The mycobiota associated to *Astragalus nebrodensis,* an endemic shrub in the Madonie Mountains (Sicily), enables this plant to survive in its harsh environment

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Although *Astragalus nebrodensis* plays a fundamental ecological role, the variety and abundance of mycorrhizal fungi associated with this species have never been observed in natural habitats. Our aim was to observe Arbuscular Mycorrhizal Fungi (AMF) in roots of *A. nebrodensis* in natural habitats and to obtain a screening of the fungal diversity occurring in them and in the soil around, considering the positive influence of mycotrophic shrub species on soil microbiota. A morphological analysis was performed on *A. nebrodensis* roots samples from the Madonie Mountains, while metabarcoding coupled with High-Throughput-Sequencing was carried out in *A. nebrodensis* roots and in the associated soil. Observations of *A. nebrodensis* roots showed typical structures of AMF such as intraradical vesicles. Sequencing revealed that Ascomycota were the most abundant phylum in both roots and soil samples, followed by Basidiomycota and Mucoromycota. *A. nebrodensis* roots host a fungal community with lower richness as compared to soil and specific taxa were differentially abundant between roots and soil. The endomycorrhizal symbiosis in *A. nebrodensis* from natural habitat is reported for the first time. The fungal diversity between the two matrices (soil vs roots) suggests the hypothesis of a specialised and well-established root microbiome in *A. nebrodensis*. The presence of many fungi associated with *A. nebrodensis* enables this plant to survive stressful conditions such as its harsh environment, and confer to this shrub an important ecological role in this Mediterranean ecosystem.

Keywords: fungal diversity, mycorrhizal fungi, soil, ITS, barcoding

Introduction

In the Mediterranean ecosystems, shrublands are among the most characteristic type of vegetation, widespread in different habitats. Each shrubby species is an important component within its plant community and plays a specific ecological role. This is due ~~Owing~~ to different factors such as the physiological, morphological, reproductive, phenological, and regenerative characters, as well as the inter-intraspecific interactions~~, each shrubby species represents an important component within the plant community and play a specific ecological role~~ (Lombardo et al. 2020). Indeed, shrubs play an important role in the nutrient cycle providing organic matter input to soils. They are able to protect watersheds from erosion and provide substrate, food, and shelter for organisms (nurse plants), ~~play an important role in the nutrient cycle providing organic matter input to soils~~ and ~~are able to protect watersheds from erosion~~ (Bochet et al. 2006). ~~Moreover~~Thus, shrubs are very important for many associated species such as mammals, birds, invertebrates, other plants ~~(that favour thanks to their role of nurse plants),~~ and their distribution also influences the variety and abundance of mycorrhizal fungi, fundamentals in ecological terms, and nutritional relationships (Kerns and Ohmann 2004).

Astragalus nebrodensis (Guss.) Strobl (Fabaceae) is a thorny perennial shrub endemic to the Madonie mountains in north Sicily (Peruzzi et al. 2015). Within the Fabaceae, it belongs to the section *Rhacophorus* Bunge (Podlech 2008). This section, ~~together with that~~as Sect. ~~of~~ *Tragacantha* DC. and Sect. *Pterophorus* Bunge, includes ~~species~~ taxa that form remarkable vegetation types in alpine and subalpine areas in SW Asia (Pirani et al. 2006) but also other orophytes ~~taxa~~ distributed in the Mediterranean region, such as *A. granatensis* Lam. in the Iberian Peninsula and Morocco, *A. creticus* Lam. and *A. dolinicola* Brullo & Giusso endemic to Crete, *A. cylleneus* Boiss & Heldr. in Greece, *A. rumelicus* Bunge in Albania and Greece, *A. psilodontius* Boiss., *A. bethlehemiticus* Boiss., and *A. argyrothamnos* (Boiss.) Greuter in Lebanon, *A. siculus* Biv. endemic to Sicily (Mt Etna) (Podlech 2008; Kurtto 2017), *A. sirinicus* Ten. in the northern, central, and southern Apennines, *A. genargenteus* Moris and *A. gennarii* Bacch. & Brullo endemic to Sardinia (Cogoni et al. 2014; Sau et al. 2014), *A. parnassi* subsp. calabricus (Fisch.) Maassoumi, endemic to Calabria (souther Italy) (Peruzzi et al. 2015).

A. nebrodensis is an orophyte ~~species~~ with a cushion-like habit, up to 60 cm high, distributed in different areas of the Madonie mountains (Portella Colla, Mt Quacella, Mt Mufara, Piano Battaglia, Piana della Canna, Mt San Salvatore, Piano Zucchi, Pizzo Carbonara) (Pignatti et al. 1980; Podlech 1986; Giardina et al. 2007; Schicchi et al. 2013; Pignatti 2017). It is a pioneer species which lives at an altitude between 1200 and 2000 m a.s.l. in the Supra-Oromediterranean bioclimatic belt (Lombardo et al. 2020). It has~~with~~ numerous adaptations typical of echinophytes orophile of the Mediterranean (Guarino et al. 2005~~), which lives at an altitude between 1200 and 2000 m (~~; Bonanno and Veneziano 2016) ~~in the Supra-Oromediterranean bioclimatic belt (Lombardo et al. 2020).~~ *A. nebrodensis* occurs on stony slopes, in clearings of beech woods or above the limit of forest vegetation, especially on windy ridges and eroded soils rich in the skeleton, especially carbonates and flaky clays (Brullo et al. 2005; Pignatti 2017). ~~It is a~~This species is characteristic of the pioneer association *Astragaletum nebrodensis* (Raimondo et al. 1992; Pignatti 2017) that evolves in the less disturbed areas towards the *Cratagetum laciniatae* (Schicchi et al. 2013). ~~Thanks to its morphological characteristics (spinescence, cushion-like growth form)~~I, ~~i~~t manages to grow in places with intense solar radiation, persistent drought, wide-ranging temperatures and strong winds thanks to its spinescence and cushion-like growth form (Guarino et al. 2005). Its thorns constitute the nucleus for the condensation of water droplets that flow along the branches and join the rootstock (Pignatti 2011), and also they ~~represent a protective strategy against~~protect plants from herbivores (Bagella et al. 2019). Moreover, its pulvines play an important ecological role, providing shelter from the strong wind for some short-cycle herbaceous plants, favouring their germination and letting a slight accumulation of organic matter (Pignatti et al. 1980; Brullo et al. 2005). Also, various insects take shelter in the cushions, taking advantage of the internal microclimate, for example the Hemiptera *Aelia rostrata* Boheman, 1852 (Pignatti et al. 1980) and the Sicilian endemic Orthoptera *Platycleis concii* Galvagni, 1959 (Massa et al. 2001). ~~It has been previously assessed that s~~Some native plant species improve the native tree establishment in Mediterranean ecosystems. ~~,~~ Tthe majority of ~~which~~ these taxa ~~are mycorrhized, could~~ act as “nurse plants" through their positive impacts on soil abiotic characteristics and microbiota, especially on mutualistic microorganisms (rhizobia and mycorrhizal fungi)~~, and sustainably improve the native tree establishment in Mediterranean ecosystems~~ (Manaut et al. 2011). ~~. Since these m~~Microbial associations sustain a vegetation cover in natural habitats, they represent a key ecological factor (Manaut et al. 2011). ~~In fact, these dual s~~Symbioses help plants to face stressful situations, such as drought, nutrient deficiency, and soil disturbance, and increase soil nitrogen content, organic matter and hydrostable soil aggregates~~. Since these microbial associations sustain a vegetation cover in natural habitats, they represent a key ecological factor (Manaut et al. 2011).~~

Although *A. nebrodensis* plays a fundamental ecological role, the variety and abundance of mycorrhizal fungi associated with this species have never been observed in natural habitats. Mycorrhized roots of *A. nebrodensis* have only been obtained in nursery, inoculating trap plants either with soil collected from the natural habitat or with the commercial *Rhizophagus irregularis* (formerly *Glomus intraradices*; Zimbardo et al. 2013). With the same artificial approach, other microbial symbionts of *A. nebrodensis* (i.e. nitrogen fixing bacteria belonging to *Mesorhizobium* spp.) have been detected by the same authors. Generally, mycorrhization in *Astragalus* species is reported in greenhouse-grown plants, as for the endangered species *Astragalus applegatei* Peck (Barroetavena et al. 1998), and often it is studied in response to stress, such as arsenic (Yizhu et al. 2020). Some data are present on the mycorrhization of *Astragalus* in different natural ecosystems: the study of *A. corrugatus* roots from a National Park in Tunisia (Neji et al. 2021), that of *A.* cfr. *arequipensis* roots from the Andes (Schmidt et al. 2008) and that of *A. adsurgens* Pall. canopy in the Mu Us sandland, China (Bai et al. 2009).

Arbuscular Mycorrhizal Fungi (AMF) are ubiquitous mutualists of most herbs, grasses but also several trees and shrubs, hornworts and liverworts (Balestrini and Lumini, 2018). These fungi are essential members of ecosystems~~,~~ because they provide inorganic nutrients from the soil to their plant hosts, obtaining reduced carbon in exchange (Lanfranco et al. 2018). For a long time placed in the

Glomeromycota phylum (Schüßler et al. 2001), AMF have recently been assigned to the subphylum Glomeromycotina thanks to an extensive phylogenomics approach, and Mortierellomycotina are considered their closest relatives (Spatafora et al. 2016).

The aim of this ~~e work~~ contribution ~~was~~ is to observe AMF fungi in roots of *A. nebrodensis* in natural habitats and to obtain a screening of the fungal diversity occurring in *A. nebrodensis* and in the soil around its roots, considering the positive influence of mycotrophic shrub species on soil microbiota (Manaut et al. 2011). Metabarcoding studies relying on High-Throughput-Sequencing and targeting the rDNA Internal Transcribed Spacer (ITS) offer an unprecedented tool to describe fungal communities (Nilsson et al. 2019a). This approach has recently been applied to describe the composition of root-associated fungi of *A. mongholicus* and their relationship with the production of secondary metabolites in the plant (Li et al. 2021).

In order to observe AMF fungi in roots of *A. nebrodensis,* a morphological analysis was performed on roots samples from the Madonie mountains, while the fungal diversity occurring in *A. nebrodensis* roots and in the associated soil, was carried out by molecular analysis of soil samples taken near the corresponding roots.

Material and Methods

Experimental site and sampling

The research focused on the area of the Madonie Mountains Regional Park (PA, Sicily, Italy) (Fig. 1). Sampling sites were selected according to the following coordinates: 37°51'52.14" N 14° 2'45.41" E (1477 m a.s.l) for *Astragalus nebrodensis*. As regards the edaphic and climatic characteristics, the Madonie Mountains Regional Park is characterized by marly limestone and dolomite associated with Mesozoic siliceous rock and arenaceous rocks originating mainly brown and lithic soils. The area is characterized by a mean annual temperature of 12.3 °C and a mean annual precipitation of 824.5 mm.

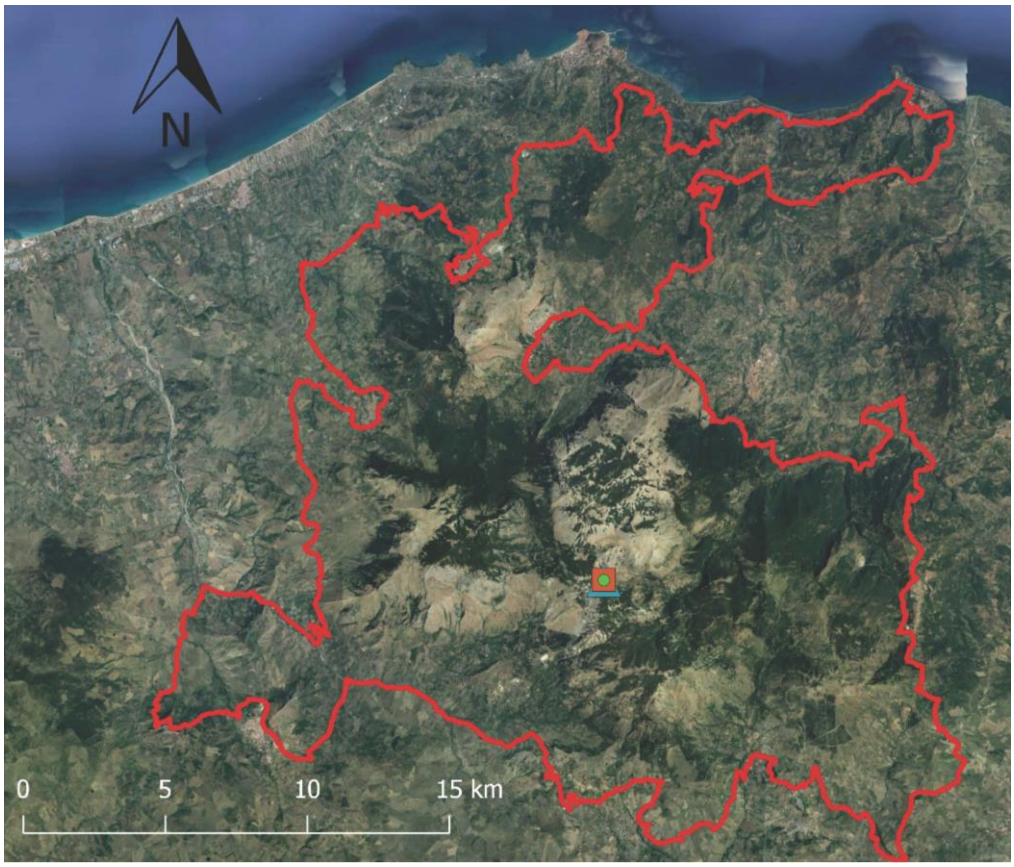

Fig. 1. Sampling sites in the Madonie Regional Park, which is delimited by the red borders. The three symbols indicate the sampling sites of the roots and the associated soil samples from three plants of *Astragalus nebrodensis*.

Samples from two compartments (roots *vs* soils) were collected in September 2019. Sampling consisted in digging to the first 5-20 cm and collecting fine feeder roots belonging to *Astragalus nebrodensis*, and a portion of soil (~ 1 Kg) surrounding the roots (Berruti et al. 2017). During the digging, the main root branches have been carefully followed and the young roots were visually recognized and collected. Three plants of *Astragalus nebrodensis* were sampled (two root samples for each plant) together with six soil samples (two under each plant) at the bottom of the plants. The soil samples (~ 300 mg) were sieved immediately at 2 mm, frozen and stored until molecular analysis. Root fragments from each plant were washed free of soil, air-dried at room temperature and immediately used for morphological analyses. The roots (~ 150 g) were stored at −20°C until used for molecular analyses.

Morphological analysis of roots

At the end of the vegetative in open field experiments (September 2019), *A. nebrodensis* roots were harvested, rid of topsoil, cleaned and stained with 0.1% (w/v) cotton blue in 80% lactic acid overnight, then destained 3 times with lactic acid for 18 h, cut into 1-cm-long segments and placed on microscope slides for morphological analysis. Approximately 25 fragments were observed under light microscope (Fig. 2).

Molecular analysis of roots and soil

In order to investigate the total fungal community, the nuclear ribosomal ITS2 region was amplified using Platinum HS PCR (Thermo Fisher Scientific) from all DNA extracts by means of a semi-nested PCR approach for root DNA and a direct approach for soil DNA. In the first PCR, the entire ITS (ITS1-5.8S-ITS2) region was amplified with the generic fungal primer pairs ITS1F-ITS4 (White et al. 1990; Gardes and Bruns, 1993).

The PCR assay for roots DNA was performed in a total reaction volume of 25 µl consisting of PCR Buffer 10X (Thermo Fisher Scientific), 0.2 mM dNTPs, 0.3 µM of the primer pair ITS1F/ITS4, 0.6 U of Taq DNA polymerase, 0.1 µg µl−1 bovine serum albumin (BSA) and 1.5 µl of target DNA (~ 5 ng). Amplifications were carried out in 0.2 ml PCR tubes using a Biometra T Gradient thermocycler in the following steps: initial denaturation of 1 min at 94°C, 30 cycles of 30 s at 94°C, annealing at 51°C for 30 s, elongation at 72 °C for 45s and a final extension of 5 min at 72°C. A negative control was included in the PCR to check for contamination. Each PCR product was checked on a 1.2 % agarose gel stained with ethidium bromide (Sigma-Aldrich). Dilutions of PCR products (1:10 and 1:100) were used as template in the semi-nested PCR with the universal forward fITS9 (GAACGCAGCRAAIIGYGA) and reverse ITS4ngs (TCCTSCGCTTATTGATATGC) primers (Ihrmark et al. 2012; Tedersoo and Smith, 2013, respectively) with overhangs.

The semi-nested PCR was carried out in a total reaction volume of 25 µl containing 2 µl of DNA (used as undiluted and 1:10), 0.2 mM dNTPs, 0.3 µM of the primers fITS9 and ITS4, 0.6 U of Taq DNA polymerase, 0.1 µg of bovine serum albumin (BSA). The semi-nested PCR cycling conditions were: an initial step at 94°C for 5 min, 35 cycles at 94°C for 40 s, 52°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 10 min. To obtain enough PCR products to be purified and sequenced, semi-nested PCR was done in triplicate.

As regards the DNA extracted from the soils, a direct PCR was carried out using PCR buffer 10X (Thermo Fisher Scientific), 0.1 mM dNTPs, 0.3 µM of the primers fITS9 and ITS4ngs, 0.6 U of Taq DNA polymerase, 0,2 µl of BSA (Thermo Fisher Scientific) and 2 µl of DNA (used undiluted, 1:10, 1:5), to obtain a final volume of 25 µl. Amplifications were carried out as for seminested PCR for roots.

All PCR products from both soil and root samples were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega), quantified with a Qubit® 2.0 Fluorometer (Invitrogen, Grand Island, USA) and then sequenced by IGA Technologies (Udine, Italy) by using Illumina run MiSeq™ with a paired end strategy (2 × 300 bp, NexteraXT index kit) and adopting a deep sequencing approach (10 million reads).

Bioinformatic analysis

The libraries were initially screened with FastQC (Andrews, 2012) for quality assessment. Cutadapt v3.4 (Martin, 2011) was used to remove forward and reverse primers, and dada2 v1.18.0 (Callahan et al. 2016) was used for quality filtering ("filterAndTrim" function with the maxEE(2,5) parameter) and the resulting reads were discarded if shorter than 165 bp. A total of $1E^8$ bases was used for denoising through the "dada" function. The denoised sequences were screened for chimeras with both *de novo* and reference-based methods, using DADA2 and the UCHIME2 algorithm (Edgar, 2016) implemented in VSEARCH v2.17.0 (Rognes et al. 2016), respectively. The UNITE v8.3 fungal ITS database (Nilsson et al. 2019b) was used as a reference set. ITSx was used to extract the ITS2 portions of each sequence. The libraries are available in the NCBI database and are included in the bioproject with code PRJNA861234 (accession numbers from SRX16441362 to SRX16441373).

VSEARCH was then used with the "–cluster-fast" option to cluster the sequences to a 97% similarity threshold. All the sequences in each OTU were annotated with BLASTn v2.11 (Camacho et al. 2009)

against the nt database. The scripts found at https://github.com/Joseph7e/Assign-Taxonomy-with-BLAST were used to parse the BLAST results. OTUs where the clustered sequences had divergent annotations were manually checked, and discarded whether a consensus annotation could not be reached. OTUs where the clustered sequences had different annotation depth within the same taxon (e.g. Glomeromycotina, Glomerales, Glomus) were resolved by keeping the highest taxonomic level (e.g. Glomeromycotina).

The OTU table was imported in R with the phyloseq v3.12 package (McMurdie and Holmes, 2013) and the counts were normalised using the median sequencing depth. The "subset_taxa" phyloseq function and the "ggstripchart" function of the ggpubr v0.4.0 package (Kassambara and Kassambara, 2020) were used to produce taxonomy barplots. The core microbiome selection was made according to Shetty et al. (2017) with the R packages phyloseq and microbiome v1.13.3 (Lahti et al. 2017). Alpha- and beta-diversity were calculated in phyloseq (The alpha diversity indices were calculated with non-normalized counts). DESeq2 v1.30.1 (Love et al. 2014) was used to estimate differential taxa abundance at a p-value threshold of 0.05. Differential abundances trees were drawn with metacoder v0.3.4 (Foster et al. 2017). In all the abundance ratios, soil abundance was picked as the numerator.

Results

AMF morphological observation

Observations of roots showed that the *Astragalus* roots were mycorrhized at the level of the cortical root parenchyma (Fig. 2). Although the arbuscles were not highlighted, other typical structures of AMF such as intraradical vesicles and extraradical mycelium were instead visualized.

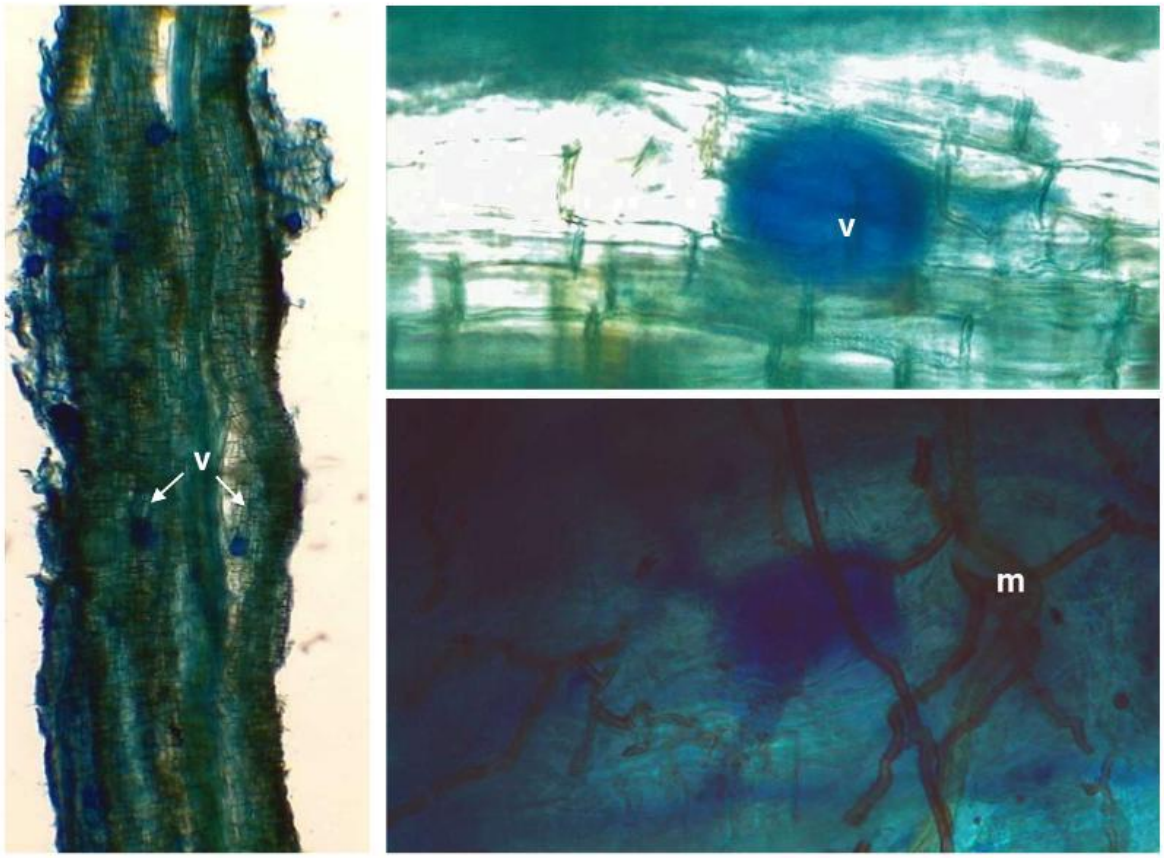

Fig. 2. Presence of vesicles (v) of arbuscular mycorrhizal fungi (AMF) inside roots of *Astragalus nebrodensis* and mycelium (m) of unidentified fungi associated with *A. nebrodensis* collected in Madonie Mountains Regional Park.

Taxonomic overview of the fungal communities

After filtering and denoising, the dataset contained 4,950 non-duplicated sequences, further clustered into 702 OTUs (Supplementary Table 1). Overall, Ascomycota were the most abundant phylum in both roots and soil samples, followed by Basidiomycota and Mucoromycota, while Chitridiomycota were scarcely represented only in soil samples (Fig. 3a). In both root and soil samples, Ascomycota (Fig. 3d) had their abundances distributed in nine main classes, with Dothideomycetes being the most abundant. Basidiomycota were dominated by Agaricomycetes (Fig. 3b), whereas the Mucoromycota community was dominated by Mortierellomycetes (Fig. 3c), followed by Glomeromycetes.

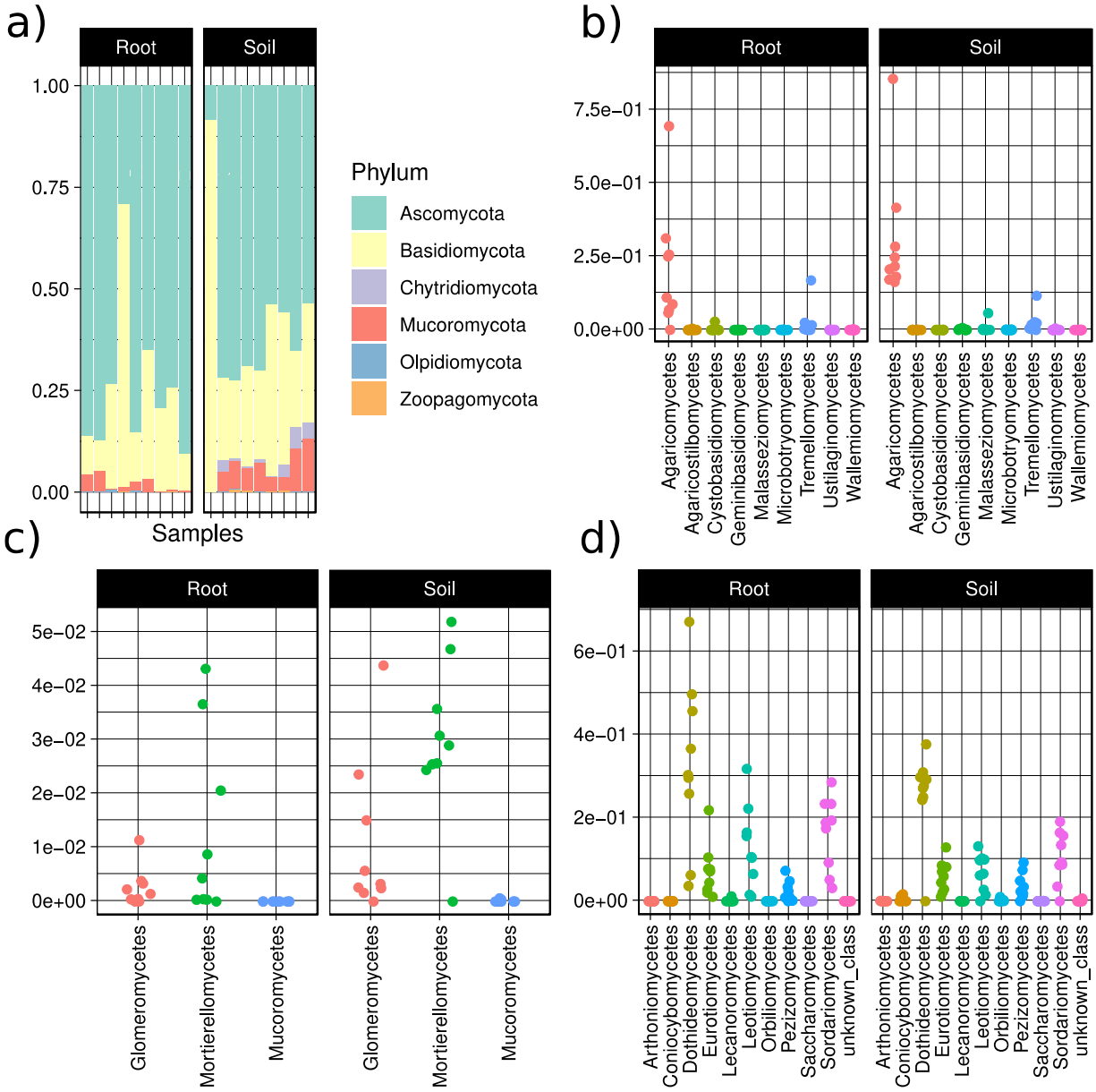

Fig. 3. Overall composition of the fungal community in the Madonie park. (a) Ascomycota had the highest relative abundance in most of the samples, over Basidiomycota and Mucoromycota (each bar represents a different sample; (b) The class Agaricomycetes was predominant in Basidiomycota, and Mortierellomycetes (c) in Mucoromycota, while Ascomycota (d) had a more even class distribution although Dothideomycetes were more abundant. Dots in (b), (c) and (d) represent samples and are distributed according to the relative abundances of each class (y axis).

Core components of the fungal communities

In order to define the core community of the whole dataset, we picked up taxa that had at least 0.01% relative abundance in at least 60% of the samples, and summarized their taxonomic affiliation at family level (Fig. 4). We found that Telephoraceae were present in a large proportion of samples at different relative abundances thresholds and that unknown families were present at more than 10 % relative abundance in nearly 100% of samples.

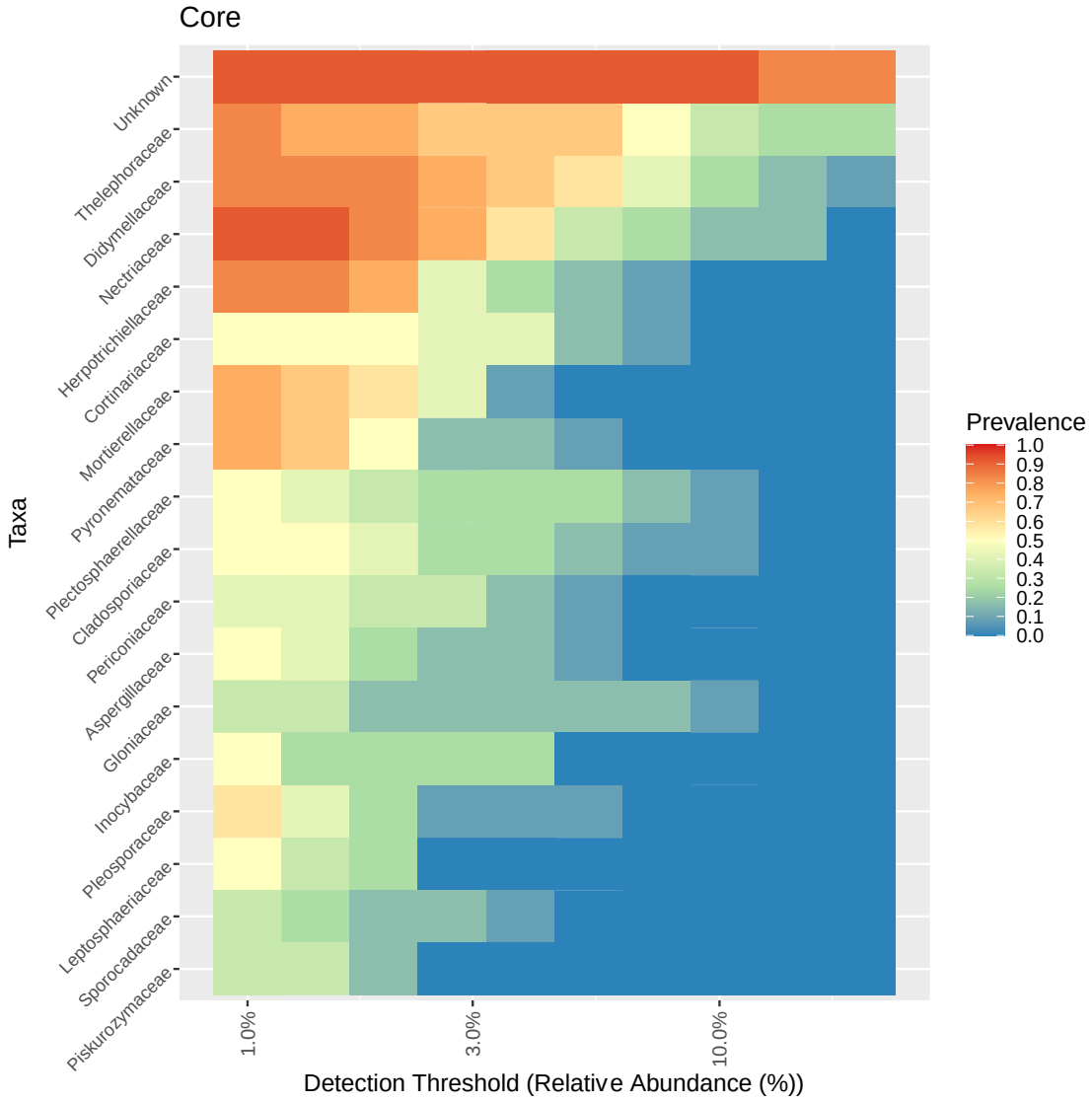

Fig. 4. Abundance and composition of the core fungal community families. Predominance was calculated using relative abundances, i.e. the abundance of each OTU was divided by the total OTUs abundances in the sample. The figure reports the fraction of samples (1 = all samples and 0 = no sample) in which a specific family had at least the relative abundance defined on the x axis. For example, unknown families and Thelephoraceae were present respectively at more than 10 % relative abundance in nearly 100% of samples, and at ~1.5 % relative abundance in ~ 90% of samples.

Alpha-, beta-diversity indices

We calculated several alpha diversity indices for the root and soil samples, and compared the subsets statistically (Fig. 5a). All the indices indicated that there were statistically significant differences between the alpha diversity values in root *vs* soil samples: roots showed lower richness than soil. We then used beta diversity indices to better visualize the compositional differences between the soil and roots fungal community (Fig. 5b). The unweighted UniFrac index (Lozupone and Knight, 2005) did not lead to a distinct separation between roots and soil samples (Fig. 5b). By contrast, such separation was visible with the use of a weighted UniFrac index, which further adds abundance data to the phylogeny-based method (Lozupone and Knight, 2005). This indicates that the differences between the two matrices (soil vs roots) are mainly driven by highly abundant taxa.

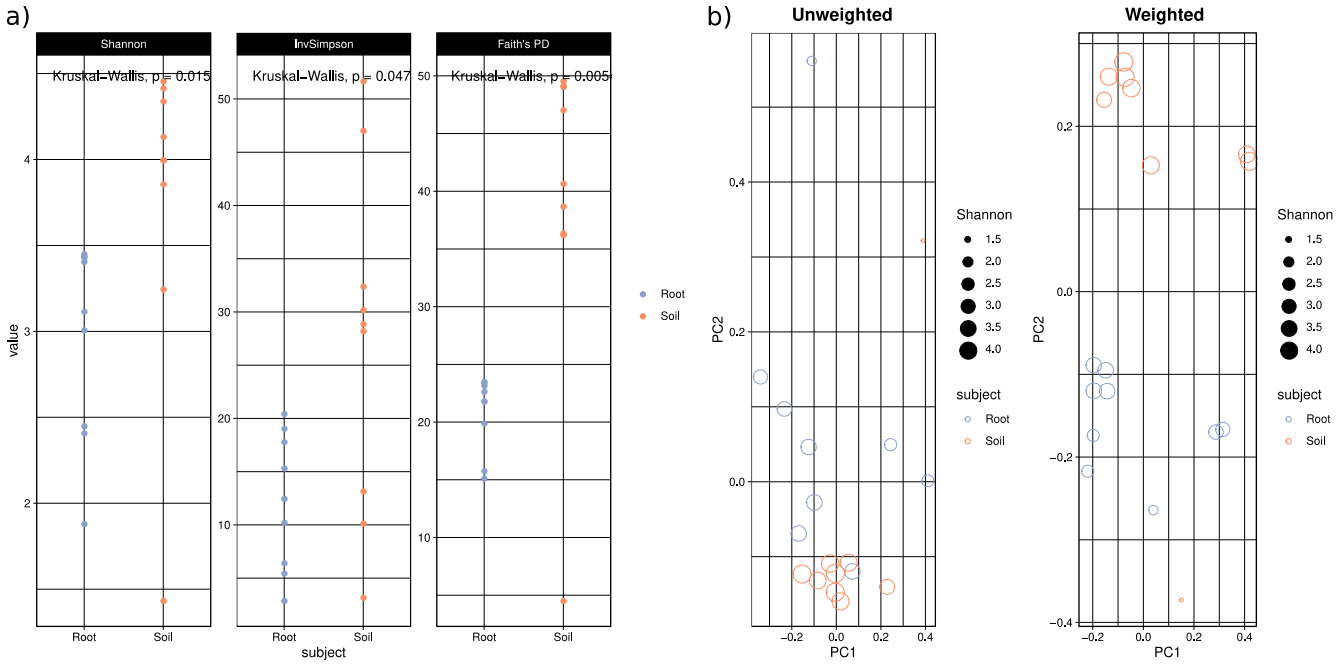

Fig. 5. Alpha and Beta diversity indices. (a) Blue and red dots represent root and soil samples, respectively, and are placed on the vertical axis based on their alpha diversity values according to specific indices (boxes). Significance values were calculated with ANOVA, at $p < 0.05$. (b) UniFrac Beta diversity indices calculated between root and soil samples. Individual shapes represent samples, and their size is proportional to the sample's Shannon alpha diversity value.

Differential taxa abundance between roots and soil

We tested whether specific taxa were differentially abundant between roots and soil. The final comparison returned differentially abundant taxa between roots and soil samples in all the phyla (Supplementary Table 2). In Ascomycetes most of the taxa were more abundant in soil, with exception of *Exophiala, Plectosphaerella cucumerina,* an unidentified taxon in *Lasiosphaeriaceae, Leptosphaeria keissleriella* and *Tuber* (Fig. 6), which were more abundant in roots. Some members of Pezizales were differentially abundant in the comparison: *Hydnobolites* was enriched in soil, while *Trichophaea* in roots. In Basidiomycetes eight taxa were more abundant in soil while only three (*Inocybe*, *Sebacina*, and an unknown taxon in *Thelephoraceae*, ectomycorrhizal fungi) in roots (Supplementary Fig. 1). Also in Mucoromycota differentially abundant taxa were found between root and soil compartments, and they were prevalently abundant in soil. *Mortierella* was abundant in soil, while *Podila* in roots. Two AMF, *Glomus indicum and Entrophospora infrequens*, were more abundant in soil, while *Rhizophagus intraradices* in roots. In Chitidriomycota only four taxa are differentially abundant in soil (*Rhizophlyctis rosea, Alogomyces tanneri, Powellomyces* and an unknown taxon in *Polychytriales*) (Supplementary Fig. 3).

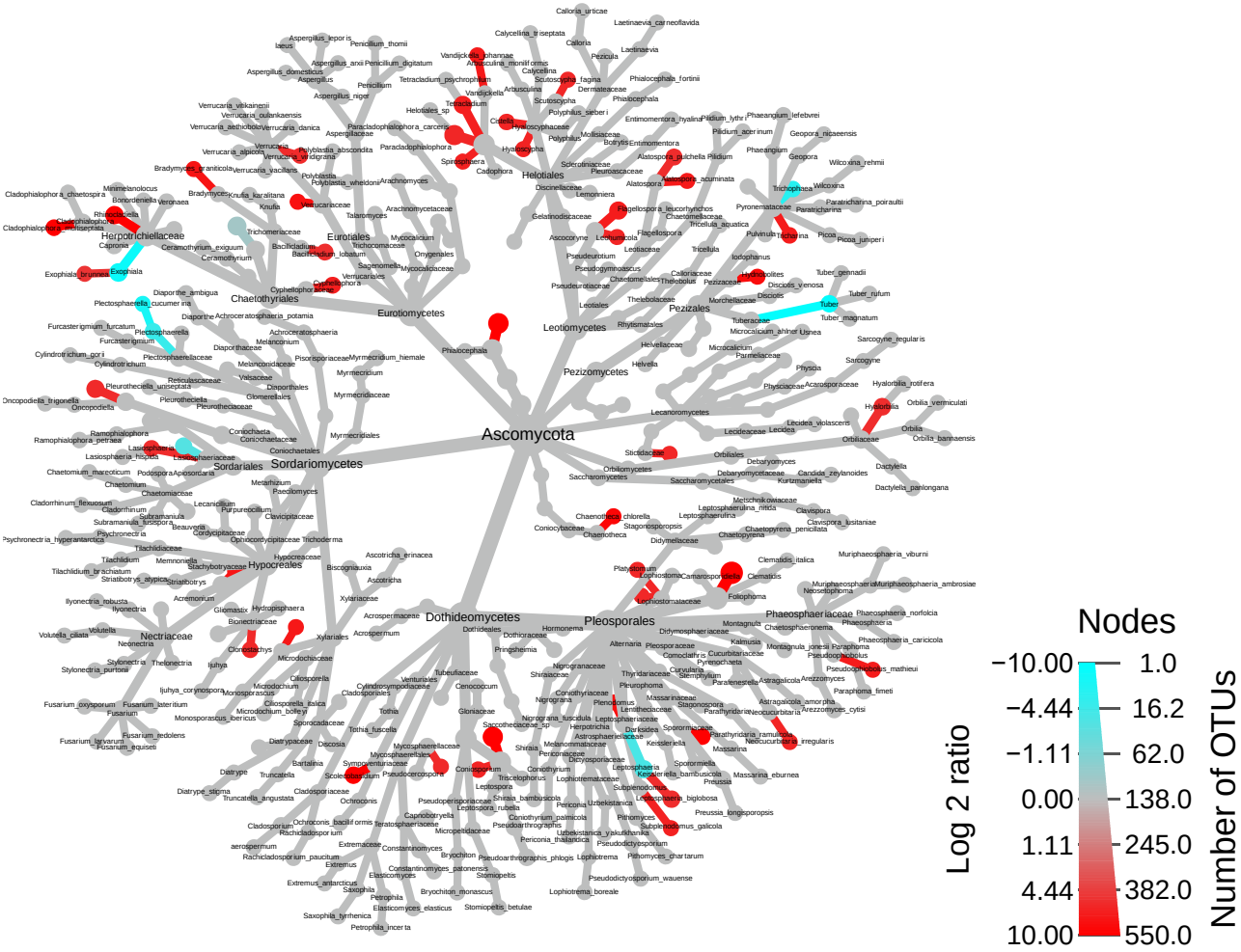

Fig. 6. Differential abundance tree showing over-represented Ascomycota taxa between soil and root samples. Red colour for nodes and edges indicates over-representation in soil, while blue indicates the opposite. Differential abundance is expressed here as the ratio of the summed log2 fold changes for each taxon.

Discussion

AMF morphological observation

In our study, the presence of endophytic and symbiotic fungi and their colonization of *Astragalus* roots were confirmed by observations at a light microscopy. To our knowledge, this is the first report of AMF colonization in *A. nebrodensis*, confirming the endomycorrhizal nature of this symbiosis.

Taxonomic overview of the fungal communities

The taxonomic overview of the fungal communities revealed that Ascomycota were dominant in both roots and soil samples. This is common in different Mediterranean habitats such as those mainly colonized by shrubs of single species as *Helianthemum almeriense* (Arenas et al. 2021) or other with a more heterogeneous landscape comprising natural cork-oak forests, pastures, managed meadow, vineyards (Orgiazzi et al. 2012), where they are mostly associated with dead plant material. The fact that Dothideomycetes resulted as the most abundant class is not surprising, considering that this represents the largest class of Ascomycetes (Hongsanan et al. 2020a). Furthermore, this is the most ecologically diverse class of fungi, comprising endophytes, epiphytes, saprobes, human and plant pathogens, lichens, and lichenicolous, nematode trapping and rock-inhabiting taxa (Hongsanan et al. 2020b). Therefore, their presence can be considered a sign of an environment with high functional heterogeneity.
Basidiomycota were the second phylum for abundance, and resulted dominated by Agaricomycetes, which was expected, due to the predominance of this class in the phylum (de Mattos-Shipley et al. 2016), and due to the fact that many fungi in this class are ectomycorrhizal (ECM). Mucoromycota, the third phylum for abundance, were dominated by Mortierellomycetes and Glomeromycetes. Both taxa contain plant endophytes (even if endophytism is facultative in Mortierellomycetes, and not common to all species; Liao et al. 2019; Lanfranco et al. 2018).

Core components of the fungal communities

The core taxa, regarded as sustainers of the community function and ecology in a specific habitat (Shade and Handelsman, 2012), revealed that unknown families were present at more than 10 % relative abundance in nearly 100% of samples. This result indicates the still little knowledge of fungi from the Madonie Park, and consequently their poor representativeness in the databases. This fact is common in places where fungal diversity has been scarcely investigated, such as Madagascar, where Ghignone et al. (2021) found many unknown fungi with the same approach used in the present work.

Alpha-, beta-diversity indices

According to several alpha diversity indices, *A. nebrodensis* roots host a fungal community with lower richness as compared to soil. This indicates that the plant operates a selection on the pool of soil microbes, compared to soil, confirming the hypothesis of a specialised and well-established root microbiome. This hypothesis is also supported by the fact that the weighted UniFrac index leads to a clear separation, between soil and root samples, that is not observed with the unweighted index; since the weighted index takes into account both taxonomic diversity and taxa abundances, this is a clear indication that fungal taxa that are poorly represented in soil, are instead abundant in roots, and make up for the largest proportion of biodiversity in those samples.

Differential taxa abundance between roots and soil

The comparison of the differentially abundant taxa between roots and soil samples revealed that most of the taxa were more abundant in soil in all the phyla. Among the few fungi which were more abundant in roots in the Ascomycota, most of them are primarily saprotrophic, inhabiting wood, dung, soil, and rotting vegetation in temperate forests (Cannon and Kirk, 2007). On the contrary, taxa belonging to *Tuber* are well known ectomycorrhizal fungi belonging to Pezizales, appreciated for their valuable aroma (Mello et al. 2006; Zambonelli et al. 2015). Other members of Pezizales, *Hydnobolites* which was enriched in soil, and *Trichophaea* in roots, are ectomycorrhizal (Miyauchi

et al. 2020; Tedersoo and Smith, 2013), suggesting that *A. nebrodensis* may develop ectomycorrhizal associations with selected, taxonomycally-related fungal partners. In addition, *Trichophaea* is placed in Pyronemataceae, a family whose members are known for their preference of burned grounds (Van Vooren et al. 2017). Also in this family, there is a taxon as *Tricharina* (saprotrophic), which was more abundant in soil. In Basidiomycota, only three taxa were more abundant in roots, *Inocybe*, *Sebacina*, and an unknown taxon in *Thelephoraceae*. All of these taxa contain ectomycorrhizal fungi with broad host spectra (Ray and Craven, 2016; Cripps et al. 2019; Miyauchi et al. 2020), which make them good candidates as *Astragalus* symbionts. *Thelephora* spp. were also detected in soil of cork oak formation in Sardinia by Orgiazzi et al. 2012. However, surprisingly, these authors retrieved them only with primers pair target ITS1 (ITS1F/ITS2) and not with the couple used for ITS2. This could demonstrate that primer pairs fITS9/ITS4 outperforms (ITS3/ITS4) to retrieve some fungal taxa (Ihrmark et al. 2012).
In Mucoromycota *Mortierella* was abundant in soil, while *Podila* in roots, confirming the facultative and specific endophytic behaviour in Mortierellaceae (Bonito et al. 2016) (Supplementary Fig. 2). Species of *Podila* are frequently isolated from forest and agricultural soil, in particular *P. minutissima* has been isolated from *Populus* roots (Bonito et al. 2016) and reported as semi-saprotrophic mycophile (saprotrophically consumes dead fungal tissue) (Rudakov, 1978). Among the Glomeromycetes, two AMF, *Glomus indicum and Entrophospora infrequens*, were more abundant in soil, while *Rhizophagus intraradices* in roots. Regarding *Glomus indicum* it should be noted that it was found, as spores, in the rhizosphere of *Euphorbia heterophylla* L. which grows naturally in the coastal sands of Alappuzha in the state of Kerala of southern India and of *Lactuca sativa* L. cultivated in Asmara, in Eritrea, in north east Africa. However, the sequence types belonging to the *G. indicum* cluster have also been documented from environmental samples mainly in the United States, Estonia and Australia, suggesting the extensive presence of the species. Also *E._infrequens* has a worldwide distribution (Oehl et al. 2011). *Rhizophagus intraradices* is one of the most detected AMF isolates in different locations throughout the world, of both stable and disturbed ecosystems (Öpik et al. 2006; Orgiazzi et al. 2012) and in many host species (Kivlin et al. 2011). This AM fungal species has a generalist and ruderal lifestyle (disturbance tolerance) as it produces large numbers of spores and extraradical mycelium (Jansa et al. 2005; Öpik et al. 2006). Our analysis on soil DNA is in agreement with such behaviour and points out the dominance of this species in plant roots also in Mediterranean environments (Lumini et al. 2010). In Chitidriomycota only four taxa, *Rhizophlyctis rosea, Alogomyces tanneri, Powellomyces* and an unknown taxon in *Polychytriales,* are differentially abundant in soil. It is worth noticing that among these fungal taxa, which are saprotrophic, *Rhizophlyctis rosea* is a common species in soils (Gleason et al. 2004) and survives stressful conditions as quiescent structures (Marano et al. 2011).

From this overview which takes a picture at the sampling time of the differential abundance between roots and soil, the roots of *A. nebrodensis* result extensively colonized by many endophytic fungi and both ecto- and endomycorrhizal fungi.
In the soil surrounding *A. nebrodensis,* mycorrhizal taxa such as *Hebeloma laterinum, Melanogaster, Lycoperdon, Tomentella and* Sebacinaceae are signs of the diversity of plant hosts in the Madonie Park, that support a diversified fungal community.
The high proportion of ectomycorrhizal Basidiomycota OTUs in this habitat is not surprising, since this is characterized by shrubs and also tree coverage. The native forest vegetation is mainly characterized by *Fagus sylvatica* L. mixed with *Acer pseudoplatanus* L., *Quercus petraea* (Mattuschka) Liebl., *llex aquifolium* L. *Fraxinus ornus* L., *Crataegus laciniata* Ucria, *Cytisus scoparius* (L.) Link, *Sorbus graeca* (Spach) Schauer and *Q. ilex*. Of considerable interest is also the presence of relict forest vegetation characterized by *Abies nebrodensis* (Lojac.) Mattei. Reafforestation with *Pinus nigra* J. F. Arnold, *Cedrus atlantica* (Endl.) Carrière and *Cedrus deodara* (D. Don) G. Don are also present in the studied area.

In conclusion, this investigation on the fungi associated with *A. nebrodensis* growing in the Madonie Mountains Regional Park is the first report showing, on one side, AMF colonization of its roots, by morphological observations, and on the other side, an overview of the total fungal biodiversity occurring in both *A. nebrodensis* roots and soil around them, by molecular analysis. The presence of many fungi associated with *A. nebrodensis* enables this plant to survive stressful conditions such as its harsh environment, and confer to this shrub an important ecological role in this Mediterranean ecosystem.

Legends

Fig. 1. Sampling sites in the Madonie Regional Park, which is delimited by the red borders. The three symbols indicate the sampling sites of the roots and the associated soil samples from three plants of *Astragalus nebrodensis*.

Fig. 2. Presence of vesicles (v) of arbuscular mycorrhizal fungi (AMF) inside roots of *Astragalus nebrodensis* and mycelium (m) of unidentified fungi associated with *A. nebrodensis* collected in Madonie Mountains Regional Park.

Fig. 3. Overall composition of the fungal community in the Madonie park. (a) Ascomycota had the highest relative abundance in most of the samples, over Basidiomycota and Mucoromycota (each bar represents a different sample; (b) The class Agaricomycetes was predominant in Basidiomycota, and Mortierellomycetes (c) in Mucoromycota, while Ascomycota (d) had a more even class distribution although Dothideomycetes were more abundant. Dots in (b), (c) and (d) represent samples and are distributed according to the relative abundances of each class (y axis).

Fig. 4. Abundance and composition of the core fungal community families. Predominance was calculated using relative abundances, i.e. the abundance of each OTU was divided by the total OTUs abundances in the sample. The figure reports the fraction of samples (1 = all samples and 0 = no sample) in which a specific family had at least the relative abundance defined on the x axis. For example, unknown families and Thelephoraceae were present respectively at more than 10 % relative abundance in nearly 100% of samples, and at ~1.5 % relative abundance in ~ 90% of samples.

Fig. 5. Alpha and Beta diversity indices. (a) Blue and red dots represent root and soil samples, respectively, and are placed on the vertical axis based on their alpha diversity values according to specific indices (boxes). Significance values were calculated with ANOVA, at $p < 0.05$. (b) UniFrac Beta diversity indices calculated between root and soil samples. Individual shapes represent samples, and their size is proportional to the sample's Shannon alpha diversity value.

Fig. 6. Differential abundance tree showing over-represented Ascomycota taxa between soil and root samples. Red colour for nodes and edges indicates over-representation in soil, while blue indicates the opposite. Differential abundance is expressed here as the ratio of the summed log2 fold changes for each taxon.

Supplementary Fig. 1-3: Differential abundance trees showing over-represented taxa between soil and root samples for Basidiomycota, Mucoromycota and Chytridiomycota, respectively.

Supplementary Tables 1-2: OTU table with raw counts for each OTU in each sample, and the related taxonomic annotations; list of differentially abundant taxa.

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Author contributions A.M. and G.V. conceived the idea. E.L., E.L. V.B. and G.D. collected the samples in the field. E.L., E.L. V.B. and A.M. processed the samples. F.V. and P.C. analysed the data. A.M. wrote the article with contribution of all authors. All authors read and approved the manuscript.

Data Availability

The libraries are available in the NCBI database and are included in the bioproject with code PRJNA861234 (accession numbers from SRX16441362 to SRX16441373).

Conflict of interest

The authors have no conflict of interest to declare.

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