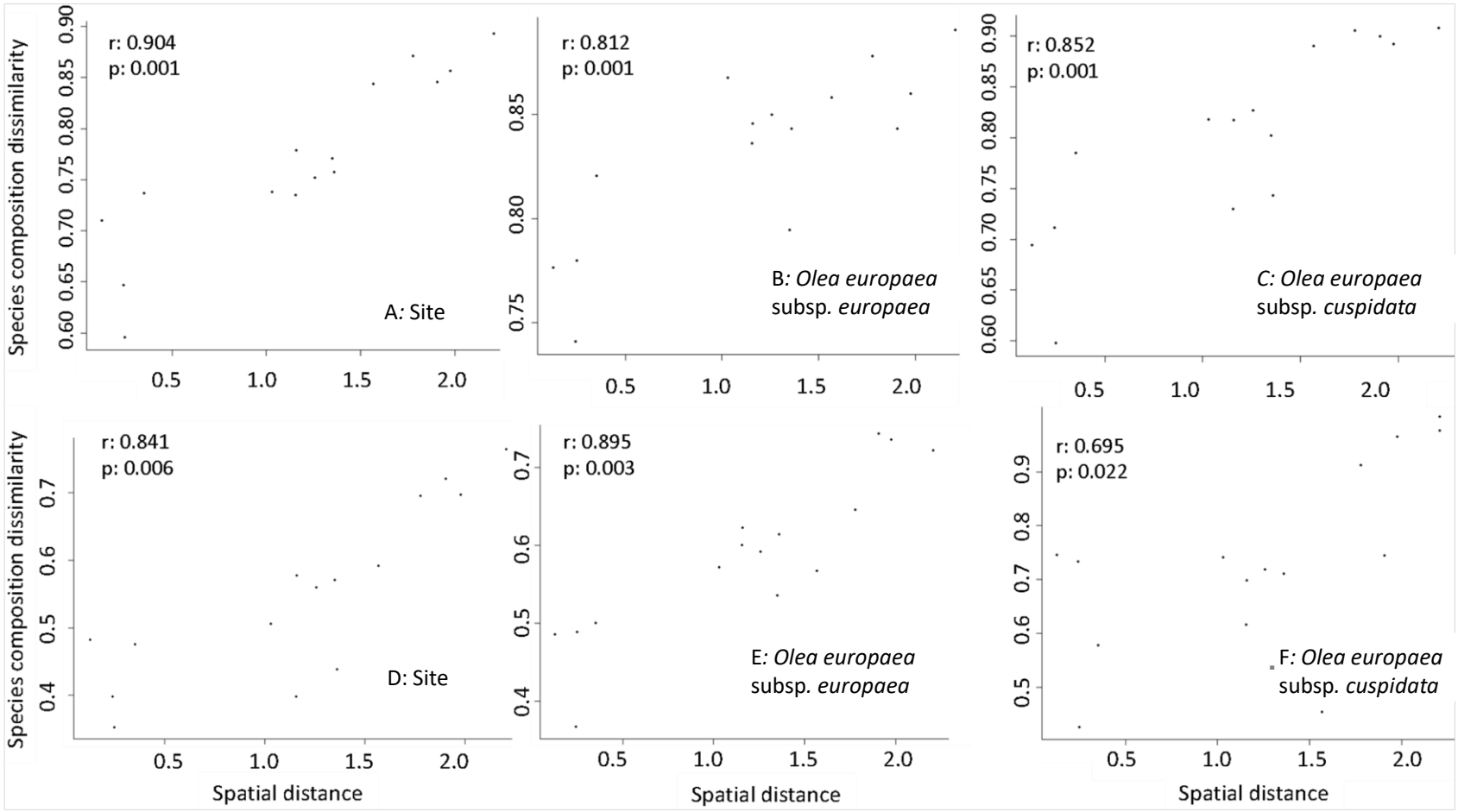

Figure 5: Correlations between geographic distance (Euclidean distance-based matrix) and fungal endophyte assemblage compositional differences based on Jaccard dissimilarity matrices (presence-absence data). Plots A, B and C are based on full fungal assemblages, while D, E and F are based on core fungal assemblages. Correlations are meaningful at $r > 0.5$ and significant when $p < 0.05$. Spatial distance, 1 = 100 km.

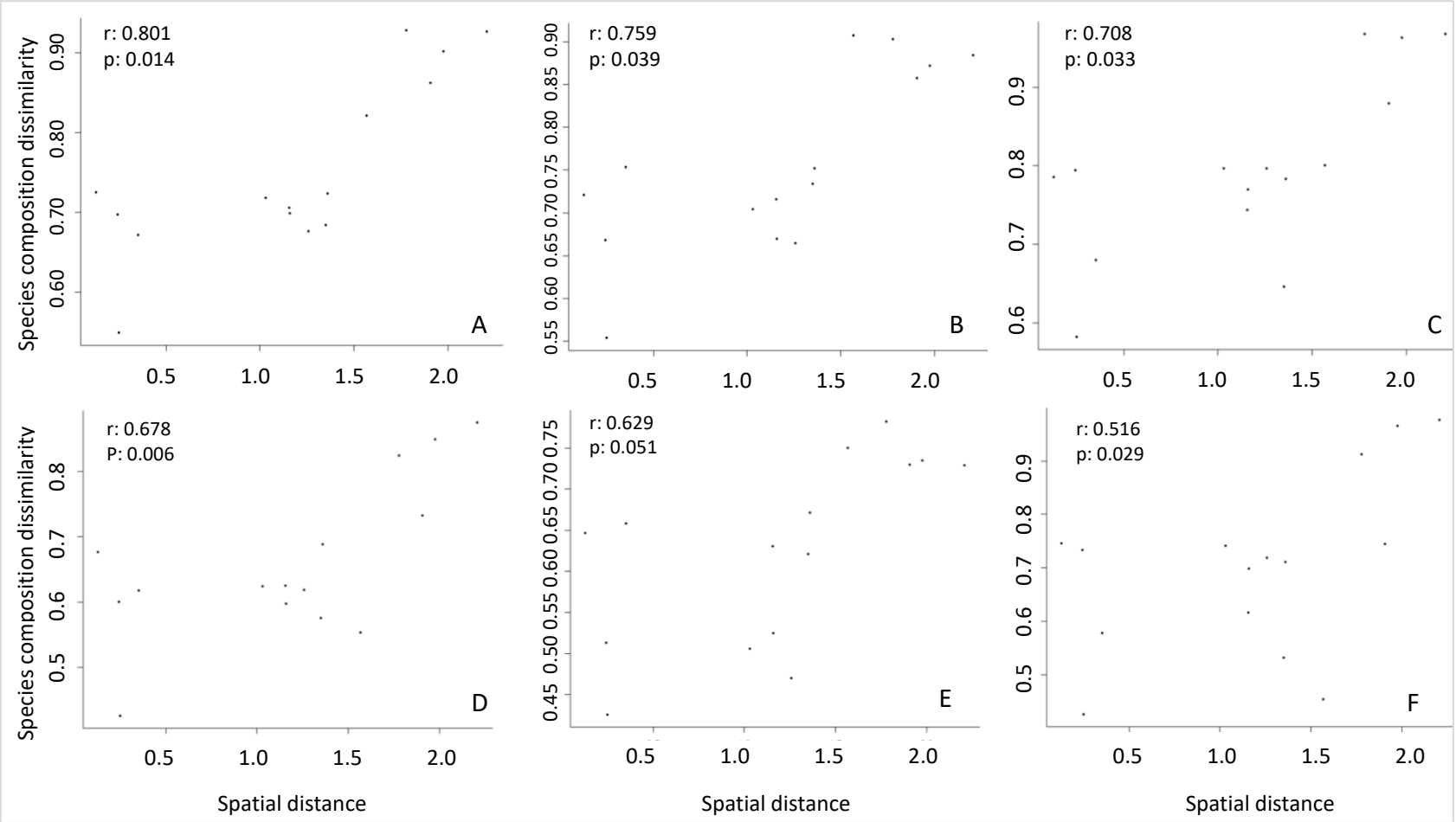

Figure 6: Correlations between geographic distance (matrix based on Euclidean distances) and fungal endophyte assemblage compositional differences measured using Bray-Curtis dissimilarity matrices (based on abundance data). Plots A, B and C are based on full fungal assemblages, while D, E and F are based on core fungal assemblages. Figures: A and D are based on assemblages per site (regardless of host identity); B and E are based on fungal assemblages of *O. europaea* subsp. *europaea* per site, and C and F are based on *O. europaea* subsp. *cuspidata* assemblages. Correlations are meaningful at $r > 0.5$ and significant when $p < 0.05$. Spatial distance, 1 = 100 km.

2.5.5 Endophytic fungal co-occurrence networks

Co-occurrence networks of fungal endophytes from the two hosts were markedly different. *Olea europaea* subsp. *europaea* twigs had fewer significant fungal co-occurrences compared to *O. europaea* subsp*. cuspidata* (Table 6, Figure 7 and 8). 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 (Table 6, Figure 8). In addition, co-occurrence calculations revealed that many of the taxa encountered within *O. europaea* subsp. *europaea* were taxa with no significant co-occurrences between samples, since only 37 of the total 189 OTUs were reflected in the network. Economically important fungi in olives often formed significant co-occurrences, e.g. *Cladosporium* species, *Phoma* Sacc. species, *Teratosphaeria* Syd. & P. Syd. And *Alternaria* Nees. species, with other taxa in respective hosts. The *O. europaea* subsp*. cuspidata* network had a higher degree of connectivity with 41 nodes having node degrees higher than the average node degree (ad= 4.44, Table 6). In contrast, *O. europaea* subsp. *europaea* had 23 nodes with a node degree higher than the average node degree (ad= 1.94, Table 6). 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. The number of nodes that formed significant co-occurrences was similar to the number of edges connecting them within the cultivated olive, while the number of connections between the African olives nodes was more than twice as high as the number of edges (Figure 8, Table 6).

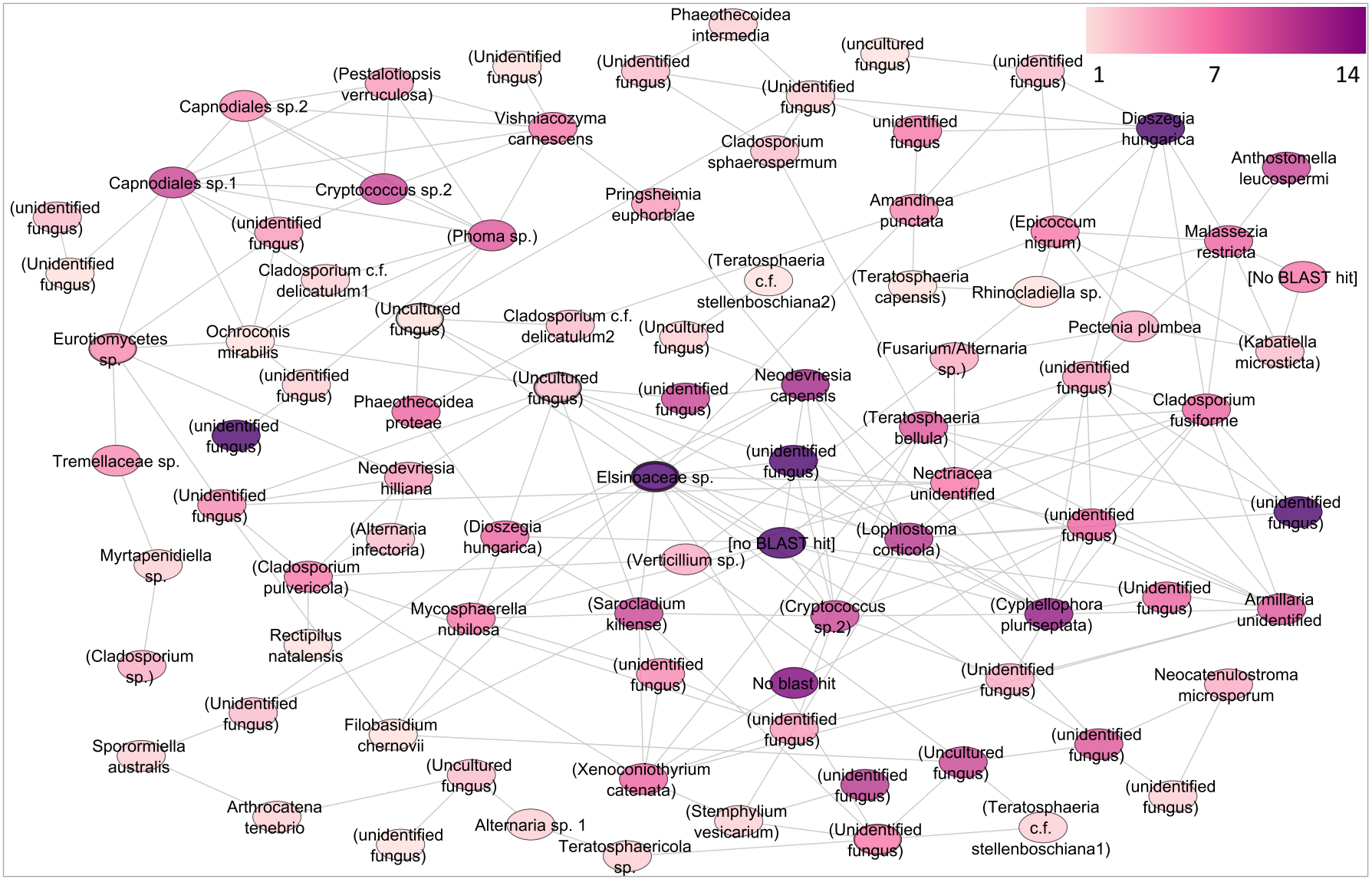

Figure 7: An ecological co-occurrence network of fungal endophytes in *Olea europaea* subsp. *cuspidata* twigs. The colour gradient of nodes is indicative of node degree, where the

darker the node the more connections it 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. The darker nodes with thick outlines have a higher probability of being hub taxa. Labels in round 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.

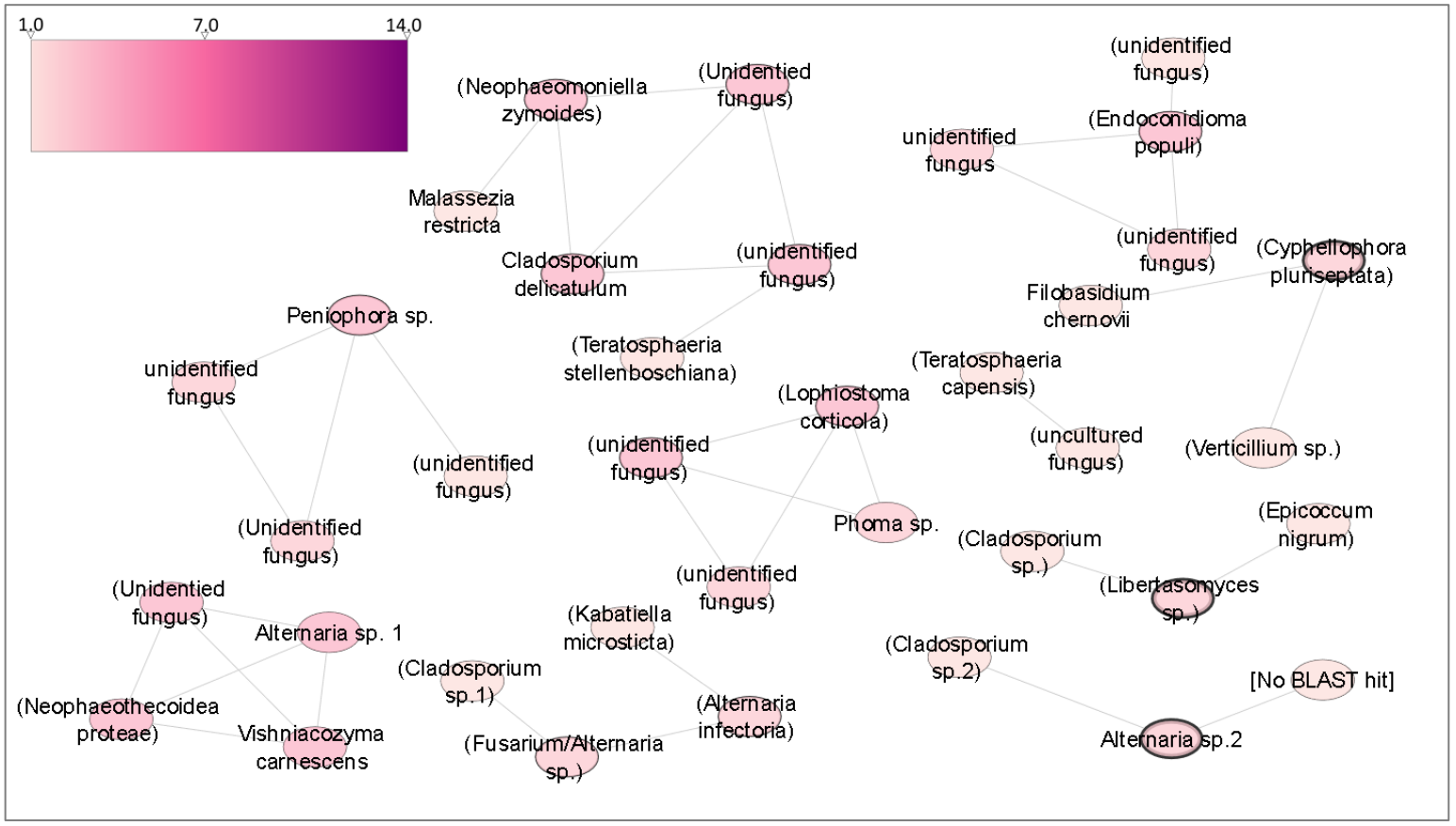

Figure 8: An ecological co-occurrence network of fungal endophyte in *Olea europaea* subsp. *europaea.* Node colour indicates node degree. Thick node outlines indicate nodes with the highest betweenness centrality. Names in round brackets originated from Genbank (NCBI) whereas those without originated from the UNITE database.

Table 6: Summary network statistics for both networks (Figure 7 and 8) 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*

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

2.6 Discussion

This work represents the first study using next generation sequencing to investigate fungal endophyte assemblages within *Olea europaea* hosts in South Africa. Although the twigs sampled in this study were asymptomatic, numerous fungal taxa with the tendency to become pathogens (latent pathogens) were recovered from *O. europaea* subsp. *europaea* and *O. europaea* subsp. *cuspidata* and may warrant further investigation. Fungal endophyte richness and abundance were higher within the native *O. europaea* subsp. *cuspidata* than in the cultivated *O. europaea* subsp. *europaea.* Sites also differed in the number of fungal endophytes within the respective hosts. Fungal assemblage composition differed significantly between the native and cultivated olives and differed significantly across the landscape with geographic distance playing a significant role in this differentiation. However, fungal endophyte assemblage composition tended to be more similar between sites for the cultivated olive than for the native subspecies. Turnover in fungal assemblages within the native host was generally similar to that within the cultivated host, but when considering core assemblages only, turnover was higher within the cultivated host. In addition, fungal assemblages within the native host were much more interconnected compared to the cultivated olive mycobiome. These data suggest that the commercial olive in South Africa house a comparably depauperate fungal endophyte assemblage, as reflected by both its core assemblage and its whole assemblage. It does, however, associate with numerous fungal endophytes, many of which it accumulates from the surrounding areas and likely from the native subspecies.

More endophyte taxa were shared between the native and non-native congeneric olive subspecies than were unique to each. This is not surprising considering similar studies on other hosts elsewhere. Results of Castañeda et al., (2018) reported that despite the diversity of the native trees sampled in Chilean forests, a large number of fungal endophytes were shared between native hosts and the neighbouring vineyard. This highlights the ease with which many endophytic fungal taxa may colonise different host

taxa in the surrounding plant communities (Bufford et al., 2016; Mehl et al., 2017; Pillay et al., 2013; Rodriguez et al., 2009). However, hosts with confamilial relatives in the introduced range have generally been found to be more likely to take up fungi from the native hosts they are related to than the hosts without relatives in the new range (Crous et al., 2017). For example, in South Africa, the invasive *Acacia* Martius and *Eucalyptus* L'Hér. species, which have confamilial relatives in the country, have acquired more native fungi than the *Pinus* species, which lack native confamilial members (Crous et al., 2017). More endophyte taxa were also shared between native and non-native congeneric *Agave* species in North and South America (Coleman-Derr et al., 2016). In the current study the two *O. europaea* subspecies were sampled where they co-occurred at sites, thus increasing contact opportunity and opportunity of fungal endophyte exchange.

The cultivated olive had a significantly lower endophyte richness and abundance, and significantly different assemblage composition to the native host. The cultivated olive also harboured fewer exclusive OTUs than the native olive. This was to be expected since some fungal associates may not have made it to the new range and some may not have survived the new environment (Hayward et al., 2015). These results suggest, and support a growing body of knowledge, that exotic species tend to lose much of their natural fungal diversity and acquire many taxa from the new surroundings (Colautti et al., 2004; Martín-García et al., 2012; Saikkonen et al., 2000). This is also seen, for example, in endophytes within native and exotic *Agave* species in South America (Coleman-Derr et al., 2016). However, an increase in residence time of an introduced host has been found to positively correlate with the number of host shifts and expansions (Desprez-Loustau et al., 2010). Thus, with increasing residence time there will likely be increased opportunities for the cultivated olive to obtain more microbial associates, as it has a fairly recent (~100 years, relative to its >100 years life span) introduction into South Africa. Nevertheless, the infrequent detection of many taxa in *O. europaea* subsp. *europaea* may suggest that, even if closely related to a native host species, it still likely represents a naïve host to many endophytic taxa. The decreased co-evolutionary history between these fungi and the European olive, relative to the native trees, may be responsible for the low occurrence of these taxa.

Co-occurrence networks revealed a dense and highly connected network of endophytes within the African olive, while fungal endophytes within the cultivated olive were very disconnected. The highly connected co-occurrence network formed by the fungal endophytes of the native olive suggests that many of the taxa likely co-occur deliberately and that these co-occurrences may play an important role in the survival and persistence of fungi and the host. The native host had more than triple the number of nodes of the

cultivated olives. In addition, most of the taxa encountered in the network of the cultivated olives also appeared in the network of the native host. Taken together this may mean that the cultivated host may have an affinity for the same fungal endophytes. Given the large number of shared taxa between the two hosts and the overlap in taxa with significant co-occurrences, the other two thirds of fungi with significant co-occurrences in the native host may still colonise the cultivated host over time if the hosts remain in contact in the field.

Network properties such as hub species, betweenness centrality and closeness centrality held no biological meaning in the cultivated olive network, since "the network" consisted of many disconnected co-occurrence subnetworks. Even so, many of the taxa that appeared in these disconnected elements are ecologically important. For example, a single *Verticillium* sp. appeared within the cultivated olive host. Species in the genus *Verticillium* Nees are amongst the most notorious plant pathogens in the world, including the serious olive wilt causal agent, *Verticillium dahliae* Kleb (Fernández-González et al., 2020; Löpez-Escudero and Mercado-Blanco, 2011). In South Africa, *V. dahliae* has been recorded as a wilt causal agent in tomatoes (Ferreira et al., 1990). Other genera with known pathogens such as *Peniophora* Cooke and *Phoma* (Taylor and Wallace, 1970; Ivic et al., 2010; Pethybridge and Hay, 2001) also formed significant co-occurrences within the cultivated olive. Members of the latter genus have been recorded as causal agents of diseases such as stem blight and leaf chlorosis in olives (Fisher et al., 1992; Rhouma et al., 2010). Widespread genera such as *Alternaria, Cladosporium* and *Epicoccum* formed significant co-occurrences in the European olive. These taxa have a global distribution and are found within a wide range of hosts including exotic species (Kosawang et al., 2018; Lou et al., 2013; Malhadas et al., 2017; Ogórek et al., 2012; Piecuch et al., 2020; Suryanarayanan et al., 2018). The sparsity of significant fungal co-occurrences may suggest that although the cultivated host takes up fungal endophytes from its surroundings these acquisitions are infrequent and largely inconsistent from one sample to the next. However, the mycobiome within this host may be incomplete with new connections still likely to establish as residence time increases.

The co-occurrence network of the fungal endophyte taxa from the native host were highly connected, indicative of a small world network (Newman, 2010; Watts and Strogatz, 1998). The three most connected nodes comprised of taxa yet to be described. The symbiotic relationship between these taxa and the African olive may play an important role to their (both host and microbe) ability to strive across the Core Cape Subregion. Given their undescribed nature and association with a poorly explored host, it is likely that they are native. *Neophaeothecoidea proteae* (Crous) Quaedvl. & Crous (=*Phaeothecoidea proteae*

Crous), *Lophiostoma corticola* (Fuckel) E.C.Y. Liew, Aptroot & K.D. Hyde and *Neodevriesia capensis* (Crous) Crous (=*Teratosphaeria capensis* Crous) formed significant co-occurrences within both hosts and were amongst potential hub species of the native olive. *Neophaeothecoidea proteae* was first described from symptomatic leaves (leaf spot) of *Protea repens* L., a native and widespread host in the Core Cape Subregion (Crous et al., 2008). *Lophiostoma corticola* has been encountered as an endophyte within olives and *Fraxinus* species (Kosawang et al., 2018; Kowalski et al., 2016; Martins et al., 2016). The increase of *L. corticola* within necrotic *Fraxinus* stems and twigs suggested that this species may also be a secondary invader or saprotroph (Kowalski et al., 2016). *Neodevriesia capensis* is found within necrotic leaves of *Protea* hosts in South Africa and *Protea repens* introduced into Portugal from South Africa (Crous et al., 2011). These fungal species appear to mainly infect hosts native to South Africa, thus suggesting that although encountered within healthy twigs, they may represent a species with important future implications for the health of the African olive.

Fungal endophyte assemblages found within tree hosts respond to external factors that are linked to geographic location. In this study, geographic location played a significant role in shaping endophytic fungal assemblages within olive twigs from both the native and the cultivated taxa. These patterns were similar to those found in fungal assemblages in olives in Portugal (Martins et al., 2016), *Quercus ilex* L. in Spain (Collado et al., 1999), and in native and non-native *Phragmites* Adans. species sampled in Michigan (Bickford et al., 2018), amongst others. The role of host locality becomes even 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. Similarly, according to Martins et al., (2016) fungal endophyte assemblages from Carrazeda de Ansiaes and Mirandela (approximately 27 km apart) were more similar to each other than to more distant sites. This is not a unique pattern to endophytes, in the Fynbos biome, soil microbial diversity also showed strong patterns of spatial correlation (Slabbert et al., 2010).

Endophytic fungi have been the subject of many studies in South African native and non-native hosts (Jami et al., 2015; Lee et al., 2004, 2005). Some fungal taxa encountered here have also been recorded in other hosts in South Africa. For example, two *Lophiostoma* species (including *L. cynaroidis* Marincowitz, M.J. Wingf. & Crous) were encountered within the sampled olives. During a survey of olive pathogens, *L. cynaroidis* was identified from a wild olive from Wellington, but no pathogenicity was reported (Spies et al., 2020). Additional taxa were encountered in olives that reside in, perhaps, the most studied fungal families in South Africa, Teratosphaeriaceae and Botryosphaeriaceae (Aylward et al., 2019; Cruywagen et

al., 2017; Osorio et al., 2017; Perez et al., 2009). For example, *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips was identified from both olive hosts. This species has also been recorded in olive nurseries and mango trees in South Africa (Mehl et al., 2017; Spies et al., 2020). *Dothiorella iberica* A.J.L. Phillips, J. Luque & A. Alves was identified from both olive hosts. This species was also previously found in *V. vinifera* in South Africa (de Wet et al., 2008) and as a causal agent of olive diseases such as twig die-back in California (Úrbez-Torres et al., 2013). Two *Teratospharia* taxa related to *Teratosphaeria stellenboschiana* (Crous) Crous were recorded within the African olive, while *T. stellenboschiana* was recorded within the cultivated olive. *Teratosphaeria stellenboschiana* is known from leaves of *Eucalyptus* species in Stellenbosch and Pretoria (Crous et al., 2009). The recovery of taxa previously recorded in other hosts in the same region of South Africa may speak to the ability of these fungi to infect multiples hosts in the same area, an ability recorded in endophytic fungi, especially of Botrysphaeriaceae and Teratosphaeriaceae (Crous et al., 2008; Mehl et al., 2017; Pillay et al., 2013). It also provides identification validation of species tentatively identified based on the short and conserved ITS marker used in metabarcoding studies.

Numerous genera identified in the current study have been previously recorded in olives in other countries. For example, *Cladosporium*, *Aureobasidium* Viala & G. Boyer, *Devriesia* Seifert & N.L. Nick., *Hormonema* Lagerb. & Melin, *Toxicocladosporium* Crous & U. Braun, *Phomopsis* (Sacc.) Sacc. and *Cryptococcus* have all been recorded from olive leaves and flowers in Italy (Abdelfattah et al., 2015). An unidentified *Cryptococcus* species was found in three *O. europaea* subsp. *europaea* samples and one *O. europaea* subsp*. cuspidata* sample from Robertson, Stellenbosch and Swellendam. *Cryptococcus gattii* (Vanbreus. & Takashio) Kwon-Chung & Boekhout has been reported in *Olea* species (Hagen et al., 2015). *Phoma* species were also identified within the cultivated olive. *Phoma incompta* Sacc. & Mart. is known to cause olive shoot necrosis in Croatia (Ivic et al., 2010). Despite the vast distances between the olive growing countries where these studies were conducted, the olive tree appears to harbour some of the same taxa (at least, at genus level). This retention might point at stringent host filtering or taxa retention since the time of separation from the home range, taxa retention was previously recorded in *Fraxinus* (Power et al., 2017).

Some endophytic fungi present conflicting lifestyles upon exiting the endophytic phase such that they can become either beneficial or harmful. Endophytic fungi that often turn to become beneficial to their host often reside in genera such as *Epicoccum*, *Cladosporium* and *Penicillium* (Dzoyem et al., 2017; Gomes et al., 2019; Khan et al., 2016). A single *Epicoccum* species (*Epicoccum nigrum*) was identified from both

hosts. *Epicoccum nigrum* (P.R. Johnst.) Qian Chen & L. Cai 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 *O. europaea* subsp*. europaea* – *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). *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 unidentified 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* Thüm. and an unidentified *Cladosporium* species have also been implicated in Algeria, Iran, Australia and Jordan as causal agents of olive fruit rots and olive leaf mould (Arnold et al., 2003; Faiza et al., 2011). Two undescribed *Cryptococcus* species formed significant co-occurrences within twigs of the native olive. A *Crytococcus* species (*Cryptococcus laurentii* Link) has been shown to be a successful biological control agent for a pathogen known to cause serious fruit decay, namely *Penicillium expansum* Link and *Monilinia fructicola* (G. Winter) Honey (Qin and Tian, 2005). 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 switch.

2.7 Conclusions

This study contributes to the growing knowledge of fungi associated with the agricultural crops cultivated in the Core Cape Subregion. Studying fungal endophyte assemblages within crops and native hosts can shed light on fungal movement and host expansion, both of which become increasingly important as global climate continues to shift. Particularly, knowledge on fungal endophytes present in crop plants is critical, especially for those that are known to shift to become pathogens and cause serious damage to crop productivity. In addition, as microbiome manipulation for the purposes of improving plant growth is gaining attention, it is important to understand fungal assemblages within agricultural crops and their closest native relatives if we are to explore their biotechnological significance in future agriculture. Although, many of the taxa present in the networks were identified up to species level, further investigations and confirmation of the presence of some of these species is required as the ITS region used in metabarcoding studies is known for its inability to discriminate closely related species (Abdelfattah

et al., 2015; Malacrinò et al., 2017). Nonetheless, the prevalence of undescribed taxa indicates a trove of potentially important fungal taxa that remains to be discovered and described. The number of taxa recovered in this study shared between the two hosts that have also been recorded in cultivated olives of the Old and New World suggests that, although physically distant, they still take up similar fungi from the surroundings. Overall, olives appear to be exposed to similar fungi as other hosts studied in the country. Thus, improving our understanding of fungal endophytes within native and cultivated hosts in the country is of great importance.

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2.9 Supplementary materials

2.9.1 Appendix A: Unbalanced alpha diversity and balanced beta diversity *post hoc* results

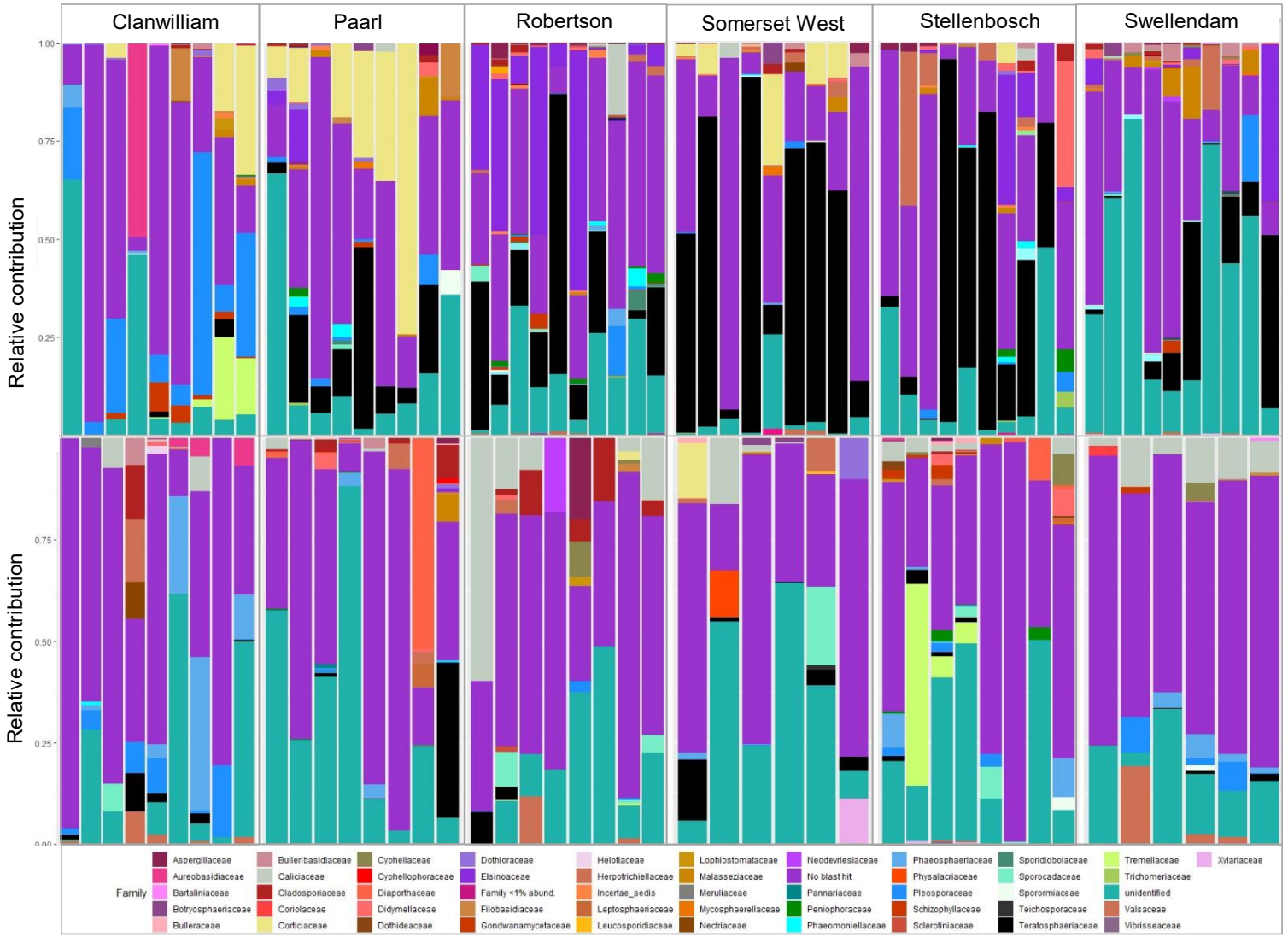

Figure S1: Stack plots of fungal endophyte families represented within *Olea europaea* subsp. *cuspidata* (top) and *Olea europaea* subsp. *europaea* (bottom) twigs (unbalanced data, n = 102) including taxa without BLAST hits and fungi that could not be placed at family level

Table S1: Linear models' *post hoc* results of fungal richness (richness, core richness, rarefied richness and core rarefied richness) between hosts and between sites. Significant when p < 0.05 (*).

		Richness			Richness (core)			Rarefied richness			Rarefied richness (core)		
variable1	variable2	SE	z-value	Pr(>|z|)	SE	z-value	Pr(>|z|)	SE	t-value	Pr(>|t|)	SE	t-value	Pr(>|t|)
O. e. subsp. europaea	*O. europaea subsp. cuspidata*	0.042	-11.7	<2e-1*6	0.270	0.932	0.351	0.815	-2.751	0.007*			
Paarl	Clanwilliam	0.075	5.265	0.001*	0.235	2.049	0.305				0.896	1.052	0.885
Robertson	Clanwilliam	0.07	9.865	0.001*	0.209	6.173	<0.001*				0.934	1.059	0.882
Somerset West	Clanwilliam	0.081	1.63	0.576	0.228	2.434	0.139				0.891	-0.593	0.99
Stellenbosch	Clanwilliam	0.074	4.92	0.001*	0.214	4.412	<0.001*				0.887	1.221	0.804
Swellendam	Clanwilliam	0.072	8.325	0.001*	0.215	5.078	<0.001*				0.939	1.085	0.871
Robertson	Paarl	0.063	4.732	0.001*	0.166	4.868	<0.001*				0.885	0.053	1
Somerset West	Paarl	0.075	-3.491	0.006*	0.190	0.388	0.999				0.741	-1.987	0.317
Stellenbosch	Paarl	0.068	-0.427	0.998	0.172	2.684	0.075				0.392	0.359	0.999
Swellendam	Paarl	0.065	3.186	0.018*	0.174	3.516	0.006*				0.890	0.085	1
Somerset West	Robertson	0.07	-7.966	0.001*	0.156	-4.707	<0.001*				0.881	-1.724	0.48
Stellenbosch	Robertson	0.062	-5.253	0.001*	0.134	-2.569	0.101				0.877	0.108	1
Swellendam	Robertson	0.059	-1.497	0.664	0.136	-1.437	0.696				0.401	0.072	1
Stellenbosch	Somerset West	0.074	3.134	0.021*	0.163	2.389	0.154				0.730	2.208	0.208
Swellendam	Somerset West	0.072	6.499	0.001*	0.164	3.271	0.013*				0.885	1.747	0.464
Swellendam	Stellenbosch	0.064	3.67	0.003*	0.144	1.034	0.903				0.882	-0.074	1

Table S2: Linear models *post hoc* results of fungal abundance (abundance, core abundance, relative abundance and core relative abundance) between hosts and between sites. Significant when $p < 0.05$ (*).

		Abundance			Abundance (core)			Relative abundance		
variable1	variable2	SE	z-value	Pr(>|z|)	SE	z value	Pr(>|z|)	SE	t-value	Pr(>|t|)
O. europaea subsp. *europaea*	*O. europaea* subsp. *cuspidata*	0.002	51.1	<2e-16*	0.005	-42.45	<2e-16*	5.616	8.092	1.07E-05*
Paarl	Clanwilliam	0.003	-98.12	<2e-16*	0.127	2.193	0.237			
Robertson	Clanwilliam	0.003	-142.7	<2e-16*	0.116	6.793	<0.001*			
Somerset West	Clanwilliam	0.003	-62.77	<2e-16*	0.135	0.068	1			
Stellenbosch	Clanwilliam	0.003	-154.8	<2e-16*	0.127	2.309	0.187			
Swellendam	Clanwilliam	0.003	-15.49	<2e-16*	0.116	6.629	<0.001*			
Robertson	Paarl	0.004	-43.98	<2e-16*	0.105	4.803	<0.001*			
Somerset West	Paarl	0.004	31.78	<2e-16*	0.127	-2.127	0.27			
Stellenbosch	Paarl	0.004	-56.65	<2e-16*	0.117	0.117	1			
Swellendam	Paarl	0.003	80.12	<2e-16*	0.106	4.628	<0.001*			
Somerset West	Robertson	0.004	74.7	<2e-16*	0.115	-6.735	<0.001*			
Stellenbosch	Robertson	0.004	-12.91	<2e-16*	0.105	-4.692	<0.001*			
Swellendam	Robertson	0.004	123.78	<2e-16*	0.092	-0.184	1			
Stellenbosch	Somerset West	0.004	-86.92	<2e-16*	0.126	2.243	0.215			
Swellendam	Somerset West	0.003	46.46	<2e-16*	0.116	6.571	<0.001*			
Swellendam	Stellenbosch	0.004	135.86	<2e-16*	0.105	4.517	<0.001*			

Table S3: Species richness and core species richness linear models *post hoc* results of the interaction between both hosts and sites. Comparisons considered significant when $p < 0.05$ (*). Oa = *O. europaea* subsp. *cuspidata* and oe = *O. europaea* subsp. *europaea*

		Richness				Richness (core)			
		Estimate	SE	z-value	Pr(>|z|)	Estimate	SE	z-value	Pr(>|z|)
oaPaarl	oaClanwilliam	-0.187	0.004	-42.017	<0.01*	0.482	0.235	2.049	0.643
oaRobertson	oaClanwilliam	-0.454	0.005	-97.261	<0.01*	1.289	0.209	6.173	<0.01*
oaSomerset_West	oaClanwilliam	-0.331	0.004	-73.605	<0.01*	0.556	0.228	2.434	0.367
oaStellenbosch	oaClanwilliam	-0.540	0.005	-112.628	<0.01*	0.944	0.214	4.412	<0.01*
oaSwellendam	oaClanwilliam	0.062	0.004	15.312	<0.01*	1.093	0.215	5.078	<0.01*
oeClanwilliam	oaClanwilliam	0.211	0.004	51.096	<0.01*	0.251	0.270	0.932	0.999
oaRobertson	oaPaarl	-0.267	0.005	-53.772	<0.01*	0.807	0.166	4.868	<0.01*
oaSomerset_West	oaPaarl	-0.144	0.005	-29.999	<0.01*	0.074	0.190	0.388	1.000
oaStellenbosch	oaPaarl	-0.353	0.005	-69.419	<0.01*	0.463	0.172	2.684	0.2209
oaSwellendam	oaPaarl	0.249	0.004	56.693	<0.01*	0.611	0.174	3.516	0.0206*
oePaarl	oaPaarl	-0.076	0.005	-15.26	<0.01*	0.036	0.202	0.179	1.000
oaSomerset_West	oaRobertson	0.123	0.005	24.502	<0.01*	-0.733	0.156	-4.707	<0.01*
oaStellenbosch	oaRobertson	-0.086	0.005	-16.291	<0.01*	-0.344	0.134	-2.569	0.2826
oaSwellendam	oaRobertson	0.516	0.005	111.867	<0.01*	-0.196	0.136	-1.437	0.952
oeRobertson	oaRobertson	0.149	0.005	28.34	<0.01*	-1.125	0.195	-5.755	<0.01*
oaStellenbosch	oaSomerset_West	-0.209	0.005	-40.699	<0.01*	0.389	0.163	2.389	0.397
oaSwellendam	oaSomerset_West	0.393	0.004	88.512	<0.01*	0.538	0.164	3.271	0.046*
oeSomerset_West	oaSomerset_West	0.537	0.005	106.138	<0.01*	-0.199	0.237	-0.837	1.000
oaSwellendam	oaStellenbosch	0.602	0.005	126.983	<0.01*	0.149	0.144	1.034	0.997
oeStellenbosch	oaStellenbosch	0.225	0.005	41.811	<0.01*	-0.492	0.181	-2.718	0.205
oeSwellendam	oaSwellendam	-0.073	0.005	-15.49	<0.01*	-0.582	0.198	-2.942	0.119

oePaarl	oeClanwilliam	-0.474	0.005	-100.358	<0.01*	0.267	0.241	1.104	0.994
oeRobertson	oeClanwilliam	-0.516	0.005	-107.739	<0.01*	-0.087	0.260	-0.335	1.000
oeSomerset_West	oeClanwilliam	-0.005	0.005	-1.14	0.9927	0.105	0.278	0.38	1.000
oeStellenbosch	oeClanwilliam	-0.525	0.005	-109.458	<0.01*	0.201	0.244	0.821	1.000
oeSwellendam	oeClanwilliam	-0.222	0.005	-46.429	<0.01*	0.260	0.256	1.013	0.997
oeRobertson	oePaarl	-0.041	0.005	-7.824	<0.01*	-0.354	0.227	-1.557	0.918
oeSomerset_West	oePaarl	0.469	0.005	89.335	<0.01*	-0.161	0.248	-0.651	1.000
oeStellenbosch	oePaarl	-0.051	0.005	-9.657	<0.01*	-0.066	0.210	-0.314	1.000
oeSwellendam	oePaarl	0.252	0.005	47.685	<0.01*	-0.007	0.223	-0.032	1.000
oeSomerset_West	oeRobertson	0.510	0.005	96.226	<0.01*	0.192	0.265	0.726	1.000
oeStellenbosch	oeRobertson	-0.010	0.005	-1.833	0.7971	0.288	0.230	1.249	0.983
oeSwellendam	oeRobertson	0.294	0.005	54.953	<0.01*	0.347	0.243	1.428	0.954
oeStellenbosch	oeSomerset_West	-0.520	0.005	-97.836	<0.01*	0.095	0.250	0.381	1.000
oeSwellendam	oeSomerset_West	-0.216	0.005	-40.873	<0.01*	0.154	0.262	0.589	1.000
oeSwellendam	oeStellenbosch	0.304	0.005	56.653	<0.01*	0.059	0.226	0.260	1.000

Table S4: Fungal abundance and core fungal abundance linear models' *post hoc* results of the interaction between both hosts and sites. Significant when $p < 0.05$ (*). Oa = *O. europaea* subsp. *cuspidata* and oe = *O. europaea* subsp. *europaea*

		Abundance				Abundance (Core)			
		Estimate	SE	z-value	Pr(>|z|)	Estimate	SE	z-value	Pr(>|z|)
oaPaarl	oaClanwilliam	0.239	0.098	2.442	0.360	1.081	0.038	28.224	0.001*
oaRobertson	oaClanwilliam	0.978	0.084	11.667	<0.01*	1.704	0.043	40.079	0.001*
oaSomerset West	oaClanwilliam	0.116	0.098	1.176	0.990	1.642	0.038	43.18	0.001*
oaStellenbosch	oaClanwilliam	0.255	0.095	2.683	0.222	1.729	0.038	45.49	0.001*
oaSwellendam	oaClanwilliam	0.748	0.087	8.624	<0.01*	1.897	0.043	44.635	0.001*
oeClanwilliam	oaClanwilliam	-0.364	0.119	-3.043	0.090	2.003	0.012	167.088	0.001*
oaRobertson	oaPaarl	0.739	0.080	9.246	<0.01*	0.624	0.037	16.893	0.001*
oaSomerset West	oaPaarl	-0.123	0.095	-1.3	0.977	0.561	0.017	33.836	0.001*
oaStellenbosch	oaPaarl	0.016	0.092	0.179	1	0.648	0.007	87.712	0.001*
oaSwellendam	oaPaarl	0.509	0.083	6.135	<0.01*	0.817	0.037	22.134	0.001*
oePaarl	oaPaarl	0.048	0.096	0.504	1	0.539	0.008	67.661	0.001*
oaSomerset West	oaRobertson	-0.862	0.080	-10.72	<0.01*	-0.062	0.037	-1.699	0.777
oaStellenbosch	oaRobertson	-0.722	0.077	-9.427	<0.01*	0.025	0.037	0.671	1.000
oaSwellendam	oaRobertson	-0.230	0.066	-3.492	0.023*	0.193	0.006	33.126	0.001*
oeRobertson	oaRobertson	-1.398	0.108	-12.96	<0.01*	-0.104	0.007	-15.949	0.001*
oaStellenbosch	oaSomerset West	0.140	0.092	1.516	0.931	0.087	0.016	5.455	0.001*
oaSwellendam	oaSomerset West	0.632	0.083	7.578	<0.01*	0.255	0.037	6.97	0.001*
oeSomerset West	oaSomerset West	-0.270	0.128	-2.103	0.604	0.508	0.006	80.433	0.001*
oaSwellendam	oaStellenbosch	0.492	0.080	6.171	<0.01*	0.168	0.037	4.602	0.001*
oeStellenbosch	oaStellenbosch	-0.022	0.095	-0.232	1	-0.012	0.007	-1.851	0.670
oeSwellendam	oaSwellendam	-0.815	0.107	-7.594	<0.01*	0.085	0.006	13.626	0.001*

oePaarl	oeClanwilliam	0.651	0.118	5.51	<0.01*	-0.383	0.037	-10.416	0.001*
oeRobertson	oeClanwilliam	-0.057	0.137	-0.412	1	-0.402	0.041	-9.703	0.001*
oeSomerset West	oeClanwilliam	0.209	0.145	1.443	0.951	0.148	0.037	4.015	0.002*
oeStellenbosch	oeClanwilliam	0.597	0.119	5.005	<0.01*	-0.286	0.036	-7.848	0.001*
oeSwellendam	oeClanwilliam	0.297	0.135	2.196	0.534	-0.021	0.041	-0.496	1.000
oeRobertson	oePaarl	-0.708	0.120	-5.878	<0.01*	-0.019	0.037	-0.506	1.000
oeSomerset West	oePaarl	-0.442	0.129	-3.418	0.028*	0.531	0.016	32.558	0.001*
oeStellenbosch	oePaarl	-0.054	0.099	-0.545	1	0.098	0.007	13.577	0.001*
oeSwellendam	oePaarl	-0.354	0.118	-3.007	0.100	0.363	0.037	9.856	0.001*
oeSomerset West	oeRobertson	0.266	0.147	1.81	0.801	0.549	0.037	14.938	0.001*
oeStellenbosch	oeRobertson	0.654	0.121	5.379	<0.01*	0.116	0.036	3.187	0.033*
oeSwellendam	oeRobertson	0.353	0.137	2.578	0.278	0.381	0.007	55.666	0.001*
oeStellenbosch	oeSomerset West	0.387	0.130	2.975	0.107	-0.433	0.014	-30.012	0.001*
oeSwellendam	oeSomerset West	0.087	0.145	0.603	1	-0.168	0.037	-4.574	0.001*
oeSwellendam	oeStellenbosch	-0.300	0.119	-2.524	0.309	0.265	0.036	7.282	0.001*

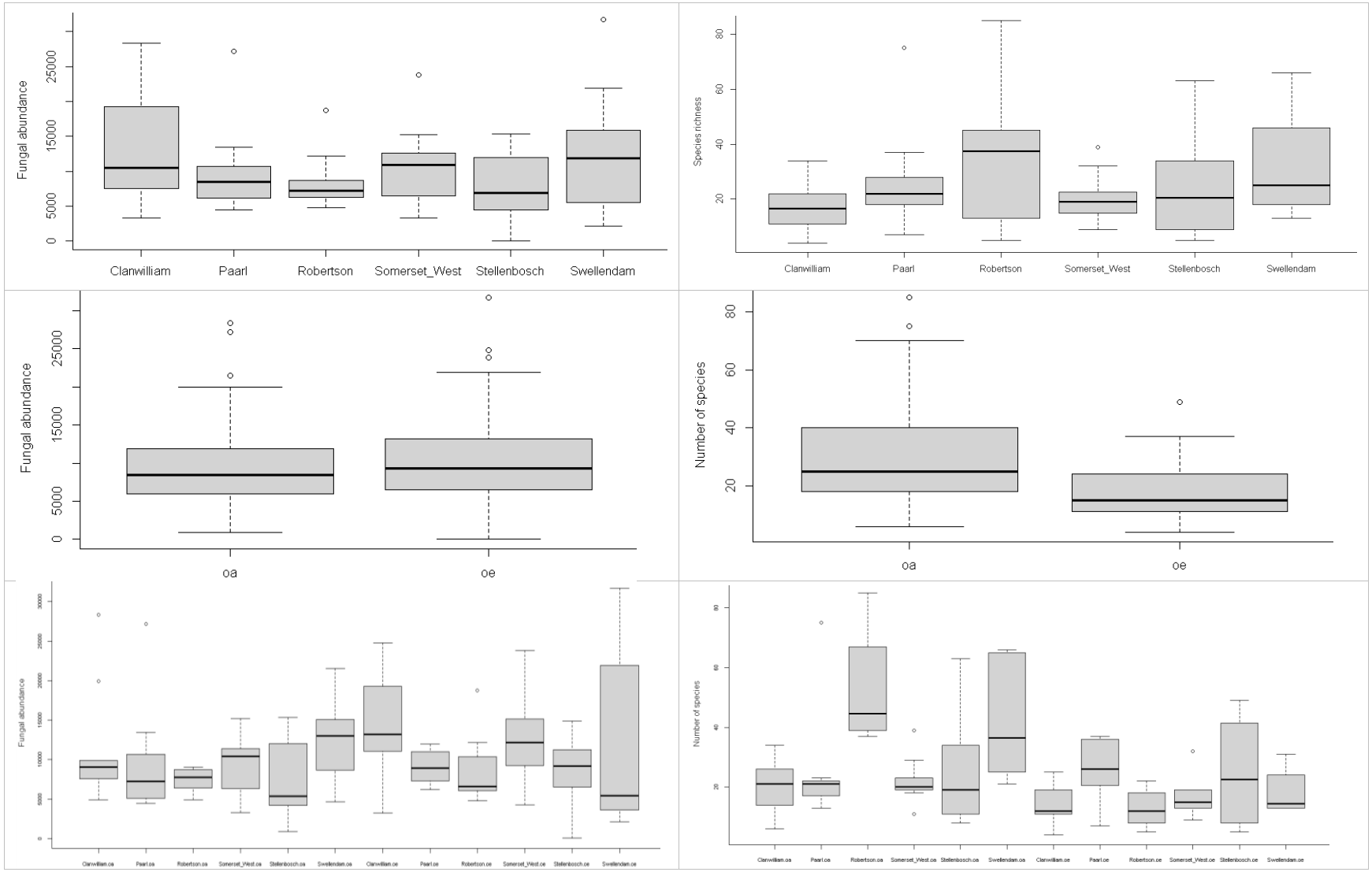

Figure S2: Box and whisker plots of fungal abundance (left) and richness (right). Oa = *Olea europaea* subsp. *cuspidata*, Oe = *Olea europaea* subsp. *europaea.* n = 102.

Table S5: PERMANOVA *post hoc* tests of reduced complete fungal assemblages and core fungal assemblages. Pairwise comparisons of fungal assemblages between sites and within sites (between hosts) are based on the balanced and reduced dataset (n =60). All bold results indicate comparisons significant at p<0.05. oe = *Olea europaea* subsp*. europaea,* oa = *Olea europaea* subsp*. cuspidata*

		Jaccard (full fungal assemblages)			Jaccard (core fungal assemblages)			Bray-Curtis (full fungal assemblages)			Bray-Curtis (core fungal assemblages)		
Groups		Site	Euro	Cusp	Site	Euro	Cusp	Site	Euro	Cusp	Site	Euro	Cusp
Clanwilliam	Paarl	0.001*	0.013*	0.011*	0.020*	0.228	0.011*	0.001*	0.035*	0.008*	0.029*	0.146*	0.032*
Clanwilliam	Robertson	0.001*	0.019*	0.010*	0.002*	0.011*	0.005*	0.001*	0.008*	0.009*	0.003*	0.032*	0.008*
Clanwilliam	Somerset West	0.001*	0.010*	0.008*	0.001*	0.023*	0.011*	0.001*	0.040*	0.010*	0.001*	0.048	0.008*
Clanwilliam	Stellenbosch	0.001*	0.011*	0.013*	0.001*	0.076	0.005*	0.001*	0.008*	0.009*	0.002*	0.059	0.005*
Clanwilliam	Swellendam	0.001*	0.011*	0.008*	0.001*	0.024*	0.01*	0.001*	0.015*	0.013*	0.001*	0.016*	0.011*
Paarl	Robertson	0.001*	0.006*	0.015*	0.004**	0.019*	0.029*	0.007*	0.032*	0.016*	0.009*	0.036*	0.038*
Paarl	Somerset West	0.014*	0.042*	0.019*	0.034*	0.164	0.043*	0.027*	0.126	0.025*	0.075	0.14	0.134
Paarl	Stellenbosch	0.176	0.272	0.048*	0.177	0.554	0.025*	0.074*	0.649	0.013*	0.211	0.711	0.018*
Paarl	Swellendam	0.001*	0.021*	0.007*	0.004*	0.081	0.007*	0.002*	0.08	0.008*	0.005*	0.105	0.012*
Robertson	Somerset West	0.001*	0.009*	0.003*	0.004*	0.034*	0.023*	0.003*	0.068	0.007*	0.004*	0.082	0.008*
Robertson	Stellenbosch	0.002*	0.007*	0.042*	0.068	0.04	0.759	0.018*	0.026*	0.154	0.159	0.07	0.586
Robertson	Swellendam	0.095	0.066	0.192	0.065	0.065	0.386	0.342	0.213	0.355	0.207	0.145	0.661
Somerset West	Stellenbosch	0.021*	0.058	0.374	0.082	0.418	0.015*	0.016*	0.162	0.044*	0.066	0.404	0.023*
Somerset West	Swellendam	0.002*	0.016*	0.006*	0.001*	0.076	0.009*	0.001*	0.021*	0.009*	0.001*	0.166	0.011*
Stellenbosch	Swellendam	0.001*	0.008*	0.057	0.026*	0.040	0.269	0.003*	0.014*	0.027*	0.017*	0.06	0.062

		Richness		Abundance		Richness (core)		Abundance (core)	
	Groups	t	P(perm)	t	P(perm)	t	P(perm)	t	P(perm)
Clanwilliam	oa, oe	1.3936	0.009*	1.5363	0.032*	1.5515	0.031*	1.5515	0.031*
Paarl	oa, oe	1.2907	0.008*	1.5915	0.012*	2.2396	0.008*	2.3403	0.011*
Robertson	oa, oe	1.6295	0.01*	1.7889	0.018*	3.233	0.013*	2.6175	0.008*
Somerset West	oa, oe	1.4785	0.006*	1.9933	0.007*	2.7817	0.01*	2.8206	0.011*
Stellenbosch	oa, oe	1.3739	0.004*	1.4929	0.006*	2.3744	0.007*	2.0853	0.009*
Swellendam	oa, oe	1.7779	0.009*	2.1835	0.008*	4.934	0.01*	3.4439	0.006*

Table S6: PERMDISP (B2). *Post hoc* pairwise comparisons within the significant factors (Table 5). Comparisons between sites, between hosts and between the interaction of site and host identity (oa = *O. europaea* subsp. *cuspidata* and oe = *O. europaea* subsp. *europaea*). The average distances around the centroid for the significant main tests are also presented. Results are considered significant when $p < 0.05$.

Site

Groups		t	p(perm)
Clanwilliam	Paarl	0.637	0.521
Clanwilliam	Robertson	0.924	0.307
Clanwilliam	Somerset West	0.875	0.335
Clanwilliam	Stellenbosch	0.49	0.582
Clanwilliam	Swellendam	2.63	0.005*
Paarl	Robertson	1.302	0.194
Paarl	Somerset West	0.103	0.906
Paarl	Stellenbosch	0.183	0.865
Paarl	Swellendam	2.64	0.011*
Robertson	Somerset West	1.568	0.08
Robertson	Stellenbosch	1.231	0.197
Robertson	Swellendam	1.357	0.106
Somerset West	Stellenbosch	0.321	0.683
Somerset West	Swellendam	3.12	0.003*
Stellenbosch	Swellendam	2.719	0.008*

		t	p(perm)
African olive	European olive	2.291	0.052

Group	Size	Ave	SE
Hosts			
oe	30	53.664	2.267
oa	30	46.019	2.449
Sites			
Clanwilliam	10	60.673	0.652
Paarl	10	61.472	1.070

Site and host interaction

Groups		t	p(perm)
oeClanwilliam	oePaarl	1.048	0.339
oeClanwilliam	oeRobertson	2.702	7.1E-2
oeClanwilliam	oeSomerset West	1.626	0.231
oeClanwilliam	oeStellenbosch	0.535	0.578
oeClanwilliam	oeSwellendam	4.257	2.1E-2*
oeClanwilliam	oaClanwilliam	1.408	0.253
oePaarl	oeRobertson	2.445	8.9E-2
oePaarl	oeSomerset West	1.223	0.359
oePaarl	oeStellenbosch	0.326	0.87
oePaarl	oeSwellendam	4.497	1.1E-2*
oePaarl	oaPaarl	3.076	5.5E-2
oeRobertson	oeSomerset West	0.698	0.592
oeRobertson	oeStellenbosch	2.285	9.5E-2
oeRobertson	oeSwellendam	0.765	0.494
oeRobertson	oaRobertson	0.619	0.661
oeSomerset West	oeStellenbosch	1.253	0.351
oeSomerset West	oeSwellendam	1.473	0.271
oeSomerset West	oaSomerset West	0.714	0.583
oeStellenbosch	oaStellenbosch	3.501	7E-3*
oeSwellendam	oaSwellendam	2.774	7.5E-2
oaClanwilliam	oaPaarl	0.905	0.499
oaClanwilliam	oaRobertson	1.527	0.295
oaClanwilliam	oaSomerset West	1.302	0.29
oaClanwilliam	oaStellenbosch	1.653	0.262
oaClanwilliam	oaSwellendam	4.604	4E-3*
oaPaarl	oaRobertson	1.057	0.476

Robertson	10	59.608	0.951	oaPaarl	oaSomerset West	0.535	0.576
Somerset West	10	61.613	0.854	oaPaarl	oaStellenbosch	1.002	0.424
Stellenbosch	10	61.216	0.895	oaPaarl	oaSwellendam	4.984	6E-3*
Swellendam	10	57.893	0.832	oaRobertson	oaSomerset West	0.785	0.615
Interactions				oaRobertson	oaStellenbosch	0.574	0.729
oeClanwilliam	5	54.394	3.571	oaRobertson	oaSwellendam	1.527	0.398
oePaarl	5	50.444	1.206	oaSomerset West	oaStellenbosch	0.445	0.698
oeRobertson	5	36.586	5.539	oaSomerset West	oaSwellendam	4.480	8E-3*
oeSomerset West	5	42.491	6.392	oaStellenbosch	oaSwellendam	4.238	8E-3*
oeStellenbosch	5	51.68	3.598				
oeSwellendam	5	31.323	4.077				
oaClanwilliam	5	45.424	5.275				
oaPaarl	5	39.823	3.235				
oaRobertson	5	30.406	8.302				
oaSomerset West	5	37.388	3.203				
oaStellenbosch	5	35.459	2.92				
oaSwellendam	5	16.757	3.31				

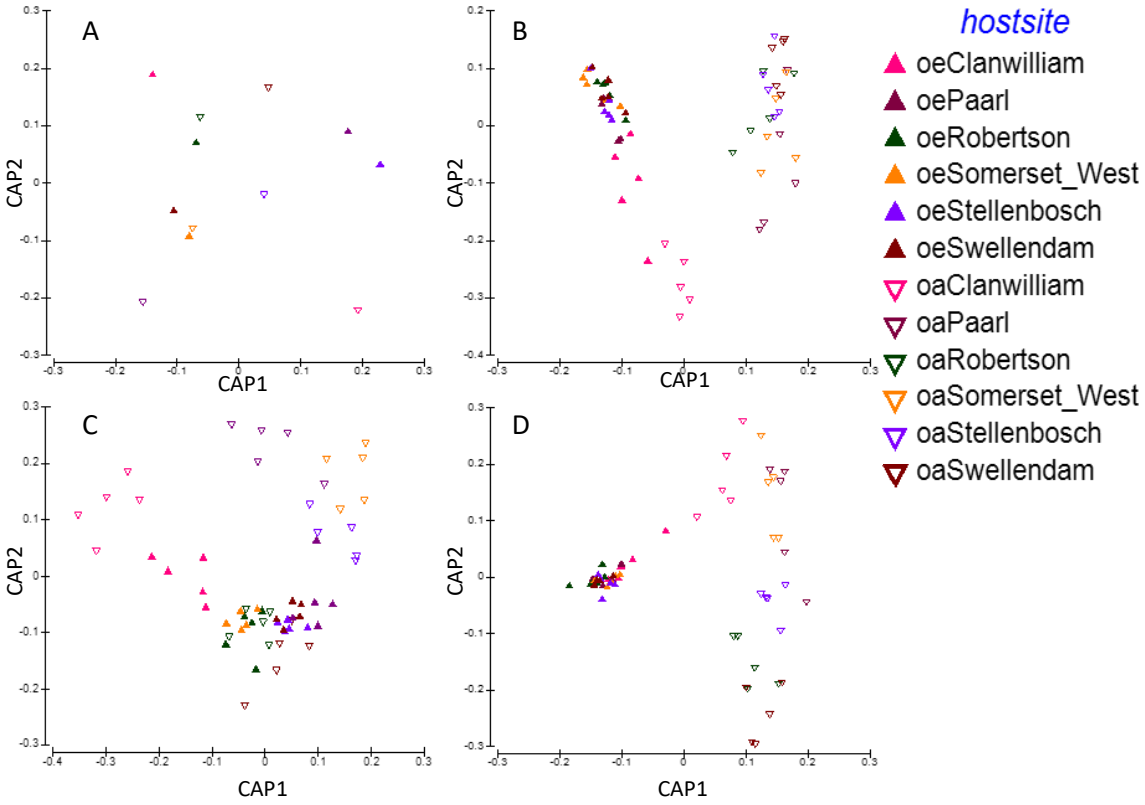

Figure S3: Canonical analysis of principal coordinates (CAP) of the site and host interaction. Ordination plots of the interaction between host (oe= *Olea europaea* subsp. *europaea*, oa= *Olea europaea* subsp. *cuspidata*) and site based on full fungal incident data (A), core fungal incident data (B), full fungal abundance (C), core fungal abundance (D). Plots are based on the balanced design, n = 60.

2.9.2 Appendix B: Supplementary information for the complete unbalanced microbial dataset (β-diversity)

Table S7: Influence of host and site on fungal endophyte diversity (n = 102). Global PERMANOVA and *post hoc* comparisons of community structure within each host (oe= *Olea europaea* subsp. *europaea*, oa= *Olea europaea* subsp. *cuspidata*) and each of the six sites, and their interaction represented by the t-statistic and p-values in brackets. Results are significant at $p < 0.05$ (*).

MAINTEST		Richness			Abundance		
Factors		F-model	Pr(>F)		F-model	Pr(>F)	
Site		2.778	0.001*		3.0287	0.001*	
Host		8.066	0.001*		11.7654	0.001*	
Interaction (site:host)		2.010	0.001*		2.0302	0.001*	
PAIRWISE PERMANOVA (*post hoc*)							
		Richness			Abundance		
Groups		both	oa	oe	both	oa	oe
		t(*p*)	t(*p*)	t(*p*)	t(*p*)	t(*p*)	t(*p*)
Stellenbosch	Paarl	1.451(0.004) *	1.431(0.005) *	1.264(0.066)	1.423(0.004) *	1.479(0.002) *	1.223(0.092)
Stellenbosch	Robertson	1.816(0.001) *	1.772(0.001) *	1.480(0.009) *	1.614(0.002) *	1.531(0.007) *	1.365(0.03) *
Stellenbosch	Swellendam	1.729(0.001) *	1.456(0.003) *	1.610(0.006) *	1.608(0.002) *	1.421(0.006) *	1.515(0.008) *
Stellenbosch	Somerset West	1.284(0.042) *	1.190(0.106)	1.234(0.107)	1.227(0.072)	1.170(0.095)	1.155(0.170)
Stellenbosch	Clanwilliam	2.489(0.001) *	1.970(0.001) *	2.987(0.001) *	2.424(0.003) *	2.016(0.001) *	1.977(0.001) *
Paarl	Robertson	1.768(0.001) *	1.741(0.002) *	1.463(0.015) *	1.650(0.001) *	1.684(0.003) *	1.287(0.062)
Paarl	Swellendam	1.925(0.001) *	1.813(0.001) *	1.508(0.008) *	1.829(0.033) *	1.825(0.001) *	1.360(0.039) *
Paarl	Somerset West	1.394(0.014) *	1.282(0.026) *	1.318(0.040*	1.292(0.001) *	1.245(0.049) *	1.151(0.162)
Paarl	Clanwilliam	2.125(0.001) *	1.528(0.002) *	1.989(0.001*	2.126(0.001) *	1.604(0.001) *	1.894(0.002) *
Robertson	Swellendam	1.386(0.026) *	1.277(0.059)	1.101(0.230)	1.311(0.053)	1.307(0.052)	1.527(0.290)
Robertson	Somerset West	1.596(0.003) *	1.723(0.002) *	1.595(0.339)	1.486(0.001) *	1.637(0.004) *	1.57(0.447)
Robertson	Clanwilliam	2.412(0.001) *	1.980(0.002) *	1.908(0.001) *	2.367(0.003) *	1.984(0.001) *	1.805(0.001) *
Swellendam	Somerset West	1.654(0.001) *	1.602(0.004) *	1.340(0.033) *	1.622(0.003) *	1.670(0.001) *	1.261(0.045) *
Swellendam	Clanwilliam	2.547(0.001) *	2.131(0.001) *	1.958(0.002) *	2.466(0.001) *	2.155(0.001) *	1.851(0.003) *
Somerset West	Clanwilliam	2.002(0.001)	1.660(0.001)	1.725(0.002)	1.946(0.001)	1.693(0.001)	1.583(0.002)

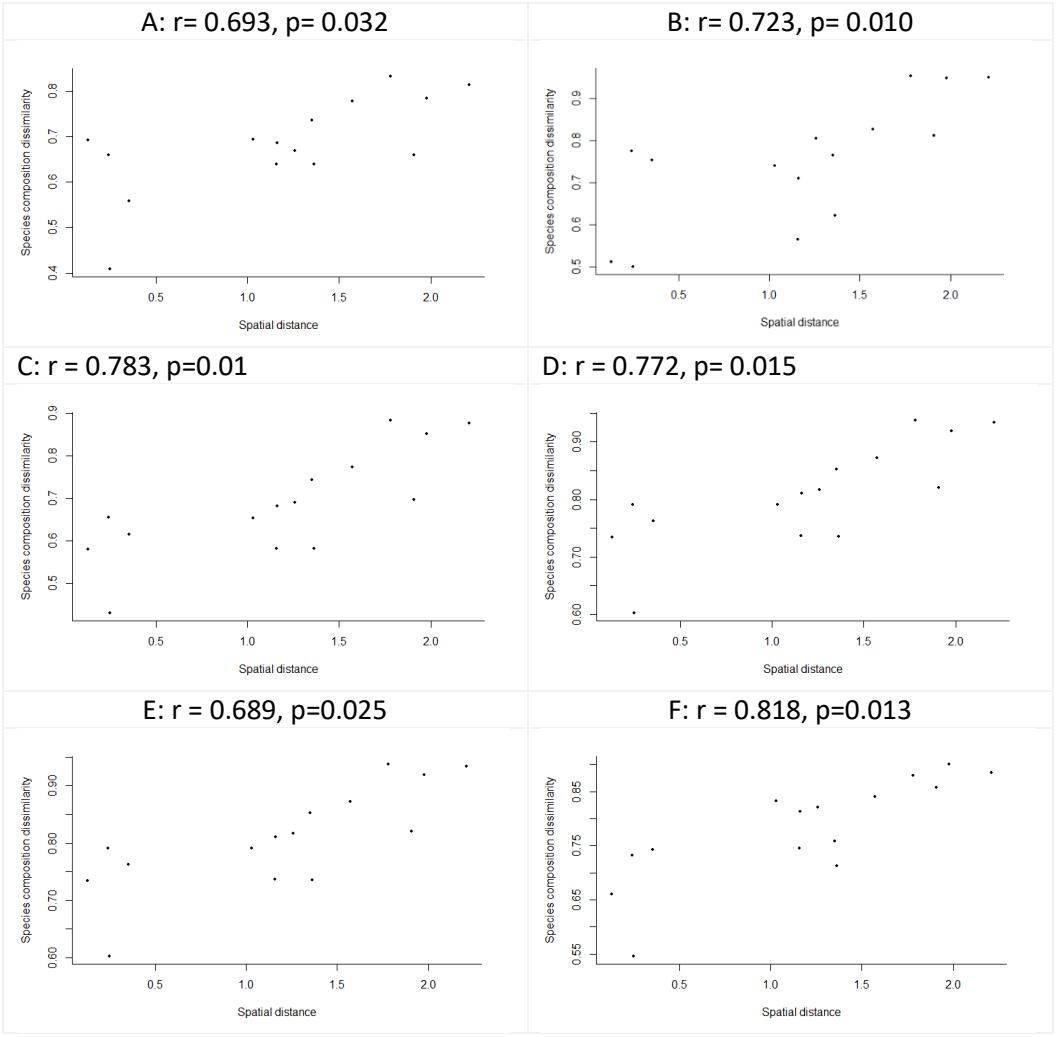

Figure S4: A Pearson's Mantel test for correlation between geographic distance (1: 100km) and assemblage dissimilar matrices. Correlation between geographic distances between sites and *O. europaea* subsp. *europaea* abundance assemblage dissimilarity (Bray-Curtis distance matrix) (**A.**) and *O. europaea* subsp. *europaea* richness assemblage dissimilarity (**B.**). *O. europaea* subsp. *cuspidata* abundance assemblage dissimilarity matrix (**C**.), *O. europaea* subsp. *cuspidata* richness community dissimilarity matrix, (**D.)**, abundance community dissimilarity matrices correlated with geographic distances between sites, (**E.**) and richness assemblage dissimilarity matrices correlated with geographic distances between sites (**F**.). Correlation between species richness and abundance dissimilarity matrices within samples per site and geographic distances between them. Dissimilarity matrices were computed using Euclidean, Bray-Curtis and Jaccard distance measures for geographic distance, fungal abundance dissimilarity matrices and fungal species richness dissimilarity matrices, respectively. Statistic significant when $p<0.05$.

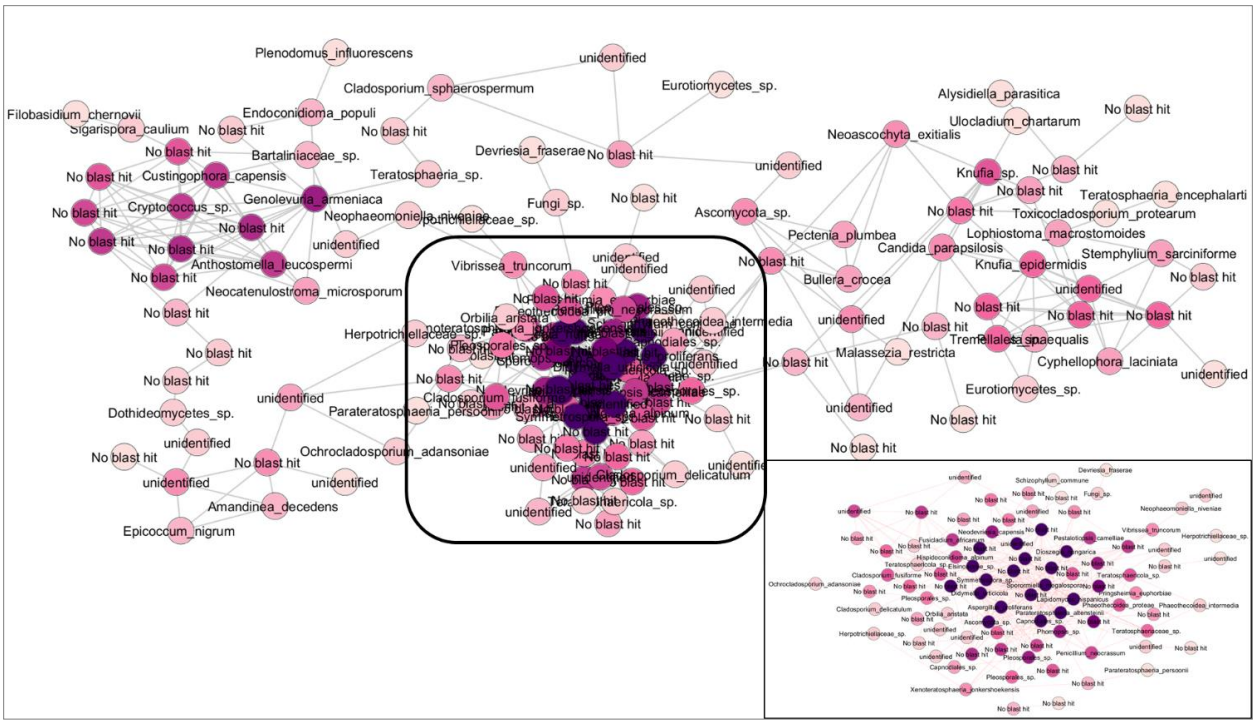

Figure S5: Co-occurrence network of endophytic fungal assemblages within *Olea europaea* subsp*. cuspidata* twigs. Purple nodes indicate node degree between 20 to 30, and light purple to light pink nodes indicate node degrees of 10 to 20. Thick node lines indicate nodes with the highest betweenness centrality. Insert shows the enlargement of the dense main hub.

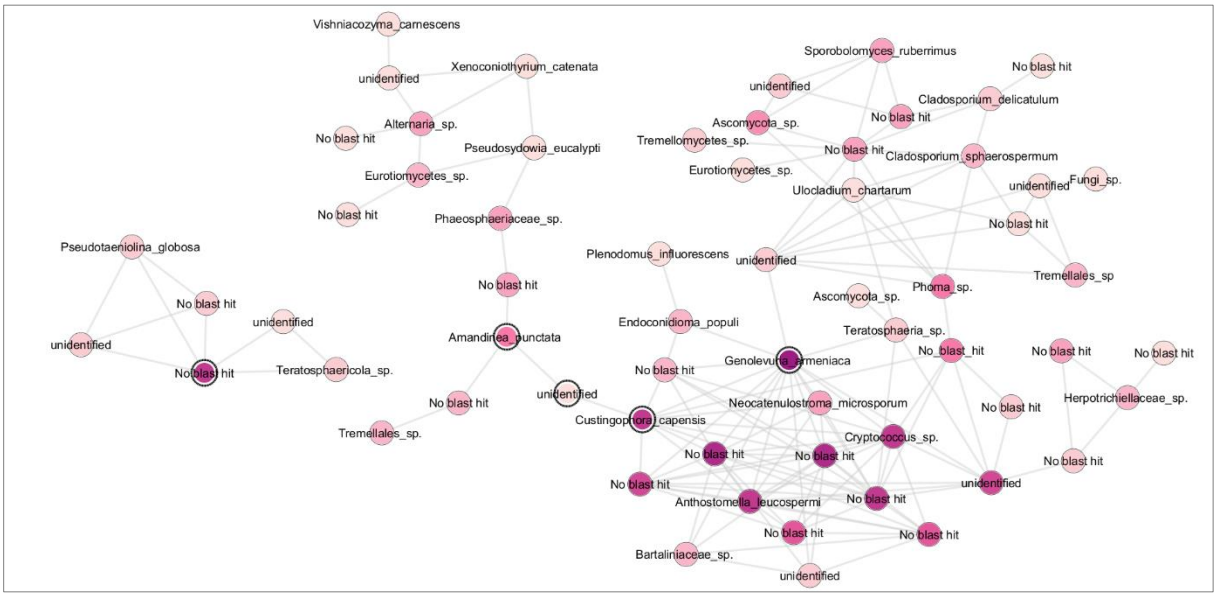

Figure S6: Co-occurrence network of endophytic fungal assemblages within *Olea europaea* subsp. *europaea* twigs. Co-occurrence network of endophytic fungal assemblages within *Olea europaea* twigs. Node shade indicate the degree of a node in relation to other nodes in the network, lightest nodes have only one neighbour and the darkest nodes have 15 connections. Thick node outlines indicate nodes with the highest betweenness centrality.

CHAPTER 3: Habitat quality has a bigger effect than surrounding vegetation on fungal endophyte assemblages within the African wild olive

3.1 Abstract

Environmental disturbances can significantly affect the cohesion of community interactions within ecosystems. Here I sought to assess how the fungal endophytes within twigs of the African olive, *Olea europaea* subsp. *cuspidata* are influenced by habitat context (planted, semi-natural and natural settings) and vegetation contrast against the neighbouring vegetation. Vegetation contrast is defined as high, medium and low when the focal tree was surrounded by vegetation dominated by non-trees (plants other than trees), non-olives and olives, respectively. Twigs from olive trees were collected from Paarl, Stellenbosch, and Somerset West in the Western Cape Province, South Africa. Fungal endophyte communities were characterised *via* DNA metabarcoding using the Illumina high-throughput sequencing technique. The effect of habitat context and vegetation contrast with surrounding vegetation on fungal endophyte alpha- and beta diversity measures were compared. In addition, co-occurrence networks were calculated to assess community cohesion under these different scenarios and to identify fungal taxa of potential ecological significance. OTU richness (but not abundance) was significantly influenced by both habitat context and vegetation contrast, and their interaction. Twigs from planted trees and twigs from medium vegetation contrast settings harboured the highest OTU richness. Only habitat context had a significant influence on fungal endophyte assemblage composition. Specifically, fungal assemblages from the natural habitat context were distinct from those originating from the planted and semi-natural contexts, while the latter two were similar. Co-occurrence network analyses revealed that significantly cohesive and diverse assemblages could only be maintained within the natural context. These findings suggest that although the African olive is widespread, the identity and composition of their associated fungal assemblages are particularly sensitive to disturbance, even in the semi-natural habitat context. This study highlights the importance of conserving natural habitats, not just for the plants, but also for the maintenance of fungal endophyte diversity and their functions.

3.2 Introduction

Human-mediated disturbances such as urbanisation, agricultural activities, biological invasions and climate change (Albrecht et al., 2007; Hanski, 2005; McKinney, 2002; Tilman et al., 2001) are driving biodiversity loss at an unprecedented rate (Komatsu et al., 2019; Ostfeld and LoGiudice, 2003). The legacies of these disturbances remain in the landscape for many years and continue to impact ecological processes (Ciccolini et al., 2015; Cortez et al., 2007; Crockatt, 2012; Foster et al., 2003; Krauss et al., 2007; Mcguire et al., 2010). Land use activities form a complex relationship with natural disturbances, which have cascading consequences such as vegetation loss, community changes, breakdown of symbioses through to changing ecosystems service delivery (Komatsu et al., 2019; Truchy et al., 2019; Vanbergen et al., 2013). Today, consequences of anthropogenic changes in landscape quality and structure have been documented for virtually all major taxonomic groups including plants, animals, bacteria and fungi (Hyvärinen et al., 2019; Leveau, 2019; Nguyen et al., 2016; Weiner et al., 2014).

Anthropogenic disturbances and their accompanying legacies on altered plant composition and ecosystem functioning are often documented (Abadie et al., 2020; Komatsu et al., 2019; Lloret and Vilà, 2003;). Increasingly, however, distinct changes in habitat quality (context) and vegetation contrast in growth forms (surrounding vegetation type) have been shown to have major implications on other taxa such as arthropods (Crouzeilles et al., 2016; Fischer and Lindenmayer, 2007). For example, Yekwayo et al., (2016) found that natural forests next to a pine plantation harboured lower arthropod diversity than natural forests next to natural grasslands in South Africa. Similarly, arthropods associated with a native tree (*Podocarpus elongatus* Aiton L'Herit. Ex Pers.) in South Africa had significantly higher diversity in natural and semi-natural contexts than in an urban planted habitat context (Swart et al., 2020). Although diversity generally decreases with exposure to anthropogenic disturbances, responses of taxa in different trophic levels can have varied responses to the same disturbance (Swart et al., 2020; Yekwayo et al., 2017, 2016). This is because niche requirements of individual species are important in determining the effect disturbance has on species within the landscape. For example, land use change generally leads to a global decline in arthropod pollinators (Clough et al., 2014; Potts et al., 2010; Steffan-Dewenter and Westphal, 2008; Vanbergen et al., 2013), with specialist taxa being more vulnerable than generalists (Winfree et al., 2011). This forms a negative feedback loop in which plants dependent on specialist pollinators also decline (Clough et al., 2014). The influence of anthropogenic disturbances on ecosystems can therefore have exceedingly complicated consequences that affect species, communities, interactions, ecological processes, and ecosystem services provided by the landscape.

To mitigate the effect of land use change on natural ecosystems, the restoration and conservation of native flora in green belts and in gardens in urban environments are advocated. This has shown some success for the recruitment of taxa dependent on native flora, such as nectar-feeding birds and pollinators (Forup et al., 2008; Frick et al., 2014; Mnisi, 2017). Records of these successes are, however, relatively few. Ecological processes such as re-establishment of biological interactions are slower to recover compared to diversity and vegetation structure (Morgan and Short, 2002; Ruiz-Jaen and Aide, 2005). The limited evidence that exists suggests that these altered environments often fail to maintain much of the specialised native fauna, instead favouring generalists species (Winfree et al., 2011). For example, in South Africa, arthropod assemblages in the native *P. elongatus* growing in green belts and planted in gardens were dominated by generalists and cosmopolitan species even though many specialist taxa are known from this species at nearby natural localities (Swart et al., 2020). The reasons behind these differences are many, but include differences in tree stress (water and pollutants), differences in the surrounding vegetation structure (vegetation contrast), and differences in trophic responses of different taxa (Swart et al., 2020). In addition, proximity of restored habitats to natural landscape can play a critical role in restoring biological interactions as the natural habitats can serve as sources (Dixon, 2009; Holzschuh et al., 2010).

Despite responses to land use change being documented for many taxa, the effect of land transformation on native plant-associated microbes have not received much attention. Plant responses to disturbances can depend on their associated fungal assemblages (Franco et al., 2017; Grilli et al., 2017). For example, fungal assemblages may shift to comprise predominantly of buffering species that shield or break down pollutants in the landscape, thus allowing the host plant to persist and appear unaffected (Deram et al., 2011; Srivastava et al., 2017; Varela et al., 2017, 2015). Conversely, abiotic legacies of disturbance may be responsible for symbiotic relationships breaking down (Boeraeve et al., 2019; Crockatt, 2012; Hewitt et al., 2016; Panayotov et al., 2017). As such, characterising fungal assemblages associated with plants and their response to land use change is crucial, as fungi can strongly influence ecosystem structure and functioning, and play a key role in many ecological services as decomposers, plant mutualists and pathogens (Orgiazzi et al., 2012; Stone et al., 2018). In addition, they are particularly sensitive to changes in their substrates, to the extent that they have been used as bioindicators of stress induced by disturbance (Abrego and Salcedo, 2014; Hewitt et al., 2016; Jumpponen and Jones, 2010; Orgiazzi et al., 2012). Saproxylic and arbuscular mycorrhizal fungi are especially popular choices as indicators of general forest health and dead wood forest soil health, respectively (Abrego and Salcedo, 2014; Gáfriková et al.,

2020; Siitonen et al., 2005). However, fungal endophytes of plants (fungi that inhabit internal tissue of asymptomatic plants), particularly those of the plant phyllosphere, are increasingly used as bioindicators in a wide range of systems (Arnold and Lutzoni, 2007; Deram et al., 2011; Jumpponen and Jones, 2010; Kandalepas et al., 2015; Lumibao et al., 2018).

The aim of this study was to characterise the fungal endophyte assemblages found within the native *Olea europaea* subsp. *cuspidata* (Wall. & G.Don) Cif. occurring in the Core Cape Subregion in the Western Cape Province of South Africa. *Olea europaea* subsp. *cuspidata* is one of six subspecies in the *Olea europaea* complex, alongside the widely cultivated *Olea europaea* subsp. *europaea* L. (Besnard et al., 2007a). *Olea europaea* subsp. *cuspidata* is a very widespread tree/shrub that grows naturally in a wide range of conditions, from the Mediterranean climate zone of the Core Cape Subregion and subtropical regions of South Africa, and extending northwards to Egypt (Besnard et al., 2007a). It is a dominant component of thicket elements, especially in native renosterveld vegetation type of the exceptionally biodiverse Fynbos Biome in the Core Cape Subregion (Manning and Goldblatt, 2012; Mucina and Rutherford et al., 2006). These plants, previously used as root grafts for cultivated olives, are important features of the natural landscape and are also used as shade trees and for ethnobotanical purposes (Aumeeruddy-Thomas et al., 2017; Besnard et al., 2007b; Long et al., 2010; Masoko and Makgapeetja, 2015). The diversity of the areas and conditions this plant grows in makes it an ideal host within which to test the influence of different surroundings and levels of disturbance on endophytic fungi.

In this chapter, the focus was on *O. europaea* subsp. *cuspidata* occurring in the Core Cape Subregion. This region has a Mediterranean climate with winters that are cold and wet, and summers that are hot and dry (Born et al., 2007). This area is of agricultural importance to South Africa (Archer et al., 2019), as such large portions of its natural systems have been transformed for agricultural purposes. Like many major metropolitan areas, Cape Town and the Cape Winelands areas have also seen an increase in urbanisation, which has further contributed to the increase of transformed natural ecosystems. The specific aim of this chapter was to investigate how different levels of disturbance (habitat context) affected fungal endophytes found within twigs of *O. europaea* subsp. *cuspidata* (the African olive). I sought to investigate how fungal endophyte assemblages differ between plants in a natural environment compared to plants that grow in semi-natural (within green belts in urban areas) and planted in completely transformed (garden) settings. In addition, I aimed to assess how structural and compositional differences in the surrounding vegetation types (vegetation contrast) affected the fungal assemblage in the twigs of the African olive. In particular, I assessed how endophytic fungal assemblages in olive twigs surrounded by

other olive trees compare to those growing with other trees (not olives) versus those growing around vegetation other than trees (growth forms other than shrubs or trees). I expected that the fungal assemblages found within African olives growing in natural settings would significantly differ from those within trees naturally growing in transformed areas or planted in these transformed habitats (Newbound et al., 2010; Tyburska et al., 2013). As endophytes can be acquired from the surrounding landscape (Giauque and Hawkes, 2016; Saikkonen et al., 2000; Shade et al., 2017), I expected that fungal endophytes within twigs from different vegetation contrast categories would significantly differ from each other.

3.3 Materials and Methods

3.3.1 Host and site selection, and sampling design

Twigs of *O. europaea* subsp. *cuspidata* were collected from Stellenbosch, Paarl and Somerset West towns. These locations were selected because fungal assemblages found within olives from these locations were not affected by the distances between sites, thus removing the effect of geographic distance as a confounding factor (Chapter 2). Trees were selected based on the habitat context they grew in (natural, semi-natural or planted), defined as follows: **Natural habitat context** was defined as when the selected tree individual grew naturally in an undisturbed area or a protected area. Trees in a **semi-natural habitat context** was defined as those that grew naturally in an area with high disturbance. For example, where olive trees grew along a riverbank in a green area in a town surrounded by roads and other urban infrastructure. Trees in a **planted habitat context** were planted in parks, roadside and gardens. Within each of these habitat context categories, tree individuals were also chosen to represent different vegetation contrasts relative to the surrounding vegetation as follows: **High vegetation contrast**, **medium vegetation contrast** or **low vegetation contrast** defined as surrounded by low shrubby and grassy vegetation, surrounded by trees of a similar height (but representing species other than olives), and those surrounded by conspecific trees, respectively (Figure 1). In total nine different categories that characterised both the habitat context and the vegetation contrast of each olive tree were sampled.

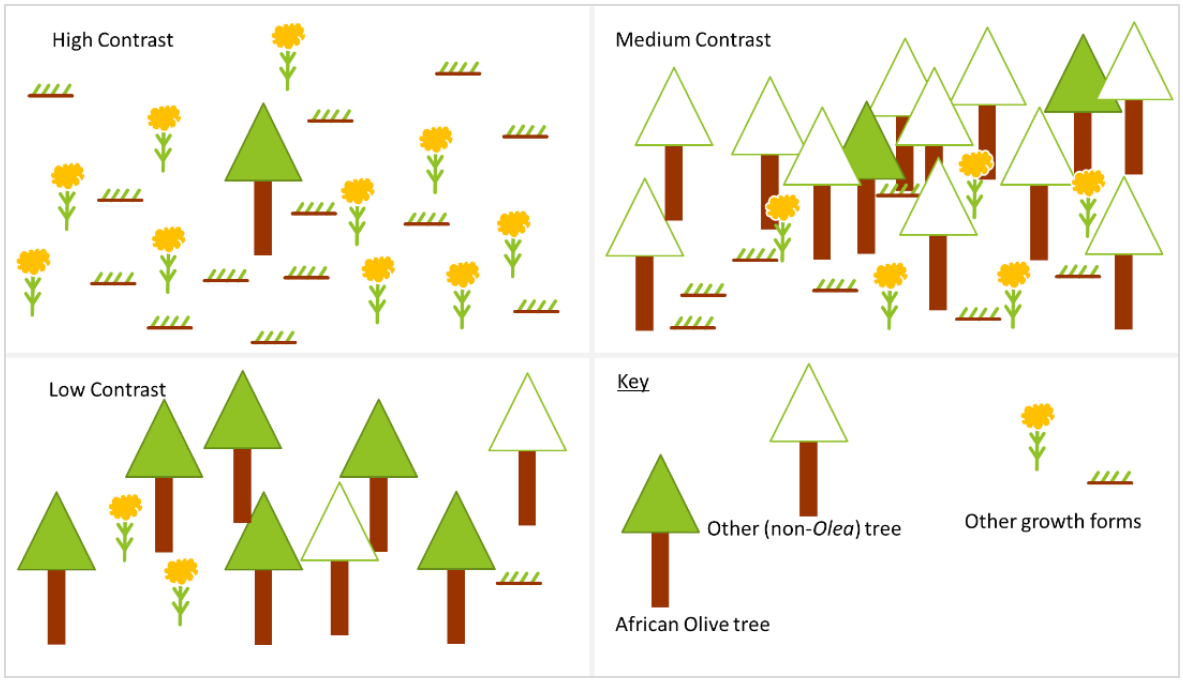

Figure 1 Depiction of the classification system used to differentiate between the three vegetation contrast types (low, medium, and high).

I collected samples from one to four trees per location such that each of the nine categories defined above contained seven samples (Table S1). Sample number per locality was dependent on the number of individuals that were located that conformed to the definitions. Tree size was standardised as much as possible by collecting samples from those with a diameter of at least 50 cm. Selected tree individuals were a minimum of 200 meters apart. From each tree individual, four asymptomatic twigs (*ca*. 5 mm in diameter and *ca*. 10 cm long) were collected from the previous growing season, one from each of the four wind directions (N, E, S and W), between April and July 2017. Samples were frozen at -80 ^{o}C prior to further processing. Twigs were surface sterilised in 70 % ethanol (45 s), household bleach (60 s), 95 % ethanol (30 s) and then rinsed in autoclaved double distilled water for 30 s (Moral et al., 2010; Slippers and Wingfield, 2007). Approximately 1 cm length of twig was aseptically excised from the middle of each twig and the four pieces per tree were combined in a single sample for DNA extraction.

3.3.2 DNA extraction

Samples were ground into fine powder using a mortar and pestle, which was cleaned with subsequent washings with 70 % ethanol, household bleach and autoclaved double distilled water between samples. DNA extraction followed a modified version of the protocol developed by Doyle and Doyle (1990) as outlined in Chapter 2.

3.3.3 Library preparation

Library preparation was conducted in two successive Polymerase Chain Reactions (PCR). Internal Transcribed Spacer (ITS) primers carrying tags with indexing primer binding sites were used during PCR1. During PCR2, indexes carrying Illumina sequencing adapters were added onto the PCR1 products. In the end, ITS amplicons carried a unique combination of tag and index (e.g. Table S2) to mark the sample they came from.

During PCR1, ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA- 3', Gardes and Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC- 3', White et al., 1990) primers modified for multiplex barcoding (metabion®, Planegg/Steinkirchen, Germany) were used to amplify fungal ITS region from total DNA extracted from the olive twigs. PCR volumes and thermal cycling conditions were identical to those followed in Chapter 2. The presence of ITS amplicons was confirmed through visualisation on an 0.8 % agarose gel by electrophoresis (Bio-budget, Technologies GmbH, Germany) supplemented by 1 µl ethidium bromide. Excess DNA, primers, primer homodimers and heterodimers were removed by washing the product following the ExoSap protocol (New England BioLabs Inc.; detailed in Chapter 2). Illumina adapters (P5 and P7) and indices were added onto PCR1 products during PCR2. Reaction volumes and thermocycling conditions were performed following the process detailed in Chapter 2. Amplicon presence was also confirmed using agarose gel using electrophoresis.

3.3.4 Sample pooling, purification, and library sequencing

The cleaned PCR amplicons carrying ITS primers and adaptors were then sequentially pooled until ITS amplicons from all 63 samples were contained in a single tube for sequencing. Prior to each pooling step, product quantity was assessed by taking photographs of the agarose gel fluorescence. Band intensities (used as proxy of molarity) were quantified using ImageJ version 1.52a (Ferreira and Rasband, 2012). Equimolar pools (with similar intensities) were combined (details are provided in Chapter 2). Then the resulting pools were purified using the CleanPCR® Kit (CleanNA, GC biotech B.V., Netherlands). After purification, the presence of the product was confirmed with another electrophoresis gel run and photograph. The purification procedure was repeated until every well only had one band visible. These were further pooled until only one superpool containing all sample amplicons remained. This tube was sent to for sequencing at the Genetics Department, Ludwig Maximilian University, Munich, using the MiSeq Reagent Kit v3 for 2 × 250 Illumina MiSeq® sequencer (Illumina Inc., San Diego, CA, USA).

3.3.5 Sequence cleaning, identification, and quantification

Once the superpool had been sequenced, a batch of sequences (containing indexes and tags) was returned. These were subjected to quality control and demultiplexing using the QIIME 1.9.1 pipeline (Caporaso et al., 2010). Quality control was conducted to identify and discard reads with low quality base calls, reads that were too short, and chimeras (Abdelfattah et al., 2018). Chimeras form when, due to sequencing jump, pieces from different products join and appear to be from the same amplicon. After demultiplexing, only forward reads were used for subsequent analyses. Sequence reads were extracted according to the forward and reverse barcodes. These sequences were separated and assigned to their corresponding sample of origin based on their tag-index combinations and the reference mapping file. Once separated, the tag-index sequences were trimmed using the FASTX-Toolkit (v. 0.0.13, http://hannonlab.cshl.edu/fastx_toolkit/) until only the ITS sequences remained. The ITS sequences were screened for possible chimeras using an abundance-based method in the USEARCH platform (Edgar, 2010). ITS sequences were then grouped into operational taxonomic units (OTU) based on sequence similarities (97 % similarity threshold) using CD-HIT-OTU (http://weizhongli-lab.org/cd-hit-otu/; Li et al., 2012; Stackebrandt and Goebel, 1994). Representative sequences were used for taxonomic placement using QIIME and the UNITE v. 7.2 database (Kõljalg et al., 2013). An OTU table was created, and abundances were inferred based on the frequency of each OTU within a sample.

3.3.6 Analyses of fungal endophyte diversity within the African olive

3.3.6.1 Alpha diversity

Fungal diversity within *O. europaea* subsp. *cuspidata* was calculated using the non-parametric Chao2 and Jackknife2 species estimators (Chao et al., 1992; Hortal et al., 2006) using Primer6 (Anderson et al., 2008). Total fungal endophyte richness and abundance were compared between the different habitat contexts and vegetation contrast levels using linear modelling procedures in R v. 3.1.2 (R Development Core Team, 2015). Measures of fungal abundance were calculated based on number of sequence reads per sample (OTU abundance). Species richness was based on 1) the number of OTUs found per sample (presence/absence) and 2) richness rarefied to samples containing the least abundant OTU (total number of reads) (Weiss et al., 2017) using *rarefy* in Vegan v. 2.5-6 (Oksanen et al., 2008). Core fungal taxa were identified as those OTUs that appeared in at least 50 % of the samples (similar to Chapter 2). A core fungal richness dataset was constructed based on the incident data of the core taxa and the core fungal

abundance dataset was based on the abundance data reduced to include only those consisting of the core OTU taxa.

OTU abundance, richness and rarefied richness were compared between habitat context categories and vegetation contrast levels using linear models. The role of habitat context and vegetation contrast in OTU richness and core richness was assessed using generalised linear modelling with a Laplace approximation fitted with a Poisson family distribution using the *lme4* package in R (Bates and Sarkar, 2007). This model was selected as the data were not normally distributed based on the Shapiro-Wilks test in *nortest* and histogram plots (Gross and Ligges, 2015). To improve the model fit and account for spatial autocorrelation, site was used as a random variable (Somerset West, Stellenbosch and Paarl). Models contained habitat context categories, vegetation contrast levels, and their interaction as fixed effects. Rarefied richness, on the other hand, was normally distributed, and showed no signs of overdispersion or spatial autocorrelations. A linear model was thus used to compare rarefied species richness between the habitat context, vegetation contrast levels and their interaction. Linear and linear mixed models were used to compare abundances and core abundances of different habitat context categories, vegetation contrast levels and their interaction. When main tests were significant, *post hoc* tests were conducted to ascertain the categories and/or levels driving the significant main effects using a conservative Tukey *post hoc* tests using the *multcomp* package (Hothorn et al., 2008). This test enabled for multiple comparisons between medians.

3.3.6.2 Beta diversity

Assemblage beta diversity analyses were conducted using Primer6 (Anderson et al., 2008). To assess beta diversity between different habitat contexts or vegetation contrasts (β1) and within habitat context categories or vegetation contrast levels (β2), permutational multivariate analyses of variance (PERMANOVA) and permutational multivariate analyses of dispersion (PERMDISP) analyses, respectively, were performed using Primer v6. In cases where a factor had a significant influence, *posterior* pair-wise comparisons were conducted to identify which levels within factors were driving the differences. To identify the axes that best represent the differences between the groupings, canonical analysis of principal coordinates (CAP) were conducted using Primer6 (Anderson and Robinson, 2006).

Using PERMANOVA, fungal endophyte compositional responses to habitat context, vegetation contrast and their interaction were investigated based on the two datasets, namely abundance, and richness. The abundance-based dataset was square-root transformed to reduce the effect of dominant OTUs and a

Bray-Curtis resemblance matrix was generated before performing a PERMANOVA with 999 permutations (Anderson, 2001). In the case of the incident-based dataset, a PERMANOVA with 999 permutations was performed on a Jaccard's dissimilarity transformed matrix (Magurran, 2004). *Post hoc* comparisons for significant effects were performed using the pairwise PERMANOVA test in Primer6. PERMDISP analyses were performed on the incident matrix (Jaccard resemblance matrix) using 999 permutations (Anderson, 2001). The beta diversity analyses were also performed to assess the effect of habitat context and vegetation contrast on the core fungal assemblages. Where PERMANOVA and PERMDISP main tests were significant, the Canonical Analysis of Principal Coordinates (CAP) procedure in Primer6 was used to find axes that best reflected these differences. To calculate similarity and dissimilarity percentages between categories and between samples within categories summary similarity percentages (SIMPER) analyses were conducted in Primer 6 and reported as accompanying summary to the networks. This was done to summarise similarities and differences between and within categories considering all taxa within the three habitat context categories.

3.3.7 Fungal endophyte co-occurrence networks

Endophytic fungal co-occurrences were calculated using the package *Hmisc* in R (Harrell and Dupont, 2007) and visualised in Cytoscape v3.7.2 (Cline et al., 2007). Significant co-occurrences were calculated based on Spearman's correlation coefficients (considered significant when Pearson's p >0.5, p<0.05) using *Hmisc* in R. Benjamini-Hochberg standard false discovery rate(FDR) correction was used to correct for multiple testing (Benjamini and Hochberg, 1995). Calculations to identify potential hubs were conducted in Cytoscape. Specifically, node degree of connectedness and betweenness centrality were highlighted on the networks. Betweenness centrality calculates which strategically placed nodes exerts the most control on the presented network (Freeman, 1977). Node degree, on the other hand, indicates how connected the node is, i.e., how many other nodes are connected to a specific node (Proulx et al., 2005).

3.4 Results

3.4.1 Fungal endophyte alpha diversity response to habitat context and vegetation contrast of olive trees

From the 63 sampled trees 491 988 sequences were obtained. The sequences belonged to 311 fungal OTUs. Species richness was significantly influenced by habitat context (Tables 1 and 2, Figure S1). Planted

olives had higher species richness than trees from the semi-natural and natural habitat contexts (Table 1). Olives growing within the natural habitat context had the lowest fungal richness according to Chao2 but had similar richness to those from the semi-natural habitat context according to Jackknife2 (Table 1). Although habitat context played a significant effect in species richness (Table 2), fungal endophyte richness was not significantly different between any of these categories (Table S3). Vegetation contrast also played a critical role in species richness within twigs (Table 2). Low vegetation contrast trees had significantly lower species richness than medium and high vegetation contrast trees (Tables 1 and 2, Figure S1). Twigs from trees growing amongst other non-olive trees (medium vegetation contrast) had the highest fungal richness (Table 1). The interaction between habitat context and vegetation contrast also significantly affected species richness within olive twigs (Table 2). The significance in the interaction was mainly driven by olive trees planted with other non-olive trees (olive tree planted in medium vegetation contrast setting) and olive trees naturally growing among other trees (Table S3, Figure S1). Specifically, fungal diversity within olive trees planted amongst other trees (planted medium) was significantly lower than that found within natural olives amongst other trees (natural medium), but higher than olive trees planted with other olives (planted low) and planted with non-tree vegetation (Table 1 and Table S3). Core fungal richness was only significantly affected by habitat context (Table 1), but none of the pairwise comparisons were significant (Table S3, Figure S2).

Table 1: Non-parametric diversity estimators (Chao2 and Jackknife2) of fungal endophyte diversity (full fungal richness (left) and core fungal richness (right)) within olive twigs from different habitat contexts (natural, semi-natural and planted), vegetation contrast (high, medium and low) and the interaction of the two factors.

		Richness			Core Richness		
Context	Contrast	Chao2	Chao (±SD)	Jackknife2	Chao2	Chao (±SD)	Jackknife2
Natural	(Regardless of contrast level)	289.167	29.843	322.450	13.500	1.323	13.989
Planted		356.558	50.994	336.582	13.083	2.506	13.500
Semi-natural		338.558	43.984	332.224	26.000	16.492	15.850
(Regardless of context level)	High	280.780	27.757	313.900	16.250	7.552	15.583
	Medium	376.821	41.805	390.479	20.000	7.483	16.000
	Low	268.329	25.505	304.610	13.250	0.729	14.083
Natural	High	215.265	44.529	183.024	29.000	23.622	18.950
Natural	Medium	331.125	160.706	193.476	22.500	17.139	15.917
Natural	Low	208.323	27.598	228.048	61.000	59.582	18.467
Planted	High	201.042	51.042	166.690	15.000	6.481	12.167

Planted	Medium	237.500	35.969	227.976	13.167	0.536	13.250
Planted	Low	175.500	31.357	168.262	14.250	3.396	15.083
Semi-natural	High	183.364	30.442	179.595	26.000	16.492	16.333
Semi-natural	Medium	356.346	91.079	270.400	17.900	6.840	19.167
Semi-natural	Low	209.524	33.836	200.071	26.000	16.492	18.167

Table 2: Summary of results of linear models of the effect of the habitat context and vegetation contrast factors on the total and the core fungal endophyte richness and abundance. Where the main effects were significant, *post hoc* test results are presented in Table S3. Tests considered significant if $p < 0.05$.

	RICHNESS			RICHNESS (Core)		
Factor	Chisq	Chi Df	p(Chi sq)	Chisq	Chi Df	p (Chi sq)
Context	59.777	6	<0.001*	13.339	6	0.040*
Contrast	65.498	6	<0.001*	6.630	6	0.357
Interaction	49.322	5	<0.001*	4.717	5	0.339
	ABUNDANCE			ABUNDANCE (Core)		
Factor	LR Stat	df	p (Chi sq)	Chisq	Chi df	p (Chi sq)
Context	5.769	6	0.450	2.064	6	0.611
Contrast	4.486	6	0.611	2.028	6	0.450
Interaction	3.283	5	0.511	2.198	5	1.000

3.4.2 Beta diversity

Habitat context was important to core and full fungal assemblages, both under Jaccard and Bray-Curtis resemblance (β1, Table 3). The significant effect of habitat context (β1 based on Jaccard resemblance) on the full and core fungal assemblages was driven by the significant differences between assemblages from the planted and the natural olives (Table S4). *Post hoc* β1 (Bray-Curtis resemblance) revealed that the whole fungal assemblages were also significantly different between planted and natural trees (Table S4). The core fungal assemblages (when considering Bray-Curtis resemblance) within the natural habitat context were distinct from the assemblages from the planted and semi-natural trees (Table S4). Ordination analyses reflected these groupings and patterns (Figure 2).

Vegetation contrast between the olive tree and the surrounding vegetation did not influence the core and full fungal assemblage composition (based on Jaccard resemblance) within olive twigs (β1, Table 3). Similarly, Bray-Curtis based resemblance β1 indicated that core fungal assemblages were not influenced by vegetation contrast between the host and its surroundings (B1, Table 3). The interaction between

vegetation contrast and habitat context (Jaccard and Bray-Curtis resemblance) did not significantly affect core and full fungal assemblage structures (β1, Table 3).

Dispersion within groups (β2) differed significantly between habitat contexts when considering the full complement of fungal taxa (Table 3). This significance was facilitated by the within groups variation in fungal assemblages from the natural habitat context, which had a significantly higher average within-group dispersion than the fungal assemblages from the planted and semi-natural habitat contexts (Table S4). In contrast, dispersion within habitat context and vegetation contrast groups (β2) was not a key determinant of core fungal dispersion. Fungal assemblages from different vegetation contrast levels had similar species turnover within vegetation contrast levels (β2). Likewise, dispersion around the centroid (β2) of the core fungal assemblages did not significantly differ according to vegetation contrast (Table 3).

Table 3: Beta diversity results based on PERMANOVA (Jaccard and Bray-Curtis resemblance) and PERMDISP (Jaccard resemblance) analyses of fungal endophyte assemblages within twigs of the African olive in response to different habitat contexts and vegetation contrasts, and the interaction between these two factors. Results considered significant if $p < 0.05$ (denoted by *). Significant *post hoc* results are reported in Table S4.

PERMANOVA (β1)

	Jaccard			Jaccard (Core)			Bray-Curtis			Bray-Curtis (Core)		
Groups	df	Pseudo-F	p	df	Pseudo-F	p	df	Pseudo-F	p	df	Pseudo-F	p
Habitat context	2	1.305	0.021*	2	2.332	0.004*	2	1.363	0.031*	2	1.919	0.016*
Vegetation contrast	2	0.958	0.628	2	0.899	0.549	2	0.850	0.771	2	0.811	0.709
Interaction	4	0.935	0.751	4	0.908	0.628	4	0.828	0.928	4	0.848	0.735

PERMDISP (β2)

	Jaccard				Jaccard (Core)			
	F	df1	df2	p	F	df1	df2	p
Habitat context	3.515	2	60	0.047*	2.750	2	60	0.115
Vegetation contrast	1.267	2	60	0.3	0.932	2	60	0.475
Interaction	0.796	8	54	0.713	0.673	8	54	0.836

Similarity percentages (SIMPER) analyses indicated that fungal endophytes from the natural and planted settings were the most dissimilar (Figure 3 insert). Fungi from planted and semi-natural habitat context twigs were the most similar. SIMPER also indicated that there was a high fungal endophyte turnover between samples even within categories. For example, on average, samples within the natural habitat

context only had 14.750 % similarity, while those from the semi-natural habitat context had 22.590 % similarity. This level of heterogeneity within the natural habitat context together with the dispersion (β2) average of 61.832 (± 0.691) suggest that this habitat harbours highly diverse assemblages even between samples (Table S4, Figure 3 insert).

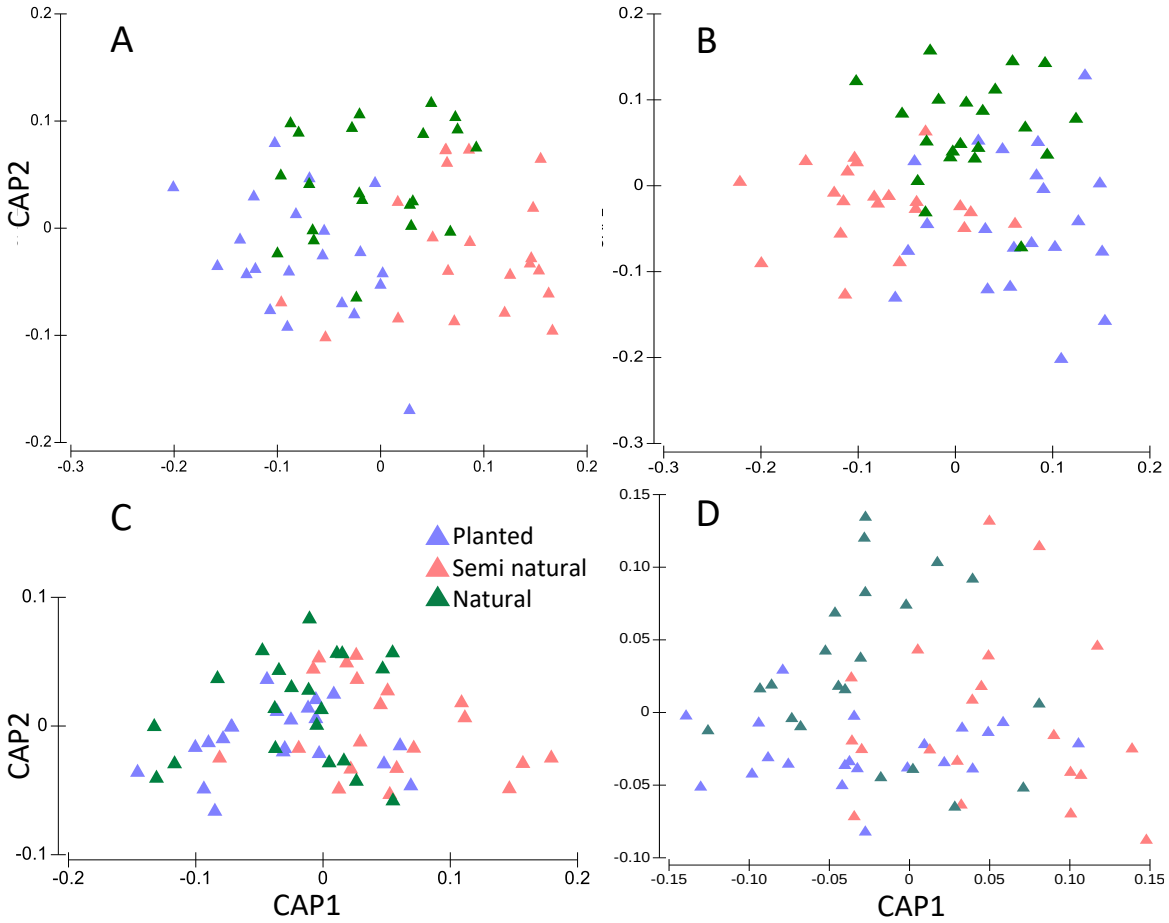

Figure 2: Canonical analysis ordination (CAP) based on Bray-Curtis (abundance data, A and C) and Jaccard (incident data, B and D) resemblance of the full (A and B) and core (C and D) fungal endophyte within the three habitat context categories (planted, semi-natural and natural).

Since only habitat context significantly influenced fungal assemblages in twigs of the African olive, co-occurrence networks are only presented for this factor. Olive twigs from natural, semi-natural and planted contexts contained 36, 25 and 16 endophytic fungal taxa, respectively, that co-occurred with each other a significant number of times (Figure 3). The co-occurrence network of OTUs within the natural context was the most connected followed by the semi-natural context network. Networks of fungal endophytes from twigs from the semi-natural and planted habitat context were very disjointed, respectively containing three and one node degree, at most. On average, nodes (OTUs) of the natural habitat context

network had 5.05 neighbours with the top three most connected nodes interacting with 13 of the other 35 nodes. Fourteen of the 36 nodes had a number of neighbours exceeding the average node degree, indicating taxa with potential to be important hub species within twigs of olive trees growing in the natural habitat context. Of the top seven key taxa, three could not be placed at genus level and the rest were identified as *Aspergillus proliferans* G. Sm., *Ulocladium chartarum* (Preuss) E.G. Simmons, *Peniophora* sp. and *Paracladophialophora* sp.

Perhaps the most noteworthy taxon was *Alternaria eureka* which was amongst the top three most connected taxa. This taxon was highly connected (nd = 9), had a high betweenness centrality (C_B= 0.6) and clustering coefficient (C_C= 0.6) indicative of a hub species that is highly connected and strategically placed to connect different sub-clusters of the main network. One of the top three highly connected taxa could not be placed at genus level, suggesting that this taxon may be undescribed.

Group	Average similarity	
Natural	14.75	
Seminatural	22.59	
Planted	20.55	
Group 1	**Group 2**	**Average dissimilarity**
Planted	Natural	83.99
Planted	Seminatural	78.89
Natural	Seminatural	81.67

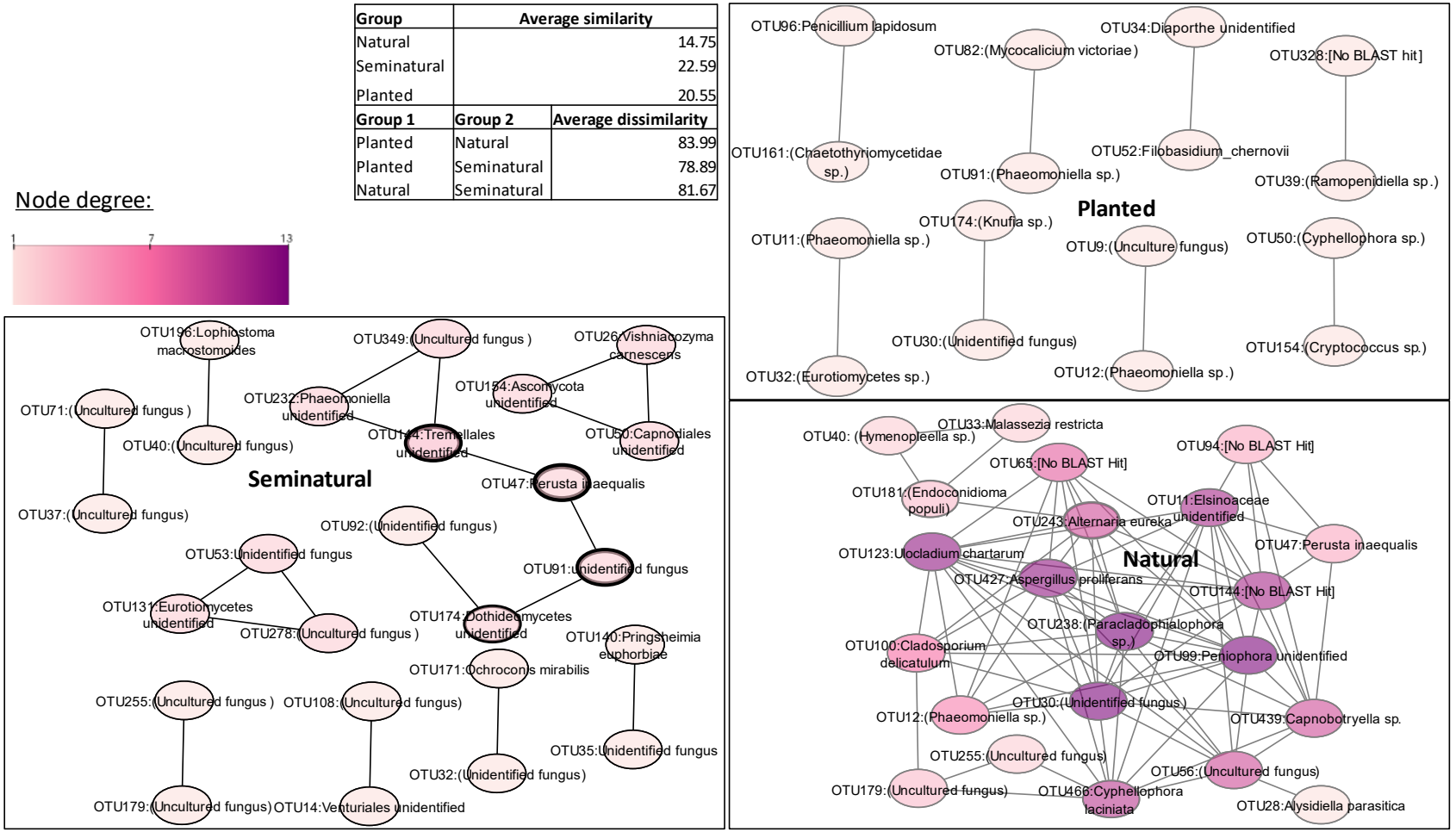

Figure 3: Co-occurrence networks highlighting significant co-occurrences of fungal endophyte taxa within twigs from planted, semi-natural and natural olive trees. Node outline thickness indicates nodes with high betweenness centrality (C_B >0.5) while shade intensity indicates node degree (nd). Average similarity and dissimilarity percentages within and between categories are summarised on the table insert. Species identifications without brackets indicate taxa that were placed using UNITE, those in round brackets represent taxa that could not be placed using the UNITE database, but which could be identified using BLAST; and those in square brackets indicate taxa that could not be placed using either database.

3.5 Discussion

This study revealed that anthropogenic activities have a significant impact on endophytic fungal assemblages within twigs of the African olive (*O. europaea* subsp. *cuspidata*) trees in the Core Cape Subregion. Fungal endophyte richness was significantly influenced by both vegetation contrast and habitat context. The number of endophytes in core assemblages increased with an increase in disturbance, which may point towards increased vulnerability to colonisation by fungi in disturbed areas. Habitat context significantly influenced fungal endophytes assemblages while vegetation contrast did not. Together these results provide a strong indication that endophyte assemblages are dominated by altered assemblages in disturbed areas. The importance of the natural habitat context for normal plant-endophyte interactions was highlighted in the co-occurrence networks of the three habitat context conditions where a highly connected fungal co-occurrence network was only attainable in the natural habitat context and lowest in the completely transformed (i.e. planted) habitat context.

Species richness within olive twigs was significantly influenced by vegetation contrast. This was consistent with arthropod diversity associated with *Podocarpus elongatus* native to the Core Cape Subregion of South Africa (Swart et al., 2020). In our study species richness was not significantly different between the habitat context categories i.e. the natural, semi-natural and planted habitat contexts. This was consistent with species richness within *P. elongatus* growing in natural conditions which was not significantly different from the trees growing in planted conditions (Swart et al., 2020). This was also consistent with patterns observed in fungal endophytes within *Spartina alterniflora* Loisel. In salt marshes affected by the Deepwater Horizon oil spill in Louisiana (United States of America), where alpha diversity was similar between plants exposed to oil spills and those that were not (Lumibao et al., 2018). This indicates that plants can host a variety of species of different taxa in almost any environment (disturbed or natural), but that the type of species they host may differ vastly. In the current study, there was an increase in the number of core taxa in the semi-natural context. As this was defined as the consistency of certain taxa within the host in different settings, it supports the hypothesis that more common species are overrepresented in the disturbed habitats, with a likely reduction in rarer native taxa.

Habitat context rather than vegetation contrast played a significant role in shaping fungal endophyte assemblages associated with *O. europaea* subsp. *cuspidata* twigs. Assemblages in the natural habitat context differed from those in the disturbed habitat contexts (semi-natural and planted). This was true

irrespective of whether the whole assemblages or just the core assemblages were considered. This indicates that even slight disturbances (such as was the case for trees growing in green belt areas) could have significant influences on endophyte assemblages. Endophytes in these, relatively natural (semi-natural), settings resembled those of trees planted in extremely transformed urban gardens. As indicated by the network analyses, the endophytes do not form cohesive assemblages in these altered ecosystems with unknown consequences to their hosts. This could be a positive emendation as was found for fungal endophyte assemblages within roots of *Arrhenatherum elatius* L. growing in soils contaminated with heavy metals that responded to changes in habitat context by shifting to taxa that could help the plant tolerate heavy metal contamination (Deram et al., 2011). Some of the species in the African olives in disturbed areas may be important for the survival of this host in disturbed habitats with different moisture regimes and influxes of pollutants.

Our results suggest that disturbance (agricultural activities and urbanisation) changes microbial assemblage interaction leading to different co-occurrence patterns between natural and disturbed conditions. Legacies of anthropogenic disturbances, such as changes in soil quality, have been implicated as reasons of plant-fungal interactions breakdown (Boeraeve et al., 2019; van Geel et al., 2018). Given the high community heterogeneity from one sample to the next within the natural context, yet the high connectivity of the network, this suggests that the fungal co-occurrences in the natural habitat context may be ecologically meaningful. Seven of the most interactive species also held the network together, amongst which were three taxa that could not be identified to the genus level. It is interesting that, the disintegrated semi-natural and planted habitat context fungal co-occurrence networks were coupled with either the demotion or absence of some of these seven taxa. The cohesion of the assemblages within the olives found in natural habitats indicates that these assemblages may serve as refugia of fungal endophyte assemblages within the African olive.

Three *Phaemoniella* taxa were abundant and co-occurred with other taxa within the twigs from the planted African olives, while only one of these was significantly associated with other taxa in olives in the natural context. Taxa in this genus are often encountered within agricultural crops, including *O. europaea* subsp. *europaea* (Carlucci et al., 2013; Chliyeh et al., 2014; Gomes et al., 2018; Moral et al., 2017; Olmo et al., 2016). In addition, *Alternaria* taxa were encountered within the African olive twigs from planted trees and within those cultivated (*O. europaea* subsp. *europaea*) in the country (Chapter 2). This genus has a global distribution and is often encountered in agricultural and forestry crops (Basson et al., 2019; Castañeda et al., 2018; Malacrinò et al., 2017). Some *Alternaria* species have also been associated with

stress tolerance in plants, including soil pollution and drought (Rodriguez and Redman, 2008). Thus, these taxa may be acquired from the surrounding plants in disturbed conditions and may not preferentially colonise the olive host in natural conditions where this host, and its surrounding vegetation, are not experiencing the same stressors as those in the semi-natural and planted habitat contexts. These taxa may be important to plants in the Core Cape Subregion as we continue to experience consequences of expanding agricultural activities and urbanisation.

3.6 Conclusion

The importance of fungal endophytes to plant health is well established (Poudel et al., 2016; Preto et al., 2017; Stone et al., 2018; van der Heijden and Hartmann, 2016). Yet, due to their sensitivity to habitat degradation, it is possible that multitudes of fungal endophytes are going extinct before they are described. Remnant natural habitats are important refugia for native taxa that may be important for ecosystems integrity and function. Thus, the sampled natural pockets may be amongst the few fungal endophyte reserves that harbour native taxa. Metabarcoding studies, such as the present study, help to identify habitats that harbour multitudes of undescribed fungi, amongst which may be the keystone fungi in plant associated microbial assemblages. Due to the conservative nature of the ITS region used in metabarcoding studies, this marker is not always ideal for separating closely related species (Abdelfattah et al., 2015; Callewaert et al., 2018; Peay et al., 2016), thus the species richness in this study may be an underrepresentation of the actual species richness within the African olive in South Africa**.** Future culture-based studies are needed to confirm the identification of taxa revealed as potential hub species and to describe those that are new species. It is important to understand how existing communities and ecosystems respond to environmental changes. For example, the α- and β- diversity results in this study suggest that although disturbance may not affect species richness, it may affect the relative representation of some taxa increasing numbers of some functional redundancies, while decreasing others. Future studies may also benefit from investigating how fungal assemblages in the Core Cape Subregion respond to urbanisation and agricultural activities separately. This can help pinpoint the exact abiotic legacies of disturbance that govern the changes in fungal assemblage structure and integrity. Understanding fungal endophyte assemblage response to disturbance will help inform landscape management decisions.

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3.8 Supplementary materials

Table S1: Sample distribution in the nine categories, divided according to the location of origin. Stellenbosch = 21, Paarl = 21 and Somerset West= 21.

	NATURAL	SEMI-NATURAL	PLANTED
LOW	Paarl (3) Stellenbosch (1) Somerset West (3)	Paarl (3) Stellenbosch (3) Somerset West (1)	Paarl (2) Stellenbosch (4) Somerset West (1)
MEDIUM	Paarl (3) Stellenbosch (1) Somerset West (3)	Paarl (3) Stellenbosch (1) Somerset West (3)	Paarl (1) Stellenbosch (3) Somerset West (3)
HIGH	Paarl (3) Stellenbosch (2) Somerset West (2)	Paarl (2) Stellenbosch (3) Somerset West (2)	Paarl (1) Stellenbosch (3) Somerset West (3)

Table S2: An example of Tag and Index combination on a single 96 well plate. Index codes correspond to proprietary Illumina ITS primers supplemented with different indices. Likewise, tag codes correspond to different sequences supplemented with adapter sequences. Each well loaded with sample DNA.

	Indices	501.1-701.1	501.2-701.2	501.3-701.3	501.4-701.4	502.1-702.1	502.2-702.2	502.3-702.3	502.4-702.4	503.1-703.1	503.2-703.2	503.3-703.3	503.4-703.4	**IC_1 ↓**
Tag	**Plate 4**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
F1.3-R1.3	**A**													
F1.4-R1.4	**B**													
F2.1-R2.1	**C**													
F2.4-R2.4	**D**													
F3.1-R3.1	**E**													
F3.2-R3.2	**F**													
F4.2-R4.2	**G**													
F4.3-R4.3	**H**													
TC_2 →														

Table S3: Linear models *post hoc* results of the significant main effects (incident-based). Naming system for interactions *post hoc* are arranged as habitat context first followed by vegetation contrast. Comparisons significant at $p < 0.05$ are indicated by *.

Context		Estimate	SE	z-value	Pr(>|z|)
Planted	Natural	0.210	0.120	1.758	0.184
Seminatural	Natural	0.261	0.116	2.245	0.064
Seminatural	Planted	0.051	0.112	0.456	0.892
Contrast					
Low	High	0.546	0.109	4.992	<1e-05*
Medium	High	-0.055	0.125	-0.441	0.898
Medium	Low	-0.601	0.111	-5.402	<1e-05*
Context (Core)					
Planted	Natural	0.511	0.231	2.212	0.069
Seminatural	Natural	0.449	0.234	1.921	0.132
Seminatural	Planted	-0.062	0.203	-0.305	0.950

Interaction		Estimate	SE	z-value	Pr(>|z|)
NaturalLow	NaturalHigh	0.546	0.109	4.992	<0.001*
NaturalMedium	NaturalHigh	-0.055	0.125	-0.441	1.000
NaturalMedium	NaturalLow	-0.601	0.111	-5.402	<0.001*
PlantedHigh	NaturalHigh	0.210	0.120	1.758	0.707
PlantedLow	NaturalLow	-0.398	0.107	-3.719	0.006*
PlantedLow	PlantedHigh	-0.063	0.115	-0.544	1.000
PlantedMedium	NaturalMedium	0.649	0.113	5.739	<0.001*
PlantedMedium	PlantedHigh	0.384	0.104	3.672	0.007*
PlantedMedium	PlantedLow	0.446	0.106	4.204	<0.001*
SeminaturalHigh	NaturalHigh	0.261	0.116	2.246	0.373
SeminaturalHigh	PlantedHigh	0.051	0.112	0.456	1.000
SeminaturalLow	NaturalLow	-0.120	0.098	-1.233	0.949
SeminaturalLow	PlantedLow	0.278	0.108	2.585	0.191
SeminaturalLow	SeminaturalHigh	0.164	0.104	1.573	0.818
SeminaturalMedium	NaturalMedium	0.542	0.112	4.823	<0.001*
SeminaturalMedium	PlantedMedium	-0.107	0.097	-1.098	0.974
SeminaturalMedium	SeminaturalHigh	0.226	0.104	2.172	0.421
SeminaturalMedium	SeminaturalLow	0.062	0.099	0.623	0.999

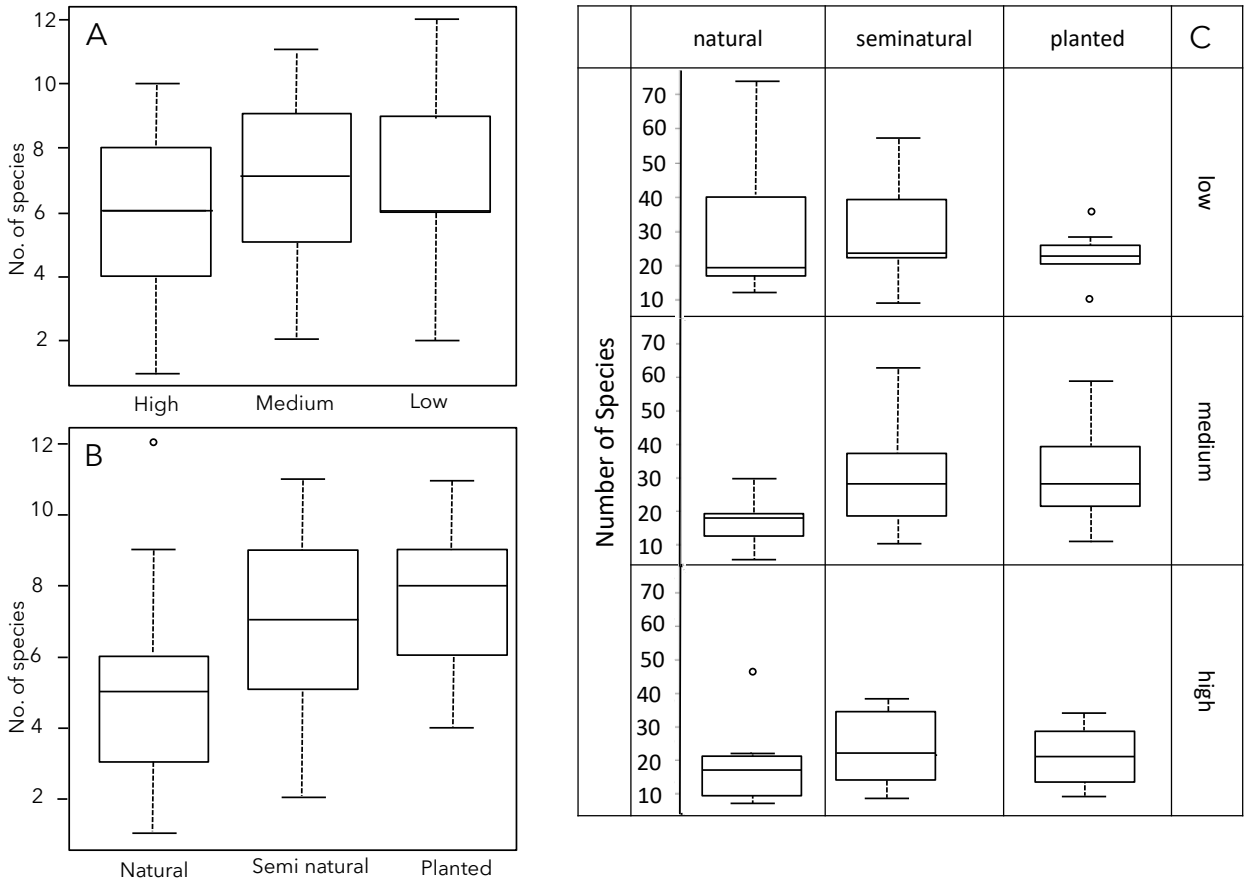


Figure S1: Box and Whisker plots of species richness between vegetation contrast levels (A), the habitat context categories (B), and the interaction between habitat context and vegetation contrast (C).

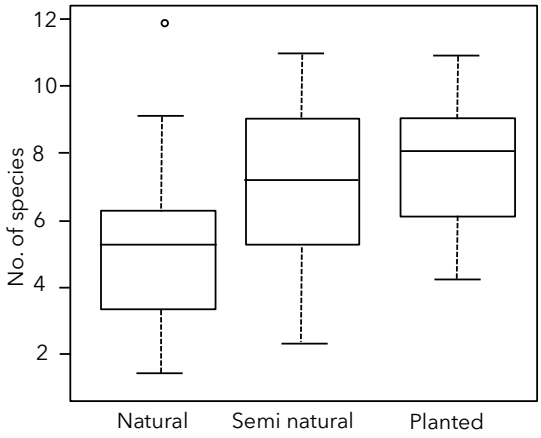


Figure S2: Box and whisker plot depicting the core OTU richness within the three habitat context categories

Table S4: Beta diversity *post hoc* comparisons of the significant Main test effects (PERMANOVA and PERMDISP, Table 3). Tests are significant at $p < 0.05$ (*). Sizes of sample distribution around the centroids are also presented.

PERMANOVA (β1)

		Jaccard		Jaccard (Core)	
Groups		t	p	t	p
Planted	Natural	1.217	0.017*	1.779	0.004*
Planted	Seminatural	1.090	0.170	1.203	0.194
Natural	Seminatural	1.115	0.103	1.534	0.017*
		Bray-Curtis		Bray-Curtis (Core)	
Groups		t	p	t	p
Planted	Natural	1.310	0.007*	1.600	0.008*
Planted	Seminatural	1.084	0.222	1.017	0.403
Natural	Seminatural	1.086	0.213	1.438	0.033*

PERMDISP (β2)- (Jaccard-based context)

		t	P(perm)
Planted	Natural	2.518	0.021*
Planted	Seminatural	0.410	0.690
Natural	Seminatural	2.260	0.025*
Group	Size	Average	SE
Planted	21	58.630	1.067
Natural	21	61.832	0.691
Seminatural	21	59.211	0.931

CHAPTER 4: Complex interactions between host species and surrounding environmental conditions dictate fungal endophyte assemblages within trees in a global biodiversity hotspot

4.1 Abstract

The important role of fungal endophytes in maintaining competitiveness of plants in their environments is becoming increasingly evident, but factors influencing these fungal endophyte assemblages are still poorly understood. Most endophytic fungi are horizontally transferred from the surrounding environment thus hosts may act as uptake filters. It is expected that closely related hosts would accrue similar fungal endophyte assemblages when growing in proximity to each other. The aim of this chapter was to determine the role of host relatedness in dictating fungal endophyte assemblages in trees/shrubs from the Core Cape Subregion, a biodiversity hotspot in South Africa. Fungal endophyte assemblages were documented using Illumina high-throughput sequencing in asymptomatic twigs from five tree species that differ in their degree of evolutionary relatedness. Endophyte richness, abundance and phylogenetic diversity were similar between all hosts but one. Fungal endophyte beta diversity differed significantly between all hosts, but this was dependent on the diversity measure used for comparisons. In cases where hosts shared similar fungal endophyte assemblages, they also shared similar habitats despite being phylogenetically distantly related. Fungal assemblage-based distance measures were not correlated to the phylogenetic distances between hosts. I conclude that tree species in this region generally host similar numbers of fungal endophytes, but that different species acquire different assemblages from the surrounding environment and act as 'uptake filters.' Closely related host species do not necessarily host similar endophyte assemblages and many endophyte taxa may be shared between phylogenetically distantly related hosts when growing in the same habitat. These results highlight the importance of the surrounding environment in dictating tree fungal endophyte assemblages.

4.2 Introduction

Host-microbe symbioses have influenced evolutionary trajectories of both hosts and microbes (Brooks et al., 2016; Groussin et al., 2017; van Oppen and Medina, 2020). For example, it has been suggested that

benefits conferred by microbes have played an important role in facilitating the shift of plants onto land (Pirozynski and Malloch, 1975). Plant-endophyte relationships are also ancient and have been dated as far back as over 400 My ago (Brundrett, 2002; Hibbett et al., 2000; Krings et al., 2007). Over time, fungal endophytes (along with other microbes) have facilitated the ability of plants to adapt to harsh conditions, such as salt marshes, coal mines, heat, drought and heavy metal contamination, amongst others (Liu et al., 2017; Márquez et al., 2007; Rodriguez et al., 2004,2009). In turn, endophytes benefit from host nutrient resources and gain shelter from fluctuating and sometimes threatening exogenous conditions, both biotic and abiotic (Hawkes et al., 2020). Together, the host and fungal endophyte may complement each other, reducing pressures faced by either and may result in novel behaviours and functions (Koskella and Bergelson, 2020).

Plants and their associated endophytes can form complex relationships ranging from harmful, to neutral or co-operative. The endophytic phase of a microbe is often temporary and can change between beneficial or harmful depending on the prevailing conditions (Fesel and Zuccaro, 2016; Thrall et al., 2007). For example, *Colletotrichum* Corda species are known as pathogens that cause disease in hosts as diverse as tea-oil trees, strawberries and olives, but can also be beneficial to other hosts (Achbani et al., 2013; Freeman et al., 2001; Li et al., 2016; Moral et al., 2017). *Colletotrichum gloeosporioides* Penz., a notorious *Olea europaea* subsp. *europaea* L. pathogen, is beneficial to tomatoes, where it confers facultative drought tolerance and leads to increased plant biomass (Rodriguez and Redman, 2008). Interestingly, when *Colletotrichum magna* (Jenkins & Winstead) Bhairi, E.P. Buckley & R.C. Staples is inoculated into different tomato cultivars it expresses a range of possible lifestyles, from pathogenic to beneficial or commensal (Rodriguez and Redman, 2008). These examples illustrate that the symbioses between hosts and fungal endophytes are delicately balanced relationships facilitated by biotic and abiotic factors that, when altered, can cause a shift in fungal lifestyle.

Both biotic and abiotic factors play a critical role in endophyte-host interactions. For example, the degree of specificity between fungal endophyte symbionts and Hawaiian native *Metrosideros polymorpha* Gaudich. (Myrtales), *Vaccinium reticulatum* Sm. (Ericales) and *Leptecophylla tameiameiae* (Cham. & Schltdl.) C.M. Weiller (Ericales) varies based on elevation (Cobian et al., 2019). Differences in host identity and soil properties were important determinants of fungal endophyte assemblages in roots of Arctic trees in Canada (Fujimura and Egger, 2012). In an earlier chapter (Chapter 3), fungal endophyte assemblages differed according to habitat quality within a single host (*Olea europaea* subsp. *cuspidata* (Wall. & G.Don) Cif.) rather than by the vegetation contrast with the surrounding vegetation.

Hosts that recently diverged tend to host similar assemblages (Higgins et al., 2007; Ragazzi et al., 2003). For example, despite the great distances between sites and different habitats, *Populus euphratica* Oliv. (from Central China) and *Populus tremula* L. (from temperate Europe) harbour conspecific xerotolerant fungal endophyte assemblages (Unterseher et al., 2012). The effect of host identity in shaping fungal endophyte assemblages, therefore, may correspond with the degree of host relatedness (Liu et al., 2019; Botnen et al., 2020). In other examples, foliar fungal endophyte assemblages of numerous hosts from different localities and habitats were found to strongly depend on host relatedness (Arnold, 2007; Hoffman and Arnold, 2008; Solis et al., 2016). Even with generalist endophytic fungi the options of hosts are often phylogenetically constrained to some degree (Gilbert and Webb, 2007; Liu et al., 2012). Thus, host preference and specificity can be a question of host taxonomic scale (e.g. host species specificity or host genus specificity).

Fungal endophytes can vary at as fine a scale as between different genotypes of the same host, e.g., cushion plants (Roy et al., 2018). In *Fraxinus excelsior* L. (the European Ash) twigs and *Populus balsamifera* L. (Balsam poplar) leaves fungal endophyte composition was sensitive to and, as result, structured by host genotypes (Bálint et al., 2013; Kosawang et al., 2019). Similarly, the mycobiomes differed between different genotypes of barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) (Sapkota et al., 2015). However, in other hosts such as seagrass (*Syringodium isoetifolium* (Asch.) Dandy), host genotype was not amongst the factors that affect endophyte assemblages (Wainwright et al., 2018). Therefore, although host genotype can be important, it appears that other additional factors may be more important.

Many endophytes are capable of inhabiting multiple hosts (Gilbert and Webb, 2007; Hersh et al., 2012). Endophytic fungal assemblages are often shared between native hosts and closely related exotic hosts (Mehl et al., 2017; Slippers et al., 2005). Although, there was a huge overlap of fungal endophyte taxa between the subspecies of *O. europaea*, fungal endophyte assemblages of *O. europaea* subsp. *europaea* and *O. europaea* subsp. *cuspidata* were distinct from each other (Chapter 2). It is possible that in the *Olea europaea* habitat context (as in Chapter 3) residence time played a bigger role in shaping fungal assemblages than host relatedness. Therefore, although host and host-related factors often shape fungal endophyte assemblages, other factors may be just as important, if not more so.

The degree of host specificity of fungal endophyte assemblages found within native host trees remains understudied. This study aimed to elucidate the importance of host phylogenetic relatedness in dictating

fungal endophyte assemblages of select trees/shrubs in a biodiversity hotspot in South Africa. As in the studies by Harrison et al., (2021) and Liu et al., (2019), fungal endophyte assemblages were expected to decrease in similarity with increasing phylogenetic distances between hosts. To this effect, the most closely related hosts, *Olea capensis* subsp. *capensis* Verdoorn and *Olea exasperata* Jacq., were expected to harbour the most similar assemblages as both of these hosts reside within the section Ligustroides in the genus *Olea* (Besnard et al., 2002; Green, 2002) despite being sampled from different biomes (Forest and Fynbos, respectively). I hypothesised that *Olinia ventosa* (L.) Cufod. Would harbour the most divergent endophyte assemblages as this host is the most phylogenetically distant taxon in this study, residing in the Myrtales as opposed to the other hosts that all belong to the Lamiales (Sebola and Balkwill, 2013) (Figure 1).

4.3 Materials and Methods

4.3.1 Site description and study species

The Harold Porter National Botanical Garden (HPNBG, -34.34985; 18.92699) is located at the edge of the Kogelberg Biosphere Reserve, which is considered the heart of the Core Cape Subregion and a global biodiversity hotspot (Rebelo et al., 2006; Silberbauer, 2013). The strategic placement of this reserve makes it critical for conservation as it includes a portion of the Afromontane Forest Biome and a portion of the iconic Fynbos Biome (Manning and Goldblatt, 2012). The tree genus *Olea* (Lamiales; Oleaceae) has evergreen representatives within natural areas of the biosphere with *O. europaea* subsp. *cuspidata* and *O. exasperata* in the Fynbos Biome, and *O. capensis* subsp. *capensis* in the Forest Biome. These taxa formed the base of the 'closely related taxa' in the present study (Figure 1). *Olea europaea* subsp. *cuspidata* colonises a wide array of habitats such as ravines, woodlands, forest edges and kloofs (Coates-Palgrave, 1977; Palmer, 1977). *Olea capensis* subsp. *capensis* grows in littoral regions and evergreen forests (Coates-Palgrave, 1977; Green, 2002). The third *Olea* species, *O. exasperata*, usually grows in sand dunes, hillsides, and open grasslands (Coates-Palgrave, 1977; Green, 2002). Thus, *O. capensis* subsp. *capensis* was collected from the evergreen forest in HPNBG, *O. exasperata* from the sand dunes immediately outside the HPNBG and *O. europaea* subsp. *cuspidata* from the forest edge just outside the HPNBG deeper into the biosphere.

An additional two evergreen non-*Olea* tree species were also sampled from the Forest Biome of the HPNBG; *Halleria lucida* L. (Lamiales; Stilbaceae) and *O. ventosa* (Myrtales; Penaeaceae) (Oxelman et al.,

2005; Sebola and Balkwill, 2013). *Halleria lucida* often grows on inland and coastal hill slopes, high mountains, stream banks and forest margins (Palmer, 1977) and *O. ventosa* is often found in evergreen forests, forest margins, rocky hillsides, and coastal scrubs (Palmer, 1977). These additional hosts were chosen for their abundance in the forest biome in HPNBG, and their increasing taxonomic distance from the *Olea* hosts (Figure 1).

4.3.2 Sampling

Asymptomatic twigs were collected from all five focal tree species and fungal endophyte assemblages were characterised using DNA metabarcoding. Specifically, twigs (5 cm length and 3 – 5 mm in diameter) were collected from the four cardinal points (one twig per side of the tree) of each of 50 randomly selected trees (10 per host) from natural areas in the HPNBG and the immediate surrounds during August in 2018. Tree individuals were at least 50 meters apart. Twigs collected from the same tree were combined in a sampling bag and stored at -80°C.

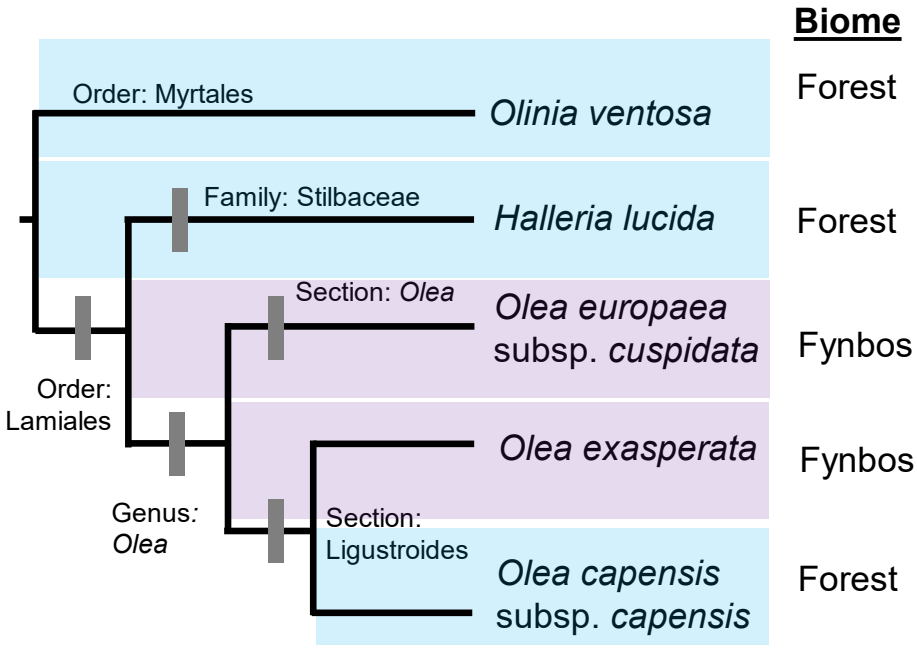


Figure 1: Phylogram of the taxonomic relations of the sampled hosts, overlain with and colour coded by their preferred Biomes. The relationships and taxonomical hierarchy were established based on Green, (2002), Coates-Palgrave, (1977), Palmer, (1977), and Sebola and Balkwill, (2013). Biome annotation is based on Mucina and Rutherford, (2006).

4.3.3 DNA extraction, host confirmation and host phylogeny

Sample processing and DNA extraction protocols followed those detailed in Chapters 2 and 3. Briefly, approximately 1 cm long pieces were excised from the middle of each twig. The four pieces collected from the same tree were surface-sterilised (Moral et al., 2010; Slippers and Wingfield, 2007) and collectively ground to a powder using a mortar and pestle. A modified Doyle and Doyle (1990) DNA extraction protocol was followed to extract total genomic DNA from the powdered twigs.

Two representative trees per host were randomly selected to confirm identity using DNA markers. Two chloroplast markers were sequenced using the TrnS (5'- GCC GCT TTA GTC CAC TCA GC-3'; Hamilton, 1999) and TrnG (5'- GAA CGA ATC ACA CTT TTA CCA -3'; Shaw et al., 2005), and PsbA (5' -CGA AGC TCC ATC TAC AAA TGG -3'; Hamilton, 1999) and TrnH (5' -ACT GCC TTG ATC CAC TTG GC-3'; Hamilton, 1999) primer pairs. The TrnS-G marker was selected because it has proven success in Oleaceae phylogenetics, specifically in *Olea* (Besnard et al., 2009). The PsbA-TrnH is known to be the most variable region in angiosperms (Byrne and Hankinson, 2012), and therefore served as an ideal marker for confirming the identity of all the hosts.

PCR reaction mixtures contained 1 µl of 2.5 mM $MgCl_2$, 0.25 µl of 10 µM of each primer, 6 µl 2X KAPA Taq ReadyMix (Kapa Biosystems, Inc., Boston, USA), 4.5 µl ddH_2O and 1 µl of 100 ng/µl template. The PCR reactions were as follows: a 95 °C for 2 min initial denaturation step, followed by 36 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 60 s) and concluding with an elongation step at 72 °C for 8 min. Once the presence of amplicons was confirmed on an agarose gel using electrophoresis, the amplicons were submitted for sequencing to the Central Analysis Facility (CAF), Stellenbosch University. Host identity was confirmed by comparing the generated sequences to those of representative samples available on GenBank (https://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Sequence Tool (BLAST).

Four markers (MatK, PsbA, rbcL, and TrnS-TrnG) were selected for host phylogenetic reconstructions (Table 1) (Olmstead et al., 2001). Maturase kinase (MatK) and RuBisCO large subunit (rbcL) sequences were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) to supplement the markers (PsbA-TrnH and TrnS-G) sequenced in the present study for host confirmation. All sequences were assessed for quality of reads, manually optimised, and aligned using BioEdit v. 7.0.5.3 (Hall, 2011). The aligned sequence files were concatenated using FASconCAT-G v1.02 (Kück and Meusemann, 2010) and converted to nexus files using DnaSP v. 6 (Librado and Rozas, 2009). The host phylogeny was reconstructed

using MrBayes v. 3.2.0 (Huelsenbeck and Ronquist, 2001). The MrBayes block was set to mixed *nst* with *invgamma* rate and run for 5 000 000 generations. The first 25 % of collected likelihood values were discarded as burn-in. Trees were sampled every 2000 trees and combined into a consensus tree (based on a 50 % majority-rule).

Table 1: Representative sequences from the five sampled hosts. Some sequences were sourced from GenBank (accession numbers included), while others were sequenced during this study (indicated by the sample number from which they originated – GenBank accession numbers pending).

	MATK	PsbAH	RbcL	TrnSG
Olea europaea subsp. *cuspidata*	AM933396.1	HM999671.1	MN017130.1~	MN017130.1~
Olea exasperata	MG255766.1~	HX11*	NC_036985.1	—
Olea capensis subsp. *capensis*	AM933412.1	OC14*	MH817925.1	AM933209.1
Halleria lucida	AF375188.1	HL11*	JX572665.1	HL11*
Olinia ventosa	JX517344.1	OV12	AF215546.1	OV11*

*: sequences generated in this study, ~: extracted from published genome sequences

4.3.4 Metabarcoding library preparation

Extracted DNA was subjected to two polymerase chain reactions. The protocols of these reactions were identical to those followed in Chapters 2 and 3. Briefly, the first PCR was conducted to amplify the fungal ITS regions using ITS1 and ITS4 (White et al., 1990; Gardes and Bruns, 1993). These primers had been modified to carry binding sites for the indexes to be added in PCR2 (Sigma-Aldrich® Munich, Germany). Reaction volumes and PCR cycle conditions are detailed in the previous chapters. Agarose gel (0.8 %; Bio-budget, Technologies GmbH, Germany) supplemented with 1 µl ethidium bromide was used to confirm the success of PCR1. Samples that failed to amplify were removed before moving forward with the protocol. An ExoSap protocol (New England BioLabs Inc.; detailed in Chapter 2) was used to purify the PCR1 product. PCR2 was conducted to add indices and Illumina adapters (P5 and P7) onto the amplicons from PCR1. Reaction volumes and thermocycling conditions are provided in Chapter 2. The success of PCR2 was also confirmed by visualisation on an agarose gel (0.8 %, supplemented with 1 µl Ethidium Bromide). PCR products were cleaned, using the ExoSap protocol, to remove unused primers. The final reaction product contained ITS amplicons carrying unique combinations of tags and indexes (with sequencing adaptors) that served as a unique identifier of the sample of origin of each amplicon.

Once the samples were tagged, sequentially equimolar pooling of the remaining 40 samples (from the original sampled 50 trees) was performed until all samples were pooled into one tube (details provided in Chapter 2). Agarose gel fluorescence photographs were taken in a dark chamber and the band intensities were used as molarity proxies. Samples with similar intensities (as quantified using ImageJ version 1.52a; Ferreira and Rasband, 2012) were combined. The resulting pools were then purified using the CleanPCR® Kit (CleanNA, GC biotech B.V.). To confirm that the cleaning procedure did not inadvertently result in the loss of the pools, another electrophoresis was conducted. These pooling steps were repeated until all products were combined into one superpool. The superpool was subjected to sequencing at the Genetics Department, Ludwig Maximilian University, Munich, using an Illumina MiSeq® sequencer (Illumina Inc., San Diego, CA, USA).

4.3.5 Metabarcoding sequence cleaning, identification and quantification

The sequencing facility returned a batch of sequences (carrying tags and indexes). Prior to any processing and separation, quality control and demultiplexing using the QIIME 1.9.1 pipeline (Caporaso et al., 2010) was performed on the batch of sequences (as described in Chapter 2). Sequence reads were extracted using the forward and reverse barcodes as identifiers. Sequence quality was evaluated in reference to a Phred quality threshold (0.35), which was used to retain those with acceptable scores and discard low quality reads using FastQC v.0.11.8 (Babraham Institute, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The remaining good quality sequences were separated and assigned to their sample of origin based on their index-tag combination. Once assigned, the tags and indexes were trimmed using the FASTX-Toolkit (v. 0.0.13, http://hannonlab.cshl.edu/fastx_toolkit/). ITS sequences were further screened to remove any chimeras using the USEARCH platform (Edgar, 2010). The remaining sequences were grouped into operational taxonomic units (OTU) based on sequence similarities using CD-HIT-OTU (http://weizhongli-lab.org/cd-hit-otu/; Li et al., 2012; Stackebrandt and Goebel, 1994). One representative sequence per OTU was extracted using QIIME for the purpose of taxonomic assignment (similarity cut-off at 97 %) using the UNITE v. 7.2 database (Kõljalg et al., 2013). Finally, an operational taxonomic unit (OTU) table containing OTU identity, abundance per sample (inferred from OTU frequency per sample), accompanying sample metadata and hierarchical taxonomic assignment was created.

4.3.6 Analyses of fungal endophytes within five hosts

4.3.6.1 Alpha diversity

Sample size (total n = 40) was unbalanced between hosts as the number of samples with fungal endophytes that failed to amplify varied between hosts. The number of successful amplifications varied: *O. capensis* subsp. *capensis* (n = 5), *H. lucida* (n = 8) and *O. ventosa* (n = 8) *O. exasperata* (n = 9), and *O. europaea* subsp. *cuspidata* (n = 10). Non-parametric species diversity estimators (Jackknife2 and Chao2) were used to calculate expected fungal endophyte richness within hosts using Primer6 (Anderson et al., 2008) and R v. 4.0.0 (R Development Core Team, 2015). Stack plots of the relative representation of the different fungal families in each sample per host were constructed using the R package, Phyloseq v. 1.28.0 (McMurdie and Holmes, 2013). Two plots were constructed, one containing only those OTUs that could be placed at least at family level (excluding the rest), and another for all OTUs recovered, regardless of whether they could be placed at family level.

Analyses of OTU abundance were based on the total number of reads per sample. However, analyses of species richness were based on both (a) the number of OTUs in each sample and (b) richness of OTUs rarefied to the smallest number of observations using *rarefy* in Vegan v. 2.5-6 (Oksanen et al., 2008; Weiss et al., 2017). Phylogenetic diversity estimates of microbial assemblages were based on Faith's phylogenetic diversity (Faith's PD), which defines diversity as the sum of the branch lengths connecting the different taxa within each host (Faith, 1992). To construct the OTU-based phylogeny for Faith's PD, representative sequences of the OTUs encountered within all samples were aligned using Mafft v. 7.245 (Katoh et al., 2002). Analyses were conducted on the entire compliment of OTUs in the samples (irrespective of sample size) to increase the likelihood of detecting differences in assemblages based on phylogenetic diversity. Due to difficulty in alignment, sequences of OTUs that had no BLAST hits were excluded from these analyses. The resulting alignment was used to construct an OTU phylogeny using MrBayes v. 3.2.0 (Huelsenbeck and Ronquist, 2001). The resulting phylogenetic tree was converted into a newick file using Figtree v.1.0.4 (Rambaut, 2009). The ultrametric-newick tree along with the abundance matrix file were used to calculate Faith's PD using *Picante* v. 1.82 package (Kembel et al., 2010). All above-mentioned alpha diversity measures were also calculated for OTUs that represented the core assemblages. Core OTUs were defined here as those that appeared in at least 50 % of all the sampled individuals of a particular host species.

To test for differences in fungal abundance, richness and phylogenetic diversity between hosts, linear regression analyses were performed using R (R Development Core Team, 2015). To test which model assumptions the data fitted, normality tests were conducted using the Nortest package (Gross and Ligges, 2015). To test whether fungal abundance was significantly different between hosts, a generalised linear model was constructed using the *lme4* package in R (Bates and Sarkar, 2007). In contrast, richness, rarefied richness, core richness, phylogenetic diversity and log transformed core abundance datasets were normally distributed, thus ANOVAs were used to assess the significant differences between hosts using *lme4*. Where the main test produced significant results, *post hoc* tests for non-parametric and parametric models were performed using conservative Tukey tests using the multcomp package (Hothorn et al., 2008) and pairwise t-tests using the package stats v. 4.0.0 (R Development Core Team, 2015), respectively.

4.3.6.2 Beta diversity

Analyses to differentiate OTU compositional assemblages were conducted using Primer6 and R. Beta diversity was assessed between hosts (β1) and within hosts (β2) using permutational multivariate analyses of variance (PERMANOVA) and permutational multivariate analyses of dispersion (PERMDISP), respectively, as outlined in previous chapters. Where host significantly influenced assemblages, pairwise comparisons were performed. PERMANOVA analyses were conducted using both a Bray-Curtis resemblance-matrix and a Jaccard resemblance-based matrix. Analyses based on the Bray-Curtis resemblance-matrix incorporate details on the number of reads per OTU in dictating assemblage compositional similarities. However, as the number of reads is an imperfect measure of OTU abundance in metabarcoding studies (Amend et al., 2010), I repeated PERMANOVA analyses using only the presence/absence OTU data. PERMDISP analyses were performed on a Jaccard resemblance-based matrix (based on presence-absence data). In addition, fungal endophyte assemblage relatedness was considered by using weighed (using abundance data) and unweighed (using incidence data) UniFrac distances to perform beta diversity analyses (β1 and β2). The UniFrac distance matrices were constructed using the whole and core microbiome datasets using the Phyloseq package in R. UniFrac distance measure incorporates the relatedness of OTUs allowing for the assessment of the tendency of closely related hosts to harbour closely related OTUs (Lozupone and Knight, 2005). UniFrac distances were calculated between samples using both OTU abundance or incidence, and the hierarchical taxonomic information of each OTU (Lozupone and Knight, 2005). PERMANOVA main tests were performed based on the UniFrac distance matrices using Primer6. When main tests were significant, *post hoc* analyses were performed. PERMDISP

main tests were conducted using Primer6 and, in cases of significant outcomes, *post hoc* analyses were also performed.

The above-mentioned beta diversity analyses may be sensitive to unequal sample sizes, thus β1 and β2 analyses were also performed on all OTUs in an adjusted sample design (n = 25). To adjust for the unequal sample sizes, the sample size per host was reduced to the smallest number of samples per host (n = 5, *O. capensis* subsp. *capensis*). To identify which samples to remove, a random number generator was used (www.randomnumbergenerator.com/). In a similar manor, core OTUs of the balanced sample design were identified and the above-mentioned β-diversity analyses were repeated.

To visualise the grouping of the fungal assemblages within hosts, Bray-Curtis, Jaccard and UniFrac distance-based non-Metric Dimensional Scaling (nMDS) plots were constructed for all above-mentioned datasets using Phyloseq. To ascertain if the vicinity of the host groupings observed in the nMDS plots were consistent with host relatedness, hierarchical clustering was used to visualise the relationships between hosts as dictated by their endophyte assemblages using the *hclust* function in the Vegan package. The congruency between host relatedness and their fungal endophyte assemblages was visualised by constructing tanglegrams (Galili, 2015; Venkatachalam and Gusfield, 2018). In the present study, the host phylogeny constructed from plastid markers was compared to hierarchical clusters from OTU distance matrices based on Bray-Curtis, Jaccard, and weighed and unweighed UniFrac distances. The assemblage-based phylogenies were constructed using the *hclust* function in the Vegan package. The plastid marker-based phylogeny was plotted alongside the OTU assemblage-based phylogenies and the corresponding host between these trees were connected to highlight congruency.

Mantel tests were conducted to ascertain whether differences between host phylogenetic distance and their mycobiomes were significantly correlated. Genetic distances between hosts were calculated based on the phylogenetic tree constructed from plastid markers using the *cophenetic* function in APE v. 5.4 package in R (Paradis and Schliep, 2018). Cophenetic distances measure differences between tips in relation to the number of nodes and edges since their last shared connection in the tree (Paradis and Schliep, 2018). The resulting distance matrices were compared with assemblage-based distance matrices using partial Mantel tests in the Vegan package. The Mantel tests were conducted using the Pearson method with 10 000 permutations. Analyses were performed using assemblage datasets constructed from OTU abundance, richness, core abundance and core richness data using Bray-Curtis and Jaccard distances for abundance and incident data, respectively. Additional Mantel tests were conducted on OTU

assemblage matrices that also considered relatedness between OTU hierarchical taxonomical placement (weighed and unweighed UniFrac) distances between hosts calculated using UniFrac in Phyloseq.

Since host identity was important for fungal assemblage differentiation, similarity percentage analyses (SIMPER) were performed using PRIMER6 to reveal which OTUs contributed to the dissimilarity between hosts. Particularly, one-way SIMPER analyses were performed using Bray-Curtis resemblance on square-root transformed abundance data (balanced and unbalanced dataset) with a 90 % cut-off for low contributions. To visualise the overlap of OTUs within hosts, Venn diagrams were constructed for the unbalanced and balanced datasets using VennDiagram v1.6.2 (Chen and Boutros, 2011) in R. Preceding OTU isolation and overlap identification were calculated using gplots v3.1.1 (Warnes, 2016) and reshape2 v1.2.2 (Wickham, 2007) packages in R.

4.4 Results

4.4.1 Fungal endophyte alpha diversity within the host plants

Fungal endophyte amplicons were successfully obtained from forty trees (out of the fifty sampled trees), from which 493 173 reads belonging to 296 OTUs were generated. One hundred and seventy-one of the OTUs belonged to 46 fungal families (Figure 2), while 125 could not be characterised at the family level (Figure s1). Once the sample sizes were reduced to balance (n = 25), 137 of the 296 OTUs remained within the 25 samples. Based on relative contributions, *Olinia ventosa* harboured the most distinct collection of families (Figure 2). Fungal families associated with *Olea capensis* subsp. *capensis* and *O. exasperata* were the most similar compared to other hosts. Notably, Teratosphaeriaceae was amongst the more dominant families in *O. europaea* subsp. *cuspidata* and *O. exasperata* hosts. Teratosphaeriaceae was also present in seven of the eight *O. ventosa* samples. A large proportion of the taxa sampled from *O. capensis* subsp. *capensis* could not be placed at the family level (Figure S1). Sporocadaceae was often found to be dominant within *H. lucida*, but rarely in any of the other four hosts. Vibrissiaceae was predominantly found within *O. ventosa.* Members of the Didymellaceae were found across all five hosts with the highest percentage coverage in *H. lucida* and *O. exasperata* and contained some economically significant taxa, namely *Epicoccum plurivorum* (P.R. Johnst.) Qian Chen & L. Cai, *Didymella calidophila* (Aveskamp, Gruyter & Verkley) Qian Chen & L. Cai and *Phoma* Sacc. Species (Chen et al., 2015).

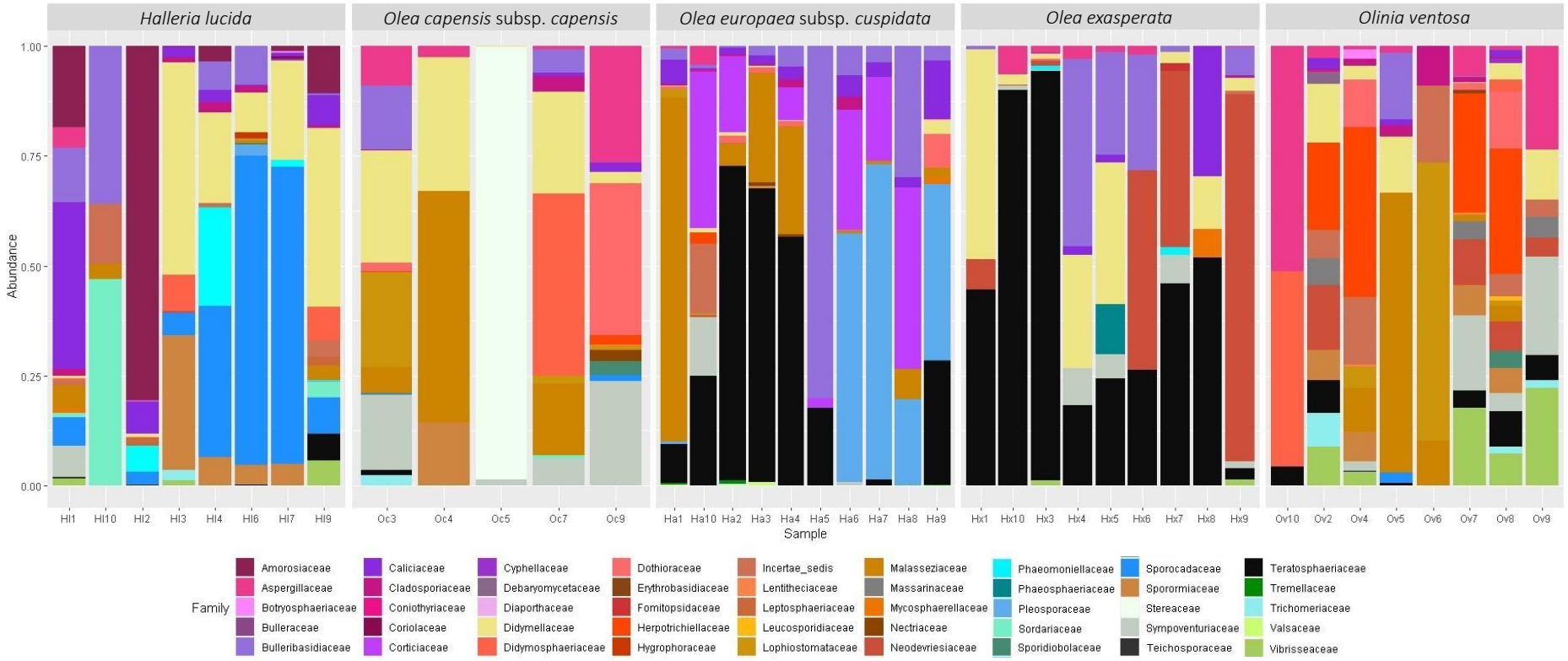


Figure 2: Stack plot capturing the relative contribution of the different fungal families (%) recovered from twigs of the five sampled hosts. Families for which identities could not be confirmed have been omitted, but these are presented in Figure S1.

Observed and estimated species richness of fungal endophytes were highest in *O. europaea* subsp. *cuspidata* followed by *O. ventosa* (Table 2). This was also true for the phylogenetic diversity of the endophyte assemblages. All other host taxa had very similar observed and estimated fungal endophyte richness and phylogenetic diversity. This pattern was also reflected when considering only the core assemblages (Table 2). Statistically, whole fungal endophyte assemblages were similar between all hosts based on all measures of alpha diversity, including abundance, richness, rarefied richness and Faith's PD (Table 3). Core fungal abundance and core rarefied richness were significantly influenced by host identity. Here, the significant differences were mainly driven by the significantly lower rarefied species richness within *O. exasperata* (Table S1, Figure 3), but core fungal abundances in this host were significantly higher than in other hosts (Figure 3).

Table 2: Species diversity estimators including Chao2 (with Standard Deviation = SD) and Jackknife2 of all fungal endophytes present in twigs of five tree hosts in the Harold Porter National Botanical Garden and surrounds. Species estimations for core assemblages (those endophytes that were present in at least 50 % of the samples per tree species) are also presented. SR = Species Richness (observed endophyte richness); PD = Faith's phylogenetic diversity.

		Whole Assemblage					Core Assemblage				
	n	SR	PD	Chao2	SD	Jackknife2	SR	PD	Chao2	SD	Jackknife2
Olea europaea subsp. *cuspidata*	10	79	20.176	229.60	34.716	239.68	17	2.973	27.50	17.139	22.972
Olea exasperata	9	40	13.478	126.08	39.575	104.18	15	2.624	34.17	17.425	20.690
Olea capensis subsp. *capensis*	5	39	15.869	125.38	22.438	123.55	14	2.888	17.13	3.656	18.917
Halleria lucida	8	63	17.749	174.28	31.818	163.11	16	2.888	20.50	7.194	20.232
Olinia ventosa	8	71	23.024	194.88	26.356	202.68	17	2.888	23.25	7.552	23.433

Table 3: Linear model analyses results of the contribution of host identity to differences in fungal endophyte richness and abundance. Linear models are based on abundance, richness, rarefied richness, and Faith's PD for fungal endophyte OTUs within the whole assemblage and the core assemblage (those endophytes that were present in at least 50 % of the samples per tree species) within hosts. Significant results ($p < 0.05$) are indicated by *.

	Whole			Core		
	Df	F-value	p	Df	F-value	p
Abundance	4	3.528~	0.474	4	4.209	0.007*
Richness	4	1.578	0.202	4	0.081	0.988
Rarefied richness	4	2.107	0.101	4	2.861	0.037*
Faith's PD	4	0.171	0.170	4	0.374	0.826

~: Chi Squared value

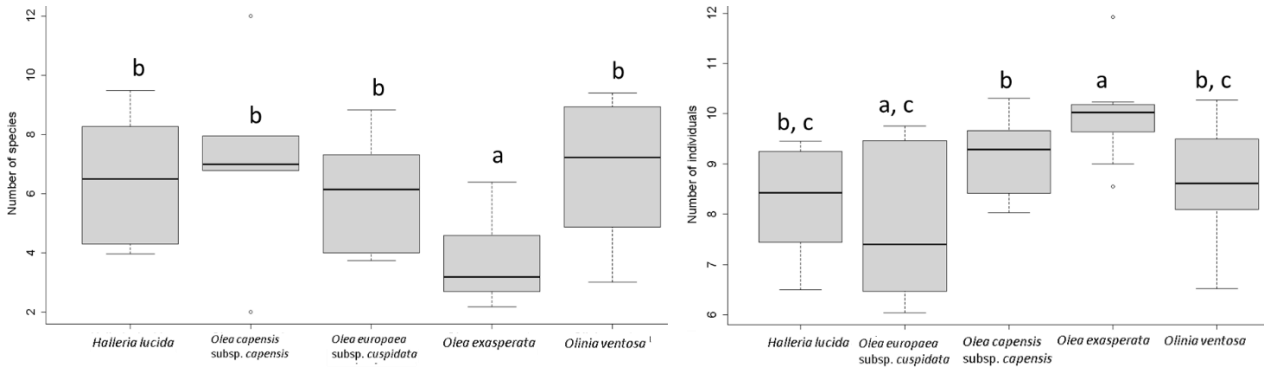


Figure 3: Boxplot representation of OTU rarefied core richness (left) and log transformed core abundance (right) within the five hosts. Small letters above each plot indicate significant differences. Differences were considered significant at $p < 0.05$, statistical values are presented in Table S1.

4.4.2 Beta diversity

Fungal endophyte composition of the full complement generally differed significantly between host species (β1). This was irrespective of the specific dataset used (whole or core assemblages) in analyses or the distance measure used (Table 4, Figure 4). In a few cases though, assemblage composition within *O. capensis* subsp. *capensis* and *O. ventosa* were not significantly different when considering the full complement of species. After reduction of the datasets to balance the sampling size, endophyte assemblage composition between different hosts remained significantly different for most tree hosts, but this was dependent on the specific distance measure used (Table 4, Figure 4). When host trees harboured similar assemblage composition, these were usually the forest hosts (*O. capensis* subsp. *capensis, H. lucida* and *O. ventosa*). *Olea*-associated fungal endophyte assemblage composition frequently differed significantly from each other. This grouping of forest species and the significant differences between *Olea* species was also evident when fungal assemblages were adjusted for fungal taxon relatedness (UniFrac measures; Table 4).

Fungal endophyte assemblage turnover within host species (β2) was not significantly affected by host identity (Table 5). This was consistently the case regardless of the sample size (balanced vs unbalanced) and the proportion of fungal endophyte assemblages assessed (whole vs core).

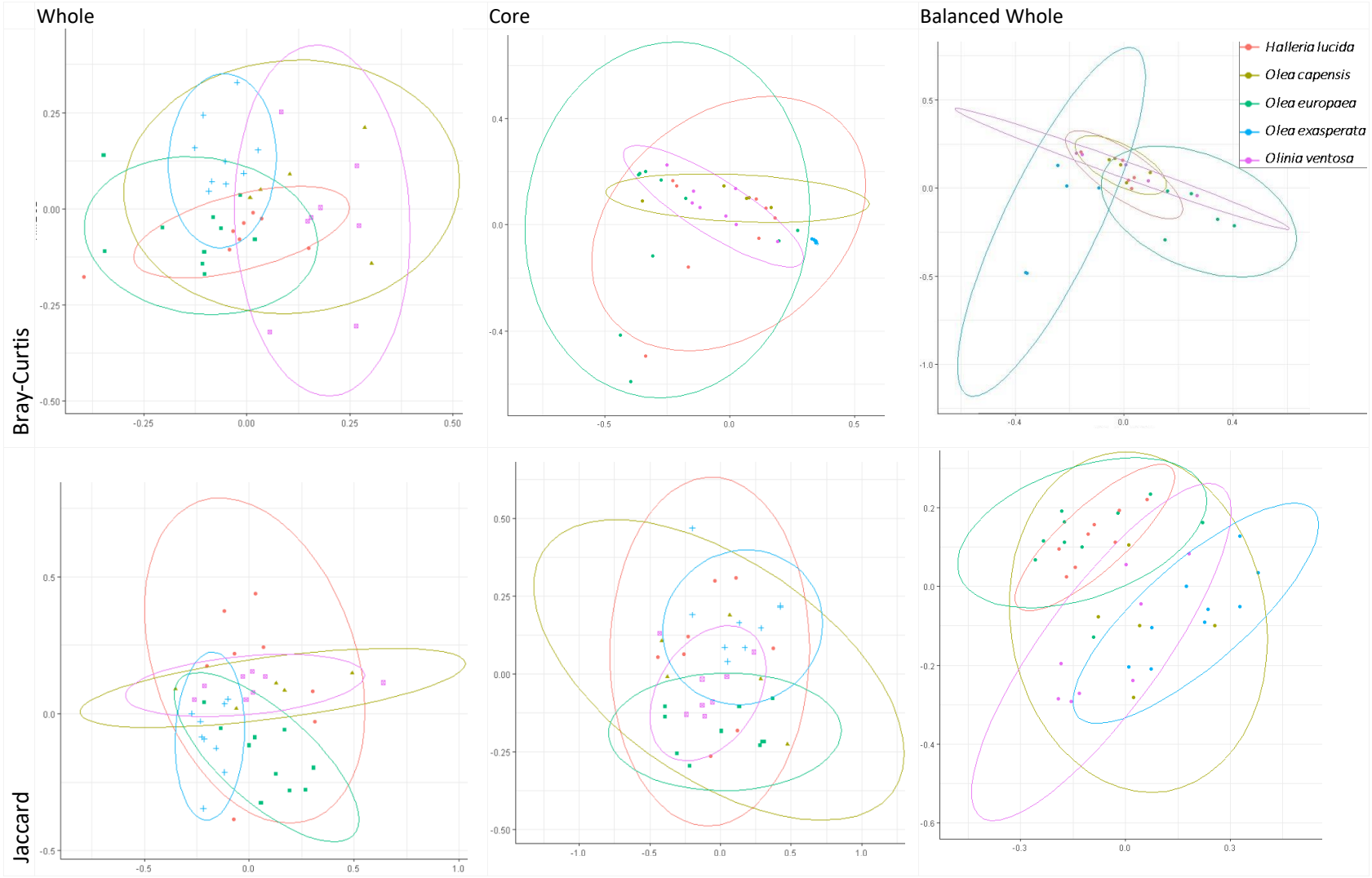
Table 4: Differences in fungal endophyte assemblages within twigs of five host tree species in and around the Harold Porter National Botanical Garden based on PERMANOVA analyses (B1) for the whole assemblage (n = 40 trees) and for the assemblages in the balanced design (n = 25 trees). Comparisons for core assemblages (those endophytes that were present in at least 50 % of the samples per tree species) are also presented. Results were considered significant at p< 0.05 and are denoted by *.

Source	Distance matrix	df	Pseudo-F	P(perm)	*Post hoc*
Whole assemblage	Bray-Curtis	4	5.257	0.001*	All different except *Olea capensis* subsp. *capensis* = *O. ventosa*
Core assemblage	Bray-Curtis	4	6.404	0.001*	All different except *O. capensis* subsp. *capensis* = *O. ventosa*
Whole assemblage	Jaccard	4	3.718	0.001*	All different
Core assemblage	Jaccard	4	4.377	0.001*	All different except *O. capensis* subsp. *capensis* = *O. ventosa*
Whole assemblage	Weighed UniFrac	4	5.764	0.001*	All different
Core assemblage	Weighed UniFrac	4	6.259	0.001*	All different
Whole assemblage	Unweighed UniFrac	4	6.763	0.001*	All different
Core assemblage	Unweighed UniFrac	4	5.276	0.001*	All different except *O. capensis* subsp. *capensis* = *Olea exasperata* and *O. ventosa*
Balanced whole assemblage	Bray-Curtis	4	2.534	0.001*	All different
Balanced core assemblage	Bray-Curtis	4	2.264	0.002*	All similar except *O. exasperata* ≠ *O. capensis* subsp. *capensis* and *O. ventosa, O. europaea* subsp. *cuspidata* ≠ *O. capensis* subsp. *capensis*, *H. lucida* ≠ *O. ventosa*
Balanced whole assemblage	Jaccard	4	2.396	0.001*	All different
Balanced core assemblage	Jaccard	4	2.151	0.007*	All similar except *O. europaea* subsp. *cuspidata* ≠ *O. capensis* subsp. *capensis* and *O. ventosa*, *O. exasperata* ≠ *O. ventosa.*

Balanced whole assemblage	Weighed UniFrac	4	1.875	0.002*	All similar except *O. europaea* subsp. *cuspidata* ≠ *O. ventosa*, *O. exasperata* ≠ *O. ventosa*, *O. capensis* subsp. *capensis* and *H. lucida*, *H. lucida* ≠ *O. ventosa*.
Balanced core assemblage	Weighed UniFrac	4	1.569	0.09	
Balanced whole assemblage	Unweighed UniFrac	4	1.572	0.001*	All similar except *O. europaea* subsp. *cuspidata* ≠ *O. ventosa*, *O. exasperata* and *O. capensis* subsp. *capensis, O. exasperata* ≠ *O. ventosa* and *O. capensis* subsp. *capensis*
Balanced core assemblage	Unweighed UniFrac	4	2.082	0.031*	All similar, except *O. europaea* subsp. *cuspidata* ≠ *O. exasperata* and *O. capensis* subsp. *capensis*, *O. exasperata* ≠ *O. ventosa*

Table 5: Within-group multivariate dispersion (B2, PERMDISP). *Post hoc* results of the significant main tests and the mean and standard errors of each host are shown. Balanced design refers to the reduced samples size per host to match the host with the smallest sample size (*Olea capensis* subsp. *capensis*, *n* = 5). Core assemblages refers to only those OTUs that appeared most frequently in samples, appearing in at least 50 % of the samples. Main tests for assemblage composition turnover within hosts, significant at $p < 0.05$.

	Distance	F-value	df1	df2	P(perm)
Whole assemblage	Jaccard	7.0334	4	35	0.288
Core assemblage	Jaccard	0.301	4	35	0.933
Whole assemblage	UniFrac	1.200	4	35	0.354
Balanced whole assemblage	UniFrac	0.655	4	20	0.778
Balanced core assemblage	UniFrac	1.467	4	20	0.458



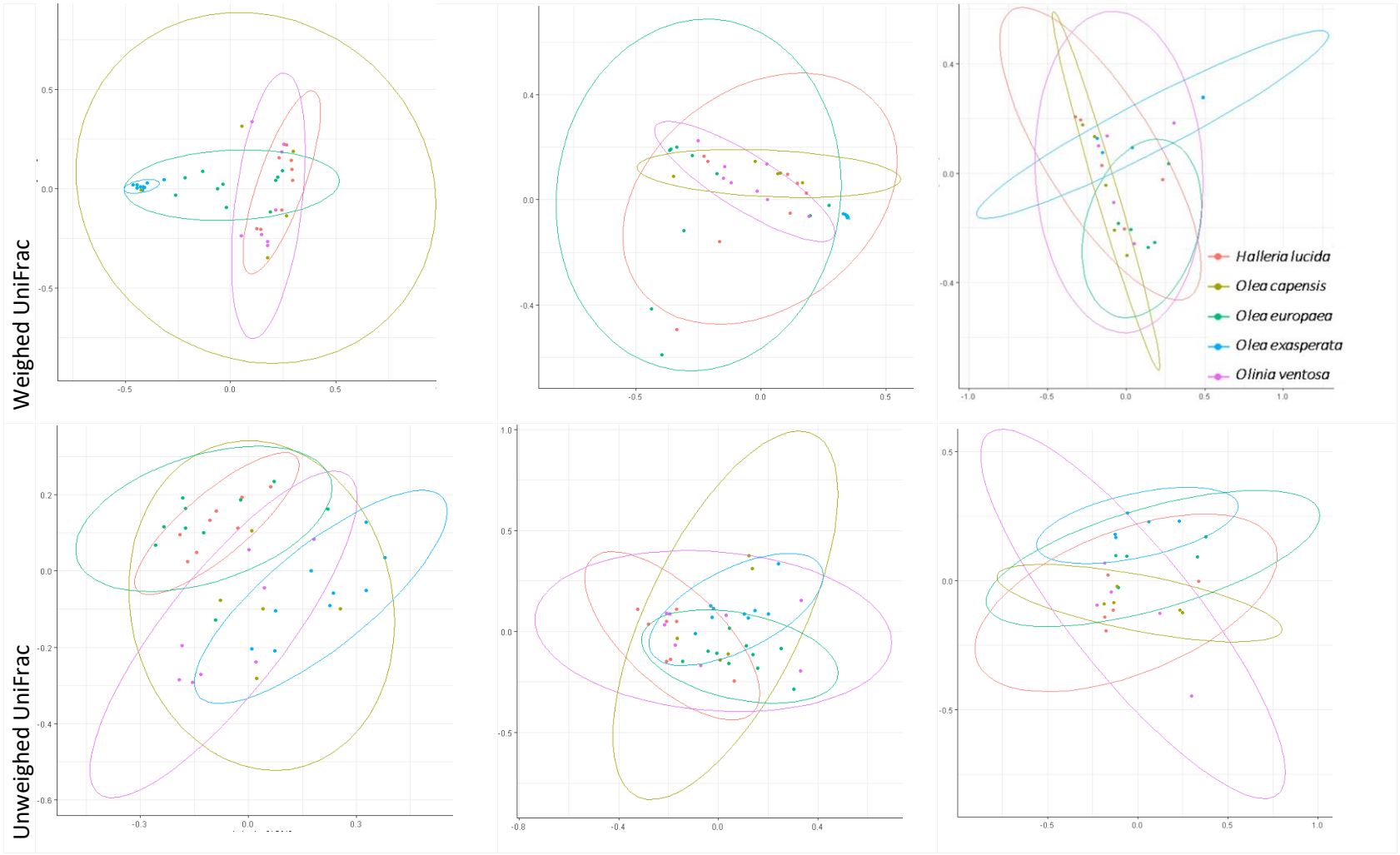


Figure 4: Non-Metric Multidimensional Scaling plots of host fungal endophyte assemblages based on Bray-Curtis, Jaccard and UniFrac (weighed and unweighed) distances. Distance matrices were constructed using balanced and unbalanced sample size and using the core fungal assemblages (fungal endophytes present in at least 50 % of the samples).

Comparisons of phylogenies representing the phylogenetic placement of the host taxa and that based on balanced fungal endophyte assemblages in the tanglegrams were incongruent (Figure 5). The forest hosts (*O. ventosa, H. lucida* and *O. capensis* subsp. *capensis*) frequently grouped together (Figure 5a, b, d, f and h), while the two non-forest *Olea* species (*O. exasperata* and *O. europaea* subsp. *cuspidata*) were basal groups that were occasionally more similar to each other than to the other three hosts (Figure 5a, b, d and f). Interestingly, despite *H. lucida* residing in the Lamiales with *Olea*, core assemblages (Jaccard distance) of *O. capensis* subsp. *capensis* were phylogenetically more like those within *O. ventosa* than within *H. lucida* (Figure 5f). Similarly, whole assemblages, as measured using Bray-Curtis distances, revealed more closely related assemblages between *O. capensis* subsp. *capensis* and *O. ventosa* than with *H. lucida* (Figure 5a). Despite being the most closely related, the Ligustroides hosts (*O. capensis* subsp. *capensis* and *O. exasperata*) only harboured closely related assemblages when incorporating fungal endophyte phylogenetic similarities and abundance data (Figure 5c). Similar to the phylogenies created from the balanced sampling design, tanglegrams constructed with the unbalanced datasets were also predominantly incongruent, with forest taxa harbouring more similar fungal endophytes than closely related hosts from different biomes (Figure S2).

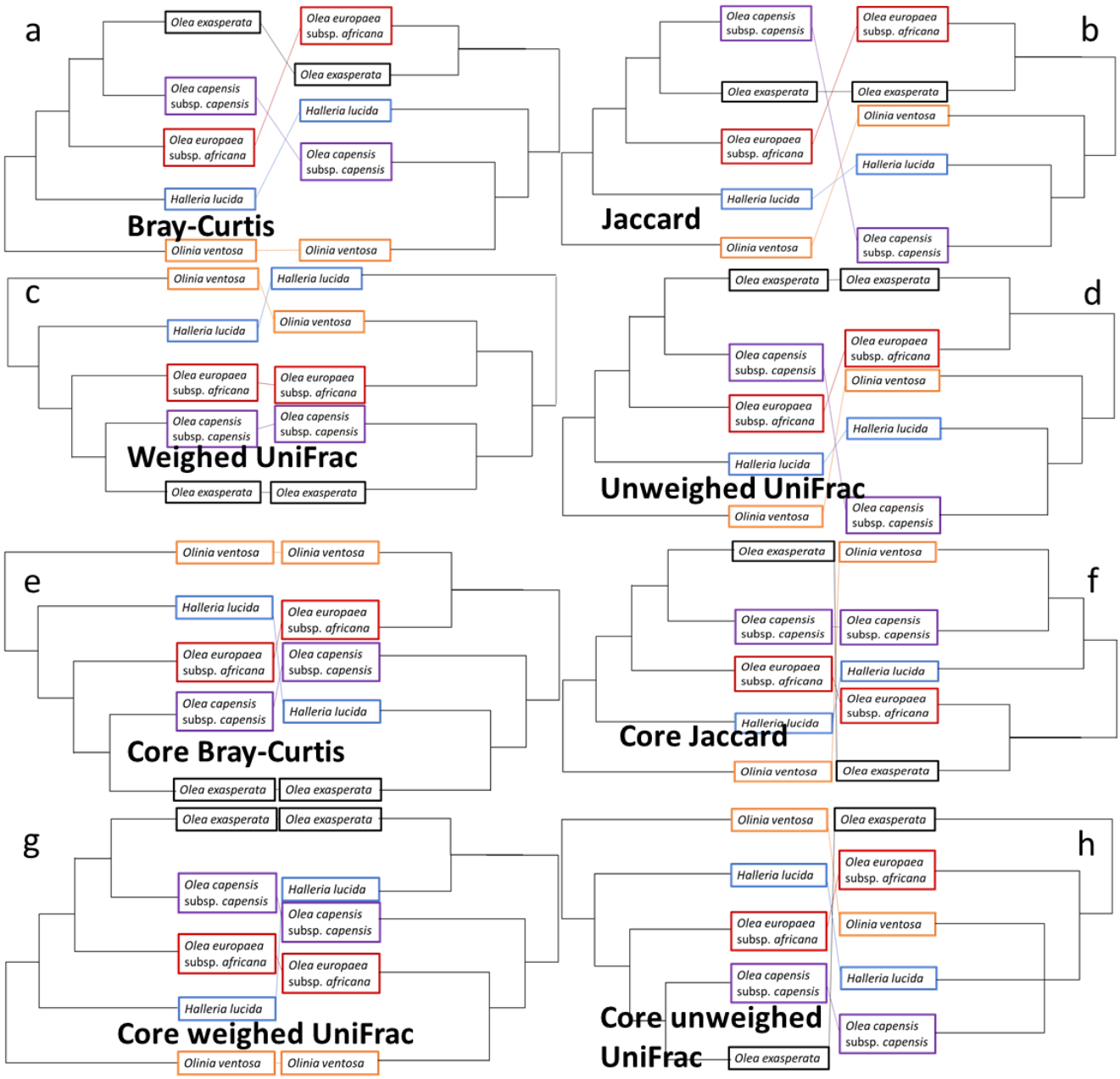


Figure 5: Host sequence-based phylogeny and fungal endophyte assemblage-based phylogenies (based on a balanced design dataset) comparisons visualised as tanglegrams. Host sequence-based trees constructed from plant-phylogenetic markers (TrnSG, MatK, PsbA-TrnH, and RbcL) are presented on the left side of each figure and fungal endophyte assemblage-based phylogenies are presented on the right side. Assemblage-based phylogenies were constructed using whole (a, b, c, and d) and core (e, f, g and h) OTU assemblages. Bray-Curtis and weighed UniFrac distance matrices were constructed based on abundance data, while Jaccard and unweighed UniFrac distance matrices were constructed based on incidence data.

Fungal assemblage-based distance matrices (Bray-Curtis, Jaccard and UniFrac distances) were not correlated to the phylogenetic distances between hosts (Table 6). This was consistently the case when considering both balanced and unbalanced sampling deigns.

Table 6: Correlations between phylogenetic distance (*'cophenetic'* distance) and fungal endophyte assemblage compositional differences based on Jaccard, Bray-Curtis and UniFrac dissimilarity matrices. Assemblage-based distances were constructed from the abundances and OTU count (richness) of all the sampled trees and the full OTU complement (whole abundance and whole richness). Core OTU abundance and richness distance matrices as calculated using Bray-Curtis, Jaccard, and weighed and unweighed UniFrac distances were also tested for correlation to host relatedness. Additionally, correlation between phylogenetic distances and OTU assemblages based on the reduced and balanced sample size (n = 25). Correlations are meaningful at $r > 0.5$ and significant when $p < 0.05$ (*).

	Distance	Mantel's R	*p*
Whole abundance	Weighed UniFrac	-0.621	0.950
Whole abundance	Bray-Curtis	-0.318	0.875
Core abundance	Weighed UniFrac	-0.594	0.883
Whole richness	Unweighed UniFrac	-0.152	0.525
Whole richness	Jaccard	-0.177	0.608
Core richness	Unweighed UniFrac	-0.684	0.908
Balanced abundance	Weighed UniFrac	0.088	0.283
Balanced abundance	Bray-Curtis	-0.570	0.833
Balanced richness	Unweighed UniFrac	-0.104	0.317
Balanced richness	Jaccard	0.316	0.225

Overall patterns of shared and unique OTUs remained largely consistent between the two datasets (balanced and unbalanced) (Figure 6). *Olea europaea* subsp. *cuspidata* harboured the highest number of unique OTUs. Very few OTUs were found within all hosts. The three *Olea* species shared less OTUs than the three distantly related forest hosts. Specifically, the three *Olea* species shared 18 and 12 OTUs (full dataset and balanced dataset, respectively), while the three forest taxa shared 28 and 17 OTUs for the two respective datasets.

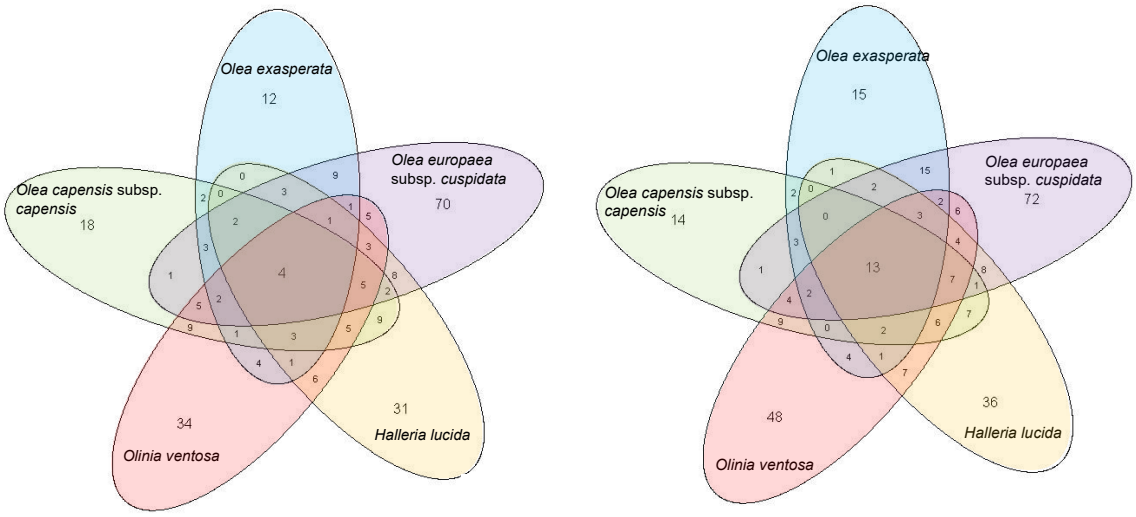


Figure 6: Venn diagram visualisation of the distribution of fungal endophyte OTUs among the examined host plants. Venn diagrams were based on the balanced (left) and unbalanced (right) datasets. Details of taxa shared between all samples (unbalanced) are given in Table S2.

Overall, average similarities between samples within hosts were below 50.000 % when the total OTU assemblage was considered (Table 7). Specifically, overall similarities of the fungal endophyte assemblages within hosts were 44.110 % (*O. exasperata*), 36.470 % (*H. lucida*), 32.920 % (*O. europaea* subsp. *cuspidata*), 27.430 % (*O. capensis* subsp. *capensis*) and 25.650 % (*O. ventosa*) (Table 2). Contribution per OTU was conservative with many OTUs contributing up to 90.000 % of the total similarities within hosts; namely 34, 31, 30, 20 and 19 OTUs contributed to *O. europaea* subsp. *cuspidata*, *O. ventosa*, *H. lucida*, *O. capensis* subsp. *capensis* and *O. exasperata*, respectively. However, when the sample size was balanced, a handful of overrepresented taxa constituted 90.000 % of the contributions, specifically, five taxa per host (*O. ventosa*, *H. lucida*, *O. capensis* subsp. *capensis* and *O. exasperata*) and three in *O. europaea* subsp. *cuspidata* accounted for 90.000 % of the similarities within hosts.

The highest contributing taxon (16.830 %) in *O. capensis* subsp. *capensis* twigs (unbalanced) was an apparently undescribed *Phoma* species (OTU68). This taxon was present in all hosts, but with lower contributions to overall similarities in the other hosts (Table S3). An undescribed taxon (OTU40) accounted for the highest similarity contribution (10.180 %) within *O. ventosa* (unbalanced) and was also found in the other forest hosts, but with a lower contribution. *Olea europaea* subsp. *cuspidata* assemblages were dominated by the highest contribution (9.870 %) of *Neophaeomoniella niveniae* (Crous) Crous (OTU26), which was also present in the rest of the hosts. The highest contributing taxon

(12.180 %) in *O. exasperata* was an undescribed taxon (OTU5) with the closest BLAST match to *Myrtapenidiella c.f*. *sporadicae* Crous. An undescribed *Aureobasidium* Viala & G. Boyer contributed the most (6.470 %) to *H. lucida* and occurred in two additional Lamiales hosts, *O. capensis* subsp. *capensis* and *O. europaea* subsp. *cuspidata* (Table S3).

Average dissimilarity in the fungal endophyte assemblages was highest between *O. europaea* subsp. *cuspidata* and *O. ventosa* (balanced and unbalanced). Average dissimilarity was lowest between *O. capensis* subsp. *capensis* and *O. ventosa* when unbalanced and lowest between *O. capensis* subsp. *capensis* and *O. exasperata* when balanced (Table 7). Interestingly, the forest Lamiales hosts, *H. lucida* and *O. capensis* subsp. *capensis*, were not the least dissimilar of the forest hosts. *Olea capensis* subsp. *capensis* was more dissimilar from *H. lucida* than it was from *O. ventosa.*

The number of taxa that contributed (90.000 % cut-off) to the dissimilarities between hosts ranged between 85 (between *O. capensis* subsp. *capensis* and *O. exasperata*) and 154 (between *O. europaea* subsp. *cuspidata* and *Olinia ventosa*) (results only presented for those taxa contributing at least 1.000% to the 90.000 %; Table S4). *Phaeothecoidea intermedia* Crous & Summerell (OTU79), *M.* c.f *sporadicae* (OTU5) and *Angustimassarina acerina* Jayasiri, Thambug., R.K. Schumach. & K.D. Hyde (OTU66) were the highest contributing OTUs to the pairwise dissimilarities between hosts contributing to three, two and two pairs of hosts each, respectively (Table S4). *Parateratosphaeria altensteinii* (Crous) Quaedvl. & Crous (OTU54), an undescribed fungus (OTU31) and *Lophiostoma cynaroidis* Marincowitz, M.J. Wingf. & Crous (OTU95) were the highest contributing taxa to dissimilarities between one pair of hosts each. Taxa contributions to dissimilarity percentages between hosts when the sample size was balanced ranged between six and eight, and all of these taxa were a subset of taxa recovered as highest contributors under the unbalanced design (results not shown).

Table 7: Summary SIMPER results of fungal endophyte percentages of average similarities and dissimilarities identified within and between the five sampled hosts, respectively. Results are based on balanced (B) and unbalanced (U) datasets. The breakdown of the contributions of each taxon to the average similarities and those contributing to average dissimilarities between hosts are presented in Tables S3 and S4. The breakdown of the taxon contribution in the balanced design-based calculation constituted a subset of the taxa in the unbalanced design, thus only the unbalanced design-based breakdown is shown.

Host			Ave. similarity (%) B	Ave. similarity (%) U
			76.340	44.110
Olea exasperata			65.820	27.430
O. capensis subsp. *capensis*			53.000	32.920
O. europaea subsp. *cuspidata*			52.060	36.470
Halleria lucida			48.000	25.650
Olinia ventosa				
Host 1	Host 2	# of OTUs	Ave. dissimilarity (%) B	Ave. dissimilarity (%) U
O. europaea subsp. *cuspidata*	*O. ventosa*	154	65.380	88.810
O. europaea subsp. *cuspidata*	*O. capensis* subsp. *capensis*	124	61.610	87.340
O. exasperata	*Olinia ventosa*	112	49.810	86.970
Halleria lucida	*Olea exasperata*	101	36.700	86.860
Olea europaea subsp. *cuspidata*	*Halleria lucida*	140	50.780	86.280
Halleria lucida	*Olinia ventosa*	125	55.590	85.350
Olea europaea subsp. *cuspidata*	*Olea exasperata*	112	45.700	83.060
Olea capensis subsp. *capensis*	*Olea exasperata*	85	32.880	82.060
Olea capensis subsp. *capensis*	*Halleria lucida*	99	41.890	80.810
Olea capensis subsp. *capensis*	*Olinia ventosa*	108	45.770	79.470

4.5 Discussion

I set out to determine the role of host relatedness as an indicator of the level of similarity in fungal endophyte taxa in five tree/shrub species in a biodiversity hotspot. The results showed that different plant species host different endophyte assemblages. This pattern was consistent across the different analyses. These results show how hosts can act as an uptake filter for fungal endophyte assemblages from the environment. There was no evidence, however, that the phylogenetic distance between hosts correlated with the degree of overlap (or similarity) in fungal endophyte assemblages. Instead, the effect of the surrounding environment may have a more substantial effect than host relatedness. Specifically, hosts from the same biome (forest or fynbos) tended to have more similar assemblages, irrespective of host relatedness. Some economically and ecological important endophytic fungal taxa encountered within the five hosts are well-known from native hosts in South Africa. Many taxa,

however, could not be characterised at family level, emphasising the extent of undiscovered fungal taxa in underexplored native hosts and regions.

Host species was important in differentiating fungal endophyte assemblages across all five hosts. Host identity is well-known to significantly influence fungal endophyte assemblages. For example, different *Agave* L. hosts species harboured significantly different assemblages in Mexico and USA (Coleman-Derr et al., 2016). Similarly, the importance of host identity to fungal endophyte assemblages has also been observed in shrubs and trees in the Sonoran Desert (Massimo et al., 2015) and for endophytes within the leaves of trees within a South American temperate rainforest (González-Teuber et al., 2020). In the latter study, the authors demonstrated that leaf resistance traits drove the significant differences in assemblages between hosts. It is expected that differences in twig traits (e.g. twig anatomy and/or physiology) between hosts in the present study may have contributed to the observed patterns. To this effect, the twigs sampled in the present study varied in scent, texture, and toughness. Their physiology may have played a role in the observed patterns. The influence of host identity on the fungal endophyte assemblages of five tree species in a temperate hardwood forest of Michigan (USA) was linked to organ pH, total phenolic content, and nitrogen (Pellitier et al., 2019). The importance of host species to fungal endophyte assemblages can be attributed to underlying host-related traits. In this sense, different hosts, or host-related traits can serve as endophyte uptake filters where only specific endophytes can be acquired and maintained in the tissues of specific host species.

In this study, co-occurring hosts (rather than taxonomically similar hosts) harboured similar fungal assemblages. This was surprising as host morphology and physiology/chemistry are expected to increase in similarity between increasingly closely related hosts. This has been seen, for example, in a set of co-occurring Asteraceae plants in a semi-arid grassland in Germany where host phylogeny was the most important predictor of fungal endophyte assemblages in roots (Wehner et al., 2014). In Canada, where closely related hosts also shared their habitat, host phylogeny also significantly contributed to fungal endophyte assemblage structures (Higgins et al., 2007). However, this link between host phylogeny and endophyte selective traits is by no means the norm. In a common garden experiment investigating foliar fungal endophyte assemblages within Asteraceae hosts, host relatedness had no influence on assemblages (Whitaker et al., 2020). These authors proposed that fungal endophytes could be more severely affected by factors related to the incredibly diverse leaf chemistry documented in the Asteraceae which, in their case, was not phylogenetically tethered (Calabria et al., 2007). It seems clear that, when hosts share the same native habitat, a close link may exist between host phylogeny and fungal endophyte assemblages. This is because closely related hosts

often share habitat preferences (Emerson and Gillespie, 2008), and may recruit and favour related fungi that share their affinity for similar environments (Horn et al., 2014). However, when related hosts possess different adaptive traits and inhabit different environments this may lead to less related microbial assemblages with the degree of dissimilarity correlated with degree of dissimilarity in habitat characteristics rather than host relatedness. In the present study, co-occurring hosts were distantly phylogenetically related, while the close relatives were found in different habitats, thus it is possible that that the apparent lack of a phylogenetic signal may be due to a dominating role of habitat-related factors. It is also plausible that the limited sample size contributed to the apparent lack of phylogenetic signal. Further studies are needed to untangle these possible reasons to ascertain the driving forces behind the patterns observed in the hosts sampled from HPNBG.

The role of different habitats in dictating fungal endophyte assemblages in this study is not unique, it has been established in a number of systems. For example, in root fungal endophytes of halophytic *Inula crithmoides* Spreng. and non-halophytic *I. viscosa* (L.) Aiton, soil salinity gradient shaped these endophytes (Maciá-Vicente et al., 2012). Edaphic properties also influenced fungal endophyte assemblages associated with hosts such as *Populus deltoides* W. Bartram ex Marshall, *Andropogon gerardii* Vitman, *Bothriochloa ischaemum* (L.) Keng, and *Cypripedium acaule* Aiton (Bonito et al., 2014; Bunch et al., 2013; Henning et al., 2021; Jia et al., 2020). In the present study edaphic factors were presumably quite dissimilar between the different habitats (Mucina and Rutherford, 2006), but this factor alone seems unlikely to influence endophyte assemblages in twigs. A more plausible explanation is that a multitude of factors that dictate host plant assemblages (e.g. moisture availability, sunlight, slope, fire) would also influence the predominant endophyte assemblages (Giauque and Hawkes, 2013; Koide et al., 2017; Qian et al., 2018; Unterseher et al., 2016; Zimmerman and Vitousek, 2012). In this sense, even if every host acts as a differential fungal endophyte uptake filter, the pool of possible endophytes that can be acquired will be constrained by the environment. This will lead to more homogenous endophyte assemblages between different plant species that inhabit similar environments.

A total of thirteen OTUs were shared across all five hosts. The most common taxon was identified as a *Phoma* sp., occurring in 33 of the 45 samples, including all five *O. capensis* subsp. *capensis* plants. An additional three OTUs, also identified as *Phoma* species, occurred in *H. lucida*, *O. exasperata* and *O. europaea* subsp. *cuspidata* (one taxon per host). *Phoma* species are notorious olive pathogens known to cause branch dieback (Rhouma et al., 2010). The genus is also known to contain numerous other disease-causing species in South Africa; *Phoma encephalarti* Negodi on *Encephalartos* (Crous et

al., 2008), *Phoma glomerata* (Corda) Wollenw. & Hochapfel*, Phoma sorghina* (Sacc.) Boerema, Dorenb. & Kesteren, and *Phoma* sp. from agricultural crops, e.g*.,* wheat (*T. aestivum*), sorghum grains (*Sorghum bicolor* (L.) Moench), olives (*O. europaea* subsp. *europaea*) and apples (*Malus domestica* L.) (Aveskamp et al., 2010; Basson et al., 2019; Chen et al., 2015; Crous et al., 1995; Pažoutová, 2009). The most common endophyte was *N. niveniae.* This species occurred in all hosts including all ten *O. europaea* subsp. *cuspidata* samples. *Neophaeomoniella niveniae* was first discovered at the Harold Porter National Botanical Garden causing leaf spots and leaf tip blight on *Nivenia stokoei* (Guth.) N.E.Br. (Crous et al., 2011; 2015). It was later found in association with trunk diseases in *O. europaea* subsp. *europaea* and *O. europaea* subsp. *cuspidata* (Spies et al., 2020).

Species-level identification of many of the taxa identified in this study was not possible. In those that could be identified, the presence of many taxa known to be plant pathogens in asymptomatic tissues adds credence to the notion that many of these fungal endophytes may be latent pathogens (Crous et al., 2011; Quaedvlieg et al., 2014). These fungal endophytes included taxa in genera such as *Neocatenulostroma*, *Parateratosphaeria* and *Xenoteratosphaeria*, which are particularly known for accommodating pathogens and saprobes (Quaedvlieg et al., 2014).

A large proportion of taxa metabarcoded within hosts were exclusively associated with a single host. A total of 185 taxa were only associated with a single host, 80 of which could not be placed at family level therefore I could not deduct anything about their possible ecological functions. Despite the availability of data on two additional *Olea* species, *O. europaea* subsp. *cuspidata* harboured the highest number of exclusive taxa (72 of the 124). The ITS marker utilised in metabarcoding studies is conservative and the amplicons are often short and rely on data from culture-based techniques for classification, which only covers a conservatively approximated ~1.5 percent of all known fungi (Bengtsson-Palme et al., 2013). It is therefore not surprising that a large number of fungal endophytes uncovered in these previously unexplored habitats and hosts appear to be new to science. Given the conservative nature of the ITS gene region, it is possible that this number of taxa thought to be unique to sampled hosts may be even higher. Additionally, the sample sizes I used were limited, thus even if the marker used was more sensitive, the fungal endophyte richness would still likely be underestimated. Protected areas preserve a wealth of information on fungal endophyte diversity and their respective ecological properties. The large number of unique taxa per host indicates that, even in a shared habitat and in related hosts, host-filtering is strong.

4.6 Conclusion

The mix of genera, habitats and the different host ranges explored allowed for a consideration of the dynamics of fungal endophyte assemblages within native tree/shrub hosts of South Africa. Although host phylogeny was not a major determinant of fungal endophyte assemblages in the Core Cape Subregion, it is apparent that fungal endophyte assemblages, much like the plant diversity in this region, are influenced by a complex interaction between biotic and abiotic factors. In the present study, at least 40 % of the taxa barcoded may represent undescribed taxa. The present study contributes at least 125 undescribed fungal endophytes from the Kogelberg Biosphere to the growing number of fungi detected at the tip of South Africa. These findings illustrate the value of fungal diversity studies in floral biodiversity hotspots for possible biotechnologically useful metabolites. The strong influence of host identity, considering the florally diverse Core Cape Subregion, suggests that fungal endophytes may be hyper-diverse in this biodiversity hotspot.

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4.1 Supplementary materials

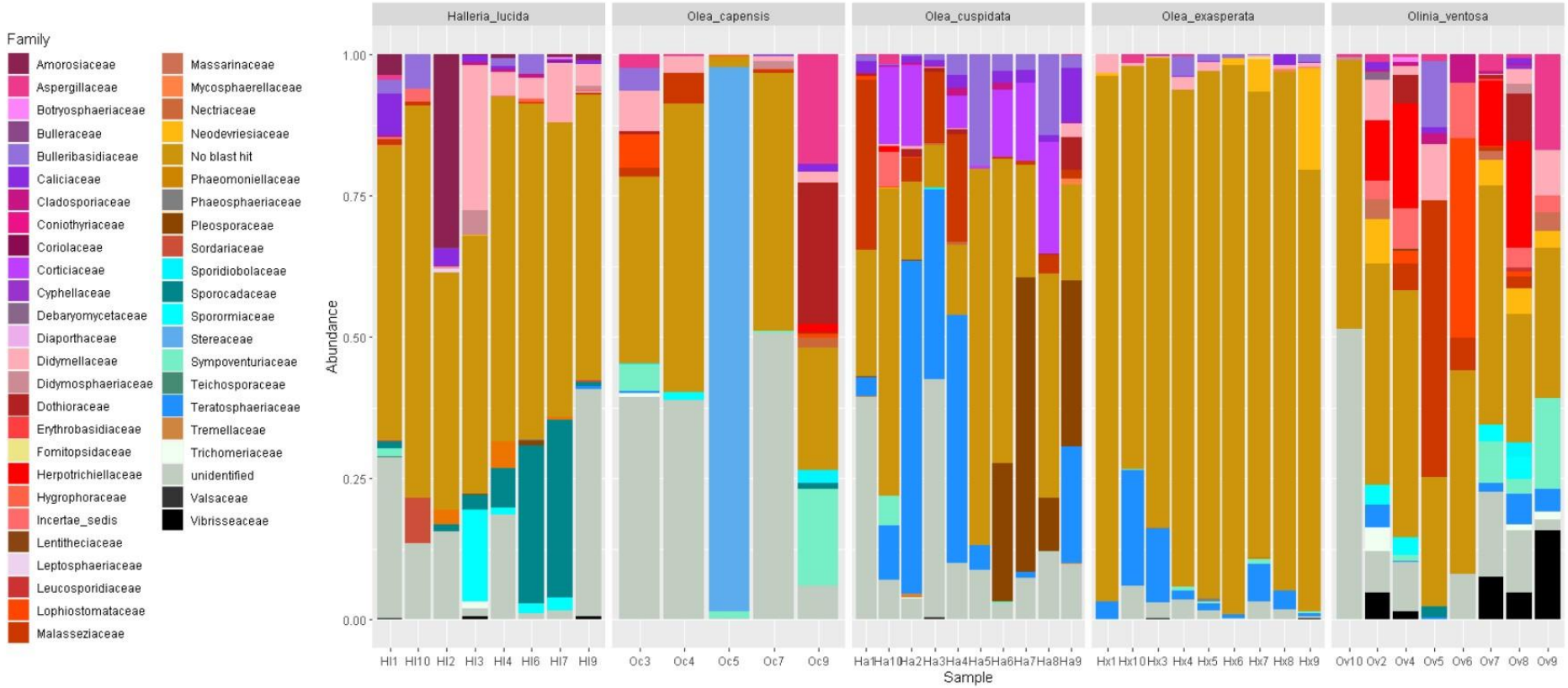

Figure S1: 100 % stack plots of relative contribution of the sampled families within samples of the five hosts, including contribution of taxa that had no blast hits and taxa that could not be placed at family level. *Olea cuspidata* = *Olea europaea* subsp. *cuspidata* and *Olea capensis* = *Olea capensis* subsp. *capensis*

Table S1: Pairwise t-test results summarizing pair-wise comparisons of core rarefied richness (left) and core abundance (right) between hosts. Results are significant when $p < 0.05$ (denoted by *).

Host 1	Host 2	adjusted *p*	adjusted *p*
Olea capensis subsp. *capensis*	*Halleria lucida*	0.587	0.480
Olea europaea subsp. *cuspidata*	*Halleria lucida*	0.753	0.097
Olea exasperata	*Halleria lucida*	0.020*	0.003*
Olinia ventosa	*Halleria lucida*	0.750	0.494
Olea europaea subsp. *cuspidata*	*Olea capensis* subsp. *capensis*	0.404	0.033*
Olea exasperata	*Olea capensis* subsp. *capensis*	0.011*	0.001*
Olinia ventosa	*Olea capensis* subsp. *capensis*	0.792	0.195
Olea exasperata	*Olea europaea* subsp. *cuspidata*	0.030*	0.119
Olinia ventosa	*Olea europaea* subsp. *cuspidata*	0.516	0.337
Olinia ventosa	*Olea exasperata*	0.009*	0.019*

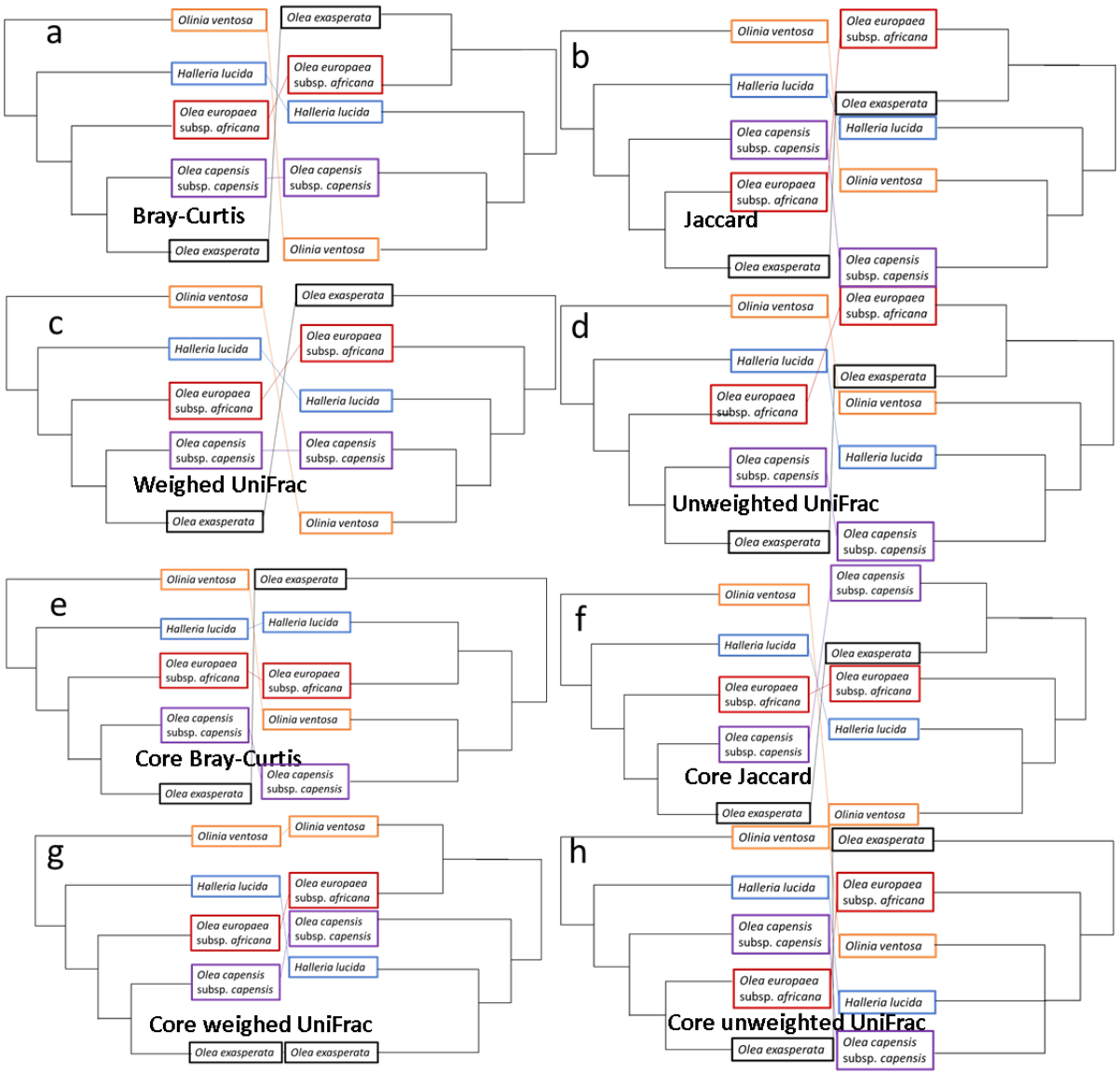

Figure S2: Comparative hierarchical clusters based on host phylogeny and fungal assemblage taxonomy. Tanglegrams constitute host phylogeny on the left with a fungal endophyte assemblage dendrograms rotated and lines drawn to connect host names to highlight congruency (if present). Plots a-d constitute assemblage dendrograms based on all assemblages from all the samples collected within each host. Plots e-h only consider the core fungal assemblages as defined as the most commonly encountered taxa within samples.

Table S2: Taxa that were shared between all hosts and the number of samples per host within which they appeared. In cases where BLAST identities, query sequence coverage (Cover%) and sequence match (identity percentage) are also reported as a measure of identification confidence. The number of a samples within each host that the taxon appeared in are also presented. *Olea europaea* = *Olea europaea* subsp. *cuspidata* and *Olea capensis* = *Olea capensis* subsp. *capensis.*

		BLAST							
Closest match	BLAST/ UNITE	Cover%	% ID	OTU	*Olea exasperata*	*Olea capensis*	*Olea europaea*	*Halleria lucida*	*Olinia ventosa*
Alternaria alternata	BLAST	100	100	OTU3	1	1	5	4	2
Aureobasidium sp.	BLAST	100	100	OTU8	3	2	7	8	1
Uncultured fungus clone	BLAST	100	96.55	OTU13	4	2	7	2	5
Cladosporium sp.	BLAST	100	100	OTU25	2	2	1	6	1
Neophaeomoniella niveniae	BLAST	100	100	OTU26	8	2	10	6	2
Uncultured fungus clone	BLAST	100	97.7	OTU48	5	4	1	1	4
Verticillium sp.	BLAST	100	100	OTU60	1	2	1	1	2
Phoma sp.	UNITE			OTU68	8	5	7	7	6
No BLAST Hit	Both			OTU88	1	1	2	2	3
Aspergillus carbonarius	UNITE			OTU103	6	4	3	3	7
No BLAST Hit	UNITE			OTU118	2	2	2	2	5
Uncultured fungus clone	BLAST	100	93.68	OTU161	4	3	5	4	3
No BLAST Hit	Both			OTU178	1	2	1	2	2

Table S3: Fungal endophyte contributions (%) to similarities observed within hosts (cut-off= 90 %).

OTUID	Closest match	*Olea exasperata*	*Olea capensis*	*Olea cuspidata*	*Halleria lucida*	*Olinia ventosa*
OTU1	*Teratosphaericola* sp.			2.630		
OTU3	*Alternaria alternata*			2.240		
OTU4	*Marchandiomyces quercinus*			5.340		
OTU5	*Myrtapenidiella c.f sporadicae*	12.180				
OTU7	*Alternaria* sp.			2.130		
OTU8	*Aureobasidium* sp.		1.490	3.880	6.470	
OTU9	Uncultured fungus clone			1.810		
OTU10	*Teratosphaeria c.f. zuluensis*			2.630		
OTU13	Uncultured fungus clone	1.620	1.460	4.340		5.910
OTU16	Uncultured fungus clone		1.240	2.480		
OTU21	*Phoma* sp.	1.460			4.350	
OTU24	*Alternaria infectoria*				1.830	
OTU25	*Amandinea punctata*		1.240		4.740	
OTU26	*Vishniacozyma carnescens*	8.750		9.870	5.040	
OTU28	*Alysidiella parasitica*					1.960
OTU29	Uncultured fungus clone				4.720	
OTU31	Uncultured fungus clone	9.350				
OTU33	Uncultured fungus clone		4.140	4.160	1.000	2.050
OTU35	Uncultured fungus clone			3.580		
OTU40	Uncultured fungus clone		4.140		3.230	10.180
OTU41	*Pestalotiopsis* sp.		4.140			
OTU42	*Neodevriesia lagerstroemiae*	4.740				
OTU44	*Foliophoma* sp.				6.470	
OTU46	*Foliophoma* sp.	5.950	1.240			0.940
OTU47	*Perusta inaequalis*					0.940
OTU48	Uncultured fungus clone	2.560	9.560			2.270
OTU50	*Cyphellophora c.f. pluriseptata*			2.320		
OTU51	*Phoma sp.*				2.950	
OTU53	Uncultured fungus clone			0.910		
OTU54	*Parateratosphaeria altensteinii*	12.180				
OTU57	Uncultured fungus clone			1.790		
OTU59	*Peniophora* sp.				1.740	

OTU60	*Verticillium* sp.		2.800			
OTU61	Uncultured fungus clone			3.230		
OTU62	Uncultured fungus clone			3.140		
OTU63	Uncultured fungus clone			2.290		3.810
OTU64	*Lophiostoma corticola*			0.850	1.780	
OTU66	*Angustimassarina acerina*				6.470	
OTU68	*Phoma* sp.	8.850	16.830	3.580	6.470	6.390
OTU71	Uncultured fungus clone			2.630		
OTU78	Uncultured fungus clone			0.920		
OTU79	*Amandinea decedens*	1.440		7.010		
OTU82	*Mycocalicium victoriae*			2.730		
OTU83	*Vibrissea sp.*					3.780
OTU85	Uncultured fungus clone			1.870		
OTU86	*Armillaria* sp.				3.290	
OTU88	*Neophaeomoniella zymoides*					1.910
OTU89	*Phaeomoniella zymoides*			2.790	1.090	
OTU92	*Furfurella c.f. nigrescens*	1.820				
OTU95	*Lophiostoma cynaroidis*		9.560			
OTU103	*Aspergillus carbonarius*	4.360	8.110			10.000
OTU112	No blast hit			0.830		
OTU113	*Neodevriesia simplex*					2.190
OTU114	*Teratosphaeria cf. bellula*			1.180		
OTU117	Uncultured fungus clone				2.070	
OTU118	*Helicoubisia c.f. coronata*		1.240			3.910
OTU119	*Preussia* sp.				3.390	1.960
OTU121	Uncultured fungus clone					0.900
OTU122	*Endosporium* sp.					2.510
OTU130	*Pestalotiopsis verruculosa*		1.490			
OTU133	*Pyrenochaeta corni*	2.560				
OTU135	*Neophaeothecoidea proteae*			0.850		
OTU142	Uncultured fungus clone					2.510
OTU146	Uncultured fungus clone					0.900
OTU160	Uncultured fungus clone					1.960
OTU161	Uncultured fungus clone	1.440	3.650	1.640	1.920	1.070
OTU164	Uncultured fungus clone			1.510		
OTU170	Uncultured fungus clone	3.110				

OTU172	*Neofusicoccum australe*		8.110		1.600	
OTU180	Uncultured fungus clone			1.150		
OTU183	Uncultured fungus clone		1.460			
OTU190	Uncultured fungus clone	4.050				
OTU191	*Penicillium* sp.					3.160
OTU192	Uncultured fungus clone					0.940
OTU196	*Lophiostoma macrostomoides*					0.940
OTU197	Uncultured fungus clone					1.160
OTU198	Uncultured fungus clone					0.940
OTU201	Uncultured fungus clone				1.590	
OTU207	*Neophaeomoniella eucalypti*			1.150		
OTU209	Uncultured fungus clone					2.420
OTU210	Uncultured fungus clone	2.880				
OTU222	Uncultured fungus clone		4.190			
OTU226	*Massarina albocarnis*					1.160
OTU259	*Symmetrospora c.f. gracilis*				2.360	
OTU313	*Paraconiothyrium* sp.				1.900	
OTU323	*Arthrocatena tenebrio*				1.920	
OTU328	No blast hit			1.110		
OTU333	*Neurospora terricola*				1.950	
OTU354	No blast hit					6.550
OTU363	*Dothiorella iberica*		4.240			
OTU391	Uncultured fungus	1.580				
OTU402	*Erythrobasidium* sp.				3.250	
OTU414	*Devriesia* sp.					4.380
OTU434	*Diaporthe anacardii*				1.800	
OTU469	Uncultured fungus				1.110	
OTU470	*Tremella sp.*				2.850	
OTU494	*Teratosphaeria knoxdaviesii*					1.180
OTU581	*Cladosporium ramotenellum*				0.950	

Table S4: OTU contribution to dissimilarity percentages between hosts. Only taxa contributing at least 1 % to the total dissimilarity are reported. The closest UNITE/BLAST matches of the OTUs are also provided. *Olea europaea* = *Olea europaea* subsp. *cuspidata* and *Olea capensis* = *Olea capensis* subsp. *capensis.*

Olea europaea and *Olea capensis*

Species	Diss	Diss/SD	Contr%	Cum.%
OTU79	1.65	1.9	1.88	1.88
OTU4	1.52	1.16	1.73	3.62
OTU48	1.48	1.21	1.69	5.31
OTU95	1.41	1.11	1.61	6.93
OTU172	1.4	1.62	1.61	8.54
OTU26	1.33	0.97	1.52	10.06
OTU103	1.2	1.16	1.37	11.43
OTU61	1.17	1.03	1.34	12.77
OTU35	1.17	1.31	1.34	14.1
OTU62	1.16	1.02	1.33	15.43
OTU89	1.08	1.01	1.24	16.67
OTU363	1.08	1.08	1.24	17.9
OTU222	1.07	1.08	1.23	19.13
OTU13	1.07	0.91	1.23	20.36
OTU82	1.07	1.01	1.22	21.58
OTU8	1.06	0.87	1.21	22.79
OTU40	1.05	1.05	1.2	23.99
OTU41	1.05	1.04	1.2	25.2
OTU63	1.03	0.82	1.18	26.38
OTU1	1.02	1.08	1.17	27.55
OTU10	1.02	1.08	1.17	28.72
OTU71	1.02	1.08	1.17	29.89
OTU3	1.02	0.81	1.17	31.05
OTU60	1	0.71	1.14	32.19
OTU7	0.98	0.85	1.12	33.32
OTU9	0.96	0.69	1.1	34.42
OTU161	0.96	0.92	1.09	35.51
OTU16	0.95	0.96	1.09	36.61
OTU33	0.95	0.79	1.09	37.69
OTU50	0.93	1.12	1.07	38.76
OTU85	0.89	0.91	1.01	39.78

Halleria lucida and *Olinia ventosa*

Species	Diss	Diss/SD	Contr%	Cum.%
OTU66	1.51	2.06	1.77	1.77
OTU8	1.35	1.55	1.58	3.35
OTU44	1.35	1.55	1.58	4.93
OTU29	1.31	1.49	1.54	6.47
OTU354	1.27	1.42	1.49	7.96
OTU25	1.24	1.32	1.46	9.42
OTU21	1.19	1.35	1.39	10.81
OTU26	1.18	1.1	1.38	12.19
OTU13	1.17	1.04	1.37	13.56
OTU103	1.16	1.07	1.36	14.92
OTU402	1.14	1.08	1.34	16.25
OTU86	1.12	1.16	1.31	17.57
OTU414	1.07	1.1	1.25	18.82
OTU51	1.06	1.17	1.24	20.06
OTU470	1.03	1.17	1.21	21.27
OTU259	1.01	0.87	1.18	22.45
OTU118	0.98	1.07	1.15	23.6
OTU119	0.97	0.93	1.14	24.74
OTU83	0.96	1.09	1.13	25.87
OTU191	0.94	0.88	1.1	26.97
OTU161	0.93	0.87	1.09	28.06
OTU333	0.93	0.85	1.08	29.14
OTU117	0.92	0.92	1.08	30.22
OTU64	0.92	0.92	1.08	31.3
OTU323	0.92	0.85	1.08	32.37
OTU33	0.9	0.84	1.05	33.42
OTU24	0.89	0.89	1.05	34.47
OTU313	0.88	0.93	1.03	35.5
OTU434	0.86	0.92	1	36.5

Olea exasperata and *Olinia ventosa*

Species	Av.Diss	Diss/SD	Contr%	Cum.%
OTU54	2.27	2.64	2.61	2.61
OTU31	2.01	1.84	2.31	4.92
OTU40	1.89	1.8	2.18	7.1
OTU5	1.87	1.84	2.15	9.25
OTU26	1.58	1.26	1.82	11.06
OTU354	1.53	1.39	1.75	12.82
OTU42	1.47	1.17	1.7	14.51
OTU190	1.37	1.21	1.57	16.08
OTU46	1.34	1.05	1.55	17.63
OTU414	1.28	1.08	1.48	19.11
OTU13	1.22	0.88	1.4	20.51
OTU170	1.22	0.98	1.4	21.91
OTU210	1.18	0.98	1.36	23.27
OTU118	1.16	1.02	1.33	24.6
OTU191	1.15	0.87	1.33	25.92
OTU64	1.14	1.09	1.32	27.24
OTU83	1.12	1.05	1.29	28.53

OTU133	1.11	1.01	1.27	29.8
OTU48	1.11	0.92	1.27	31.07
OTU161	1.01	0.89	1.16	32.23
OTU88	1.01	0.74	1.16	33.39
OTU142	1	0.88	1.15	34.54
OTU209	1	0.85	1.15	35.69
OTU33	0.99	0.72	1.13	36.82
OTU122	0.98	0.92	1.13	37.95
OTU92	0.97	0.8	1.11	39.06
OTU103	0.94	0.7	1.08	40.15
OTU391	0.91	0.79	1.05	41.2
OTU113	0.91	0.9	1.05	42.25
OTU21	0.89	0.86	1.02	43.27

Olea europaea *Halleria lucida*

ID	Diss	Diss/SD	Contr%	Cum.%
OTU79	1.46	2.06	1.69	1.69
OTU44	1.41	1.98	1.63	3.32
OTU66	1.41	1.98	1.63	4.96
OTU4	1.31	1.24	1.51	6.47
OTU29	1.22	1.46	1.42	7.89
OTU25	1.17	1.33	1.36	9.25
OTU21	1.12	1.34	1.29	10.54
OTU13	1.1	1.17	1.28	11.82
OTU402	1.06	1.05	1.23	13.05
OTU35	1.05	1.35	1.21	14.26
OTU86	1.04	1.14	1.21	15.47
OTU40	1.03	1.01	1.19	16.66
OTU61	1.03	1.07	1.19	17.85
OTU62	1.02	1.06	1.18	19.03
OTU119	0.99	1.04	1.15	20.18
OTU51	0.98	1.15	1.14	21.32
OTU470	0.97	1.15	1.12	22.44
OTU82	0.94	1.05	1.09	23.53
OTU33	0.93	0.95	1.08	24.61
OTU259	0.91	0.85	1.06	25.67
OTU10	0.91	1.11	1.06	26.73
OTU71	0.91	1.11	1.06	27.79
OTU1	0.9	1.06	1.04	28.83
OTU63	0.9	0.87	1.04	29.87
OTU89	0.89	0.9	1.03	30.89
OTU16	0.88	1.13	1.02	31.91
OTU3	0.87	0.86	1.01	32.92
OTU161	0.86	0.85	1	33.93

Olea europaea and *Olinia ventosa*

ID	Diss	Diss/SD	Contr%	Cum.%
OTU79	1.5	1.94	1.69	1.69
OTU40	1.42	1.47	1.6	3.29
OTU26	1.38	1.32	1.55	4.84
OTU4	1.35	1.2	1.53	6.36
OTU354	1.22	1.34	1.38	7.74
OTU103	1.21	1.14	1.37	9.11
OTU8	1.08	1.08	1.21	10.32
OTU61	1.06	1.05	1.19	11.52
OTU414	1.03	1.06	1.16	12.67
OTU35	1.02	1.15	1.15	13.82
OTU62	1	0.97	1.13	14.95
OTU89	0.98	1.03	1.11	16.06
OTU82	0.97	1.03	1.09	17.15
OTU33	0.94	0.92	1.06	18.21
OTU83	0.94	1.12	1.06	19.27
OTU10	0.94	1.08	1.05	20.32
OTU71	0.94	1.08	1.05	21.38
OTU118	0.93	1	1.05	22.43
OTU63	0.93	0.85	1.04	23.47
OTU1	0.92	1.04	1.04	24.51
OTU191	0.9	0.84	1.02	25.53
OTU3	0.9	0.85	1.02	26.54
OTU16	0.9	1.11	1.01	27.55
OTU7	0.89	0.87	1	28.55
OTU64	0.89	0.95	1	29.55

Olea capensis *Olinia ventosa*

ID	Diss	Diss/SD	Contr%	Cum.%
OTU95	1.48	1.26	1.87	1.87
OTU354	1.45	1.35	1.83	3.69
OTU172	1.39	1.31	1.75	5.44
OTU414	1.22	1.05	1.54	6.98
OTU48	1.19	0.85	1.49	8.47
OTU13	1.18	0.87	1.48	9.96

OTU222	1.16	1.1	1.46	11.41
OTU363	1.15	1	1.44	12.86
OTU41	1.14	0.99	1.43	14.29
OTU64	1.11	1.11	1.39	15.69
OTU33	1.1	0.87	1.39	17.07
OTU83	1.1	1.12	1.38	18.45
OTU191	1.09	0.84	1.38	19.83
OTU60	1.07	0.79	1.35	21.18
OTU118	1.07	0.93	1.35	22.53
OTU161	1.05	0.96	1.32	23.85
OTU40	1.01	0.74	1.28	25.12
OTU209	0.99	0.84	1.24	26.37
OTU88	0.98	0.73	1.23	27.6
OTU142	0.96	0.86	1.2	28.8
OTU122	0.94	0.9	1.18	29.98
OTU119	0.9	0.89	1.14	31.11
OTU130	0.9	0.76	1.13	32.25
OTU8	0.89	0.77	1.12	33.36
OTU183	0.88	0.83	1.11	34.48
OTU46	0.88	0.9	1.11	35.58
OTU47	0.88	0.9	1.1	36.69
OTU113	0.87	0.88	1.09	37.78
OTU26	0.87	0.81	1.09	38.87
OTU28	0.86	0.94	1.09	39.96
OTU386	0.84	0.86	1.06	41.02
OTU312	0.84	0.75	1.05	42.07
OTU178	0.83	0.85	1.04	43.11

Olea exasperata and *Olea capensis*

ID	Diss	Diss/SD	Contr%	Cum.%
OTU5	2.57	2.43	3.14	3.14
OTU54	2.57	2.43	3.14	6.27
OTU95	2.04	1.4	2.49	8.76
OTU31	1.87	1.24	2.27	11.03
OTU42	1.67	1.14	2.03	13.06
OTU26	1.61	1	1.96	15.02
OTU172	1.55	1.19	1.89	16.91
OTU190	1.53	1.19	1.86	18.77
OTU46	1.45	0.99	1.76	20.54
OTU170	1.37	0.97	1.67	22.21
OTU363	1.36	1.14	1.66	23.87
OTU222	1.35	1.13	1.65	25.51
OTU60	1.35	0.74	1.64	27.16
OTU33	1.34	1.13	1.64	28.79
OTU40	1.34	1.13	1.64	30.43
OTU41	1.34	1.13	1.64	32.07
OTU48	1.32	0.79	1.61	33.68
OTU210	1.31	0.93	1.59	35.27
OTU133	1.23	1.01	1.5	36.77
OTU161	1.22	0.97	1.49	38.26
OTU13	1.2	0.89	1.46	39.72
OTU8	1.16	0.82	1.42	41.13
OTU103	1.14	0.74	1.39	42.52
OTU92	1.09	0.78	1.33	43.85
OTU25	1.04	0.78	1.27	45.12
OTU98	1.03	0.72	1.25	46.38
OTU391	1.03	0.77	1.25	47.63
OTU16	0.97	0.86	1.18	48.81
OTU118	0.95	0.86	1.16	49.97
OTU21	0.95	0.83	1.16	51.13
OTU79	0.95	0.82	1.16	52.29
OTU130	0.94	0.77	1.15	53.44
OTU183	0.93	0.77	1.14	54.57
OTU19	0.91	0.53	1.11	55.69
OTU50	0.89	0.85	1.08	56.77
OTU47	0.88	0.83	1.07	57.84
OTU178	0.88	0.83	1.07	58.91
OTU373	0.83	0.79	1.01	59.92
OTU263	0.82	0.79	1	60.92

Olea europaea and *Olea exasperata*

ID	Diss	Diss/SD	Contr%	Cum.%
OTU31	1.72	1.46	2.07	2.07
OTU4	1.6	1.21	1.92	3.99
OTU5	1.57	1.54	1.88	5.88
OTU54	1.57	1.54	1.88	7.76
OTU46	1.5	1.45	1.81	9.57
OTU33	1.37	1.24	1.65	11.22
OTU42	1.31	1.07	1.57	12.79
OTU190	1.26	1.17	1.52	14.31
OTU61	1.23	1.06	1.48	15.79
OTU35	1.22	1.34	1.47	17.26
OTU62	1.22	1.04	1.47	18.73

OTU79	1.18	0.96	1.42	20.15
OTU103	1.18	0.99	1.42	21.57
OTU8	1.14	0.95	1.38	22.95
OTU89	1.13	1.03	1.36	24.31
OTU82	1.12	1.03	1.35	25.66
OTU170	1.11	0.94	1.34	26.99
OTU13	1.1	0.91	1.33	28.32
OTU63	1.09	0.85	1.31	29.63
OTU210	1.09	0.95	1.31	30.94
OTU3	1.08	0.83	1.3	32.23
OTU1	1.07	1.1	1.29	33.52
OTU10	1.07	1.1	1.29	34.81
OTU71	1.04	1.01	1.26	36.06
OTU7	1.03	0.87	1.24	37.3
OTU133	1.02	0.99	1.23	38.53
OTU48	1.02	0.98	1.23	39.76
OTU9	1.02	0.71	1.22	40.98
OTU16	1.01	1.03	1.21	42.2
OTU50	0.98	0.97	1.18	43.38
OTU161	0.97	0.91	1.17	44.55
OTU85	0.93	0.92	1.12	45.67
OTU92	0.91	0.8	1.1	46.77
OTU57	0.91	0.91	1.09	47.86
OTU164	0.9	0.74	1.09	48.95
OTU68	0.9	0.66	1.08	50.03
OTU391	0.84	0.77	1.02	51.05

	Halleria lucida		*Olea capensis*	
ID	**Diss**	**Diss/SD**	**Contr%**	**Cum.%**
OTU66	1.66	2.08	2.06	2.06
OTU48	1.53	1.27	1.89	3.94
OTU95	1.51	1.26	1.87	5.81
OTU29	1.44	1.5	1.79	7.6
OTU44	1.44	1.43	1.78	9.38
OTU21	1.37	1.53	1.69	11.08
OTU402	1.27	1.04	1.57	12.64
OTU86	1.23	1.17	1.53	14.17
OTU26	1.2	0.93	1.49	15.66
OTU8	1.17	1.03	1.45	17.11
OTU103	1.17	1.09	1.45	18.56
OTU119	1.16	1.04	1.44	20
OTU25	1.15	1.01	1.42	21.42
OTU259	1.13	0.85	1.4	22.82
OTU222	1.12	1.13	1.39	24.2
OTU51	1.1	1.07	1.37	25.57
OTU363	1.1	1.07	1.36	26.93
OTU470	1.09	1.07	1.34	28.28
OTU172	1.07	0.95	1.32	29.6
OTU33	1.06	0.89	1.32	30.91
OTU41	1.04	0.98	1.29	32.2
OTU161	1.04	0.85	1.29	33.49
OTU333	1.03	0.83	1.28	34.77
OTU60	1.03	0.76	1.27	36.04

OTU323	1.02	0.82	1.27	37.31
OTU117	1.02	0.92	1.26	38.57
OTU40	1.02	0.82	1.26	39.83
OTU24	0.98	0.91	1.21	41.04
OTU313	0.96	0.93	1.19	42.23
OTU434	0.94	0.92	1.16	43.4
OTU64	0.93	0.92	1.15	44.55
OTU59	0.92	0.92	1.13	45.68
OTU130	0.88	0.84	1.09	46.77
OTU201	0.86	0.95	1.06	47.83
OTU46	0.84	0.86	1.03	48.86
OTU89	0.84	0.66	1.03	49.9
OTU13	0.82	0.8	1.02	50.91
OTU3	0.81	0.8	1	51.91

Halleria lucida *Olea exasperata*

ID	**Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
OTU5	2.17	2.91	2.5	2.5
OTU54	2.17	2.91	2.5	5
OTU31	1.92	1.94	2.21	7.22
OTU44	1.74	2.17	2.01	9.22
OTU66	1.74	2.17	2.01	11.23
OTU29	1.51	1.54	1.74	12.97
OTU42	1.41	1.2	1.63	14.6
OTU46	1.34	1.18	1.55	16.15
OTU402	1.33	1.08	1.53	17.68

OTU40	1.33	1.08	1.53	19.2
OTU25	1.33	1.18	1.53	20.73
OTU119	1.32	1.2	1.52	22.25
OTU190	1.32	1.24	1.52	23.77
OTU8	1.32	1.16	1.52	25.28
OTU86	1.3	1.19	1.49	26.77
OTU51	1.21	1.2	1.39	28.17
OTU470	1.18	1.2	1.36	29.53
OTU103	1.18	0.99	1.36	30.89
OTU259	1.17	0.87	1.35	32.24
OTU170	1.17	1	1.35	33.59
OTU21	1.14	1	1.31	34.9
OTU210	1.13	1	1.31	36.21
OTU161	1.09	0.87	1.25	37.46
OTU333	1.08	0.85	1.25	38.71
OTU323	1.08	0.85	1.24	39.95
OTU117	1.07	0.94	1.23	41.18
OTU133	1.07	1.03	1.23	42.41
OTU48	1.06	1.01	1.22	43.63
OTU313	1.01	0.95	1.17	44.8
OTU172	0.99	0.9	1.14	45.94
OTU24	0.99	0.95	1.14	47.08
OTU64	0.99	0.94	1.14	48.22
OTU434	0.98	0.94	1.13	49.35
OTU59	0.96	0.94	1.11	50.46
OTU92	0.93	0.81	1.07	51.53
OTU13	0.91	0.86	1.05	52.58
OTU201	0.9	0.96	1.03	53.61
OTU89	0.88	0.68	1.01	54.62
OTU391	0.88	0.8	1.01	55.64

Simper-OTUs closest BLAST and/or UNITE matches

OTU1	*Teratosphaericola sp.*	UNITE
OTU3	*Alternaria alternata*	BLAST
OTU4	*Marchandiomyces quercinus*	UNITE
OTU5	*Myrtapenidiella c.f sporadicae*	BLAST
OTU7	*Alternaria sp.*	UNITE
OTU8	*Aureobasidium sp.*	BLAST
OTU9	Uncultured fungus clone	BLAST
OTU10	*Teratosphaeria c.f. zuluensis*	BLAST
OTU13	Uncultured fungus clone	BLAST
OTU16	Uncultured fungus clone	BLAST
OTU19	*Stereum hirsutum*	UNITE
OTU21	*Phoma sp.*	BLAST
OTU24	*Alternaria infectoria*	BLAST
OTU25	*Cladosporium sp.*	BLAST
OTU26	*Neophaeomoniella niveniae*	BLAST
OTU28	*Alysidiella parasitica*	UNITE
OTU29	Uncultured fungus clone	BLAST
OTU31	Uncultured fungus clone	BLAST
OTU33	Uncultured fungus clone	BLAST
OTU35	Uncultured fungus clone	BLAST
OTU40	Uncultured fungus clone	BLAST
OTU41	*Pestalotiopsis sp.*	BLAST
OTU42	*Neodevriesia lagerstroemiae*	UNITE
OTU44	*Foliophoma sp.*	BLAST
OTU46	*Dendrophoma pleurospora*	BLAST
OTU47	*Perusta inaequalis*	UNITE
OTU48	Uncultured fungus clone	BLAST
OTU50	*Cyphellophora c.f. pluriseptata*	BLAST
OTU51	*Phoma sp.*	BLAST

OTU54	*Parateratosphaeria altensteinii*	UNITE
OTU57	Uncultured fungus clone	BLAST
OTU59	*Peniophora sp.*	BLAST
OTU60	*Verticillium sp.*	BLAST
OTU61	Uncultured fungus clone	BLAST
OTU62	Uncultured fungus clone	BLAST
OTU63	Uncultured fungus clone	BLAST
OTU64	*Lophiostoma corticola*	BLAST
OTU66	*Angustimassarina acerina*	UNITE
OTU68	*Phoma sp.*	UNITE
OTU71	Uncultured fungus clone	BLAST
OTU79	*Phaeothecoidea intermedia*	BLAST
OTU82	*Mycocalicium victoriae*	BLAST
OTU83	*Vibrissea sp.*	UNITE
OTU85	Uncultured fungus clone	BLAST
OTU86	*Plenodomus c.f. influorescens*	UNITE
OTU88	*Neophaeomoniella zymoides*	BLAST
OTU89	*Phaeomoniella zymoides*	BLAST
OTU92	*Furfurella c.f. nigrescens*	BLAST
OTU95	*Lophiostoma cynaroidis*	UNITE
OTU98	*Neocatenulostroma microsporum*	UNITE
OTU103	*Aspergillus carbonarius*	UNITE
OTU113	*Neodevriesia simplex*	UNITE
OTU117	Uncultured fungus clone	BLAST
OTU118	*Helicoubisia c.f. coronata*	BLAST
OTU119	*Preussia sp.*	UNITE
OTU122	*Endosporium sp.*	UNITE
OTU130	*Pestalotiopsis verruculosa*	BLAST
OTU133	*Pyrenochaeta corni*	BLAST

CHAPTER 5: General Discussion and Future Prospects

5.1 *Olea europaea* L. taxonomy and significance

The olive complex, *Olea europaea* L. (Lamiales, Oleaceae), accommodates six subspecies including the commercial *Olea europaea* subsp. *europaea* L. (Besnard et al., 2002; Kaniewski et al., 2012). The remaining taxa are the wild *Olea europaea* subsp. *cuspidata* (Wall. Ex G.Don) Cif., *Olea europaea* subsp. *laperrinei* (Batt. & Trab.) Cif., *Olea europaea* subsp. *guanchica* P.Vargas et al., *Olea europaea* subsp. *maroccana* (Greut. & Burd.) P. Vargas et al., and *Olea europaea* subsp. *cerasiformis* G.Kunkel & Sunding (Besnard et al., 2002; Green, 2002; Kaniewski et al., 2012). *Olea europaea* subsp. *cuspidata* is native to South Africa (with a distribution range extending outside the continent) growing across a variety of habitats such as ravines, woodlands, forest edges and kloofs (Green and Kupicha, 1979; Coates-Palgrave, 1977; van der Vossen and Umali, 2001). It is amongst the most widespread species in South Africa (Green, 2002). *Olea europaea* subsp. *cuspidata* produces useful timber, but also have important ethnobotanical and ecological uses (Masoko and Makgapeetja, 2015; Palmer, 1977). In South Africa, it is used to treat ailments such as black quarter in cattle (Mthi et al., 2018). Since 1854, it has been venerated as a potent treatment for malaria (Altinyay et al., 2011). *Olea europaea* subsp. *europaea,* a culturally and commercially significant feature of the Mediterranean region, is cultivated in South Africa with the industry mainly based in and around the Boland region (Agricultural Research Council and Directorate Plant Production, 2010; Costa, 1998; Zohary and Spiegel-Roy, 1975). The South African olive industry is becoming increasingly important as the demand for olive products continues to grow globally (Agricultural Research Council and Directorate Plant Production, 2010).

Microbial organisms (such as fungal endophytes) play an important role in plant health, both as pathogens and in plant protection (Gao et al., 2010). Both *Olea europaea* subsp. *cuspidata* and *O. europaea* subsp. *europaea* are plagued by numerous pathogens (Manca et al., 2020; Raimondo and Carlucci, 2021; Spies et al., 2020). Little is known about the endophytes of *Olea europaea* in South Africa, despite the value of both subspecies. The main aim of this PhD was focused on exploring the diversity and ecology of fungal endophytes associated with *Olea europaea* subsp. *cuspidata* and its relatives in South Africa.

5.2 Fungal endophytes of *Olea europaea* subsp. *cuspidata* and *O. europaea* subsp. *europaea* from six sites in the Core Cape Subregion of South Africa (Chapter 2)

Fungal endophyte assemblages of *Olea europaea* have been extensively studied globally. Most of the available research focused on the commercially significant subspecies, *O. europaea* subsp. *europaea* (Martins et al., 2016; 2021; Moral et al., 2010; Nicoletti et al., 2020). However, the mycobiome of the lesser-known *Olea europaea* subsp. *cuspidata* remains unstudied. The overarching aim of this thesis was to elucidate and compare, using high-throughput sequencing (metabarcoding), the ecology of endophytic fungi within twigs of *O. europaea* in the Core Cape Subregion of South Africa.

The first data chapter (Chapter 2) focused on the impact of geographic location and host identity on fungal endophytes of *O. europaea* subsp. *cuspidata* and *O. europaea* subsp. *europaea*. Fungal endophyte assemblages of twigs collected from both hosts in six locations in the Core Cape Subregion were assessed using Illumina based metabarcoding of the ITS region. Fungal endophyte richness was significantly higher in the native African olive than in the cultivated European olive. Mantel test results revealed that there is a stronger positive geographic signal in *O. europaea* susp. *cuspidata* than in *Olea europaea* susp. *europaea.* This supports previous findings of a strong geographic signal in *O. europaea* subsp. *europaea* from Portugal (Martins et al., 2016). Similar to findings on four congeneric grass species in the USA (Lyons et al., 2021), host identity significantly influenced fungal endophyte assemblages. Many of the fungal endophytes found within the European olive were well-known species, while many of the taxa within the African olive were unknown fungi. The highly connected microbial network within the African olive was suggestive of a collective of fungal endophytes similarly adapted to the host physiological conditions or the environmental conditions faced by the host. The abundance of fungal endophytes with known pathogenic effects on cultivated olives elsewhere in the world was alarming.

5.2.1 Bridging the gap: Fungal endophytes in *Olea europaea* subsp. *europaea* (globally), fungal endophytes in South Africa and fungal endophytes in *Olea europaea* in South Africa

Host range expansion of fungal endophytes (including latent pathogens) has been recorded between native and related introduced hosts (Crous et al., 2017; Gioia et al., 2020; Mehl et al., 2017). It is possible that some fungal endophytes within either olive host may shift hosts or expand their ranges

with unknown consequences. Although host shift or expansions were not established in this study, I did find some fungal endophytes shared between hosts studied here, between European olives elsewhere and our African olives, and within our European olives (in South Africa) and elsewhere.

The identification of fungal endophytes within the olives (cultivated and native) in South Africa connects very important fungal ecology research foci. It connects what is known of fungal endophytes within plants in South Africa with that of fungal endophytes in cultivated olives in other olive growing countries. Some fungi recovered within the European olive in South Africa have been detected in this host in other olive growing countries. For example, species in *Cladosporium* Link and *Aureobasidium* Viala & G. Boyer were amongst taxa found across all examined olive cultivars in Spain (Costa et al., 2021). Different *Alternaria* Nees species have also been recorded as pathogens of *O. europaea* subsp. *europaea* (Chliyeh et al., 2014; Costa et al., 2021; Gomes et al., 2019; Malhadas et al., 2017). In the Core Cape Subregion, three different *Alternaria* species were identified across multiple sites within both hosts. Fungal endophyte taxa recorded in the second chapter matched those previously recorded in native hosts in South Africa. For example, *Lophiostoma cynaroidis* Marincowitz, M.J. Wingf. & Crous, previously recorded within *Protea cynaroides* (L.) L. and *O. europaea* subsp. *cuspidata* (Marincowitz et al., 2008; Spies et al., 2020), were detected within both hosts from multiple sites. Teratosphaeriaceae and Botryosphaeriaceae are amongst the most well studied fungal endophyte families in South Africa because they include many latent pathogens (Aylward et al., 2019; Crous and Wingfield et al., 1996; Jami et al., 2015;). I detected four Botryosphaeriaceae and 19 Teratosphaeriaceae species among the twigs of both species studied. Some of these Botryosphaeriaceae species are well known pathogens of olives in other countries (Moral et al., 2017; Úrbez-Torres et al., 2013). The number and identity of shared taxa recovered in the present study suggests that, when exposed, the cultivated and native olives of South Africa may face some of the same risks that other olives in other countries and other hosts in South Africa face. These results fill in some knowledge gaps on the ecology of microbes within economically and ecologically important olive trees. Alarmingly, although the sampled twigs were visibly healthy, their fungal assemblages consisted of numerous latent pathogens that require further investigation. This is critical to future risk assessments focused on early detection of potential pathogens and monitoring for conditions known to trigger pathogenicity in such latent pathogens.

The results of this thesis contribute towards growing research efforts to identify the increasing number of potential pathogens associated with olive orchards (Chliyeh et al., 2014; Costa et al., 2021; Nicoletti et al., 2020; Preto et al., 2017). The use fungal endophytes to aid in disease management is

increasingly being explored (Landum et al., 2016; Malhadas et al., 2017; Poveda and Baptista, 2021). The urgent need for alternatives to chemical-based treatments for crop diseases necessitates better understanding of the olive microbiome as an additional biological resource (Aktar et al., 2009; Pingali and Gerpacio, 1997). The *Xylella fastidiosa* Wells. crisis that decimated olive trees in Europe has put further strain on the industry and contributed to the growing attention given to microbial assemblages of olives (Bucci, 2018; Giampetruzzi et al., 2020). The importance of fungal endophyte assemblages to disease management is, at least in part, hinted at by the different assemblages found within olive cultivars that have different tolerance levels to certain olive pathogens (Costa et al., 2021; Martins et al., 2021). Thus, the microbial ecology reported in this PhD is not only of value to South Africa, but also to olive researchers globally.

5.2.2 Going the distance: The relevance of the European cultivated, European wild and South African wild olives to each other

The role of geographic factors is very important as it influences exposure to certain microbes. For example, South Africa is very far away from the native range of the cultivated olive. This can be advantageous as our orchards are at a lower risk of exposure to dangerous pathogens affecting the European olive populations, such as the *X. fastidiosa* outbreak. This geographic isolation of the introduced host from its native range does, however, expose it to *Olea* species native to the introduced range and their associated endophytes, from which it would otherwise have been naturally isolated. This exposes both host trees to each other's microbial associates with may pose a threat to all hosts involved. The African olive associates with a wide range of fungal endophytes that are of unknown ecology both in this host and in the European olive. This presents the opportunity to assess fungal endophytes within these hosts for novel beneficial associations that may hold the key to controlling some of the known pathogens. At a time when researchers are actively seeking biological solutions to existing problems, South African populations present additional avenues for natural management options.

The practise of exploring native relatives for solutions to problems of the cultivated olives is not novel. The wild European olive, *O. europaea* subsp. e*uropaea* var. *sylvestris* Miller, has previously been screened for bacterial endophytes to be used for the control of the *O. europaea* subsp. e*uropaea* pathogen, *Verticillium dahliae* Kleb. (Aranda et al., 2011). The wild European olive and the wild African olives are not just of value to the cultivated European olive because of their microbial associates, they are also recommended as candidates for genetic improvement of their cultivated relative, *O. europaea*

subsp. *europaea* (Hannachi et al., 2009). The multifaceted value of the African olive necessitates an urgent and in-depth understanding of its molecular biology and ecology- and, by extension, that of their associated microbial assemblages.

5.3 Response of fungal endophyte assemblages associated with *Olea europaea* subsp. *cuspidata* to habitat quality and different growth forms of neighbouring plants (Chapter 3)

Olea europaea subsp. *cuspidata* is amongst the most widespread plants in South Africa and inhabits a diverse range of habitats ranging from pristine to heavily disturbed, traversing many biomes and provincial boundaries (Coates-Palgrave, 1977; Palmer, 1977). This enabled me to study the effect of level of habitat degradation and neighbouring vegetation types on the fungal endophyte assemblages within twigs of the African olive. Twigs were sampled from different habitats, ranging from natural (undisturbed), semi-natural (naturally occurring plant in transformed areas) to completely disturbed (trees planted in disturbed areas). Within these habitat contexts, different vegetation contrast levels were established; namely, low (olive tree growing amongst olive trees), medium (olive trees growing amongst other trees), and high (olive trees growing amid other vegetation types).

The results revealed that, although *O. europaea* subsp. *cuspidata* is highly adaptive and resilient, the fungal endophyte assemblages were particularly sensitive to differences in habitat quality, but not to vegetation contrast levels. This was curious, as I expected that the surrounding vegetation would matter, given that in woody plants many fungal endophytes are horizontally transferred from the surrounding host sources (Rodriguez et al., 2009). Additionally, spore deposition in a forest-agriculture landscape has been found to greatly depend on vegetation type rather than distance or weather (Redondo et al., 2020). Similarly, in beachgrass species in Oregon the type of external sources of propagules were a stronger determinant of colonisation of most endophyte taxa than disturbance (David et al., 2017). It is possible that the ability to maintain effectively unchanged endophytic assemblages in African olives growing in different vegetation contrasts may be related to the natural ability of this plant to thrive in diverse habitats. The influence of habitat quality on endophyte diversity is well-established in literature (Jumpponen and Jones, 2010; Matsumura and Fukuda, 2013). Surprisingly, in the African olives fungal endophyte assemblages were more diverse within the planted habitat context, followed by the semi-natural habitat context and the lowest in the natural habitat context. Contrary to the African olives results, the adverse effect of urbanisation on fungal endophyte

assemblages leading to lower diversity in urban areas compared to rural natural sites has been previously recorded (Matsumura and Fukuda, 2013). Similarly, diversity of mycorrhizal fungi differed significantly between urban and non-urban habitats, with the former harbouring significantly reduced fungal diversity than the latter (Bainard et al., 2011). Despite the natural olives harbouring lower species richness, their fungal endophyte assemblages were more cohesive and connected in the network. These plants also harboured a remarkably high species turnover from one tree to the next. The networks progressively disintegrated as the conditions deviated from the natural habitat context. This network disintegration may be linked to the loss in species richness resulting from stress experienced by the hosts in the disturbed habitats. It may also suggest that the high richness in planted olives is due to the inability of this host to effectively filter what fungal endophytes it acquires in stressful conditions.

A mix of well-known and poorly understood fungal taxa with beneficial and pathogenic effects were identified across the sampled categories. More *Phaeomoniella* Crous & W. Gams taxa were recovered from the planted habitat context than in any other context. *Phaeomoniella* pathogens have been reported in South Africa and in cultivated olives in other countries (Carlucci et al., 2013; Gomes et al., 2019; Moral et al., 2017; Úrbez-Torres et al., 2013). In addition, various taxa known to cause core rot in apples in South Africa were identified in the African olive plants. These included members of the genera *Alternaria*, *Penicillium* Link, *Ulocladium* and *Aspergillus* P. Micheli ex Haller (Basson et al., 2019). Many more undescribed taxa formed significant co-occurrences in the networks of the seminatural habitat context than in the other contexts. Further investigations into the fungal endophyte assemblages of wild olive twigs growing in the seminatural habitat context will be required to gain a better understanding of these fungal endophytes and their role to adaptation in this habitat context.

5.3.1 Restoration potential of disturbed habitats

The inability of the African olive to retain cohesive fungal endophyte assemblages in disturbed habitats indicates that even though some plants manage to thrive under disturbed conditions, their associated microbes may be adversely affected. Given the critical role of microbes to plant health and resilience (Newbound et al., 2010), the loss of key taxa may complicate restoration efforts in seminatural and completely transformed habitats. The importance of fungal mutualism to plant recovery in restored dunes has been documented (Gooden et al., 2020; Sikes et al., 2016). Particularly, Gooden et al., (2020) found that recolonisation by dark septate fungi was reduced even 30 years after coastal dune

reconstruction. This was thought to be related to low restoration potential and ecosystem function. In a restored prairie, fungal endophyte association with an invasive tall fescue increased its invasiveness and hindered native plant establishment (Moore et al., 2019). Fungal endophyte assemblages of the African olive growing in habitats of different quality presented a rare opportunity to simultaneously assess the response of fungal endophytes to different levels of disturbance and vegetation contrasts without the confounding effect of time- and host-related factors. Elucidating fungal endophyte assemblages within the natural habitat can help inform landscape management decisions, including restoration efforts of disturbed habitats in the Core Cape Subregion of South Africa.

5.4 Host relationships and their influence on the associated fungal endophyte assemblages (Chapter 4)

In addition to *O. europaea* subsp. *cuspidata*, three *Olea* species are native to South Africa; namely, *Olea exasperata* Jacq., *Olea capensis* L. and *Olea woodiana* Knobl. (Besnard et al., 2002; Green, 2002). *Olea capensis* is further classified into three subspecies, only *O. capensis* subsp. *capensis* and *O. capensis* subsp. *macrocarpa* occur within the Core Cape Subregion, while *O. capensis* subsp. *enervesis* occurs outside the Core Cape Subregion (Green, 2002). *Olea woodiana* occurs in the northern provinces of the country (Green, 2002). The aim of the fourth chapter was to test if host identity and phylogenetic relatedness mattered to the fungal endophyte assemblages of the Core Cape Subregion *Olea* native; namely, *O. europaea* subsp. *cuspidata*, *O. capensis* subsp. *capensis* and *O. exasperata*. *Olea capensis* subsp. *capensis* and *O. exasperata* were the most closely related, residing in section Ligustroides, while *O. europaea* subsp. *cuspidata* belongs to section *Olea* of the *Olea* genus. To expand the phylogenetic distance between hosts, additional samples were collected from ecologically important non-*Olea* species, *Halleria lucida* L. (order Lamiales) and *Olinia ventosa* (L.) Cufod. (Order Myrtales). *Olea capensis* subsp. *capensis*, *H. lucida* and *O. ventosa* were sampled from the Harold Porter National Botanical Garden (Betty's Bay). *Olea exasperata* was collected from the dunes immediately outside Harold Porter Botanical Garden, while *O. europaea* subsp. *cuspidata* was collected further inland. Both sites also fall within the Kogelberg Biosphere within which Harold Porter Botanical Garden falls.

The results revealed that fungal endophyte assemblages were significantly influenced by host identity, but this was not correlated with phylogenetic distances between hosts. Beta diversity measures revealed a significant grouping of the botanical garden samples, while assemblages in *O. europaea*

subsp. *cuspidata* and *O. exasperata* often differed from each other and from the botanical garden samples. This suggested a strong influence of habitat preference and, possibly, a spatial influence. A spatial influence has been established in fungal endophytes of *O. europaea* (Martins et al., 2016; Chapter 2). The influence of host-related factors such as host variety has also been recorded within *O. europaea* (Costa et al., 2021). It was not surprising that the different *Olea* species hosted significantly different assemblages given that even within the two taxa of *Olea europaea*, *O. europaea* subsp. *europaea* and *O. europaea* subsp. *cuspidata*, host identity determined fungal endophyte assemblages (Chapter 2). The significant differences in assemblages in these hosts could be indicative of coevolutionary ties between hosts and endophytes.

5.5 Moving towards integrated approaches

Given the number of pathogens affecting both the wild and cultivated olives (Spies et al., 2020; van Dyk et al., 2021), there is an urgent need to invest time and resources into improving our understanding of fungal endophyte assemblages within olives in South Africa. To this end, the ecological patterns uncovered here can be used to inform research efforts into the functional significance of these microbes. For example, numerous taxa within the healthy twigs overlapped with the taxa identified as trunk pathogens in these hosts. Simultaneously investigating the metacommunities, metatranscriptomes, and metaproteomes of symptomatic and asymptomatic organs will shed light on the processes that govern pathogenic presentation of latent fungal endophytes. A recent metatranscriptomic investigation of the molecular consequences of a *Verticillium dahliae* infection revealed a very complex multi-organism (including endophytes that commonly associate with the cultivated olives) interaction and attack that govern the devastating olive wilt that results from this infection (Martí et al., 2020). Unravelling infection processes such as those of the *Verticillium* wilt may hold answers to key microbial taxa important to disease management. Additionally, this may shed light on the initiation of pathogenicity in latent pathogens.

5.6 Conclusion

This PhD characterised fungal endophyte assemblages within native hosts and how they compare to those in an agriculturally important non-native congeneric relative. The outcomes of the assessment of fungal endophyte response to different levels of disturbance and surrounding vegetation provides invaluable insight into the factors that influence fungal endophyte assemblages within a native host exposed to different types and levels of land use activities. This is important information to consider

in the context of our shrinking areas of protected biodiversity hotspots. The outcomes of this PhD indicate that we should not only worry about the threatened flora lost in this way, but also about the microbes associated with plants of Least Concern status on the IUCN list. Restoration potential of some disturbed habitats may depend on our understanding of their associated microbes and the ecological functions they serve. As our biodiversity hotspots continue to shrink our microbial diversity may be disappearing with them. This also erodes our opportunity to understand and apply it as a valuable resource for crop diseases management. While diseases and their associated microbes are typically obvious, potentially beneficial microbes are poorly characterised and require extensive screening and experimentation to identify. Thus, pre-emptive studies focusing on microbial ecology are important to gain an advantage that, should the need arise, not only allow for a speedy identification of the problem, but also possible solutions. Knowing the host preferences, geographic distribution and disturbance tolerance of these fungal endophytes will contribute critical insight as we move towards organic solutions for agricultural issues. *Olea europaea* subsp. *cuspidata* is a dynamic plant that hosts species rich fungal endophyte assemblages, which are sensitive to disturbance. These assemblages further include many undescribed species that merit further investigation. Having access to both *Olea europaea* hosts growing in close proximity and the baseline information about their fungal endophytes opens endless opportunities to study and experiment with different ways to utilise fungal endophytes to enhance plant health in these host plants. Perhaps more importantly, this dissertation demonstrates for the first time, the value of Next Generation Sequencing to fungal endophyte ecology of agriculturally and ecologically important plants in South Africa.

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