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

2 **Symptoms**

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12

13 **Abstract**

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

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

3

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

5

6 **Introduction**

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

17 All Citrus species appear to be susceptible to CGS but the disease symptoms are more severe
18 in lemon, grapefruit, and their hybrids [1, 6–8]. Among sweet oranges, early ripening cultivars are
19 the most susceptible, whereas Valencia-like cultivars show less intense symptoms [2, 6]. Whiteside
20 [4] also reported symptoms on other genera of the family Rutaceae, including Poncirus, Fortunella,
21 Murraya, and Aeglopsis. The disease has been reported within citrus-growing areas in at least 14
22 countries on 6 continents including Italy.

23 Symptoms of CGS were first reported on citrus in Florida [9]. The cause of this disease was
24 unknown for a longtime, and it was thought to be a consequence of nutritional problems or the rust
25 mite *Phyllocoptruta oleivora* [10]. The involvement of a Cercosporoid fungus was firstly
26 hypothesized in Japan where the fungus *Mycosphaerella horii* was isolated from symptomatic leaves
27 [11]. The fungus *Zasmidium citri-griseum* (syn. *Stenella citri-grisea*, teleomorph *Mycosphaerella*
28 *citri*) was first proposed as the causal agent of CGS by Whiteside [5] and is now generally recognized
29 as the causal agent of CGS [1]. Ascospores of *M. citri* are produced in pseudothecia in decomposing
30 leaf litter on the ground [4]. When the pseudothecia mature, ascospores are ejected following a

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

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

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

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

25

26 **Materials and Methods**

27 *Sampling and DNA Extractions*

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

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

15

16 *Fungal DNA Amplification and Sequencing*

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

22 PCR reactions were conducted in a total volume of 25 µl containing 2.5 µl of 10× reaction
23 buffer, 0.25 µl of each primer ITS3-ITS4 (10 µM), 0.1 µl of AccuPrime Taq DNA Polymerase High
24 Fidelity (Invitrogen, CA, USA), and 1 µl of DNA template (10 ng/µl). Reactions were incubated in an
25 Eppendorf Mastercycler gradient (Hamburg, Germany) for 1 min at 94 °C followed by 30 cycles of
26 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
27 72 °C. For each sample, amplicons from the tree sub-samples were pooled and purified using the
28 Agencourt AMPure XP system (Beckman Coulter, Inc.). The concentration and quality of the purified
29 amplicons were evaluated by agarose gel electrophoresis. Amplicons were sequenced by Macrogen
30 Inc. (Seoul, Korea) using the 454 GS FLX + System (Roche Diagnostics Corporation).

1 *Data Analysis and Statistics*

2 The bioinformatics pipeline, QIIME v. 1.8 [23], was used to process and analyze the obtained
3 sequence data. Preliminary processing of data included de-multiplexing and quality filtering with a
4 minimum quality score of 25, a minimum/maximum length of 150/1000, and a maximum number
5 of homopolymer bases of 6. Sequences were denoised using the denoise wrapper [24] and the ITS2
6 region was extracted using ITSx application [25]. Chimeric sequences were identified and filtered
7 using the USEARCH 6.1 software [26]. Sequences were clustered at 99 % similarity threshold using
8 USEARCH 6.1 software, and the most abundant sequences in each operational taxonomic unit (OTU)
9 were selected as representative sequences. These sequences were then used for the taxonomy
10 assignment. OTUs were picked using the UNITE dynamic database released on January 8,
11 2015(<http://unite.ut.ee/>) as a reference database. The same database was also used for taxonomy
12 assignments using the BLAST algorithm [27] at a similarity threshold of 0.97.

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

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

24 25 *Identification of Fungal Taxa*

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

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

8 **Results**

9 *Fungal Diversity and Richness*

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

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

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

26 *Fungal Community Structure*

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

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

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

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

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

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

1 with validated reference sequences [37] enabled its identification as *Cladophialophora protea* (Fig. 2 6b).

3 The genus *Cladosporium* had a RA of 5.0 % and was represented by a single ST (Fig. 4b). 4 According to the phylogenetic analysis, this ST clustered within the *Cladosporium cladosporoides* 5 complex (Fig. 6c), but its identification at the species level was not possible due to the complexity 6 of the genus and the existence of many taxa with identical ITS2 sequences [38].

7 The other two STs, having a cumulative RA of 3.1 %, were associated to the genus 8 *Cryptococcus* (Fig. 4b). According to BLAST search, the most abundant ST had a 100 % similarity to 9 an uncultured fungus clone (EU486124) detected in the intestine of a dog with inflammatory bowel 10 disease and 99 % similarity with *Cryptococcus carnescens* (KT819336) detected in seaweed in New 11 Zealand. *Cryptococcus* was mainly present in Lane Late (7.70 %) compared to Tangelo (1.00 %) and 12 Tarocco scirè (0.70 %).

13 The genus *Stagonospora* was represented by a single ST with an RA of 2.50 % (Fig. 4b). The 14 identification of this genus must be considered with caution since according to BLAST analyses the 15 most closely related sequences of this genus had only 94 % of homology. On the other hand, a 97 % 16 of homology was determined in relation to an uncultured fungus from cactus [39]. Similarly, 17 sequences associated to the genus *Strelitziana* (RA 1.80 %) had the highest homology (94 %) with a 18 sequence of *Strelitziana malaysiana*.

19 The genus *Colletotrichum* had an RA of 1.4 % and was represented by three STs (Fig. 4b). 20 According to the phylogenetic analyses, the most abundant ST was identified as *C. gloeosporioides* 21 sensu str. [40] (Fig. 6d). The other two STs clustered within the *Colletotrichum boninense* species 22 complex [40, 41]. One of these STs was associated to *Colletotrichum karstii* and *Colletotrichum* 23 *phyllanthi* while the second one did not show high identity with any of the currently known species 24 within this species complex [40] (Fig. 6e).

25 Lastly, four STs having an RA of 1.3 % were associated with the genus *Devriesia*. Phylogenetic 26 analysis along with reference sequences [42] enable the identification of the most abundant STs 27 identified as *Devriesia fraseriae* and *Devriesia hilliana*, respectively (Fig. 6f). The other two STs 28 clearly clustered within the genus but were not identified at the species level. Many other fungal 29 taxa were detected with an RA less than or equal to 1 % and cumulatively represented 9.40 % of all 30 detected sequences (Fig. 4b).

1 Discussion

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

9 The most abundant ST of the Mycosphaerellaceae family was associated to the species *R.*
10 *brunnea*. This species was originally described to cause leaf spot in *Tussilago farfara*, a perennial
11 herbaceous plant of the family Asteraceae native to Europe and parts of western and central Asia
12 [44] (Fig. 5). Information about *R. brunnea* is very limited and its taxonomic association seems to be
13 still uncertain. That was in part due to existence of several synonyms that are associated with this
14 fungus. For instance, according to the NCBI Taxonomy
15 (<http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/>), *R. brunnea* is a synonym of
16 *Ramularia grevilleana* (anamorph of *Mycosphaerella fragariae*) which is the causal agent of
17 Strawberry leaf spot [45]. Other less abundant sequences of the Mycosphaerellaceae were
18 associated to different species of the genera *Mycosphaerella*, *Septoria*, and *Dissoconium* or were
19 thought to represent new putative species related to the genus *Pseudocercospora*.

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

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

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

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

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

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

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

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

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

18 Other detected sequences were associated to the genus *Devriesia* and in particular to *D.*
19 *fraseriae* and *D. hilliana*. The first fungus, which represented 1.3 % of citrus leaves, was originally
20 isolated in 2010 in Australia, and it was recently found as the most abundant fungi on olive leaves
21 [30]. It was also detected in the olive fruit fly *Bactrocera oleae* [62, 63], but it has never been
22 reported on citrus. Although little is known about *D. hilliana*, it was originally isolated from leaves
23 of *Macrozamia communis* in New Zeal and little is known about its biological and ecological role
24 [35].

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

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

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10

11 **References**

- 12 1. Mondal S, Timmer L (2006) Greasy spot, a serious endemic problem for citrus production in the
13 Caribbean Basin. *Plant Dis* 90(5): 532–538
- 14 2. Whiteside J (1988) Greasy spot and greasy spot rind blotch. *Compendium of Citrus Diseases*.
15 Whiteside, JO, Garnsey, SM and Timmer, LW (Eds). APS, St Paul, Minnesota, USA, pp 15-17
- 16 3. Whiteside J (1983) Fungicidal effects of some acaricides on *Mycosphaerella citri*. *Plant Dis*
17 67(8):864–866
- 18 4. Whiteside J (1972) Histopathology of citrus greasy spot and identification of the causal fungus.
19 *Phytopathology* 62(2):260–263
- 20 5. Whiteside J (1970) Etiology and epidemiology of citrus greasy spot. *Phytopathology*
21 60(10):1409–1414
- 22 6. Timmer L, Gottwald T (2000) Greasy spot and similar diseases. *Compendium of citrus diseases'*
23 *Second edition*. LW Timmer, SM Garnsey, JH Graham (Eds). pp 25-28
- 24 7. Mondal S, Timmer L (2002) Environmental factors affecting pseudothecial development and
25 ascospore production of *Mycosphaerella citri*, the cause of citrus greasy spot. *Phytopathology*
26 92(12):1267–1275
- 27 8. Timmer L, Roberts P, Darhower H, Bushong P, Stover E, Peever T, Ibáñez A (2000) Epidemiology
28 and control of citrus greasy spot in different citrus-growing areas in Florida. *Plant Dis* 84(12):1294–
29 1298

- 1 9. Fawcett HS (1915) Citrus diseases of Florida and Cuba compared with those of California. Calif
2 Agric Exp Stn Bull B262:153–211
- 3 10. Thompson W (1948) Greasy spot on citrus leaves. Citrus Industry 29(4):20–22
- 4 11. Yamada S (1956) Studies on the greasy spot (black melanose) of citrus.
5 II. Morphological characteristics of the causal fungus (*Mycosphaerella horii* Hara). Horticulture
6 Division, National Tokai-kinki Agricultural Experiment Station, Okitsu Japan. Bulletin 3:49–62
- 7 12. Whiteside J (1970) Effect of fungicides applied to citrus trees on perithecial development by the
8 greasy spot fungus in detached leaves. Plant Dis Rep 54:865–869
- 9 13. Hidalgo H, Sutton TB, Arauz F (1997) Epidemiology and control of citrus greasy spot on Valencia
10 orange in the humid tropics of Costa Rica. Plant Dis 81(9):1015–1022
- 11 14. Haggag W (2012) First report of *Mycosphaerella citri*, the agent of greasy spot of orange in Egypt.
12 J Plant Pathol 94(4): pS4.92.
- 13 15. Wellings C (1981) Pathogenicity of fungi associated with citrus greasy spot in New South Wales.
14 Trans Br Mycol Soc 76(3):495–499
- 15 16. Huang F, Groenewald J, Zhu L, Crous P, Li H (2015) Cercosporoid diseases of citrus. Mycologia
16 107(6):1151–1171
- 17 17. Grasso S, Catara A (1982) Osservazioni su intumescenze gommose delle foglie di agrumi [dovute
18 a funghi patogeni]. Informatore fitopatologico 32:43–46
- 19 18. Grasso FM, Bella P, Grasso S, Catara A (2008) New or re-emerging fungal citrus diseases in the
20 Mediterranean. Integr Contr Citrus Fruit Crops 38:301–304
- 21 19. Biasi A, De Patrizio A, Faedda R, Schena L, Pane A, Cacciola S, Magnano di San Lio G (2012)
22 *Septoria citri* is a common pathogen of citrus in Southern Italy. J Plant Pathol 94(4)
- 23 20. Wang W-L, Xu S-Y, Ren Z-G, Tao L, Jiang J-W, Zheng S-S (2015) Application of metagenomics in
24 the human gut microbiome. World J Gastroenterol 21(3):803–814
- 25 21. Xu X, Passey T, Wei F, Saville R, Harrison RJ (2015) Amplicon based metagenomics identified
26 candidate organisms in soils that caused yield decline in strawberry. Horticulture Research
27 2:15022
- 28 22. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal
29 RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18:315–322

- 1 23. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG,
2 Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA,
3 McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann
4 J, Yatsunencko T, Zaneveld J, Knight R (2010) QIIME allows analysis of highthroughput community
5 sequencing data. *Nat Methods* 7(5):335– 336. doi:[10.1038/nmeth.f.303](https://doi.org/10.1038/nmeth.f.303)
- 6 24. Reeder J, Knight R (2010) Rapidly denoising pyrosequencing amplicon reads by exploiting rank-
7 abundance distributions. *Nat Methods* 7(9):668–669. doi:[10.1038/nmeth0910-668b](https://doi.org/10.1038/nmeth0910-668b)
- 8 25. Bengtsson-Palme J, Ryberg M, Hartmann M, Branco S, Wang Z, Godhe A, De Wit P, Sánchez-
9 García M, Ebersberger I, de Sousa F, Amend A, Jumpponen A, Unterseher M, Kristiansson E,
10 Abarenkov K, Bertrand YJK, Sanli K, Eriksson KM, Vik U, Veldre V, Nilsson RH (2013) Improved
11 software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and
12 other eukaryotes for analysis of environmental sequencing data.
- 13 26. *Methods Ecol Evol* 4(10):914–919. doi:[10.1111/2041-210X.12073](https://doi.org/10.1111/2041-210X.12073) 26. Edgar RC (2010) Search
14 and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26(19):2460–2461.
15 doi:[10.1093/bioinformatics/btq461](https://doi.org/10.1093/bioinformatics/btq461)
- 16 27. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J*
17 *Mol Biol* 215(3):403–410, [http://dx.doi.org/10.1016/S0022-2836\(05\)80360-2](http://dx.doi.org/10.1016/S0022-2836(05)80360-2)
- 18 28. Bray JR, Curtis JT (1957) An ordination of the upland forest communities of southern Wisconsin.
19 *Ecol Monogr* 27(4):325–349
- 20 29. Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial
21 communities. *Appl Environ Microbiol* 71(12):8228–8235. doi:[10.1128/AEM.71.12.8228-](https://doi.org/10.1128/AEM.71.12.8228-8235.2005)
22 [8235.2005](https://doi.org/10.1128/AEM.71.12.8228-8235.2005)
- 23 30. Abdelfattah A, Nicosia MGLD, Cacciola SO, Droby S, Schena L (2015) Metabarcoding analysis of
24 fungal diversity in the phyllosphere and carposphere of olive (*Olea europaea*). *PLoS ONE* 10(7),
25 e0131069
- 26 31. Abdelfattah A, Wisniewski M, Droby S, Schena L (2016) Spatial and compositional variation in
27 the fungal communities of organic and conventionally grown apple fruit at the consumer point-
28 of-purchase. *Horticulture Research* 3:16047
- 29 32. Abdelfattah A, Wisniewski M, Li Destri Nicosia MG, Cacciola SO, Schena L (2016) Metagenomic
30 analysis of fungal diversity on strawberry plants and the effect of management practices on the

- 1 fungal community structure of aerial organs. PLoS ONE 11(8), e0160470.
2 doi:[10.1371/journal.pone.0160470](https://doi.org/10.1371/journal.pone.0160470)
- 3 33. Prigigallo MI, Abdelfattah A, Cacciola SO, Faedda R, Sanzani SM, Cooke DE, Schena L (2016)
4 Metabarcoding analysis of Phytophthora diversity using genus-specific primers and 454
5 pyrosequencing. Phytopathology 106(3):305–313. doi:[10.1094/PHYTO-07-15-0167-R](https://doi.org/10.1094/PHYTO-07-15-0167-R)
- 6 34. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary
7 genetics analysis version 6.0. Mol Biol Evol 30(12):2725–2729. doi:[10.1093/molbev/mst197](https://doi.org/10.1093/molbev/mst197)
- 8 35. Crous PW, Schoch CL, Hyde KD, Wood AR, Gueidan C, deHoog GS, Groenewald JZ (2009)
9 Phylogenetic lineages in the Capnodiales. Stud Mycol 64:17–47S17. doi:[10.3114/sim.2009.64.02](https://doi.org/10.3114/sim.2009.64.02)
- 10 36. Réblová M, Untereiner WA, Réblová K (2013) Novel evolutionary lineages revealed in the
11 Chaetothyriales (Fungi) based on multigene phylogenetic analyses and comparison of ITS
12 secondary structure. PLoS ONE 8(5), e63547
- 13 37. Liu X-Y, Udayanga D, Luo Z-L, Chen L-J, Zhou D-Q, Su HY, Hyde KD (2015) Backbone tree for
14 Chaetothyriales with four new species of Minimelanolocus from aquatic habitats. Fungal biology
15 119(11):1046–1062
- 16 38. Bensch K, Groenewald J, Braun U, Dijksterhuis J, de Jesús Y-MM, Crous P (2015) Common but
17 different: the expanding realm of Cladosporium. Stud Mycol 82:23–74
- 18 39. Fonseca-Garcia C, Coleman-Derr D, Garrido E, Visel A, Tringe S, Partida-Martinez L (2015) The
19 Cacti microbiome interplay between habitat-filtering and host-specificity. Front Microbiol 7:150
- 20 40. Weir B, Johnston P, Damm U (2012) The Colletotrichum gloeosporioides species complex. Stud
21 Mycol 73(1):115–180
- 22 41. Damm U, Cannon P, Woudenberg J, Johnston P, Weir B, Tan Y, Shivas R, Crous P (2012) The
23 Colletotrichum boninense species complex. Stud Mycol 73(1):1–36
- 24 42. Li W, Xiao Y, Wang C, Dang J, Chen C, Gao L, Batzer JC, Sun G, Gleason ML (2013) A new species
25 of Devriesia causing sooty blotch and flyspeck on rubber trees in China. Mycol Prog 12(4): 733–
26 738. doi:[10.1007/s11557-012-0885-z](https://doi.org/10.1007/s11557-012-0885-z)
- 27 43. Mondal S, Timmer L (2003) Effect of urea, CaCO₃, and dolomite on pseudothecial development
28 and ascospore production of Mycosphaerella citri. Plant Dis 87(5):478–483
- 29 44. Wolf FA (1912) The brown leaf spot of Colt's Foot, Tussilago Farfara L. Annales Mycologici 10:65–
30 67

- 1 45. Paulus AO (1990) Fungal diseases of strawberry. HortSci 25(8):885–889
- 2 46. Crous P, Wood A, Okada G, Groenewald J (2008) Foliicolous microfungi occurring on
3 Encephalartos. Persoonia-Mol Phylogeny Evol Fungi 21(1):135–146
- 4 47. Swart L, Crous P, Denman S, Palm M (1998) Fungi occurring on Proteaceae. I. S Afr J Bot
5 64(2):137–145
- 6 48. Crous PW, Schubert K, Braun U, De Hoog G, Hocking A, Shin H-D, Groenewald J (2007)
7 Opportunistic, human-pathogenic species in the Herpotrichiellaceae are phenotypically similar
8 to saprobic or phytopathogenic species in the Venturiaceae. Stud Mycol 58:185–217
- 9 49. Bensch K, Braun U, Groenewald JZ, Crous PW (2012) The genus Cladosporium. Stud Mycol 72:1–
10 401
- 11 50. Tashiro N, Noguchi M, Ide Y, Kuchiki F (2013) Sooty spot caused by Cladosporium cladosporioides
12 in postharvest Satsuma mandarin grown in heated greenhouses. J Gen Plant Pathol 79(2):158–
13 161
- 14 51. Huang F, Chen G, Hou X, Fu Y, Cai L, Hyde K, Li H (2013) Colletotrichum species associated with
15 cultivated citrus in China. Fungal Divers 61(1):61–74
- 16 52. Benyahia H, Ifi A, Smaili C, Afellah M, Lamsetef Y, Timmer L (2003) First report of Colletotrichum
17 gloeosporioides causing withertip on twigs and tear stain on fruit of citrus in Morocco. Plant
18 Pathol 52(6):798–798
- 19 53. Peng L, Yang Y, Hyde KD, Bahkali AH, Liu Z (2012) Colletotrichum species on citrus leaves in
20 Guizhou and Yunnan provinces, China. Cryptogam Mycol 33(3):267–283
- 21 54. McGovern RJ, Seijo TE, Hendricks K, Roberts PD (2012) New report of Colletotrichum
22 gloeosporioides causing postbloom fruit drop on citrus in Bermuda. Can J Plant Pathol
23 34(2):187–194
- 24 55. Moges AD, Admassu B, Belew D, Yesuf M, Njuguna J, Kyalo M, Ghimire SR (2016) Development
25 of microsatellite markers and analysis of genetic diversity and population structure of
26 *Colletotrichum gloeosporioides* from Ethiopia. PLoS ONE 11(3), e0151257
- 27 56. Timmer LW, Garnsey SM, Graham JH (2000) Compendium of citrus diseases, 2nd edn. American
28 Phytopathological Society (APS Press), St. Paul, MN

- 1 57. Rhaiem A, Taylor PWJ (2016) *Colletotrichum gloeosporioides* associated
2 with anthracnose symptoms on citrus, a new report for Tunisia. *Eur J Plant Pathol* 146(1):219–224.
3 doi:[10.1007/s10658-016-0907-9](https://doi.org/10.1007/s10658-016-0907-9)
- 4 58. Migheli Q, Cacciola SO, Balmas V, Pane A, Ezra D, Magnano di San Lio G (2009) Mal secco disease
5 caused by *Phoma tracheiphila*: a potential threat to lemon production worldwide.
6 *Plant Dis* 93(9):852–867
- 7 59. Nigro F, Ippolito A, Salerno M (2011) Mal secco disease of citrus: a journey through a century of
8 research. *J Plant Pathol* 93(3):523–560
- 9 60. Schena L, Mosca S, Cacciola S, Faedda R, Sanzani S, Agosteo G, Sergeeva V, Magnano di San Lio
10 G (2014) Species of the *Colletotrichum gloeosporioides* and *C. boninense* complexes associated
11 with olive anthracnose. *Plant Pathol* 63(2):437–446
- 12 61. Mosca S, Li Destri Nicosia MG, Cacciola SO, Schena L (2014) Molecular analysis of *Colletotrichum*
13 species in the carposphere and phyllosphere of olive. *PLoS ONE* 9(12), e114031.
14 doi:[10.1371/journal.pone.0114031](https://doi.org/10.1371/journal.pone.0114031)
- 15 62. Malacrinò A, Schena L, Campolo O, Laudani F, Palmeri V (2015) Molecular analysis of the fungal
16 microbiome associated with the olive fruit fly *Bactrocera oleae*. *Fungal Ecol* 18:67–74, [http://dx.](http://dx.doi.org/10.1016/j.funeco.2015.08.006)
17 [doi.org/10.1016/j.funeco.2015.08.006](http://dx.doi.org/10.1016/j.funeco.2015.08.006)
- 18 63. Malacrinò A, Schena L, Campolo O, Laudani F, Mosca S, Giunti G, Strano CP, Palmeri V (2017) A
19 Metabarcoding Survey on the Fungal Microbiota Associated to the Olive Fruit Fly. *Microb Ecol.*
20 doi:[10.1007/s00248-016-0864-z](https://doi.org/10.1007/s00248-016-0864-z)

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1 **Table 1.** Summary of analyses and results of metabarcoding surveys conducted with citrus
 2 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 |

9
 10 ^aLeaf samples comprised leaves with (Sy) and without (As) typical greasy spot symptoms.

11 ^bMIDs: multiplex identifiers

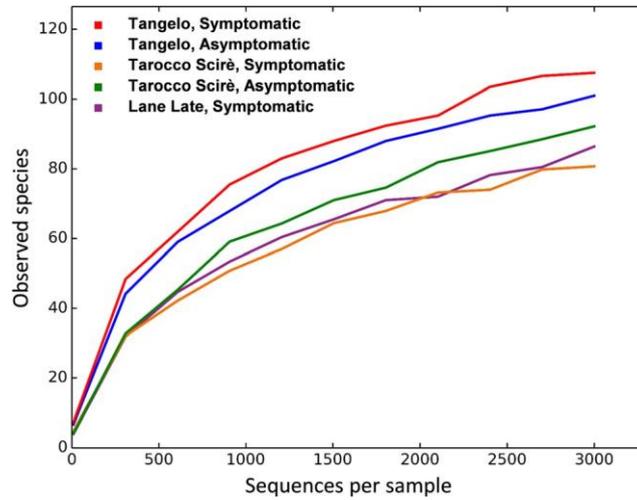
12 ^cTotal number of detected OTUs

13 ^dNumber of OTUs detected with an even sequencing depth of 3000 sequences

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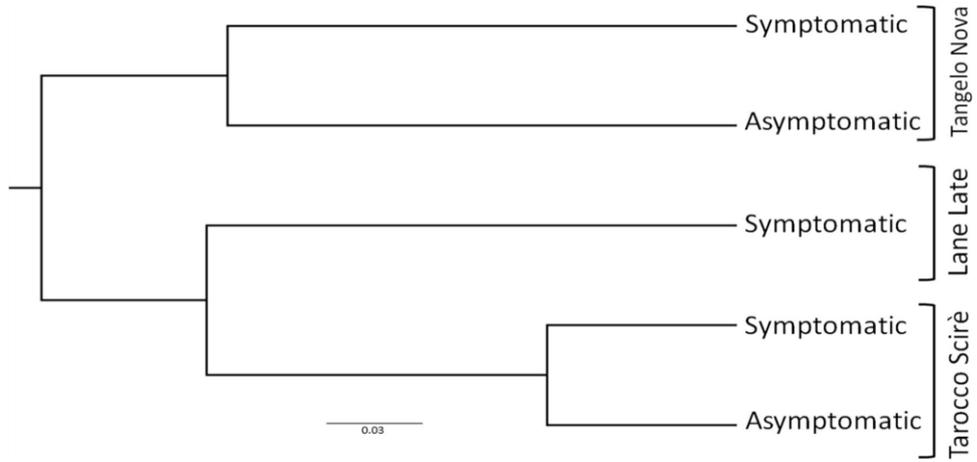
10 **Fig. 1.** Citrus leaves of Tarocco Scirè (left), Lane Late (Middle), and Tangelo (right) showing greasy
11 spot-like symptoms, collected in Sicily, southern Italy.



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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.

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

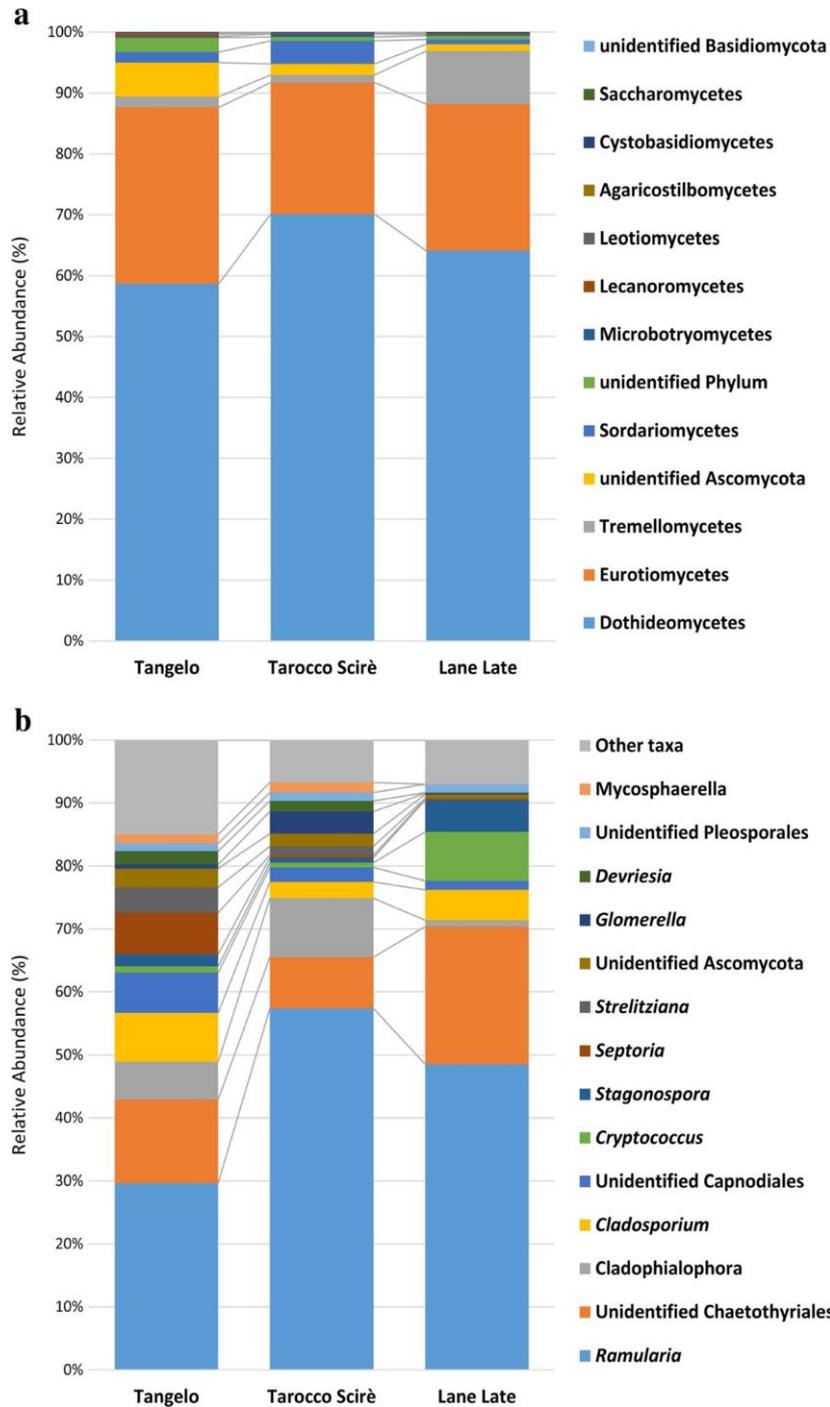
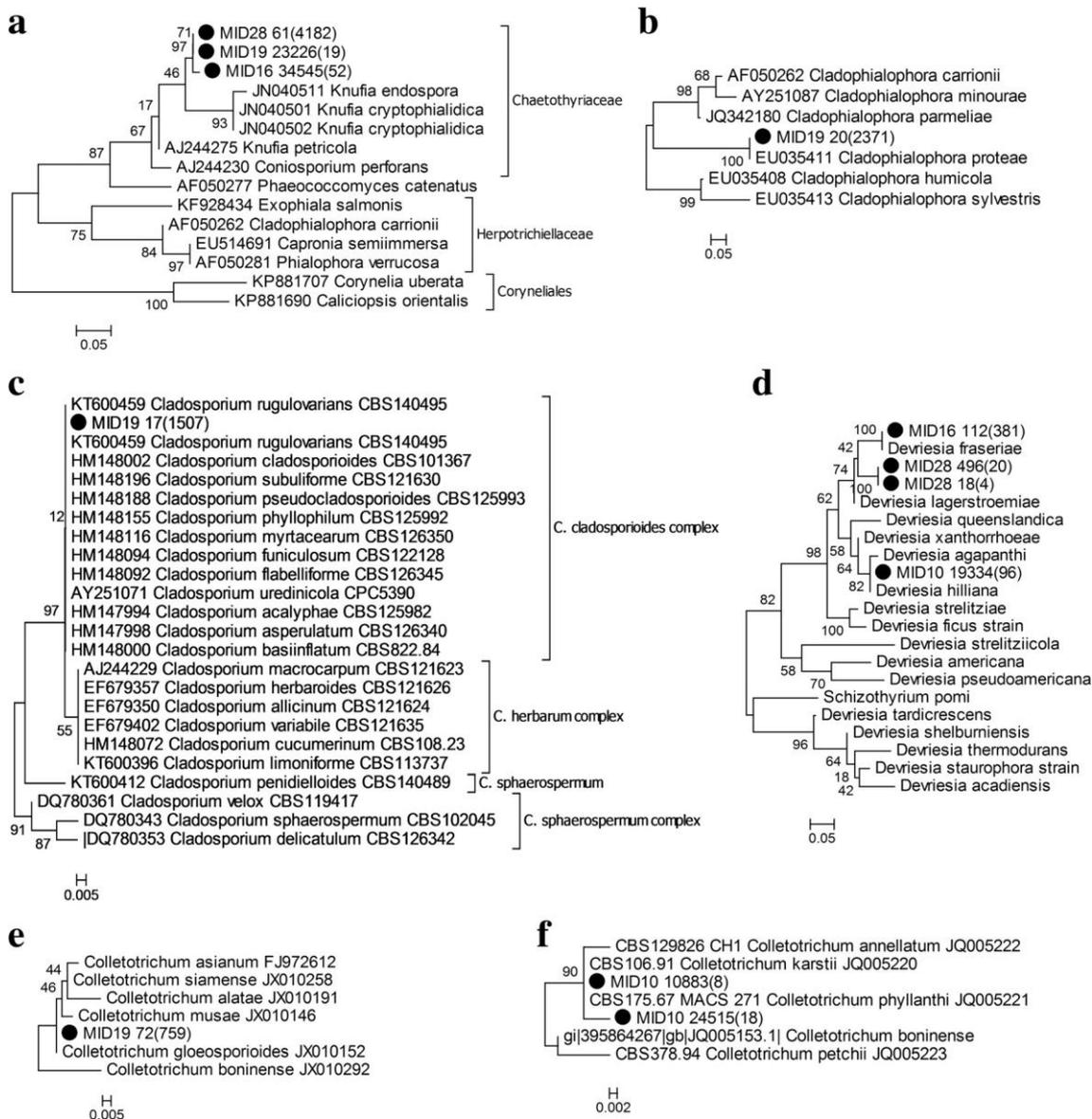


Fig. 4. Relative abundance of fungal classes (a) and genera (b) detected in leaves of sweet orange (cv Tarocco Scirè and Lane Late) and Tangelo Nova. In b, fungal genera representing less than 1 % of the total relative abundance are reported as "Bother taxa".



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.



1

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