Analysis of the Fungal Diversity in Citrus Leaves with Greasy Spot Disease Symptoms

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Abstract

Citrus greasy spot (CGS) is a disease of citrus with worldwide distribution and recent surveys have revealed a high level of incidence and severity of symptoms of the disease in Sicily, southern Italy. Although *Mycosphaerella citri* (anamorph *Zasmidium citri-griseum*) and other related species are generally considered as causal agents, the etiology of CGS is still unclear. Here, we report the use of an amplicon metagenomic approach to investigate the fungal communities on citrus leaves symptomatic or asymptomatic for CGS from an orchard in Sicily showing typical CGS symptoms. A total of 35,537 high-quality chimeric free reads were obtained and assigned to 176 operational taxonomic units (OTUs), clustered at 99 % similarity threshold. Data revealed a dominating presence of the phylum Ascomycota (92.6 %) over other fungal phyla. No significant difference was observed between symptomatic and asymptomatic leaves according to both alpha and beta diversity analyses. The family Mycosphaerellaceae was the most abundant and was represented by the genera *Ramularia, Mycosphaerella*, and *Septoria* with 44.8, 2.4, and 1.7 % of the total detected sequences, respectively. However, none of the species currently reported as causal agents of CGS was detected in the present study. The most abundant sequence type (ST) was associated to *Ramularia brunnea*, a species originally described to cause leaf spot in a perennial herbaceous plant of the family Asteraceae. Results exclude that CGS symptoms observed in Sicily are caused by *Z.*

citri-griseum and, moreover, they indicate that a considerable part of the fungal diversity in citrus leaves is still unknown.

Keywords: *Amplicon metagenomics*, *Zasmidium* spp., *Mycosphaerella* spp., *Metabarcoding*, NGS

Introduction

Citrus greasy spot (CGS) is a disease of citrus that affects leaves and, less frequently, fruits of several Rutaceae species [1]. Leaf symptoms appear as yellow, darkbrown, or black lesions occurring first on the underside of mature citrus leaves. As the lesions develop on the underside of the leaves, they gradually become darker and a corresponding chlorotic spot appears on the upper leaf surface. Lesions are more yellowish and diffuse on lemon *(Citrus limon*) and grapefruit (*Citrus paradisi*) and more raised and darker on tangerines (*Citrustangerina*). Affectedleavesfall prematurely from the tree in fall and in winter resulting in reduced tree vigor and yield. On grapefruit, small, black, necrotic spots are produced on the fruit surface with the surrounding area retaining a green color, causing a symptom referred to as greasy spot rind blotch. Rind blotch is a significant problem on grapefruits produced for fresh market [1–6].

All Citrus species appear to be susceptible to CGS but the disease symptoms are more severe in lemon, grapefruit, and their hybrids [1, 6–8]. Among sweet oranges, early ripening cultivars are the most susceptible, whereas Valencia-like cultivars show less intense symptoms [2, 6]. Whiteside [4] also reported symptoms on other genera of the family Rutaceae, including Poncirus, Fortunella, Murraya, and Aeglopsis. The disease has been reported within citrus-growing areas in at least 14 countries on 6 continents including Italy.

Symptoms of CGS were first reported on citrus in Florida [9]. The cause of this disease was unknown for a longtime, and it was thought to be a consequence of nutritional problems or the rust mite *Phyllocoptruta oleivora* [10]. The involvement of a Cercosporoid fungus was firstly hypothesized in Japan where the fungus *Mycosphaerella horii* was isolated from symptomatic leaves [11]. The fungus *Zasmidium citri-griseum* (syn. *Stenella citri-grisea*, teleomorph *Mycosphaerella citri*) was first proposed as the causal agent of CGS by Whiteside [5] and is now generally recognized as the causal agent of CGS [1]. Ascospores of *M. citri* are produced in pseudothecia in decomposing leaf litter on the ground [4]. When the pseudothecia mature, ascospores are ejected following a

wetting period and are dispersed by air [1]. Since infection occurs through stomata, only ascospores deposited on the underside of the leaf germinate and penetrate into the mesophyll after the formation of an appressoria. Colonization of the leaf occursveryslowly, and symptoms appear only after 45 to 60 days, even on highly susceptible species under optimal conditions [1].

Several fungi can be isolated from CGS-like lesions and even reproduce the same symptoms [6, 11–14]. Among others, the fungi *Septoria citri* and *Colletotrichum gloeosporioides* are frequently isolated from diseased leaves but Koch's postulates were not fully satisfied [15]. More recently, four Zasmidium species, including *Zasmidium indonesianum* in Indonesia, *Zasmidium fructicola*, and *Zasmidium fructigenum* in China, and *Z. citrigriseum* in many different countries, have been isolated from symptomatic leaves of several citrus species [16]. In Italy, the disease was first reported in Calabria (Southern Italy) more than 30 years ago and more recently an outbreak has been observed in Sicily [17, 18]. Although *Mycosphaerella* sp. and *S. citri* were reported to be associated with symptoms of the disease [17, 19], no convincing evidence of the pathogenicity of these fungi has been provided and no detailed analyses to determine the etiology of the disease have ever been performed.

In recent years, next-generation sequencing (NGS), together with the emergence of metagenomic approaches, has made it easier to comprehensively analyze microbial communities on or in any type of matrix including plant tissues. These techniques have been also proved as a powerful tool to determine the relationship of specific microorganisms to health and disease conditions in a number of different environments including humans [20, 21].

The aim of the present study was to use an amplicon metagenomic approach to characterize fungal communities associated to leaves in citrus orchards located in Sicily showing typical CGS symptoms in order to determine any correlation with the presence of *Z.citri-griseum* and/or other Cercosporoid fungi.

Materials and Methods

Sampling and DNA Extractions

Samples were collected in March 2015 from symptomatic and asymptomatic leaves of a mid-ripening sweet orange (*Citrus × sinensis*) cultivar ("Tarocco Scirè") and the tangelo hybrid "Nova" (*Citrus × tangelo*) as well as from symptomatic leaves of a late-ripening sweet orange cultivar ("Lane

Late"). Symptomatic leaves exhibited typical symptoms of CGS (Fig. 1) as described in literature (Mondal and Timmer [1]). Leaf samples were collected in a citrus orchard of approximately 50 Ha, located south-west of Catania (Mineo), Italy, GPS coordinates (37° 19′ 38.5" N 14° 41′ 11.5" E). Three sub-samples of both symptomatic and asymptomatic leaves of "Tarocco Scirè" and "Nova" as well as three subsamples of symptomatic leaves of "Lane Late" were individually collected. Only symptomatic leaves of BLane Late^ were collected due to difficulty in finding completely asymptomatic leaves. Sub-samples, each comprising 50 leaves collected from 10 trees according to a complete randomized block design, were kept in sterile plastic bags in a thermally insulated container for approximately 4–5 h until lyophilization (Labconco Corp., Kansas City, MO). Freeze-dried samples were stored at −20 °C and homogenized by grinding under liquid nitrogen. Total DNA was extracted from each subsample using 0.02 g of homogenized tissue and the DNeasy Plant Mini kit according to the manufacturer (QIAGEN, Dusseldorf, Germany). The quantity and quality of extracted DNA were determined using a Nanodrop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE).

Fungal DNA Amplification and Sequencing

DNA extracts from each sub-sample were amplified in triplicate using the universal fungal primers ITS3-ITS4 targeting the ITS2 region of ribosomal DNA [22]. Both primers were modified to construct fusion primers appropriate for 454 sequencing with adapters sequences A and B, key sequences, and multiplex identifiers (MIDs) (http://www.454.com/). Five different MIDs were utilized to label different samples (Table 1).

PCR reactions were conducted in a total volume of 25 µl containing 2.5 µl of 10× reaction buffer, 0.25 µl of each primer ITS3-ITS4 (10 µM), 0.1 µl of AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen, CA, USA), and 1 µl of DNA template (10 ng/µl). Reactions were incubated in an Eppendorf Mastercycler gradient (Hamburg, Germany) for 1 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 68 °C. All reactions ended with a final extension of 1 min at 72 °C. For each sample, amplicons from the tree sub-samples were pooled and purified using the Agencourt AMPure XP system (Beckman Coulter, Inc.). The concentration and quality of the purified amplicons were evaluated by agarose gel electrophoresis. Amplicons were sequenced by Macrogen Inc. (Seoul, Korea) using the 454 GS FLX + System (Roche Diagnostics Corporation).

Data Analysis and Statistics

The bioinformatics pipeline, QIIME v. 1.8 [23], was used to process and analyze the obtained sequence data. Preliminary processing of data included de-multiplexing and quality filtering with a minimum quality score of 25, a minimum/maximum length of 150/1000, and a maximum number of homopolymer bases of 6. Sequences were denoised using the denoise wrapper [24] and the ITS2 region was extracted using ITSx application [25]. Chimeric sequences were identified and filtered using the USEARCH 6.1 software [26]. Sequences were clustered at 99 % similarity threshold using USEARCH 6.1 software, and the most abundant sequences in each operational taxonomic unit (OTU) were selected as representative sequences. These sequences were then used for the taxonomy assignment. OTUs were picked using the UNITE dynamic database released on January 8, 2015(http://unite.ut.ee/) asa reference database. The same database was also used for taxonomy assignments using the BLAST algorithm [27] at a similarity threshold of 0.97.

For downstream analysis, the OTU table was rarefied at an even depth to reduce biases in sequencing depth. Alpha diversity was calculated using both Shannon and Chao1 estimates and results were compared using a two-sample t test based on non-parametric (Monte Carlo) methods with 999 permutations.

The Bray Curtis method [28] was utilized to evaluate βdiversity [29] and then visulaized in (Unweighted Pair Group Method with Arithmetic Mean) UPGMA plots. A distancebased redundancy analysis (db-RDA) and Permanova as implemented in QIIME v. 1.8 was utilized to relate the fungal community composition to sample types and to evaluate differences between symptomatic and asymptomatic leaves and differences among varieties. Additionally, a Monte Carlo permutation test was used to determine experimental variables significantly contributing to the observed variance in fungal communities.

Identification of Fungal Taxa

In order to confirm the accuracy of QIIME taxonomic assignments, sequences associated with the most abundant OTUs were extracted and introduced into ElimDupes (http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html) to detect identical sequences and determine their frequency within each OTU. Unique representative sequences, defined as sequence types (STs) [30–33, 31], were analyzed along with genetically closely related reference sequences of

the same taxa to determine their phylogenetic affiliation and enable their identification with the highest possible level of accuracy. To this aim, local databases of validated reference sequences were created with priority given to sequences from specific recent taxonomic studies. For each selected taxon, STs identified in the present study and reference sequences were aligned using MUSCLE and introduced into MEGA for phylogenetic analysis utilizing the Maximum Likelihood method [34]. Analyses were performed with 500 bootstrap replications.

Results

Fungal Diversity and Richness

A total of 35,537 reads were recovered after quality evaluations (length trimming, denoising, ITS2 extraction, and chimeric sequence exclusion), and assigned to 176 OTUs clustered at a 99 % similarity threshold. Considering an even depth of 3000 sequences per sample, the number of OTUs ranged between 80.7 in BLane late^ orange and 107.5 in BNova^ tangelo (Table 1).

The rarefaction analysis indicated that the sequencing depth had been saturated for all of the analyzed samples and that the great majority of OTUs had been detected (Fig. 2). According to α-diversity, based on Shannon's Diversity Index and Chao1 estimate, a similar level of fungal diversity was present in symptomatic and asymptomatic leaves (P = 0.891).

Furthermore, β-diversity, calculated using distance-based redundancy analysis (db-RDA) and Permanova based on Bray Curtis dissimilarity, did not reveal any significant difference between symptomatic and asymptomatic leaves (P = 0.8) (Fig. 3). Concerning the investigated citrus species, a higher number of OTUs was detected on BNova^ hybrid tangelo as compared to both orange varieties (BTarocco Scirè^ and BLane Late^) (Table 1). Furthermore, samples from the two orange varieties and from BNova^ were clearly differentiated in the UPGMA plot, regardless of being symptomatic or not (Fig. 3).

Fungal Community Structure

Regardless of the presence of symptoms and citrus species or variety, the phylum Ascomycota dominated, representing 92.6 % of the total number of the detected sequences or relative abundance (RA). This was followed by the phylum Basidiomycota (RA 4.3 %), and then unidentified fungi (RA 1.2 %). Within the phylum Ascomycota, the class Dothideomycetes (RA 63 %)

was the most abundant followed by Eurotiomycetes (RA 24.4 %) (Fig. 4a). The high incidence of the former class was primarily due to the abundance of the order Capnodiales, and more precisely the family Mycosphaerellaceae. Within the class Eurotiomycetes, only fungi associated to the family Chaetothyriales were detected (Fig. 4a). Other non-identified fungi were associated to the order Capnodiales (RA 5 %), and Pleosporales (RA 1.2 %) or to the phylum Ascomycota (RA 1.8 %).

Sequences associated to the family Mycosphaerellaceae were the most abundant and were represented by *Ramularia* spp., *Septoria* spp., and *Mycosphaerella* spp., with 44.8, 2.4, and 1.0 % of the total detected sequences, respectively (Fig. 4b). Since the identification at the species level of sequences clustering within this family was not possible with QIIME analyses, nine representative STs were identified and phylogenetically analyzed along with validated reference ITS sequences of the order Capnodiales, including all *Zasmidium* species that are currently reported as causal agents of CGS [16, 35]. According to this analysis, the most abundant ST, representing 92.9 % of the sequences clustering in the family Mycosphaerellaceae, was associated to *Ramularia brunnea*.

Another ST was associated to reference isolates of *Mycosphaerella africana*, *Mycosphaerella ellipsoidea*, and *Mycosphaerella keniensis*, three species characterized by identical ITS2 sequences. Similarly, other STs were found to be related to *R. rumofaciens* and *Mycosphaerella graminicola* or were associated to three different species of Septoria spp. (*Septoria senecionis, Septoria convolvuli*, and *Septoria apiicola*) because of identical or very similar ITS sequences (Fig. 5). The least abundant STwas identified as *Dissoconium commune* (Fig. 5) while the identification of other STs clustering within the Mycosphaerellaceae family was not possible because of the absence of closely related sequences in genetic databases. In particular, one ST that accounted for 4.2 % of the sequences was found to be somehow related to the genera *Pseudocercospora* and may represent a new species still unknown to the scientific community (Fig. 5).

The second most abundant group of sequences had a relative abundance (RA) of 14.3 % and was represented by three STs related to the order Chaetothyriales (Fig. 4b). According to the phylogenetic analysis with reference sequences of Chaetothyriales [36], these three STs clustered together within the family Chaeothyriaceae but were phylogenetically distant from all currently reported species in GenBank, being with Knufia the most closely related genus (Fig. 6a).

Another group of sequences represented by a single ST had RA of 5.4 % and was associated with the genus Cladophialophora (Fig. 4b). In particular, the phylogenetic analysis of this ST along

with validated reference sequences [37] enabled its identification as Cladophialophora protea (Fig. 6b).

The genus *Cladosporium* had a RA of 5.0 % and was represented by a single ST (Fig. 4b). According to the phylogenetic analysis, this ST clustered within the *Cladosporium cladosporoides* complex (Fig. 6c), but its identification at the species level was not possible due to the complexity of the genus and the existence of many taxa with identical ITS2 sequences [38].

The other two STs, having a cumulative RA of 3.1 %, were associated to the genus Cryptococcus (Fig. 4b). According to BLAST search, the most abundant ST had a 100 % similarity to an uncultured fungus clone (EU486124) detected in the intestine of a dog with inflammatory bowel disease and 99 % similarity with Cryptococcus carnescens (KT819336) detected in seaweed in New Zealand. Cryptococcus was mainly present in Lane Late (7.70 %) compared to Tangelo (1.00 %) and Tarocco scirè (0.70 %).

The genus *Stagonospora* was represented by a single ST with an RA of 2.50 % (Fig. 4b). The identification of this genus must be considered with caution since according to BLAST analyses the most closely related sequences of this genus had only 94 % of homology. On the other hand, a 97 % ofhomology was determined in relationto anuncultured fungus from cactus [39]. Similarly, sequences associated to the genus *Strelitziana* (RA 1.80 %) had the highest homology (94 %) with a sequence of *Strelitziana malaysiana*.

The genus *Colletotrichum* had an RA of 1.4 % and was represented by three STs (Fig. 4b). According to the phylogenetic analyses, the most abundant ST was identified as *C. gloeosporioides* sensu str. [40] (Fig. 6d). The other two STs clustered within the *Colletotrichum boninense* species complex [40, 41]. One of these STs was associated to Colletotrichum karstii and *Colletotrichum phyllanthi* while the second one did not show high identity with any of the currently known species within this species complex [40] (Fig. 6e).

Lastly, four STs having an RA of 1.3 % were associated with the genus *Devriesia*. Phylogenetic analysis along with reference sequences [42] enable the identification of the most abundant STs identified as *Devriesia fraseriae* and *Devriesia hilliana*, respectively (Fig. 6f). The other two STs clearly clustered within the genus but were not identified at the species level. Many other fungal taxa were detected with an RA less than or equal to 1 % and cumulatively represented 9.40 % of all detected sequences (Fig. 4b).

Discussion

Results of the present study indicate that citrus leaves support a high level of fungal diversity, many of which are facultative plant pathogens. In general, Mycosphaerellaceae species were more abundant than any of the other identified taxa. Noteworthy, the family Mycosphaerellaceae contains all fungal species so far associated to CGS disease [1, 6, 8, 16, 43]. Unexpectedly, none of the detected sequences clustered with reference species currently reported as possible causal agents of CGS, including *Mycosphaerella horii* [11], *Z. citri-griseum* [1] and *Z. indonesianum, Z. fructicola*, and *Z. fructigenum* [16].

The most abundant ST of the Mycosphaerellaceae family was associated to the species R. brunnea. This species was originally described to cause leaf spot in *Tussilago farfara*, a perennial herbaceous plant of the family Asteraceae native to Europe and parts of western and central Asia [44] (Fig. 5). Information about *R. brunnea* is very limited and its taxonomic association seems to be still uncertain. That was in part due to existence of several synonyms that are associated with this fungus. For instance, according to the NCBI Taxonomy (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome. html/), R. brunnea is a synonym of *Ramularia grevilleana* (anamorph of *Mycosphaerella fragariae*) which is the causal agent of Strawberry leaf spot [45]. Other less abundant sequences of the Mycosphaerellaceae were associated to different species of the genera *Mycosphaerella, Septoria*, and *Dissoconium* or were thought to represent new putative species related to the genus *Pseudocercospora*.

Nevertheless, the species identification designated in the current study must be viewed with caution since it is only based on the phylogenetic analysis of the ITS2 sequence and because of the complex taxonomy of Mycosphaerellaceae [35]. Yet, these results clearly demonstrate that none of the currently reported causal agents of CGS is involved in the disease in the investigated area in Southern Italy. On the other hand, the abundant presence of sequences clustering with the family Mycosphaerellaceae may suggest the involvement of other Cercosporoid fungi as the causal agent of CGS disease.

Although our data did not show significant differences between symptomatic and asymptomatic leaves, the putative pathogen responsible for CGS may be present in asymptomatic leaves as quiescent or latent infections as it had been previously observed with olive anthracnose [30]. This latter consideration is particularly relevant for Cercosporoid fungi considering their typical

long incubation period [1]. Obviously, specific investigations to isolate and fulfill Koch's postulates are needed to confirm the involvement of these fungi in CGS. However, the long incubation period and difficulties in isolating these slowly growing fungi may greatly complicate such analyses [15]. Indeed, previous attempts to isolate fungal species from orange leaves collected in the same geographic area and showing symptoms closely resembling those of greasy spot disease did not enable the isolation of Mycosphaerella species [19].

Chaetothyriaceae, the second most abundant group of fungi found in this study, are known as epiphytes and can be saprophytic or biotrophic colonizers of leaves and bark. Even though the detected sequences clustered together within this family, they were phylogenetically distant from all currently reported species.

Other non-identified fungi detected with a lower frequency were associated to the phylum Ascomycota or to the order *Capnodiales* and *Pleosporales*, but their accurate identification was not possible due to the lack of closely related sequences in GenBank databases. Similarly, some detected sequences were associated to the genera *Cryptococcus, Stagonospora*, and *Strelitziana*; however, their identification must be considered with caution since according to BLAST analyses, the most closely related sequences had a very low level of homology. All these sequences and many others detected with a low frequency are likely to represent still unknown species and indicate that a considerable portion of the fungal diversity of citrusleaveshas yet to be characterized. Inthis context,further investigations are worthwhile since currently available data do not enable supported speculations on their role in the citrus phyllosphere.

A widely detected group of sequences was identified as *C. protea*. This species was originally isolatedfrom the woody shrub Protea cynaroides, on which it was assumed to be pathogenic, although no inoculation tests have ever been conducted to confirm this hypothesis [46, 47]. C. protea also occurs on dead leaf tissues of the cycad Encephalartos altensteinii [47, 48]. Regardless, there are no records of this species on citrus plants.

The detection of sequences clustering within the *C. cladosporoides* complex was not surprising since the genus *Cladosporium* represents one of the most common fungi of the dematiaceous hyphomycetes [38]. It comprises human and plant pathogens, as well as beneficial fungi [49]. Recently, it was abundantly detected in the olive phyllosphere and it was hypothesized to be involved in the sooty mold symptoms [30]. Indeed, it is widely reported that these fungi can

grow on the surface of leaves and other plant organs covered with insect or physiological honey dew in different plant species including citrus [50].

The genus *Colletotrichum* was represented by a low percentage of sequences associated to *C. gloeosporioides sensu str.* and to two different species of the *C. boninense* species complex (*C. karstii* and *C. phyllanthi*). *Colletotrichum* species are commonly known to be associated to Citrus plants as saprobes, important pre-harvest and post-harvest pathogens, and endophytes [51]. In particular, *C. gloeosporioides* is a cosmopolitan and the most frequently isolated species from citrus; it has been associated to common diseases of fruit, leaves, and twigs collectively named anthracnose [52–57]. However, on citrus, this fungus behaves prevalently as an opportunistic pathogen and colonizes tissues killed or weakened by either more aggressive fungal pathogens such as *Plenodomus tracheiphilus*, the causal agent of mal secco disease of citrus [58, 59], or abiotic agents such as hail or frost. Although *C. karstii* and *C. phyllanthi* cannot be differentiated using the ITS2 region as barcode gene, detected sequences are likely to belong to *C. karstii* since it has been found on citrus in Italy since 1992 [60]. Like *C. gloeosporioides*, it has a wide geographical distribution and is a weak opportunistic pathogen of citrus [51]. The same Colletotrichum species were found in the phyllosphere of olive and other fruits, using both a traditional isolation method and metagenomic approaches [30,32,60, 61].

Other detected sequences were associated to the genus *Devriesia* and in particular to *D. fraseriae* and *D. hilliana*. The first fungus, which represented 1.3 % of citrus leaves, was originally isolated in 2010 in Australia, and it was recently found as the most abundant fungi on olive leaves [30]. It was also detected in the olive fruit fly *Bactrocera oleae* [62, 63], but it has never been reported on citrus. Although little is known about *D. hilliana*, it was originally isolated from leaves of *Macrozamia communis* in New Zeal and little is known about its biological and ecological role [35].

In conclusion, citrus leaves appeared to have more fungal diversity than ever expected. Although Mycosphaerellaceae were the most abundant fungi, a large portion of the detected sequences were not associated with any of the currently known fungal species, indicating that a considerable portion of the detected fungal diversity has yet to be characterized. Even though the present investigation did not provide definitive results about the etiology of CGS in the investigated area, it clearly showed that *Z.citri-griseum* and other fungal species reported to be causal agent of

CGS are not present in the investigated area in Sicily, southern Italy. On the other hand, the abundant detection of *R. brunnea* and other Cercosporoid fungi may suggest their involvement in CGS. Although a more extended survey of citrus orchards in Sicily, including also disease-free citrus growing areas, could be useful to confirm this hypothesis, our study demonstrates the potential use of metagenomic approaches to study the etiology of complex plant diseases.

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Table 1. Summary of analyses and results of metabarcoding surveys conducted with citrus Leaves

Leaf samples[a]	MIDs[b]	OTUs (total)[c]	OTUs (3000)[d]	Shannon	chao1
Nova hybrid tangelo (Sy)	MID7	113	107.5	4.53	135.8
Nova hybrid tangelo (As)	MID10	124	101.0	4.03	128.2
Lane late orange (Sy)	MID16	108	80.7	2.80	122.3
Tarocco Scirè orange (As)	MID19	133	92.2	2.81	120.2
Tarocco Scirè orange (Sy)	MID28	112	86.4	2.81	119.7

[a]Leaf samples comprised leaves with (Sy) and without (As) typical greasy spot symptoms.
[b]MIDs: multiplex identifiers
[c]Total number of detected OTUs
[d]Number of OTUs detected with an even sequencing depth of 3000 sequences

Fig. 1. Citrus leaves of Tarocco Scirè (left), Lane Late (Middle), and Tangelo (right) showing greasy spot-like symptoms, collected in Sicily, southern Italy.

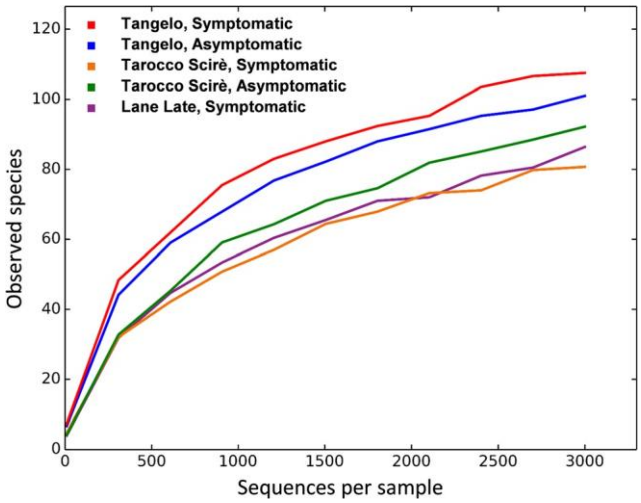

Fig. 2. Species accumulation curves, rarefied at 3000 sequences, determined for citrus samples investigated in the present study phylogenetic analysis utilizing the Maximum Likelihood method [34]. Analyses were performed with 500 bootstrap replications.

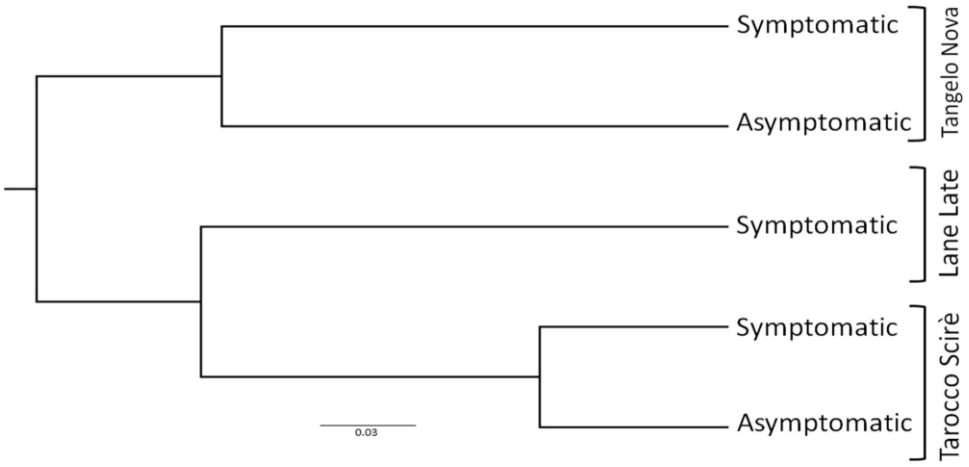

Fig. 3. UPGMA dendrogram constructed using β-diversity results based on Bray Curtis dissimilarity metrics of the studied citrus samples.

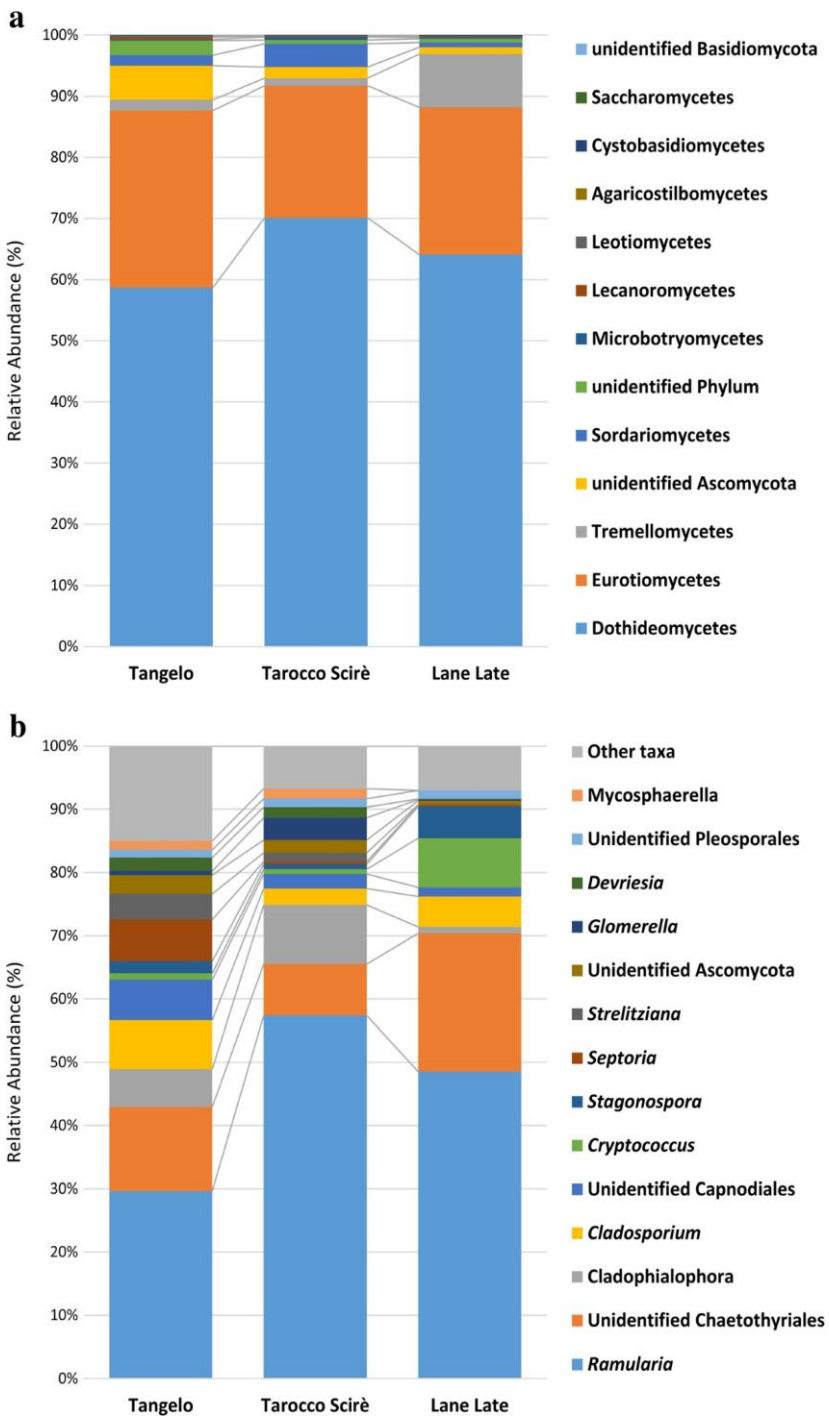

Fig. 4. Relative abundance of fungal classes (a) and genera (b) detected in leaves of sweet orange (cv Tarocco Scirè and Lane Late) and Tangelo Nova. In b, fungal genera representing less than 1 % of the total relative abundance are reported as "Bother taxa".

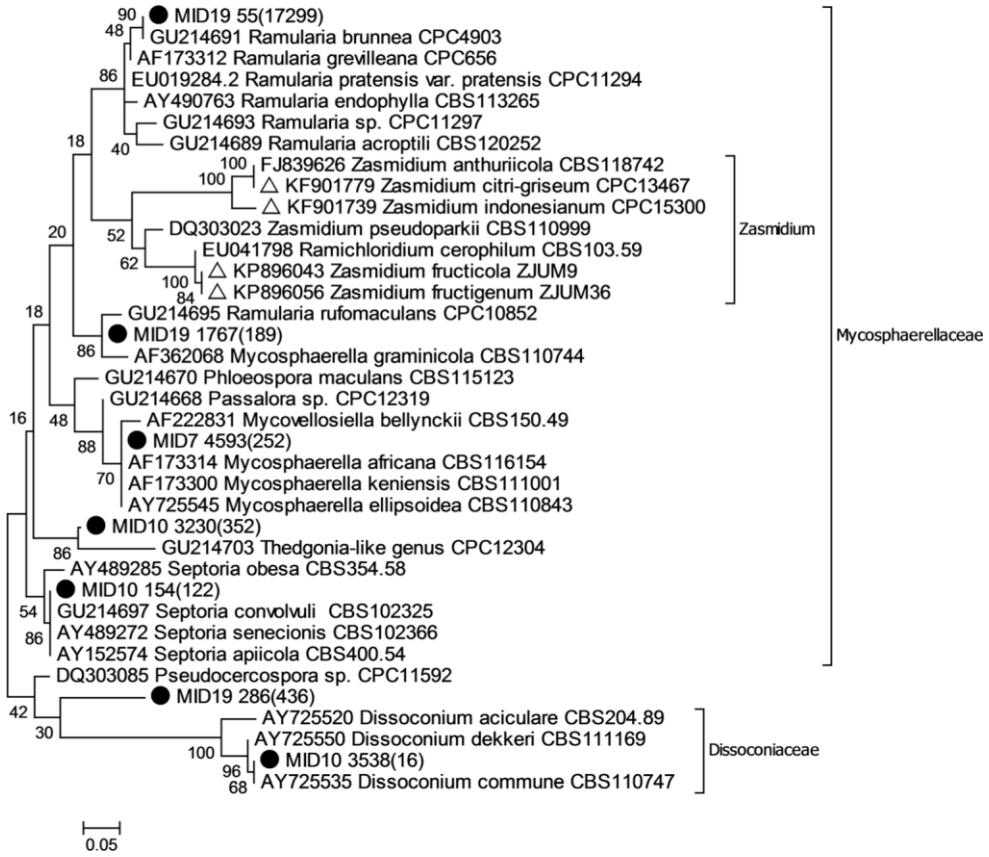

Fig. 5. Phylogenetic trees built using unique representative of sequence types (STs) detected in the present study and validated reference sequences of the family Mycosphaerellaceae [16, 35]. Representative STs and sequences of species reported as causal agents of citrus greasy spot were highlighted with black dots and empty triangles, respectively. Numbers in parentheses along with STs (MIDs) indicate the number of sequences represented by each ST. Numbers on nodes represent the posterior probabilities for the maximum likelihood method.

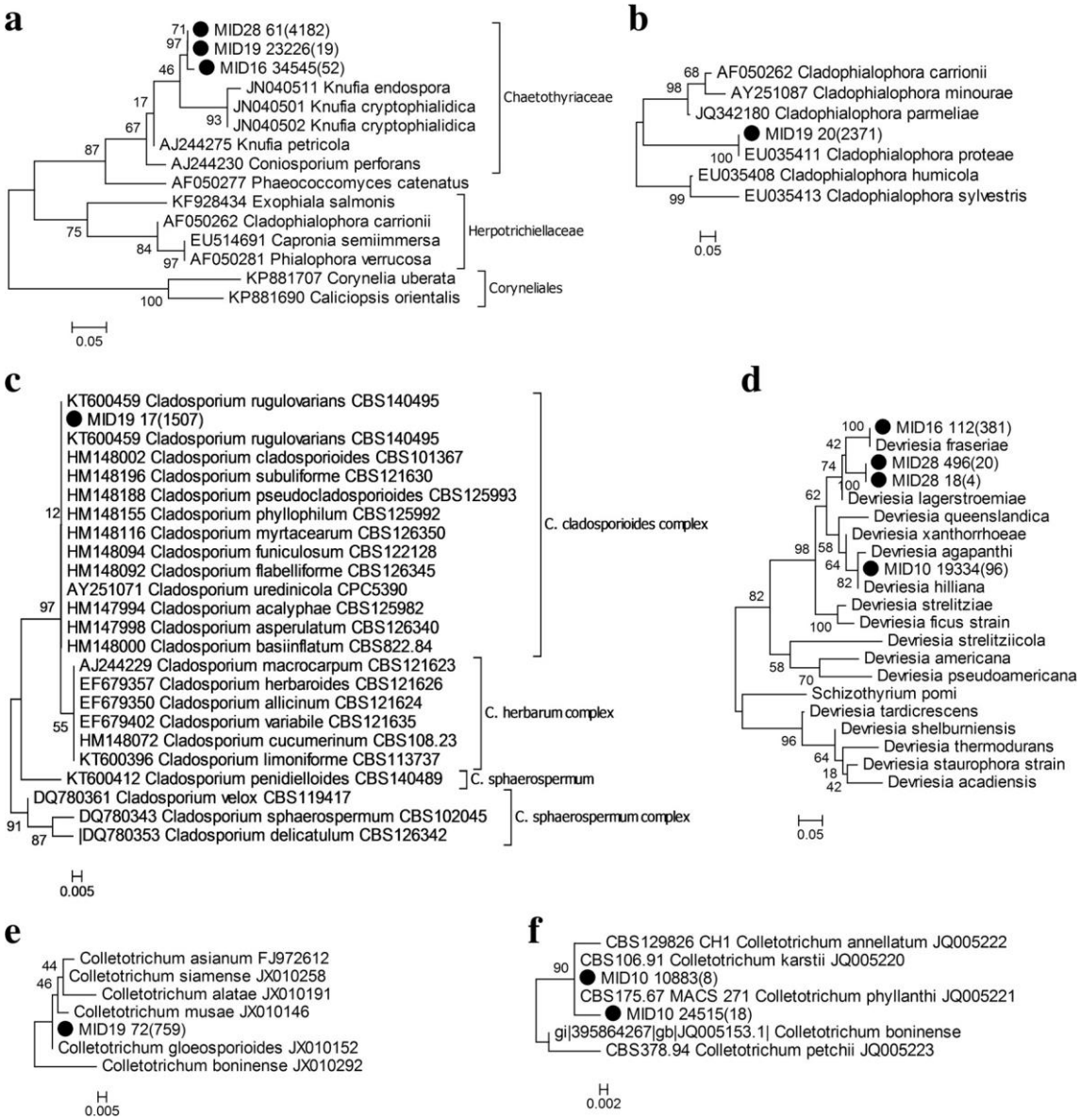

Fig. 6. Phylogenetic trees built using unique representative of sequence types (STs) detected in the present study and validated reference sequences of the order *Chaetothyriales* [36] (a), *Cladophialophora* spp. [37] (b), *Cladosporium* spp. [38] (c), *Devriesia* spp. [42] (d), *Colletotrichum gloeosporioides sensu lato* [40] (e), and *Colletotrichum boninense sensu lato* [41] (f). Representative STs and sequences of species reported as causal agents of citrus greasy spot were highlighted with black dots and empty triangles, respectively. Numbers in parentheses along with STs (MIDs) indicate the number of sequences represented by each ST. Numbers on nodes represent the posterior probabilities for the maximum likelihood method.