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INSECTS AND FUNGI: ECOLOGICAL INTERACTIONS AND FUNCTIONAL BIODIVERSITY

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Chapter 1

Interactions between insects and microorganisms in the era of 'omics' sciences: an outline

Microbial ecology and 'omics' sciences

Microbes are the main drivers of several fundamental physical, chemical and biological phenomena [1]. The study of their ecology is widely spreading all around the scientific community. In particular, during the last decade, culture-independent techniques to study microbial communities (microbiota) and their genes (microbiome), gained a great deal of attention [2]. Several studies shed light on microbial assemblies associated to human body, leading to important discoveries on diseases, disorders and human development, as well as new sources of bioactive compounds able to revolutionize entire fields [2, 3]. However, human microbiology has been just the beginning, and this field expanded within other research topics, from marine ecology to food science and insect science [4-6].

This expansion was boosted mainly after the diffusion of 'omics' techniques in microbial ecology, which allowed to study with increasing details the response of microbes to environmental factors. These are high-throughput, data-driven, holistic and top-down methodologies that aim to help in the functional characterization of cell biology and its response to external factors. The main aim of these technologies is the characterization of entire genomes, transcriptomes, protein bulks, metabolites, and so on (reviewed in [7]). In this context, High Throughput Sequencing (HTS) techniques represent a powerful tool to unveil the role of genes, and subsequently transcripts, proteins and metabolites, of entire microbial communities [8]. These technologies have been rapidly changed over the last years, changing also the way to analyse microbial communities, which previously relied on fragment cloning and Sanger sequencing. This field was firstly revolutionized by the benchtop sequencer Roche 454 GS-FLX/+, which have been lead the field in the last years, but now it is going to be phased-out [9]. Thereafter, Illumina and Life Technologies released the machines that are now commonly used in characterizing microbial communities, such as MiSeq, NextSeq and HiSeq (Illumina), IonTorrent and IonProton (Life Technologies). Furthermore, there is growing interest in using longer reads (>600 bp), providing much more reliable taxonomic classification, with the use of PacBio RSII (Pacific Biosciences) and Oxford Nanopore Technologies.

The main way these technologies have been exploited in microbial ecology is to perform meta'omics studies, aiming at identifying microbial communities, their genes along with their expression, the metabolic pathways and functions in environmental and uncultured samples [8]. Among these, metagenomics refers to the sequencing of the DNA of whole communities, aiming to understand their composition by analysing the whole metagenome through shotgun sequencing, or more frequently, targeting a specific region of the rRNA gene as barcode (16S for bacteria, 18S for eukaryotes and ITS2 for fungi as examples) with the approach called metabarcoding [8, 10, 11].

Metabarcoding: an overview

The term metabarcoding was introducted by Taberlet *et al*. [11], defining it as a "*high-throughput multispecies (or higher-level taxon) identification using the total and typically degraded DNA extracted from an environmental sample*". This technique is the evolution of more classical DNA barcoding that rely on the use of a series of genes, conserved at species level, used to perform molecular taxonomic identification [12]. The metabarcoding mainly diffused among different disciplines following the development of HTS machines, that are capable to generate millions of short reads, allowing a complete reconstruction of the microbial community with bioinformatics analyses. The characterization of a microbial community with this approach can be achieved through these steps: (i) sampling; (ii) DNA extraction; (iii) targeted amplification; (iv) sequencing; (v) data analysis.

Sampling is a crucial step in this process, as an adequate and even collection of matrices to be analysed can ensure a correct reconstruction of the microbial community. As well, DNA extraction and PCR steps must be conducted with particular care, as microbes are present everywhere in the environment, and a careless handling of samples can result in carrying unwanted microorganisms over the whole analysis, to steps where it would not be possible to distinguish them from those extracted from samples. As reported above, sequencing could be performed with different technologies, spanning in terms of number of reads from Roche 454 (up to 700,000) to Illumina HiSeq (up to 300 millions), that impact on the full reconstruction of the microbial community and on the number of samples that can be multiplexed together. Several works describe these procedures in details, explaining how to deal with approaches in different contexts and in dealing with different techniques [9, 13-15].

Data analysis is the final step of the procedure, and it is often time and power consuming, as it is very computing intensive. This step relies on different bioinformatics tools, such as QIIME, MOTHUR and OBITOOLS among the most commonly used [16-18]. The entire analysis could be divided into two main parts:

1. Generation of OTU table. This part involves the direct handling of sequence data, filtering low quality information, grouping sequences by similarity and assigning a name to each bin of similar sequences.
2. Statistical analysis. Once we have what so called 'OTU table' (described below), it is possible to perform any kind of statistical analysis on the dataset.

The first part could be divided into the following steps: (i) demultiplexing; (ii) quality filtering; (iii) OTU picking; (iv) representative set picking; (v) taxonomy assignment; (vi) OTU table building. The demultiplexing step aims to assign reads to the respective sample. Since all samples are pooled together before sequencing, each sample is tagged during PCR by adding a single or a double barcode of 6/8 bp named GoLay barcode [19]. Afterwards, the bioinformatics software can read and use barcodes to associate each read to the proper sample.

The purpose of quality filtering is to discard reads with low quality base calls, chimeras, and short reads that could lead to misinterpretation of the final results. For 454 pyrosequencing, this step includes also the denoising procedure, which aim to reduce the impact of sequencing errors due to long homopolymers. Once quality filtered, reads are clusterized using a wide variety of algorithms. Most of them rely on the selection of seed sequences from data to generate clusters that contain similar sequences according to a preselected threshold of similarity (commonly 97%). After generating these clusters, singletons (clusters containing only 1 sequence) are usually discarded to avoid misleading results due to sequencing errors, and a representative set of sequences is generated. This set include one representative sequence for each cluster, and it is useful to speed up the taxonomy assignment, which is performed against a set of pre-built databases such as Greengenes for 16S, SILVA for 18S and UNITE for ITS2 [20-22]. Alternatively, custom databases can be also used. The first part of the procedure ends with building up the OTU table. This file contains the number of reads for each OTU within each sample, and allows a tremendous wide range of statistical tests among samples. To complete this part, the representative set of sequences can be aligned and phylogenetically analysed. These steps can be useful for further data analyses.

The second part can be carried out using the same software packages, or in union with R statistical software plugged with a very diverse set of packages like 'vegan' or 'Bioconductor' [23-25]. This part of the analysis commonly includes alpha diversity, beta diversity, and statistical comparison. The first one aims to analyse the diversity within each samples, whereas the beta diversity analyse the diversity among different samples, comparing them with multivariate procedures such as PCA, PCoA, NMDS and so on. These statistical analyses could be coupled with other approaches in order to highlight differences and similarities, such as ANOSIM and PERMANOVA. Further insights can be achieved by computing the network of OTU occurrence in order to represent graphically

the relationship among microorganisms in the analysed samples and highlight interactions otherwise difficult to represent.

Insects and microorganisms

Both insects and microorganisms stand out for abundance, number and diversity of species that co-occur in multiple habitats, performing a wide variety of interactions between them [26]. In recent years, the microbial community associated to different insect hosts has gained a great deal of attention. This was due mainly to the discovery of their helpfulness toward hosts, allowing them to exploit new food sources, improving insects' resistance to stress and affecting gene flow [27, 28]. Accordingly to the current knowledge all insects host microorganisms, sometimes with symbiotic relationship or with a less dependent interaction, but with the clear evidence that in most of the cases microbes can influence different insects' traits [29].

This field have been attracted a great deal of attention in recent years, mainly because of the spread of technologies that can allow these analyses. Furthermore, this research can pose the basis of further studies on higher animals. Indeed, microorganisms can be found everywhere in our globe, so the association with microbes is widely spread among eukaryotes [30]. As it happened in the past for studies on *Drosophila*, *Apis mellifera* and other model organisms, research on these insects can help to understand how humans deal with their microbiota [29].

In insects, bacteria are widely known for protection towards natural enemies and other microorganisms (e.g. *Hamiltonella defensa*, *Regiella insecticola*), excluding parasitoids from the host and/or producing secondary metabolites that complete insect's immune system [31, 32]. Furthermore, microorganisms can play a fundamental role in insects' nutrition, sometimes in a dependent symbiotic way, making available several nutrients or regulating their allocation [29]. Furthermore, insects can exploit microorganisms to regulate their relationship with plants. The psyllid *Bactericerca cockerelli* that exploit its symbiont to modulate plant defensive gene expression [33], bark and ambrosia beetles that exploit fungi for dietary needs but also to overcome plant defences [34], and the maintenance of leaf green islands, fundamental for leaf miners' survival, by symbiotic bacteria [35] are just few examples of the plasticity of these interactions [36]. Furthermore, these mutualistic relationships boosted invasion processes of pests in new environments, sometimes resulting in increasing virulence of insects [36-38]. After an adequate study on the insights and effects of these interactions, clearly appear the possibility to manipulate these microbes to improve pest control, hold the spread of invasive species, and exploit them for industrial purposes.

Therefore, it is clear that bacterial symbionts play a key role in insects' lifestyle, so that their absence would likely produce severe effects on host nutrition,

reproduction, fitness, behaviour and survival [39, 40]. Recent advances suggest a key role of fungi on insects' biology, however our knowledge on the fungal biodiversity associated to insects is not so broad as it is for bacteria [39]. Well-known cases of mutualistic insect-fungus associations occur among different taxa and in different ways, such as bark beetles and ambrosia beetles, fungus farming ants and termites, yeasts found in insects' gut, wood wasps and gall midges [41-43]. On the other hand, there are cases of antagonistic relationships between insect and fungi, as it occurs for entomopathogenic fungi such as *Bauveria* spp. and *Metarhizium* spp [44]. Further interactions include also peculiar multitrophic relationships, like the ability of *Metarhizium* to transfer nitrogen from infected larvae of *Galleria mellonella* to plants [45], phenomenon similarly reported for the ectomycorrhizal fungus *Laccaria bicolor* in white pine [46]. Also gene horizontal transfer between the two kingdoms has been reported in aphids [47]. However, few studied focused on studying the entire fungal microbiota associated to insects, and to date collected information are restricted to Collembola [48], Lepidoptera [49], Coleoptera [50-52] and Diptera [53].

Aim of this work

It clearly appears that the relationship between insects and fungi is worth to be investigated, since published works suggest that this relationship is of fundamental ecological importance. Results from these studies can answer to numerous questions and bring to new important results of basic and applied interest. Furthermore, we have very few information on how insects and fungi interact. As highlighted above, the HTS technologies and in particular metabarcoding, can help us to understand how microbial communities are assembled, how they answer to different environmental conditions and to suggest how they can interact with other organisms, including insects.

This work focused on this specific topic, bringing to front three important unanswered questions in ecology:

1. Which fungi are associated to insect pests? The olive fruit fly was used as model system to deeply investigate the composition of fungal communities and formulate supported speculations on their ecological roles in relationship to the insect. Particular attention was given to plant pathogenic fungi and on the possible role of the fly as a vector (Chapter 2 and 3).
2. It is known that port of entry, like international harbours, play a fundamental role in the introduction of alien species. Can these ports be also point of entry of known and unknown plant pathogens through the introduction of insects? To answer this question, samples of introduced bark and ambrosia beetles from three Italian international harbours were analysed (Chapter 4).
3. Recently, a lot of research work has been made to understand how aboveground and belowground microbial communities interact in agricultural and natural

ecosystems. Available data suggest the existence of a mutual interaction between insects and soil microbial communities but this hypothesis has never been fully demonstrated. Therefore, the questions are: can insects impact on soil microbial communities? Is it possible to have a vice-versa effect? To answer these questions, a model with aphids feeding on plants grown on soil hosting different microbial communities was investigated. The results, somehow unexpected, opened a new window on the fascinating field of the plant-insect-microorganisms interactions (Chapter 5).

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Chapter 2

Molecular analysis of the fungal microbiome associated with the olive fruit fly *Bactrocera oleae*

Abstract

In this study a molecular approach was performed to investigate the fungal microbiome associated with *Bactrocera oleae* Rossi, a major key pest of *Olea europea* L., using the ITS2 region of the ribosomal DNA (rDNA) as barcode gene. Amplicons were cloned and a representative number of sequenced fragments were used as barcode genes for the identification of fungi. The analysis of the detected sequence types (STs) enabled the identification of a total of 34 phylotypes which were associated with 10 fungal species, 3 species complexes and 8 genera. Three phylotypes remained unresolved within the order *Saccharomycetales* and the phylum *Ascomycota* because of the lack of closely related sequences in GenBank. *Cladosporium* was the most abundantly detected genus, followed by *Alternaria* and *Aureobasidium*, well-known components of olive sooty moulds. Interestingly, *Colletotrichum sp.* and other fungal plant pathogens were also detected, leading to potential new insights on their epidemiology.

Keywords: *Cladosporium*, *Alternaria*, *Aureobasidium*, *Colletotrichum*

Introduction

Among eukaryotes, insects and fungi stand out for abundance, number and diversity of species that co-occur in multiple habitats, performing a wide variety of interactions between them [1]. Fungi, with 99,000 known species, have been found in almost all habitats and are associated with a wide variety of organisms becoming often essential to their survival [2]. Well-known cases of mutualistic insect-fungus associations occur among different taxa and in different ways, such as bark and ambrosia beetles, fungus farming ants and termites, yeasts found in insects' gut, wood wasps and gall midges [3-5]. On the other hand, there are cases of antagonistic relationships between insect and fungi, as it occurs for entomopathogenic fungi such as *Beauveria spp.* and *Metarhizium spp*. Particular insects' behaviors may work as antifungal treatments, as it occurs in ants and termites that exploit self- and allo-grooming to clean themselves from fungal spores [6].

Bactrocera oleae (Rossi), the olive fruit fly, is a key pest of *Olea europea* particularly in the Mediterranean area where more of the 90% of worldwide olive cultivation takes place. This pest can develop 2-5 generations/year, and due to the feeding activity of larval instars, and in particular producing the exit holes from fruits, it is capable to strongly affect quality and quantity of the olive production [7]. Control strategies rely on foliage spraying using chemical insecticides, or baiting using poisoned protein hydrolyzate. To this purpose, attention was devoted to the development of forecasting models that could help reducing environmental and economic impact, increasing the performance of treatments [8, 9]. Recently, new control methods based on the use of symbionts as control factors are emerging [10-13]. New developments in this sense could greatly benefit from a better understanding of the microbial communities associated with *B. oleae* [14]. Indeed a number of studies have been recently conducted to investigate bacterial communities while very little information is available on fungi [14].

Another relevant aspect is the possible interaction between the olive fruit fly and major olive fungal pathogens. Particularly interesting is the possible interaction of olive fruit fly with fungal pathogens responsible of significant damages on fruits. Among these, different species of the genus *Colletotrichum* are causal agents of olive anthracnose and may have a great economic impact, by severely affecting both fruit yield and quality of oil [15, 16]. Furthermore, different fungal species belonging to the family *Botryosphaeriaceae* along with species of the genera *Fusarium* and *Alternaria* may be involved in olive drupe rots [17]. All these fungi share at least a part of their life cycle with the olive fruit fly, since they mainly affect fruits from the beginning of olives ripening, and could be potentially favored by insects that may act as carriers. Furthermore, ovipositing wounds may enhance the infection process of fungi, although wounds are not essential for the infection of hemibiotroph pathogens like *Colletotrichum* spp. [18, 19]. Iannotta and co-workers [20] revealed the existence of a

correlation between *Botryosphaeria* olive rots, formerly associated with the fungus *Camarosporium dalmaticum,* and olive fly infestations, but did not provide any proof about the role of the insect in favoring fungal infections.

In the present study the total fungal community associated with *B. oleae* was characterized, in order to acquire qualitative and quantitative information about the fungal microbiome in male and female individuals of this key pest for olive production.

Materials and methods

Sampling

Samples were collected in the middle of November 2013, in six fields of approximately 1 Ha each. Sampling sites were representative of a 100 Ha wide area of olive groves located in Gioia Tauro, Calabria, Southern Italy (38° 23' 30'' N, 15° 56' 7'' E). Investigated olive orchards were almost homogeneous for ecological conditions (300m a.s.l., southern exposition, 5-10% of slope and type of natural vegetation), age (50-70 years old), cultivar (Ottobratica) and planting pattern (10×10 m). All orchards were managed following organic farming regulation.

A total of 128 specimens of *B. oleae* (61 males and 67 females) were individually collected in sterile plastic vials and kept at low temperature (~5°C) for maximum 4-5 hours, waiting for lyophilization performed in laboratory.

DNA extraction and PCR amplification of fungal DNA

Bactrocera flies were crushed in an extraction buffer (10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) with the aid of a bead mill homogenizer. The mixture was treated with Proteinase K following producer's protocol (5Prime GmbH, Germany) and total DNA was extracted as described by Schena and Cooke [21]. Purified DNA was analyzed by electrophoresis in TBE buffer and 1.5% agarose gel stained with GelRed™ nucleic acid stain (Biotium, USA) and observed through UV light using Gel Doc™ (Bio Rad, USA). Furthermore, DNA concentration and quality was assessed measuring the absorbance at 260, 280 and 230 nm by means of a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., USA).

PCR reactions were conducted in a total volume of 25 µl and contained 1 µl (about 50 µg) of extracted DNA, 1X Taq buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 40 µM dNTPs, 1 unit of Taq polymerase and 0.5 µM of primers (ITS3 and ITS4) targeting the fungal ITS2 region of the rDNA [22, 23]. Amplifications were performed in a Mastercycler Ep Gradient S (Eppendorf, Germany) set at 94°C for 3 minutes, 94°C for 30s, 55°C for 30s and 72°C for 30s, repeated 35 times, and ended with

10 minutes of extension at 72°C. A non-template control in which target DNA was replaced by nuclease-free water was included in all PCR reactions. PCR products were analyzed by electrophoresis as described above.

Cloning and sequencing of PCR fragments

PCR products from male and female flies were pooled according to the sex, purified using the magnetic-bead system Agencourt AMPure XP purification kit (Beckman Coulter, USA) and cloned into competent cells of *Escherichia coli* using the pGEM-T Easy Vector System (Promega, Switzerland). Four hundred randomly-selected clones (285 from females and 115 from male specimens) were directly used in PCR reactions (colony PCR) with ITS3 and ITS4 primers, as previously described. Amplified products were analyzed by electrophoresis and single bands of the expected size were sequenced in both directions by Macrogen Europe (Amsterdam, The Netherlands).

Data analysis

Sequence data obtained for both male and female flies were accurately checked for quality, edited and assembled using CHROMASPRO v. 1.5 software (http://www.technelysium.com.au/). Sequences that resulted unreliable, poor in quality or with doubtful bases were sequenced again. Before analyses, sequences of primers were detected and trimmed with TAGCLEANER [24]. The complete panel of sequences was analyzed with the software ElimDupes (http://www.hiv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html) to identify multiple identical sequences and determine sequence type (ST), defined as the distinct and reproducible representative ITS2 sequences recovered in this study. In order to reduce the risk of errors due to artifacts during PCR and/or plasmid replication, only STs represented by at least two sequences were considered for further analyses.

Sequence types were preliminarily assigned to a taxonomic group using the UNITE database [25] and the bioinformatic pipeline QIIME 1.8.0 [26]. Since the UNITE databases enabled a reliable identification of fungi only at the genus level [27], identified STs were also analyzed along with genetically closely related reference sequences in order to determine their phylogenetic collocation and enable their identification at the highest possible level of accuracy. This analysis was possible for fungal genera for which comprehensive databases of validated reference sequences were available and comprised *Colletotrichum acutatum* sensu lato [28], *Pseudocercospora* spp. [29], *Devriesia* spp. [30], *Cladosporium* spp. [31], *Aureobasidium* spp. [32], *Alternaria* spp. [33], *Cochliobolus* spp. [34], *Leptosphaerulina* spp. [35] and *Lecanicillium* spp. [36]. When none of the above sequences from validated reference panels was identical to those identified in the present study, more closely related sequences were searched by

MegaBLAST against GenBank database with default parameters, after accurate evaluation of their reliability.

For each genus, selected reference sequences and STs were aligned using MUSCLE [37] and phylogenetically analyzed with RAxML 8.0.0 using a GTR + Γ model [38]. When specific panels of validated sequences were not available, detected STs were analyzed, and identified, only through a BLAST query.The relative abundance of detected taxa in male and female flies was determined, in terms of incidence of sequences associated to each *taxa*, after taxonomy assignment. Data were subjected to the calculation of Shannon-Weaver Diversity Index, Equitability Index, and Species Accumulation Curves with PAST statistical software in order to retrieve information about the diversity and the evenness of the fungal communities found on both male and female flies [39].

Results

Identification of Sequence Types (STs)

The PCR amplification of total DNA extracted from individually collected flies with fungal primers produced a fragment of the expected size (≅350 bp) from most analyzed samples. After cloning, a total of 381 reliable sequences were obtained from 400 randomly selected colonies. These sequences collapsed into 34 unique STs mostly belonging to the phylum *Ascomycota*, and in a single case to the phylum *Basidiomycota*. A single sequence was not associated with the Kingdom fungi and did not match with any currently available sequence in GenBank. Accumulation curves of identified taxa showed the achievement of the saturation zone for male and female samples, suggesting that the analysis of clones was deeper enough to detect most of fungal biodiversity associated with these insects (Fig. 1).

Among detected fungal STs, 20 were phylogenetically analyzed along with validated reference sequences and were identified to the species level or associated with a restricted number of related species (Fig. 2; Table 1). Among these, 9 species within the genus *Cladosporium* were the most abundantly detected and accounted 82.0% of the total sequenced clones. Five and 2 STs were associated with the species complexes of *Cladosporium cladosporioides* and *C. herbarum*, respectively (Fig. 2a). Furthermore, a ST represented by 3 sequences (CLA1) was identified as *Cladosporium velox*, while a ST represented by 4 sequences (CLA2) remained unresolved within the genus *Cladosporium* (Fig. 2a).

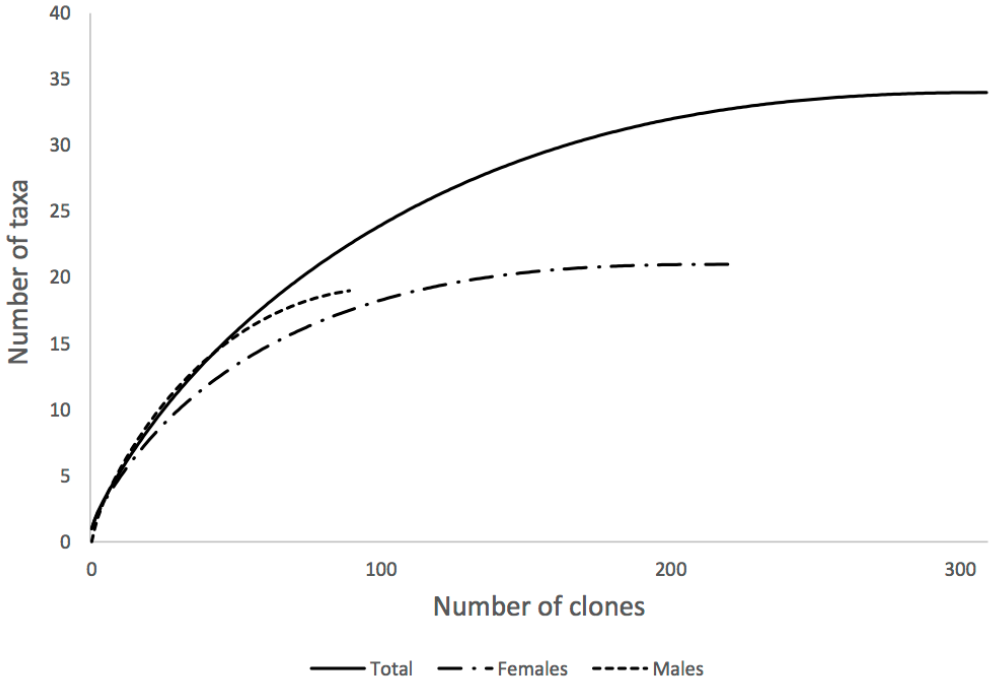


FIGURE 1. SPECIES ACCUMULATION CURVES. SPECIES ACCUMULATION CURVES OF TAXA FOUND AT INCREASING NUMBER OF CLONES ANALYSED, DETERMINED FOR MALE, FEMALES AND BOTH MALE AND FEMALE FLIES TOGETHER.

Single STs were unambiguously identified as *Devriesia frasarie* (DEVR1), *Leptosphaerulina chartarum* (LEPTO1), *Aureobasidium pullulans* (AUREOB1), *Aureobasidium namibiae* (AUREOB2) and *Pseudocercospora cladosporioides* (PSEUDOC1) (Fig. 2b, 2c, 2d). Two STs (COCHL1, COCHL2) were associated *Bipolaris cynodontis*, although one of the two STs was slightly different as compared to the closest reference sequence (Fig. 2f). Another ST (COLL1) was associated with two different species, *C. acutatum s.s.* and *C. cosmi*, that are characterized by identical ITS2 sequences (Fig. 2h). Similarly two STs (LECAN1 and LECAN2) clustered with two different species (*Lecanicillium aphanocladii* and *L. dimorphum*) (Fig. 2i). Finally, two STs (ALTER1, ALTER2) were associated with *Alternaria* Sect. *alternata* but it was not possible to discriminate within this section because it contains species characterized by identical or very similar ITS2 regions [33] (Fig. 2e).

The other thirteen STs were only analyzed by means of MegaBLAST analysis because of the lack of validated reference sequences (Table 1). Great precaution was taken in the identification process because of unreliable annotations of sequences in public DNA repositories, which remain an obstacle to all sequence-based species identifications [40]. Indeed the BLAST identification of taxa was considered reliable only when a consistent number of sequences from different sources was available. According

to these analyses a single ST (EPIC1) of the phylum *Ascomycota* was identified as *Epicoccum nigrum* with a 100% of identity after BLAST analyses. Using the same method a ST of the phylum Basidiomycota was identified as *Hannaella oryzae*. In contrast, other STs of the phylum Ascomycota were only identified at the level of genus (*Aspergillus*, *Penicillium*, *Hansfordia, Taphrina, Toxicocladosporium* and *Rachicladosporium*) because identical or very similar GenBank sequences were shared by different species within each genus. Finally, three STs (SACCH1, ASCH1 and ASCH2) were significantly different from all currently available fungal ITS2 sequences in databases. For these STs it was only possible to establish their affinity with the order Saccharomycetales and the phylum Ascomycota, respectively. Similar indexes of diversity and equitability were revealed for male and female flies indicating a high degree of diversity and a low equitability, probably due to the great abundance of *Cladosporium* spp. (Table 2).

TABLE 1. LIST OF ITS2 SEQUENCE TYPES (STS) IDENTIFIED IN MALE AND FEMALE FLIES OF *BACTROCERA OLEAE* ALONG WITH FUNGAL SPECIES OR GENERA ASSOCIATED TO EACH ST, GENBANK ACCESSION NUMBERS FOR SEQUENCES AND REFERENCES FOR VALIDATED SEQUENCES PANELS UTILIZED IN PHYLOGENETIC ANALYSES FOR FUNGAL IDENTIFICATION (CFR. FIG. 2). N.A.= NOT AVAILABLE

STs	Identified species/genera	GenBank Acc n.	Reference
ALTER1	*Alternaria* sp. sect. Alternata	KM975297	Woudenberg et al. (2013)
ALTER2		KM975298	
AUREOB1	*Aureobasidium pullulans*	KM975314	Zalar et al. (2008)
AUREOB2	*Aureobasidium namibiae*	KM975315	
CLA1	*Cladosporium velox*	KM975305	Bensch et al. 2012
CLA2	*Cladosporium sp.*	KM975303	
CLA3	*Cladosporium cladosporioides* species complex	KM975299	
CLA4		KM975300	
CLA5		KM975304	
CLA6		KM975301	
CLA7		KM975302	
CLA8	*Cladosporium herbarum* species complex	KM975307	
CLA9	*Cladosporium herbarum species complex*	KM975306	
COCHL1	*Cochliobolus cynodontis*	KM975311	Manamgoda et al. (2012)
COCHL2		KM975312	
COLL1	*Colletotrichum acutatum s.s.*	KM975310	Damm et al. (2012)
DEVR1	*Devriesia fraseriae*	KM975308	Crous et al. (2010)

STs	Identified species/genera	GenBank Acc n.	Reference
LECAN1	*Lecanicillium sp.*	KP167624	Zare and Gams (2008)
LECAN2		KP167625	
LEPTO1	*Leptosphaerulina chartarum*	KM975309	Aveskamp et al. (2013)
PSEUDOC1	*Pseudocercospora cladosporioides*	KM975313	Crous et al. (2013)
ASPER1	*Aspergillus sp.*	KP167632	N.A.
HANS1	*Hansfordia sp.*	KP167626	N.A.
HANS2		KP167627	
PENIC1	*Penicillium sp.*	KP167631	N.A.
PENIC2		KP167633	
RACH1	*Rachicladosporium sp.*	KP167638	N.A.
TAPHR1	*Taphrina sp.*	KP167629	N.A.
TOXIC1	*Toxicocladosporium sp.*	KP167637	N.A.
EPIC1	*Epicoccum nigrum*	KP167635	N.A.
HANN1	*Hannaella oryzae*	KP167630	N.A.
ASCH1	*Ascomycota*	KP167636	N.A.
ASCH2		KP167634	
SACCH1	*Saccaromycetales*	KP167628	N.A.

TABLE 2. DIVERSITY INDICES FOR FUNGAL COMMUNITIES ASSOCIATED TO *BACTROCERA OLEAE*.

	Shannon-Weaver Diversity Index	Equitability Index
Females	0.944	0.357
Males	0.942	0.401
Total	**1.002**	**0.3346**

Relative abundance of detected STs

The analysis of the complete panel of detected STs enabled the identification of 34 phylotypes that were associated with 10 fungal species, 3 species complexes and 8 genera. Furthermore, three fungal phylotypes were associated with the order Saccharomycetales and with the phylum Ascomycota. Among detected phylotypes the genus *Cladosporium* was the most abundant and on the whole accounted for 82.0% of the sequenced clones. Within this genus a phylotype identified as *C. cladosporioides* species complex was the most abundant accounting for 56.4 and 45.0% of the sequences in female and male flies, respectively (Fig. 3). Another *Cladosporium* phylotype identified at the level of genus accounted for 19.4 and 21.7% of the clones while *C. herbarum*

species complex represented 5.0 and 11.6% of the sequences in male and female, respectively. *Cladosporium velox* was detected with a low frequency only in male flies.

Apart from *Cladosporium* spp., a phylotype associated with *Alternaria* sect. *alternata* was the second most commonly detected and was particularly abundant in female flies (7.2%). *Pseudocercospora cladosporioides* was quite abundant in female (3.9%) while was not detected in male flies (Fig. 2). Similarly a phylotype represented by a single ST (COLL1) associated with *C. acutatum* s.s. and *C. cosmi*, was only detected in female flies and represented a small part of the population (0.55%). Other quite abundantly detected phylotypes were associated with *A. namibiae* (detected on both male and female) and *A. pullulans* (detected only on male flies).

All other detected phylotypes represented a small portion of the population (0.6-2.9%) and were detected on both male and females (*Penicillium* spp.), only on females (*Lecanicillium* spp., *Epicoccum* spp., *Cochliobolus* spp., *Leptosphaerulina* spp., and *Devriesia* spp.) or only on male (*Hannaella* spp., *Taphrina* spp., *Hansfordia* spp. and *Aspergillus* spp.) flies (Fig. 3).

Discussion

In recent years, the microbial community associated with different insect hosts has gained a great deal of attention [41, 42]. In the present study, a molecular approach based on the use of fungal specific primers (ITS3-ITS4) was utilized to identify and determine the relative abundance of fungal species associated with male and female flies of *B. oleae.* The use of the fungal ITS2 region of the rDNA as a barcode gene for *in situ* species identifications is widely accepted although it is not always discriminant among closely related species [22, 43, 44]. Investigations conducted in the present study confirmed the validity of this marker to study fungi associated with *B. oleae* since analyses yielded only fungal sequences and enabled a good level of discrimination among taxa given that most STs were associated with specific fungal species. Indeed, the analysis of the complete panel of detected STs enabled the identification of a total of 34 phylotypes that were associated with 10 fungal species, 3 species complexes and 8 genera. Three phylotypes remained unresolved within the order *Saccharomycetales* and the phylum *Ascomycota* because of the lack of closely related sequences in GenBank. Furthermore, the species accumulation curves revealed that most of fungal biodiversity associated with *B. oleae* was revealed.

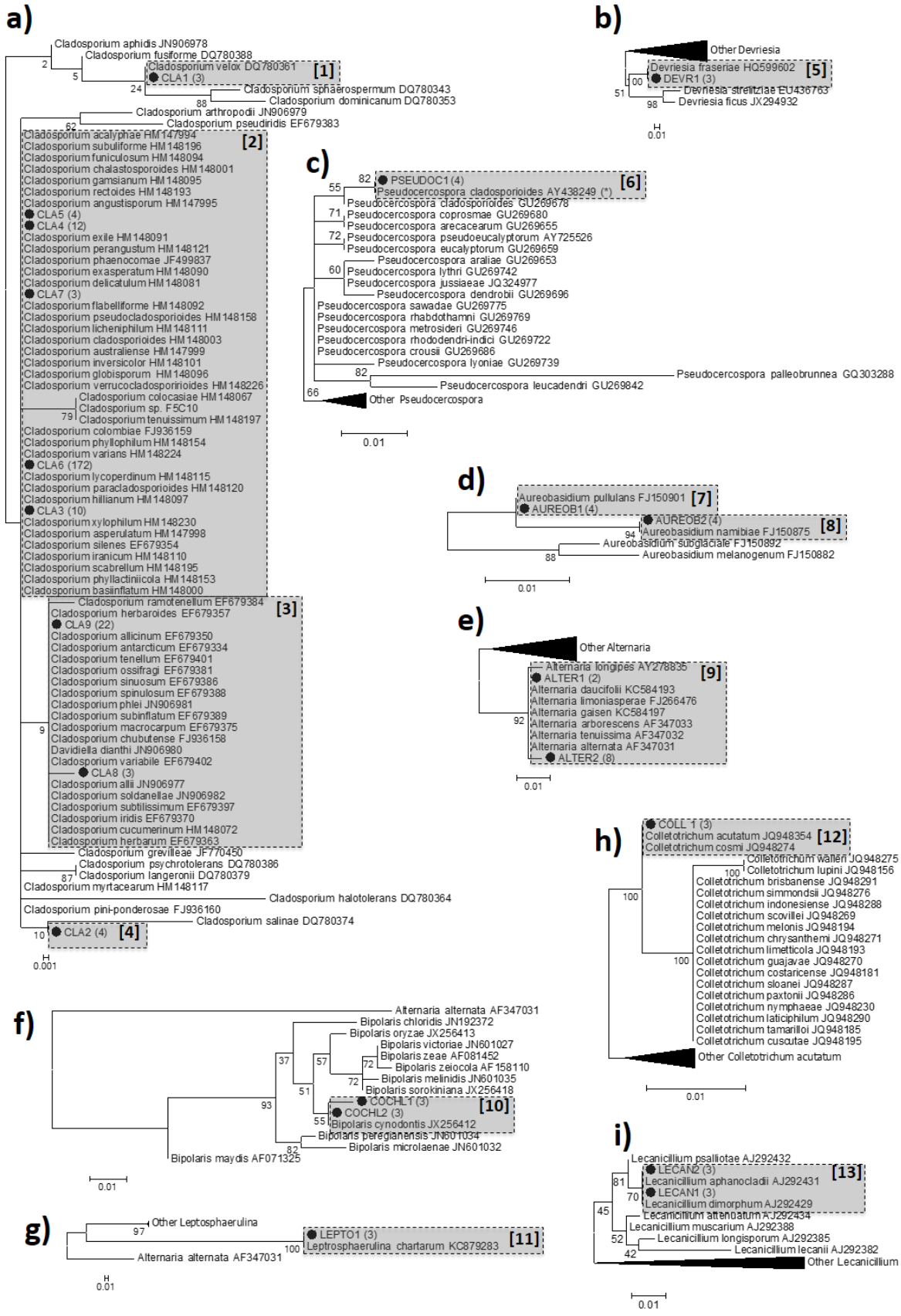
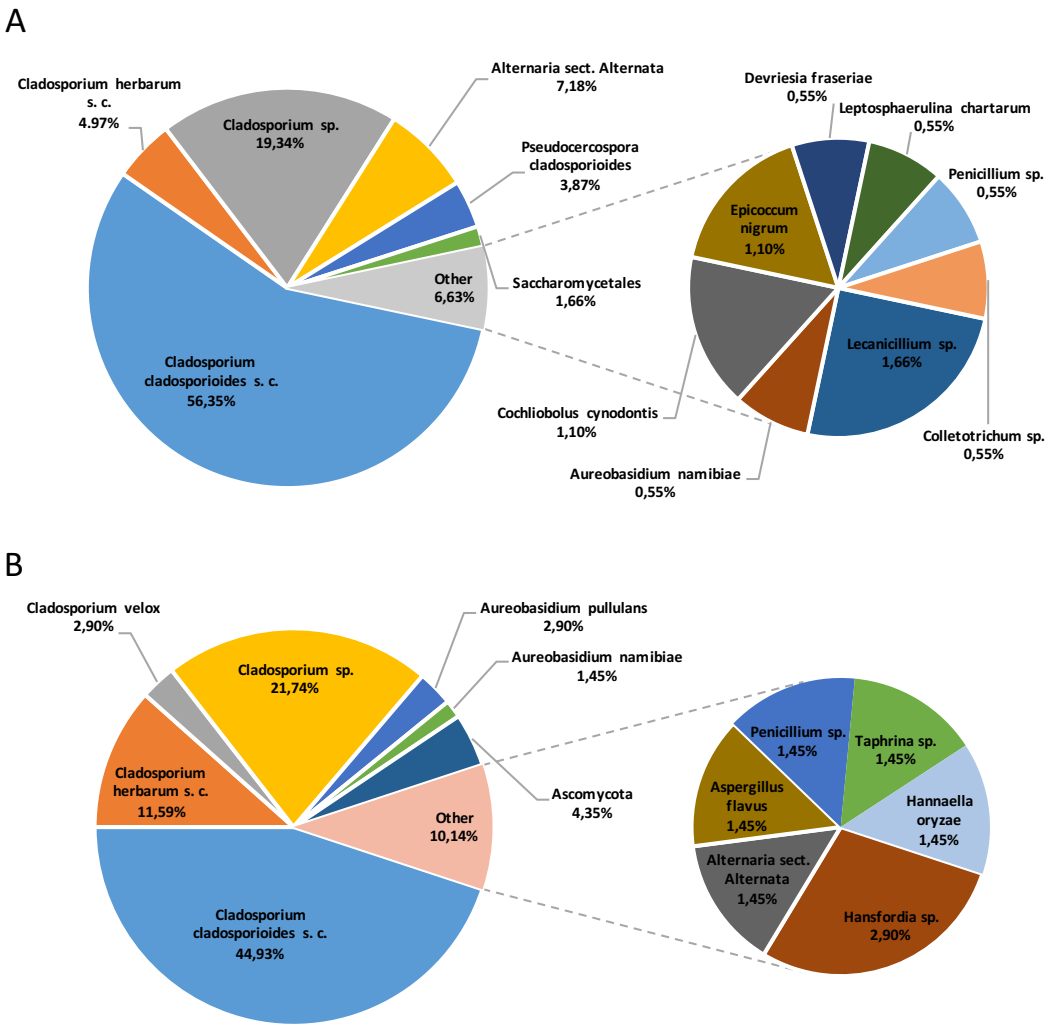

a)
Cladosporium aphidis JN906978
Cladosporium fusiforme DQ780388
Cladosporium velox DQ780361
CLA1 (3)
[1]
Cladosporium sphaerospermum DQ780343
Cladosporium dominicanum DQ780353
Cladosporium arthropodii JN906979
Cladosporium pseudiridis EF679383
Cladosporium acalyphae HM147994
Cladosporium subuliforme HM148196
Cladosporium funiculosum HM148094
Cladosporium chalastosporoides HM148001
Cladosporium gamsianum HM148095
Cladosporium rectoides HM148193
Cladosporium angustisporum HM147995
CLA5 (4)
CLA4 (12)
Cladosporium exile HM148091
Cladosporium perangustum HM148121
Cladosporium phaenocomae JF499837
Cladosporium exasperatum HM148090
Cladosporium delicatulum HM148081
CLA7 (3)
Cladosporium flabelliforme HM148092
Cladosporium pseudocladosporioides HM148158
Cladosporium licheniphilum HM148111
Cladosporium cladosporioides HM148003
Cladosporium australiense HM147999
Cladosporium inversicolor HM148101
Cladosporium globisporum HM148096
Cladosporium verrucocladosporiioides HM148226
[2]
Cladosporium colocasiae HM148067
Cladosporium sp. F5C10
Cladosporium tenuissimum HM148197
Cladosporium colombiae FJ936159
Cladosporium phyllophilum HM148154
Cladosporium varians HM148224
CLA6 (172)
Cladosporium lycoperdinum HM148115
Cladosporium paracladosporioides HM148120
Cladosporium hillianum HM148097
CLA3 (10)
Cladosporium xylophilum HM148230
Cladosporium asperulatum HM147998
Cladosporium silenes EF679354
Cladosporium iranicum HM148110
Cladosporium scabrellum HM148195
Cladosporium phyllactiniicola HM148153
Cladosporium basiinflatum HM148000
Cladosporium ramotenellum EF679384
Cladosporium herbaroides EF679357
CLA9 (22)
Cladosporium allicinum EF679350
Cladosporium antarcticum EF679334
Cladosporium tenellum EF679401
Cladosporium ossifragi EF679381
Cladosporium sinuosum EF679386
Cladosporium spinulosum EF679388
Cladosporium phlei JN906981
Cladosporium subinflatum EF679389
Cladosporium macrocarpum EF679375
Cladosporium chubutense FJ936158
Davidiella dianthi JN906980
Cladosporium variabile EF679402
CLA8 (3)
Cladosporium allii JN906977
Cladosporium soldanellae JN906982
Cladosporium subtilissimum EF679397
Cladosporium iridis EF679370
Cladosporium cucumerinum HM148072
Cladosporium herbarum EF679363
[3]
Cladosporium gevilleae JF770450
Cladosporium psychrotolerans DQ780386
Cladosporium langeronii DQ780379
Cladosporium myrtacearum HM148117
Cladosporium halotolerans DQ780364
Cladosporium pini-ponderosae FJ936160
Cladosporium salinae DQ780374
CLA2 (4)
[4]
0.001
b)
Other Devriesia
Devriesia fraseriae HQ599602
DEVR1 (3)
[5]
Devriesia strelitziae EU436763
Devriesia ficus JX294932
0.01
c)
PSEUDOC1 (4)
Pseudocercospora cladosporioides AY438249 (*)
[6]
Pseudocercospora cladosporioides GU269678
Pseudocercospora coprosmae GU269680
Pseudocercospora arecacearum GU269655
Pseudocercospora pseudoeucalyptorum AY725526
Pseudocercospora eucalyptorum GU269659
Pseudocercospora araliae GU269653
Pseudocercospora lythri GU269742
Pseudocercospora jussiaeae JQ324977
Pseudocercospora dendrobii GU269696
Pseudocercospora sawadae GU269775
Pseudocercospora rhabdothamni GU269769
Pseudocercospora metrosideri GU269746
Pseudocercospora rhododendri-indici GU269722
Pseudocercospora crousii GU269686
Pseudocercospora lyoniae GU269739
Pseudocercospora palleobrunnea GQ303288
Pseudocercospora leucadendri GU269842
Other Pseudocercospora
0.01
d)
Aureobasidium pullulans FJ150901
AUREOB1 (4)
[7]
AUREOB2 (4)
Aureobasidium namibiae FJ150875
[8]
Aureobasidium subglaciale FJ150892
Aureobasidium melanogenum FJ150882
0.01
e)
Other Alternaria
Alternaria longipes AY278835
ALTER1 (2)
[9]
Alternaria daucifolii KC584193
Alternaria limoniasperae FJ266476
Alternaria gaisen KC584197
Alternaria arborescens AF347033
Alternaria tenuissima AF347032
Alternaria alternata AF347031
ALTER2 (8)
0.01
h)
COLL 1 (3)
Colletotrichum acutatum JQ948354
Colletotrichum cosmi JQ948274
[12]
Colletotrichum walleri JQ948275
Colletotrichum lupini JQ948156
Colletotrichum brisbanense JQ948291
Colletotrichum simmondsii JQ948276
Colletotrichum indonesiense JQ948288
Colletotrichum scovillei JQ948269
Colletotrichum melonis JQ948194
Colletotrichum chrysanthemi JQ948271
Colletotrichum limetticola JQ948193
Colletotrichum guajavae JQ948270
Colletotrichum costaricense JQ948181
Colletotrichum sloanei JQ948287
Colletotrichum paxtonii JQ948286
Colletotrichum nymphaeae JQ948230
Colletotrichum laticiphilum JQ948290
Colletotrichum tamarilloi JQ948185
Colletotrichum cuscutae JQ948195
Other Colletotrichum acutatum
0.01
f)
Alternaria alternata AF347031
Bipolaris chloridis JN192372
Bipolaris oryzae JX256413
Bipolaris victoriae JN601027
Bipolaris zeae AF081452
Bipolaris zeicola AF158110
Bipolaris melinidis JN601035
Bipolaris sorokiniana JX256418
COCHL1 (3)
COCHL2 (3)
[10]
Bipolaris cynodontis JX256412
Bipolaris peregianensis JN601034
Bipolaris microlaenae JN601032
Bipolaris maydis AF071325
0.01
g)
Other Leptosphaerulina
LEPTO1 (3)
Leptrosphaerulina chartarum KC879283
[11]
Alternaria alternata AF347031
0.01
i)
Lecanicillium psalliotae AJ292432
LECAN2 (3)
Lecanicillium aphanocladii AJ292431
LECAN1 (3)
[13]
Lecanicillium dimorphum AJ292429
Lecanicillium attenuatum AJ292434
Lecanicillium muscarium AJ292388
Lecanicillium longisporum AJ292385
Lecanicillium lecanii AJ292382
Other Lecanicillium
0.01

FIGURE 2. PHYLOGENETIC TREES (←). TREES BUILT USING ITS2 SEQUENCE TYPES (STS) DETECTED ON *B. OLEAE* FLIES (•) TOGETHER WITH REFERENCE SEQUENCES OF *CLADOSPORIUM* SPP. (A - BENSCH *ET AL.*, 2013); *DEVRIESIA* SPP. (B - LI *ET AL.*, 2013), *PSEUDOCERCOSPORA* SPP. (C - CROUS *ET AL.*, 2013), *AUREOBASIDIUM* SPP. (D - ZALAR *ET AL.*, 2008), *ALTERNARIA* SPP. (E - WOUDENBERG *ET AL.*, 2013), *COCHLIOBOLUS* SPP. (F - MANAMGODA *ET AL.*, 2012), *LEPTOSPHAERULINA* SPP. (G - AVESKAMP *ET AL.*, 2010), *COLLETOTRICHUM ACUTATUM SENSU LATO* (H - DAMM *ET AL.*, 2012) AND LECANICILLIUM SPP. (I – ZARE AND GAMS 2008). WITHIN EACH TREE, DETECTED STS WERE ASSOCIATED TO DIFFERENT *TAXA* (GREY BOXES) INCLUDING *CLADOSPORIUM VELOX* [1], *CLADOSPORIUM CLADOSPORIOIDES* SPECIES COMPLEX [2], *CLADOSPORIUM HERBARUM* SPECIES COMPLEX [3], *CLADOSPORIUM* SP. [4], *DEVRIESIA FRASERIAE* [5], *PSEUDOCERCOSPORA CLADOSPORIOIDES* [6], *AUREOBASIDIUM PULLULANS* [7], *AUREOBASIDIUM NAMIBIAE* [8], *ALTERNARIA* SECT. ALTERNATA [9], *BIPOLARIS CYNODONTIS* [10], *LEPTOSPHAERUMINA CHARTARUM* [11], *COLLETOTRICHUM* SP. [12], AND *LECANICILLIUM* SP. [13]. FEW ADDITIONAL SEQUENCES OF *PSEUDOCERCOSPORA CLADOSPORIOIDES* (*) WERE SOURCED IN GENBANK BECAUSE DETERMINED FOR ISOLATES OBTAINED FROM OLIVE. NUMBERS IN BRACKETS ALONG STS INDICATE THE NUMBER SEQUENCES REPRESENTED BY EACH ST. NUMBERS ON NODES REPRESENT THE POSTERIOR PROBABILITIES FOR THE MAXIMUM LIKELIHOOD METHOD.

FIGURE 3. RELATIVE ABUNDANCE OF EACH FUNGAL TAXA (↓). RELATIVE ABUNDANCE OF FUNGAL TAXA DETECTED IN FEMALE (A) AND MALE (B) FLIES OF *BACTROCERA OLEAE* (COMPARE WITH FIG. 2).



A conventional cloning and Sanger sequencing approach was utilized to determine STs. Although this technique enable a much lower coverage of the genetic diversity as compared to second generation sequencing strategies an important advantage of the Sanger approach is the high reliability of sequences especially if, as in the present study, both strands of DNA (forward and reverse) are determined. This aspect is particularly important for the identification of fungi in light of the high number of species that are sometimes characterized by identical or very similar ITS sequences [28, 45, 46]. It is possible to anticipate the future use of more variable markers as a barcode genes used in fungal metagenomic analyses to enable a higher level of discrimination among species [28, 45]. However, the single copy nature of most alternative markers genes may provide lower levels of sensitivity as compared to the multi-copy ITS regions. Furthermore, the lack of reliable universal primers and the lower number of available reference sequences in genetic databases may pose an obstacle [44].

The genus *Cladosporium* was the fungal group with the highest relative abundance on the olive fly *B. oleae*. Furthermore, we also detected *D. fraseriae* that is a cladosporium-like fungus [30, 47]. The presence of *Cladosporium* spp. on *B. oleae* flies was partially expected since it represents a group of fungi with cosmopolitan distribution and is commonly encountered as endophytes in the phyllosphere of many plant species. It is known that *Cladosporium* spp., together with fungi of the genera *Aureobasidium*, *Alternaria* and *Epicoccum* are the main inhabitants of plants phylloplane and carpoplane and the most abundant and frequent representatives of sooty moulds communities [17, 48, 49]. There are also evidence of the involvement of this heterogeneous genus in secondary plant diseases [31]. On the other hand, specific strains of *Cladosporium* spp. have been proposed as an effective biocontrol agent against homopteran insects [50] as well as against *Spodoptera litura* [51] and *Helicoverpa armigera* (Hübner). However, the much higher relative abundance of *Cladosporium* over other sooty molds fungi may suggest a specific interaction between *B. oleae* and *Cladosporium* spp. Indeed currently available information on fungal species associated with the olive phyllosphere and carposphere does not suggest a prevalence of *Cladosporium* spp. over other fungal species like *A. pullulans* [17]. Interestingly, we also detected a phylotype of the genus *Hansfordia* that comprises species reported as hyperparasites of *Cladosporium* spp.

Apart from *Cladosporium* spp., *Alternaria* sect. *Alternata* was the second most commonly detected fungus and was particularly abundant in female as compared to male flies. The section *Alternata* comprises about 60 ubiquitous species, including *Alternaria alternata* a common fungus on several matrices and widely known as a facultative pathogen responsible for diseases on both olive leaves and fruits [33, 52]. This fungus is common on olive drupes and although it generally acts as a saprophyte it may cause severe infections and in favourable environmental conditions can reduce the

quality and yield of both fruit and oil [17]. The relative abundance of *Alternaria* on females as compared to males flies suggests a potential role of the insect behavior during oviposition and/or feeding in the acquisition of the fungus. Similarly, a well-known olive pathogen, *P. cladosporioides* was only detected in females. This pathogen is the causal agent of olive cercosporiosis a disease primarily affecting leaves but it can also infect fruits [53]. Among plant pathogens, relevant was also the detection of a *Colletotrichum* phylotype associated with *C. acutatum s.s.* and *C. cosmi*. Although the ITS2 region of these species do not enable their discrimination [28] it is very likely that the detected ST belonged to *C. acutatum s.s.* that is a known causal agent of olive anthracnose. It is the prevalent species in Australian and South African olive groves and in a recent study it has been found to be widely spread in Italy [15, 54]. Interestingly, *C. godetiae* was not detected in *B. oleae* even though it is widely spread in the investigated area [54]. Although STs associated with fungal plant pathogens represented a small proportion of the detected sequences, our data demonstrate the presence on fungal pathogens on the body of *B. oleae* indicating that the insect may act as a carrier. This finding is relevant because even few propagules may have an important impact on the epidemiology of a fungal disease. For example, it has been reported that *Colletotrichum* species can produce a large number of conidia in conducive environmental conditions and that only one infected drupe per tree can result in 100% affected fruits [55]. Consequently, few new infections determined by propagules carried by the fly may represent a well-distributed source of inoculum for new infections. Furthermore, the olive fly is also likely to produce infections by creating wounds on olive fruits with both sterile and fertile punctures. These fungi could be also transmitted by parasitoids of *B. oleae*, during oviposition, as suggested for *Lasioptera berlesiana* Paoli during the parasitization of the olive fruit fly larvae [56].

Among detected phylotypes worth mentioning is the genus *Lecanicillium* spp. since it comprises several insect pathogens. Several other fungal phylotypes were detected on the body of *B. oleae* but it is difficult to formulate hypothesis on their role/relevance. However the simple fact that they are there, on the body surface of the fly or in the intestine, may be relevant on an ecological point of view. This is particularly true for fungal phylotypes remained unresolved within the order Saccaromicetales and the phylum Ascomycota and which may represent still unknown fungal species.

In conclusion, the present study opened a window on the fungal community associated with *B. oleae* and more generally to insects. Our data showed the existence of rich fungal populations with a number of different species. Some fungi could interact with the fly, while others may be present on the fly just as contaminants. The study of these aspects is worthy of further investigations and the use of second generation sequencing approaches may greatly contribute to this scope by significantly increasing the level of coverage of the analyses.

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Chapter 3

A metabarcoding survey on the fungal microbiota associated to the olive fruit fly

Abstract

The occurrence of interaction between insects and fungi is interesting from an ecological point of view, particularly when these interactions involve insect pests and plant pathogens within an agroecosystem. In this study, we aimed to perform an accurate analysis on the fungal microbiota associated to *Bactrocera oleae* (Rossi) through a metabarcoding approach based on 454 pyrosequencing. From this analysis we retrieved 43,549 reads that clustered into 128 OTUs, of which 29 resulted the "core" associate fungi of *B. oleae*. This fungal community was mainly represented by sooty mould fungi, such as *Cladosporium* spp., *Alternaria* spp. and *Aureobasidium* spp., by plant pathogens like *Colletotrichum* spp. and *Pseudocercospora* spp., along with several other less abundant taxa whose ecology is unclear in most of the cases. Our findings lead to new insights into the microbial ecology of this specific ecological niche, enabling the understanding of a complex network of interactions within the olive agroecosystem.

Keywords: 454 Pyrosequencing; High Throughput Sequencing; *Bactrocera oleae*; *Cladosporium*; *Colletotrichum*; *Pseudocercospora*

Introduction

Insects and fungi can co-occur in multiple habitats performing a wide variety of reciprocal interactions, from mutualistic symbiosis to antagonistic activities [1, 2]. These interactions are interesting from an ecological point of view, especially when associations between insect pests and plant pathogenic fungi occur within an agroecosystem. Indeed, herbivores can be attracted, repelled or can act indifferently toward tissues infected by fungal pathogens [3, 4]. One of the examples of insect-fungi association involves scolytid beetles with many different fungal species, including symbionts and plant pathogens, which those insects can exploit as food source [2, 5]. Furthermore, phytopathogenic fungi can be enhanced by the damages caused by insect herbivores during feeding and/or oviposition [3]. Antagonistic interactions are also known to occur and entomopathogenic fungi such as *Metarhizium brunneum* have been also exploited in pest management strategies [6, 7].

Under this perspective, the olive tree cultivation appears of particular interest, mainly because of its wide diffusion within Mediterranean Basin and the yearly increase of areas cultivated with this crop. Unfortunately, the key pest *Bactrocera oleae* (Rossi) (olive fruit fly) is often associated to olive trees, which larvae can affect quality and quantity of olives and oil [8-10]. Control of this insect, as well as other tephritid pests, is very complex and generally relies on integrated pest management (IPM) strategies which include synthetic insecticides, repellent minerals, baited traps and biocontrol agents [11-15]. Moreover, serious olive diseases can be caused by fungal plant pathogens including *Spilocaea oleagina*, *Colletotrichum* spp. and *Pseudocercospora* spp. [16].

In a recent companion study, we investigated the fungal microbial community of olive fruit flies using a molecular method based on the amplification of the Internal Transcribed Spacer 2 (ITS2) region of the ribosomal DNA (rDNA) with universal primers, the cloning of amplicons and the Sanger sequencing of a representative number of clones [17]. In that study, we provided inedited information about the *B. oleae* fungal microbiota, that was dominated by fungi associated to the olive sooty moulds like *Cladosporium* spp., *Alternaria* spp. and *Aureobasidium* spp. [18]. Furthermore, relevant fungal pathogens including *Colletotrichum* spp. and *Pseucercospora cladosporioides* were also detected [17, 19, 20]. The presence of these fungi on the body of the olive fruit fly is likely to affect their epidemiology, as they can exploit insects as carrier to spread [3, 4]. In particular, pathogens affecting fruit such as *Colletotrichum* species may be enhanced during the infection process through ovipositing wounds [4].

In recent years, High Throughput Sequencing (HTS) technologies, combined with amplicon targeted sequencing, made easier to comprehensively study the microbial communities on any type of matrix [21-23]. The main advantage of this technique, over culture-dependent methods, is the ability to theoretically detect all organisms that

possesses the targeted barcode gene. This includes uncultivable organisms and rare taxa that are usually not detected by culturing techniques and less powerful approaches based on fragment cloning and Sanger sequencing [17]. The ITS regions of the ribosomal rDNAare the most used DNA barcodes in fungal metabarcoding since they can be easily amplified and sequenced with universal primers, and their sequences are highly represented in genetic databases [24, 25]. The choice of using either ITS1 or ITS2 is optional since these regions share many properties, enabling similar discrimination levels, although the ITS2 is generally preferred due to its wider diffusion in public databases. Furthermore, performing the metabarcoding only on ITS2 region led to a series of advantages, mainly due to the low variability of its length (reduced sequencing bias). In this way, it is also possible to avoid the amplification of the highly conserved 5.8S region, that could lead to the formation of chimeric ITS1-ITS2 amplicons from different species [26, 27]. A major drawback of the ITS regions as barcode genes concern difficulties in discriminating phylogenetically related fungal species, that may have almost identical sequences but completely different ecology, including pathogenicity. This disadvantage may be partially solved by combining bioinformatics and phylogenetic analysis of unique representative sequences, along with validated reference sequences [23, 28, 29]. These analyses may enable the exploitation of all available genetic variations within the ITS2 region and the identification of detected taxa with the highest possible level of accuracy [23, 29].

The aim of this study was the accurate investigation of the whole fungal microbiota associated to male and female individuals of the olive fruit fly, using an amplicon metabarcoding approach based on 454 pyrosequencing. The use of a HTS approach yielded a much wider range of amplicons compared to our previous study and enabled an in depth analysis of the fungal diversity associated to *B. oleae*. This approach was combined with specific phylogenetic analyses, in order to enable the best possible identification of major detected taxa, giving particular attention to plant pathogens.

Materials and methods

Sampling and DNA extraction

Samples were collected in the beginning of November 2014 in Rizziconi, Calabria, Italy (38°40' N, 15°92' E) in sites randomly selected among olive orchards within a 100 hectares wide area. Sampling sites (6 in total, approximately 1 Ha each) were characterized by a similar natural vegetation and homogeneous ecological conditions (300 m a.s.l., southern exposition, 5-10% of slope), olive tree age (50-70 years old), cultivar (Ottobratica) and planting pattern (10×10 m). Thirty insects (15 males and 15 females) were individually collected using sterile plastic vials from each sampling site in order to get a total of 180 specimens. Insects were kept at low temperature (≈5°C) for a maximum of 5 hours and then stored at -80°C. Each insect was crushed in the extraction

buffer (10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) with the aid of a bead mill homogenizer (5 min at 30 Hz), and the mixture was treated with Proteinase K (5Prime GmbH, Germany) following the producer's protocol. Total DNA was extracted and analysed by electrophoresis as described by Schena and Cooke [30]. DNA concentration and quality was assessed by means of a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA).

454 GS FLX+ library preparation

Libraries for 454 GS FLX+ sequencing were prepared using fusion primers (http://www.454.com/) targeting the fungal ITS2 region of the rDNA [26]. PCR reactions (total volume of 25 µl) were conducted using 1 µl of extracted DNA (≈50 µg), 1X Taq buffer, 1.5 mM $MgCl_2$, 40 µM dNTPs, 1 unit of Taq polymerase (AccuPrime™, Thermo Fisher) and 0.5 µM of each primer (ITS3 and ITS4) [31]. Both primers were modified to construct fusion primers appropriate for 454 sequencing with adapter sequences A and B, a key sequence and multiplex identifiers (MIDs) (http://www.454.com/). A total of 12 MIDs (MID 1-5-6-7-9-10-11-12-14-15-19-20) were used to identify male and female specimens from each sampling site. Amplifications were performed in a Mastercycler Ep Gradient S (Eppendorf, Germany) set at 94°C for 3 minutes followed by 35 cycles of 94°C for 30s, 55°C for 30s and 72°C for 30s and by a final extension of 10 minutes at 72°C. The fungal ITS2 region was amplified in triplicate from single flies in order to decrease the stochastic variability among reactions [32]. A non-template control in which target DNA was replaced by nuclease-free water was included in all PCR reactions. PCR products were analysed by electrophoresis as described above, and purified using Agencourt AMPure XP kit (Beckman Coulter Inc., CA, USA). For each sex and sampling site amplicons were pooled together and their concentration was measured by means of Qbit instrument (Applied Biosystems), and normalized to a concentration of 1 ng/µl with molecular biology grade water. Ten µl of each pooled sample were sequenced by Macrogen Inc. (Seoul, Korea) on 1/8 of a sequencing plate on 454 GS FLX+ System (454 Life Sciences, Branford, CT, USA).

HTS data processing

Raw sequencing data were processed using QIIME 1.8.0 [33], setting the minimum quality score to 25. Furthermore, mismatches in the primer sequence were not allowed and sequences <150 bp, >1000bp, containing homopolymers >10 bp and with >6 ambiguous bases, were discarded. Reads were denoised using denoise wrapper [34], and chimeric sequences were identified using USEARCH 6.1 algorithm [35] combining a reference-based with a de novo detection. ITSx was used to extract ITS2 sequences [36]. Reads were then clustered into Operational Taxonomic Units (OTUs) using the BLAST method, with 0.99 similarity threshold to the UNITE dynamic reference

database [24] accessed on January 2016 (http://unite.ut.ee/). When reads failed to hit to reference database, sequences were clustered as de novo. Singletons were discarded from analyses. The UNITE database was employed, using BLAST algorithm, for the taxonomic identifications of OTU representative sequences.

The method described by Magurran and Anderson [37] was applied to decompose the Species Abundance Distribution (SAD) and identify core and satellite OTUs, associated to our samples. The threshold between the two categories was set as the number of samples at which the SAD fitted a log-normal distribution. The core dataset was employed for taxonomic and ecological aspects, while the whole community composition was used in alpha and beta diversity analysis. Alpha diversity was estimated through the Species Accumulation Curves (SAC) and a set of diversity indices (Shannon-Weaver and Equitability). The beta diversity was tested through a Principal Coordinates Analysis (PCoA) approach with 95% confidence ellipses, supporting these results with a PERMANOVA non-parametric approach determined with 999 permutations. Analyses were performed with QIIME 1.8.0 [33] and Microsoft® Excel® 2013.

Taxonomic identification of core taxa

Since the UNITE database enabled a reliable identification of fungi only at the genus level [38], all OTUs were manually re-checked for their identity using BLAST searches of GenBank and Fungal Barcoding Databases (http://www.fungalbarcoding.org/ - accessed on January 2016). Furthermore, some "core" OTUs were phylogenetically analysed along with related reference sequences to enable their identification with highest possible level of accuracy. This latter approach was only possible when comprehensive dataset of validated reference sequences were available in literature (see Fig. S1). OTUs associated to "Uncultured fungi" were not analysed. For each fungal genus, sequences were aligned using MUSCLE [39] and phylogenetically analysed with MEGA6 [40] using a Maximum Likelihood approach with a Tamura-Nei substitution model and a Gamma distributed substitution rate (1000 bootstraps for each analysis). Taxa for which it was not possible to use this approach, the identification was performed through a BLAST search, considering as reliable the identifications with a minimum query cover of 90% and a percentage of identity greater than 95%.

Results

After processing raw pyrosequencing data (quality filtering, denoising, chimera discarding), we retrieved 43,549 reads with an average of 3,660 sequences per sample and a mean length of 251 bp. Almost all reads (99.74%) were associated to the kingdom fungi. With a 0.99 cutoff, and without considering clusters with less than 5 sequences, a total of 128 OTUs were identified. As shown in Fig. 1, the Species Accumulation Curve (SAC) tend to flatten as the number of analysed sequences increased, indicating that the sequencing was deep enough to detect most fungal diversity. Alpha diversity indices revealed a high diversity and a low equitability of fungal taxa associated to the olive fruit fly (Tab. 1). A slight higher diversity was revealed in males as compared to females (Table 1), but Beta diversity analyses did not show significant differences according to both PCoA (Fig. S2) and PERMANOVA analyses (PseudoF = 1.13; *P* = 0.20).

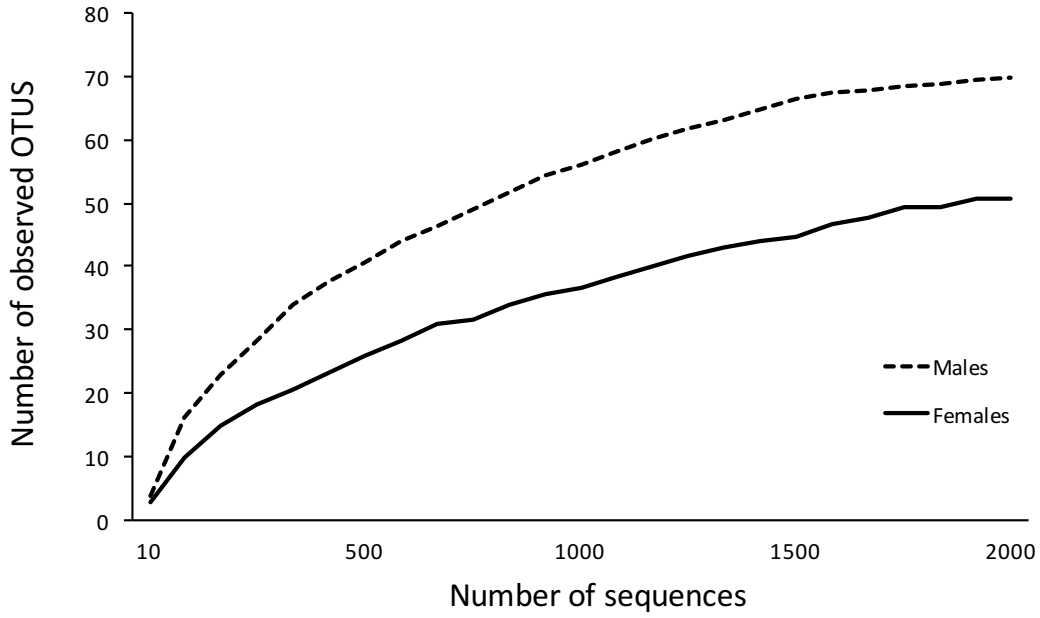

FIG. 1 – RAREFIED SPECIES ACCUMULATION CURVES (SAC), FOR BOTH MALE AND FEMALE SPECIMENS.

TABLE 1 – DIVERSITY INDICES OF FUNGAL COMMUNITIES ASSOCIATED TO *B. OLEAE*

	Shannon-Weaver index	Equitability index
Female	1.145	0.2544
Male	1.682	0.3501
Total	1.528	0.3149

The SAD analysis (Fig. 2), allowed the identification of 29 "core" OTUs (persistence ≥6 samples - goodness of fit χ^2=17.54; *P* = 0.17). These sequences represented the 89.7% of the total fungal sequences retrieved in this study. According to BLAST and phylogenetic analyses 5 OTUs were identified at the species level, 8 were assigned to species-complexes and 16 were only identified at the genus level (Table 2). On the other hand, the satellite part of SAD included 99 OTUs, mainly represented by fungi belonging to *Hannaella oryzae*, *Alternaria* sp., *Penicillium* sp., *Fusarium* sp., *Cladosporium* sp., several unidentified taxa and other little characterized fungi, with an unclear ecological role (Tab. S1).

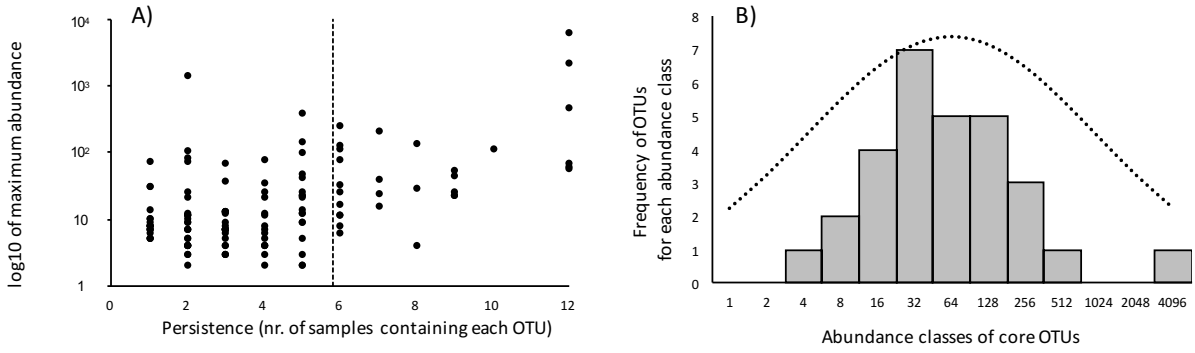

FIG. 2 - SEPARATION OF CORE AND SATELLITE OTUS OF THE FUNGAL COMMUNITY ASSOCIATED TO *B. OLEAE*. SPECIES ABUNDANCE DISTRIBUTION (A) BASED ON ABUNDANCE/PERSISTENCE OF OTUS, WITH DASHED LINES ON THE THRESHOLD BETWEEN CORE AND SATELLITE OTUS SET THROUGH PROCESS ITERATED UNTIL THE ABUNDANCE CLASSES DISTRIBUTION AND (B) FIT A LOG-NORMAL DISTRIBUTION.

The genus *Cladosporium* represented the 77.81±5.34% of core OTUs sequences, with a total of 30,102 reads clustering into 6 OTUs. Among them, 3 OTUs were associated to the *Cladosporium cladosporioides* and one to the *Cladosporium herbarum* species complexes, respectively (Table 2; Fig. S1). The remaining 2 OTUs clustered within the genus but did not match any currently known taxa (Fig. S1). A total of 4,344 sequences (9.78±3.93%) clustering in a single OTU was associated to *Alternaria* sect. Alternata. Also the genus *Aureobasidium* was well represented (4.64±1.34%), with a single OTU identified as *Aureobasidium pullulans*. Finally, 1 OTU was identified as *Leptosphaerulina chartarum* (0.57±0.21%) and another one was associated to the genus *Devriesia* (0.61±0.07%) (Fig. S1).

Putative causal agents of plant diseases were also identified among core OTUs. Specifically, we retrieved 2 OTUs belonging to the genus *Colletotrichum*. One of these (COL1 – 0.72±0.41%) was associated with *C. acutatum sensu stricto* and *C. cosmi*, while the other one (COL2 – 1.12±0.84%) clustered together with *C. gloeosporioides* (Fig. S1).

Another OTU accounting for the 0.36±0.16% of sequences was associated to *P. cladosporioides*. Finally a group of sequences clustering in a single OTU were associated to the genus *Botrytis* (0.51±0.17%). The remaining 15 OTUs were only analysed through BLAST because of the lack of validated reference sequences (Tab. 3). According to this analysis, they were associated to 4 species and 10 genera (Table 2).

TABLE 2 – FUNGAL TAXA ASSOCIATED TO CORE OTUS DETECTED IN THE PRESENT STUDY AND CORRESPONDING RELATIVE ABUNDANCE (RA). §TAXA MARKED WITH (P) WERE IDENTIFIED ACCORDING TO BOTH BLAST AND PHYLOGENETIC ANALYSES. OTHER TAXA WERE ONLY IDENTIFIED ACCORDING TO BLAST ANALYSES

Taxa§	**OTU**	**RA (%±SE)**
(P) *Cladosporium cladosporioides* s.c.	CLA1	76.28±5.23
	CLA2	0.77±0.22
	CLA3	0.15±0.11
(P) *Alternaria* sect. Alternata	ALT1	9.78±3.93
(P) *Aureobasidium pullulans*	AUR1	4.64±1.34
Epicoccum nigrum	EPI1	1.24±0.23
(P) *Colletotrichum gloeosporioides sensu str.*	COL2	1.12±0.84
Hansfordia pulvinata	HAN2	1.03±0.48
(P) *Colletotrichum acutatum sensu str.*	COL1	0.72±0.41
(P) *Devriesia* sp.	DEV1	0.61±0.07
(P) *Leptosphaerulina chartarum*	LEP1	0.57±0.21
(P) *Botrytis sp.*	BOT1	0.51±0.17
Rachicladosporium sp.	RAC1	0.46±0.25
(P) *Pseudocercospora cladosporioides*	PSE1	0.36±0.16
(P) *Cladosporium herbarum* s.c.	CLA4	0.33±0.19
Tecaphora sp.	TEC1	0.28±0.1
Mycocalicium victoriae	MYC1	0.25±0.09
Quambalaria sp.	QUA1	0.23±0.14
Nigrospora sp.	NIG1	0.18±0.06
Malassezia sp.	MAL1	0.16±0.08
(P) *Cladosporium* sp.	CLA5	0.15±0.13
(P) *Cladosporium* sp.	CLA6	0.13±0.06
Aspergillus sp.	ASP1	0.09±0.04
Penicillium sp.	PEN1	0.09±0.05
Periconia sp.	PER2	0.08±0.04
Periconia sp.	PER1	0.07±0.03
Aspergillus tritici	ASP2	0.06±0.02

Discussion

Olive is one of the most important crop around the world, and *B. oleae* represents the most harmful pest, able to damage up to 100% of the production [8, 41]. Our previous survey investigated on the fungal microbiota of the olive fruit fly, providing preliminary data based on a limited number of sequences [17]. Indeed, the use of a high-throughput culture-independent sequencing approach provided a much high number of reads and, as confirmed by rarefaction curves, enabled the analysis of the whole fungal diversity associated to *B. oleae*. Furthermore, the lack of the cloning step needed in the Sanger sequencing, allowed a more accurate quantitative analysis in term of relative abundance of each detected taxon. In agreement with previous reports, coupling of QIIME analysis together with the identification through BLAST and phylogenetic analysis, was useful to identify taxa with the highest possible level of accuracy for the targeted fragment [23, 29]. According to these analyses, the 454 pyrosequencing confirmed all fungal genera and species detected with the cloning/Sanger sequencing approach, along with new previously undetected taxa. Considering both core and satellite OTUs a rich fungal community was revealed and comprised fungi belonging to sooty moulds, plant pathogens, and other mycetes with an undisclosed ecological role.

According to the earlier findings [17], the genus *Cladosporium* represented the wider part of retrieved sequences, although its relative abundance (76%) was slightly lower as compared to our previous study [17]. Within this genus, we identified OTUs belonging to two species complexes (*C. cladosporioides* and *C. herbarum*), and other 2 OTUs that may represent unknown species since they did not cluster with none of the currently available sequences for this genus. The abundant presence of *Cladosporium* spp. was expected, since it is one of the most common inhabitant of plants phylloplane and carpoplane [42]. Sequences associated to *Alternaria* sect. Alternata were also widely detected in our study (about 9% of sequences). This section of *Alternaria* represents not only a widely known component of sooty moulds, but also a facultative pathogen of both olive leaves and fruits [43, 44]. Moreover, we retrieved sequences that were associated to *A. pullulans*, *Devriesia* sp. and *Epicoccum nigrum*, which together with *Cladosporium* spp. and *Alternaria* spp. are the main representatives of sooty moulds fungal communities [18, 42, 45].

A comparison of our results with those reported by Abdelfattah *et al*. [29], that investigated the fungal diversity associated to olive leaves, flowers and fruit collected in the same area, year and period, highlights an unexpected differential pattern of relative abundance. Indeed, in our study, the sequences associated to the genus *Cladosporium* were up to 42 times greater than that reported by Abdelfattah and co-workers. Otherwise, sequences associated to the genus *Alternaria* showed the same pattern, resulting up to 49 times more abundant on the body of *B. oleae* than that in the olive phyllosphere. On the other hand, reads associated to the genus *Aureobasidium* were

more abundant on leaves and fruits than on insects, with values up to 10 times greater on plant than on *B. oleae*. These results are of particular interest from an ecological point of view suggesting a specific association between *B. oleae* and some specific fungal genera. Further analyses involving a simultaneous sampling from both plant tissues and insects are needed to evaluate the spatio-temporal association of the fungal microbiota between plant and fly.

This survey highlighted also the association between *B. oleae* and fungi belonging to the genus *Colletotrichum*. Specifically, we retrieved 2 OTUs, of which one (COL1) was associated to *C. acutatum* s.s. and *C. cosmi*, and the second one (COL2) to *C. gloeosporioides*. Although the ITS2 region does not enable the discrimination of very closely related species such as *C. acutatum* s.s. and *C. cosmi*, the first species was also detected in our previous investigation and, being widely diffused in the olive phylloplane of the investigated area, it is likely to be actual detected species [46, 47]. *Colletotrichum acutatum* s.s. is an aggressive pathogen responsible for olive anthracnose, but it was considered absent in Italy until few years ago [19, 47]. The detection of this species in most of the investigated samples (core OTU) indicate that the olive fruit fly may have contributed to its spread. Another species, *C. godetiae,* was the most important causal agent of olive anthracnose Italy until few years ago, but it was not detected on the olive fly even if it was widely diffused in olive orchards of the investigated area [47, 48]. Further investigations are worthwhile to understand why *C. acutatum s.s.*, and not *C. godetiae*, was associated to the olive fruit fly. Indeed, the olive fruit fly is likely to act as carrier of *C. acutatum* spores, supporting the spread of the conidia between olive groves, and helping the infective process by creating wounds on olive fruits, with both sterile and fertile punctures. Unlikely *C. acutatum s.s., C. gloeosporioides* is considered of secondary importance as olive anthracnose agent [49], and it was not detected using the cloning/Sanger sequencing, probably because of the lower sensitivity of the method. Among putative plant pathogens, we also retrieved sequences associated to *Aspergillus* spp., *Botrytis* sp., *Fusarium* sp. and *Pseudocercospora cladosporioides*. In particular, fungi of the genus *Fusarium* have been reported being responsible of olive fruit rots [18]. Similarly, *P. cladosporioides* is the agent of olive cercosporiosis and can affect leaves and fruits [20].

Furthermore, several other fungal taxa associated to *B. oleae* were detected and identified at species or genus level. The determination of such a high genetic variability represents an important advancement in the study of the complex interactions between olive fruit fly and fungi, although currently available data does not support speculations on their role and/or on the relevance of their presence on the insect. A part of retrieved sequences was classified as “Unknown fungus.” This can be due to a series of factors, including the high presence of sequences with unsettled nomenclature in public databases. Furthermore, many fungal species associated to insects are still unknown or their ITS barcode is still publicly unavailable.

In the previous survey [17], it was argued about the differential community composition between male and female specimens. However, in this study we did not reveal such difference, which probably arose from the low accurateness of the used method for quantitative analyses and the limited number of sequences analysed.

The results of the present study highlight the need of further investigations to assess the ecological role of identified taxa. Particularly interesting is the difference in the presence of fungi, such as *Cladosporium*, *Alternaria*, *Aureobasidium* and *Devriesia*, found on insects and olive trees in the same sites and time [29]. Several other fungal phylotypes were detected associated of *B. oleae*, on its body surface or retrieved from the intestinal lumen, which may result relevant from an ecological point of view. This opens new ways for the definition of the microbial ecology of this particular ecological niche, in particular taking into account the presence of plant pathogens associated to this pest (e.g. *Pseudocercospora* spp. and *Colletotrichum* spp.). This study represents a further step to define the ecological role of the olive fruit fly, not only as direct source of damage, but also as a major component of olive agroecosystem and as a vector of plant pathogenic fungi. The knowledge of the composition and the dynamics of fungal communities within the olive agroecosystem, could be pivotal in shaping the future generation of pest management and control strategies.

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Chapter 4

Fungal communities associated to bark and ambrosia beetles trapped at international harbours

Abstract

Bark and ambrosia beetles are widely diffused pests, known to establish trophic relationships with fungi often agents of plants and timber diseases. *Orthotomicus erosus* (Wollaston) and *Xyleborinus saxesenii* (Ratzeburg) are, respectively, a bark and an ambrosia beetle originally Palaearctic and now cosmopolitan, frequently trapped at international harbours because they could be easily moved around the world within woody materials. Here, we investigated their associated fungal communities, which may be moved along with their hosts, without strictly focusing on nutritional symbionts, well investigated in previous works. Targeting the ITS2 region of the fungal rDNA through pyrosequencing, we retrieved taxa associated to known agents of plant and timber diseases, together with taxa never previously associated to these beetles, and sequence clusters not linked to any known fungus. Our findings suggest that surveillance at harbours can be successfully extended to the fungi associated to wood beetles, and may reveal potential novel and unknown plant pathogens.

Keywords: *Orthotomicus erosus*, *Xyleborinus saxesenii*, Ophiostomataceae, *Geosmithia*, ITS2

Introduction

The existence of specific associations between insects and fungi has been widely documented for a number of different taxa, ranging from mutualistic symbiosis to antagonistic activity but, in most of the cases, their nature is still unknown to the scientific community [1, 2]. These associations become of particular interest when they involve plant pathogens, as it occurs in wood-boring beetles. In particular, bark and ambrosia beetles (Coleoptera; Curculionidae; Scolytinae) are known to be associated with different fungal taxa, including plant pathogens and wood rots [1, 3-5]. This particular association was considered functional to the beetle establishment [6-8], although the ecological role of these fungi has been questioned [9]. As globalization is leading to a sharp increase in the number of wood-boring beetles moved outside their native range, one could raise the question whether these fungal associates may be carried by these insects and become invasive, causing severe damage to invaded ecosystems [4, 10]. Examples come from the elm bark beetles *Scolytus* spp. and fungi belonging to the genus *Ophiostoma*, which have been destructive to elms in both North America and Europe [11], and from *Xyleborus glabratus* and *Raffaelea lauricola*, which caused extensive mortality of redbay and other species of Lauraceae in the USA [12].

The association with fungi is usually different between bark and ambrosia beetles. Bark beetles build galleries in the phloem, from which they take most of nutrients, and exploit fungi to supplement their diet [3, 13-15]. Ambrosia beetles, instead, dig their galleries in the xylem and feed on fungi cultivated on the galleries' walls [3]. These differences are not always clear, as some ambrosia beetles infest also phloem-sapwood interface [16]. In both groups, fungi can be transported in specialized structures called mycangia [17-20], in the gut [21], or phoretically on the beetle exoskeleton [14, 22]. Fungal symbionts are usually vertically transmitted from one generation to the next, but horizontal transmission from one species to another has also been demonstrated to occur in both bark and ambrosia beetles [5, 9, 23]. The same phenomenon can involve symbionts acting as plant pathogens [5]. Such a transmission can occur when a species interacts with another species' brood gallery and its associated fungi, or via fungus-feeding phoretic mites [1, 24, 25].

Bark and ambrosia beetles can be also easily moved around the world within wood-packaging materials [26, 27], wood chips [28], and logs [29]. Harbours, receiving large amounts of imported commodities, represent the most likely points of entry for exotic species [30-32]. Since the risk of new introductions has strongly increased in the last decades [33-35], specific preventive measures have been taken to prevent the arrival and establishment of exotic species, including international standards [36] and early-detection programs carried out at harbours using baited traps [27, 37, 38]. These traps can capture not only a number of exotic species, but also several native species, which could have arrived either from the natural areas surrounding harbours or from

the wood packaging materials used for shipping [27, 37]. When introduced in a new environment they can shift their associated microbiome and then redistribute themselves together with associated microorganisms in both their native and invaded ranges [39, 40]. This suggests that human-mediated movement of woody materials can mix up individuals of a given species, and its associate organisms.

In this study we characterized the fungal community associated with a bark and an ambrosia beetle species frequently trapped at three Italian international harbours [27, 37], with a focus on non-nutritional fungi. In particular, the work aimed at disclosing potential plant threatening agents and provide novel important details on their diffusion pathways and ecology. The study did not focus on primary symbionts nutritionally associated to ambrosia beetles, since they are widely described in literature and harmless to plants [41, 42]. Analyses were conducted on the bark beetle *Orthotomicus erosus* (Wollaston) and the ambrosia beetle *Xyleborinus saxesenii* (Ratzeburg). These species were selected because: (i) they represented the most commonly trapped species at Italian harbours for two consecutive years [27, 37]; (ii) they are native to Europe but they have been introduced in several other countries [26, 43-45]; (iii) they are representative of in-out travelling populations, and/or of those in which horizontal transfer may have occurred [39]. A culture-independent high-throughput metabarcoding approach, based on fungal ITS2 region and 454 pyrosequencing, was performed to analyse both *O. erosus* and *X. saxesenii* associates. Fungal communities were analysed according to the beetle species and the ports in which they were trapped.

Materials and methods

Orthotomicus erosus and *Xyleborinus saxesenii* trapping

The individuals of *O. erosus* and *X. saxesenii* analysed in the present study were collected in 2013, during a nationwide trapping program carried out at the main Italian harbours and aimed at improving the early-detection of alien wood-boring beetles [27]. At each site, three 12-unit black multiple-funnel traps (Econex, Murcia, Spain) were placed within the harbour area, hanging them about 2 m above the ground. Traps were baited with a multi-lure blend composed of: (−)-α-pinene, ipsenol, ipsdienol, 2-methyl-3-buten-2-ol, and ethanol (Contech Enterprises Inc., Victoria, BC, Canada). Collection cups were sprayed with an insecticide (FERAG IDTM – SEDQ, Spain) to quickly kill the insects, and no liquid was added. In this way, samples were collected completely dry and cross-contamination risks were kept as low as possible, although we are conscious that fungal spores could have moved among species. Furthermore, there is no evidence in our results of cross-contamination between insect species, since their fungal communities are different, and their shared core taxa are known as ubiquitous fungi (see Results). Traps were checked biweekly, and trapped beetles were

sorted by species and preserved at −80°C in Eppendorf 1.5 ml tubes, filled with 95% ethanol. A sufficient number of individuals of each species to allow for analyses were collected in three international harbours (Marghera - 45° 43’ N, 12° 31’ E, Ravenna - 44° 49’ N, 12° 28’ E, and Salerno - 40° 67’ N, 14° 64’ E), which are known to import large amount of solid commodities from foreign countries [46].

DNA extraction and library preparation

DNA was extracted from samples made of 10 randomly selected specimens, with a total of 3 replicates from each harbour for each beetle species (total of 18 samples). Before DNA extraction, ethanol used to store the samples was completely evaporated using a vacuum evaporator (Eppendorf® Concentrator Plus, Hamburg, Germany). Each sample (10 insects) was crushed in an extraction buffer (10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) with the aid of a bead mill homogenizer, and the mixture was then treated with Proteinase K (5Prime GmbH, Germany) following the producer’s protocol. Total DNA was extracted as described by Schena and Cooke [47], and analysed by electrophoresis in TBE buffer and 1.5% agarose gel stained with GelRed™ nucleic acid stain (Biotium, USA) and visualized with UV light using a Gel Doc™ system (Bio Rad, USA). DNA concentration and quality was assessed by means of a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., USA).

Libraries for 454 GS FLX+ sequencing were built using fusion primers (http://www.454.com/) targeting the fungal ITS2 region of the ribosomal DNA (rDNA). PCR reactions were conducted in a total volume of 25 µl and contained 1 µl of extracted DNA (about 50 µg), 1X Taq buffer, 1.5 mM MgCl2, 40 µM dNTPs, 1 unit of Taq polymerase (AccuPrime™, Thermo Fisher) and 0.5 µM of primers (ITS3 and ITS4 – White, Bruns, Lee and Taylor [48]). Amplifications were performed in a Mastercycler Ep Gradient S (Eppendorf, Germany) set at 94°C for 3 minutes, 94°C for 30s, 55°C for 30s and 72°C for 30s, repeated 35 times, and ended with 10 minutes of extension at 72°C. A non-template control, in which target DNA was replaced by nuclease-free water, was included in all PCR reactions. PCR products were analysed by electrophoresis as described above, and purified using Agencourt AMPure XP kit (Beckman Coulter Inc., CA, USA). Samples were amplified in triplicate, in order to decrease the stochastic variability among reactions [49]. The concentration of PCR products in each sample was measured with Qbit Instrument (Thermo Fisher Scientific, USA), and normalized diluting amplicons in molecular biology grade water. Ten µl of each purified sample were pooled together and sequenced by Macrogen Inc. (Seoul, Korea) on one 1/8th regions of a sequencing plate on a 454 GS FLX+ System (454 Life Sciences, Branford, CT, USA).

Data processing

Raw sequencing data were processed using QIIME 1.8.0 [50], setting the minimum quality score to 25 and without allowing mismatches in the primer sequence. Sequences <150 bp, >1000bp, containing homopolymers >10 bp and with >6 ambiguous bases were discarded. Reads were denoised using denoise wrapper [51], and chimeric sequences were removed using USEARCH 6.1 algorithm [52] combining a reference-based with a *de novo* detection method. ITS2 sequences were extracted using ITSx [53], as it is known that conserved flanking regions can lead to errors in clustering, taxonomic and similarity results [18, 54]. Reads were then clustered into Operational Taxonomic Units (OTUs) using BLAST method, with 0.99 similarity threshold to the UNITE dynamic reference database [55] accessed on March 2015 (http://unite.ut.ee/). When reads failed to hit to the reference database, sequences were clustered as *de novo*, and singletons were discarded from analyses. The UNITE database was employed, using BLAST algorithm, for the taxonomic identifications of representative sequences of each detected OTU.

The most commonly associated OTUs in each analysed sample were identified using the method of core/satellite taxa as described by Magurran and Henderson [56]. This approach involves an iterative process to decompose the Species Abundance Distribution (SAD) into two parts: core OTUs and satellite OTUs. The threshold between the two categories was set at the number of samples at which the SAD fitted a log-normal distribution. The core dataset was employed for taxonomic and ecological aspects, while the whole community composition was used in alpha and beta diversity analyses.

The alpha diversity was estimated through the Species Accumulation Curves (SAC) and a set of diversity indices (Dominance, Shannon and Chao1), calculated for both insect species. The beta diversity was tested through a Principal Coordinates Analysis (PCoA) with 95% confidence ellipses, supporting these results with a PERMANOVA non-parametric approach determined with 999 permutations. All analyses were performed with QIIME [50] and Microsoft® Excel® 2013.

Taxonomic identification of core taxa

Since the UNITE databases enabled a reliable identification of fungi only at the genus level [57], representative sequences of all detected OTUs were further analysed by means of MegaBLAST search. Furthermore, OTUs classified as “core” OTUs and putative plant pathogens were analysed along with validated reference sequences of closely related species, in order to determine their phylogenetic placement at the highest possible level of accuracy (Malacrinò, Schena, Campolo, Laudani and Palmeri [2], Abdelfattah, Nicosia, Cacciola, Droby and Schena [58] – Fig. S1). OTUs associated to “Uncultured fungi” were not phylogenetically analysed. Specifically, we analysed with

the phylogenetic approach all sequences belonging to the genera *Alternaria*, *Aspergillus*, *Aureobasidium*, *Boeremia*, *Botrytis*, *Cladosporium*, *Devriesia*, *Geosmitia*, *Ophiostoma*, and *Stemphylium*. For each fungal genus, sequences were aligned using MUSCLE [59] and phylogenetically analysed with RAxML 8.0.0 using a GTR + Γ model [60].

Results

General results

In total, 59,247 reads were retrieved after quality filtering, denoising and chimera discarding. Sequences were demultiplexed, obtaining an average read count of 3,291 and a mean length of 250bp. Using a 0.99 cut-off, and deleting singletons from the analyses, a total of 294 OTUs were retrieved. The flattening of Species Accumulation Curve (SAC), at increasing number of analysed sequences, indicated a sufficient sequencing depth to reconstruct the fungal community of both Scolytinae species (Fig. 1 A-B). The fungal communities of both beetles had similar values of diversity indices (Tab. 1), and a clear clustering of fungal communities was revealed when samples were labelled according to the beetle species (Fig. 2), while they did not differ among the three harbours. These results were supported by a PERMANOVA analysis of the dataset, that highlighted differences between insect species (PseudoF = 8.295; P = 0.01) but not among sampling harbours within each species (PseudoF = 1.482; P = 0.15).

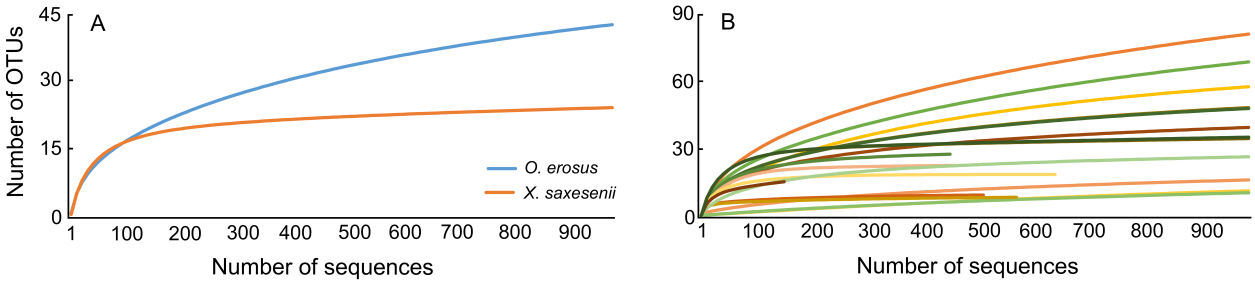

FIG. 1 RAREFIED SPECIES ACCUMULATION CURVES (SAC) REPORTED FOR: (A) EACH BEETLE SPECIES AND (B) FOR EACH OF THE 18 SAMPLES (3 HARBOURS X 2 SPECIES X 3 REPLICATES).

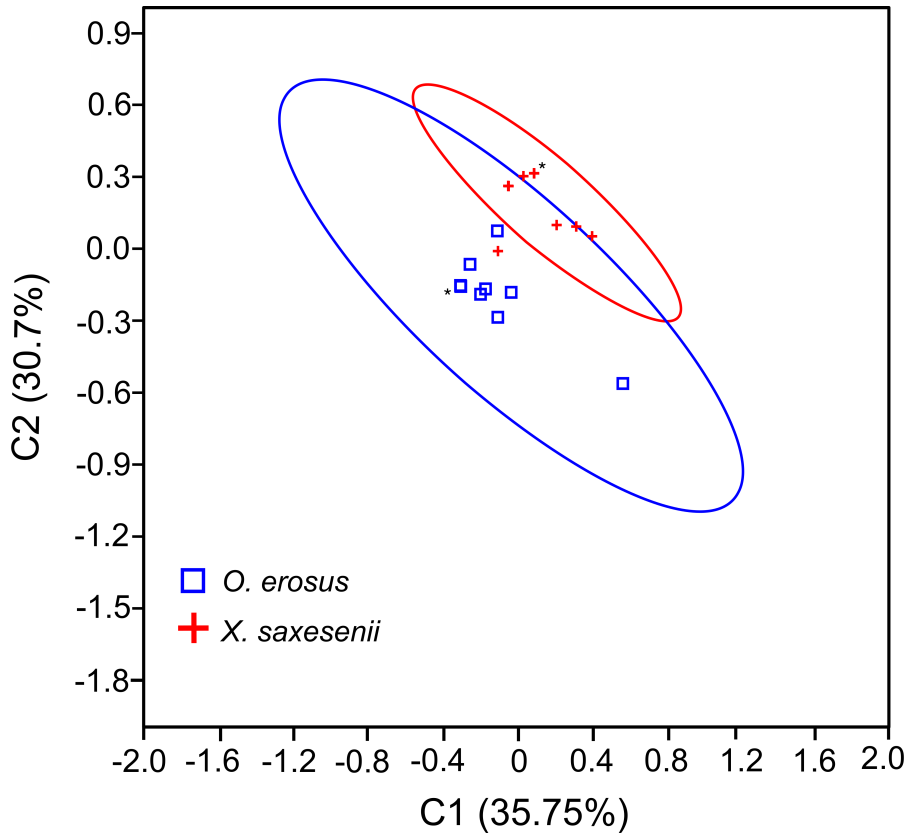

FIG. 2 PRINCIPAL COORDINATES ANALYSIS (PCOA) RESULTS OF FUNGAL COMMUNITY DIVERSITY ANALYSIS OF *ORTHOTOMICUS EROSUS* (BLUE — *N* = 9) AND *XYLEBORINUS SAXESENII* (RED — *N* = 9). POINTS MARKED WITH (*) REPRESENT SAMPLES WITH SIMILAR COMPOSITION OF FUNGAL COMMUNITY, RESULTING IN OVERLAPPING POINTS.

TABLE 1 DIVERSITY INDICES ABOUT THE FUNGAL COMMUNITY ASSOCIATED TO *ORTHOTOMICUS EROSUS* AND *XYLEBORINUS SAXESENII*.

Species	Observed OTUs	Dominance (D)	Shannon (H)	Chao1
O. erosus	18.56±3.19	0.23±0.07	2.05±0.26	18.79±3.24
X. saxesenii	45.56±7.85	0.37±0.12	1.84±0.38	51.10±9.09

Fungal communities associated to *Orthotomicus erosus* and *Xyleborinus saxesenii*

One hundred sixty-nine fungal OTUs were identified in *O. erosus*. The analysis of SAD divided the dataset into core (log-normal, goodness of fit χ^2=6.308; *P* = 0.70) and satellite taxa, classifying as core those OTUs with a persistence of ≥6 samples (Fig. 3 A-B). This approach suggested 20 OTUs as core taxa associated to this bark beetle, accounting for 79.70±5.41% of sequences. Inside the core taxa, 51.40±10.92% of the

sequences were identified at least at genus level, 35.67±7.79% were associated to different yeast genera and 12.39±3.81% matched sequences from "Unknown fungi". Filamentous fungi identified at genus or species level included: *Aspergillus* spp., *Devriesia* sp., *Geosmithia* sp., *Stemphylium* sp., *Fusarium* sp. (*incarnatum-equiseti* species complex), *Ophiostoma pulvinisporum*, *Alternaria* sp., *Botrytis* sp. and *Boeremia* sp. (Tab. 2). Among yeasts, we retrieved sequences that matched with the following genera: *Ogataea* sp., *Sporobolomyces* sp., *Pichia* sp., *Myxozyma* sp., *Rhodosporium* sp., *Rhodotorula* sp. The phylogenetic analysis enabled the identification of OTUs *OPH2*, *OPH3* and *OPH4* as *Ophiostoma pulvinisporum*, *O. saponiodorum* and *O. rectangulosporium*, respectively (Fig. S1H), while the cluster *OPH1* remained unresolved within the genus *Ophiostoma*. Furthermore, the OTU *ALT1* was associated to *Alternaria* sect. Alternata (Fig. S1A).

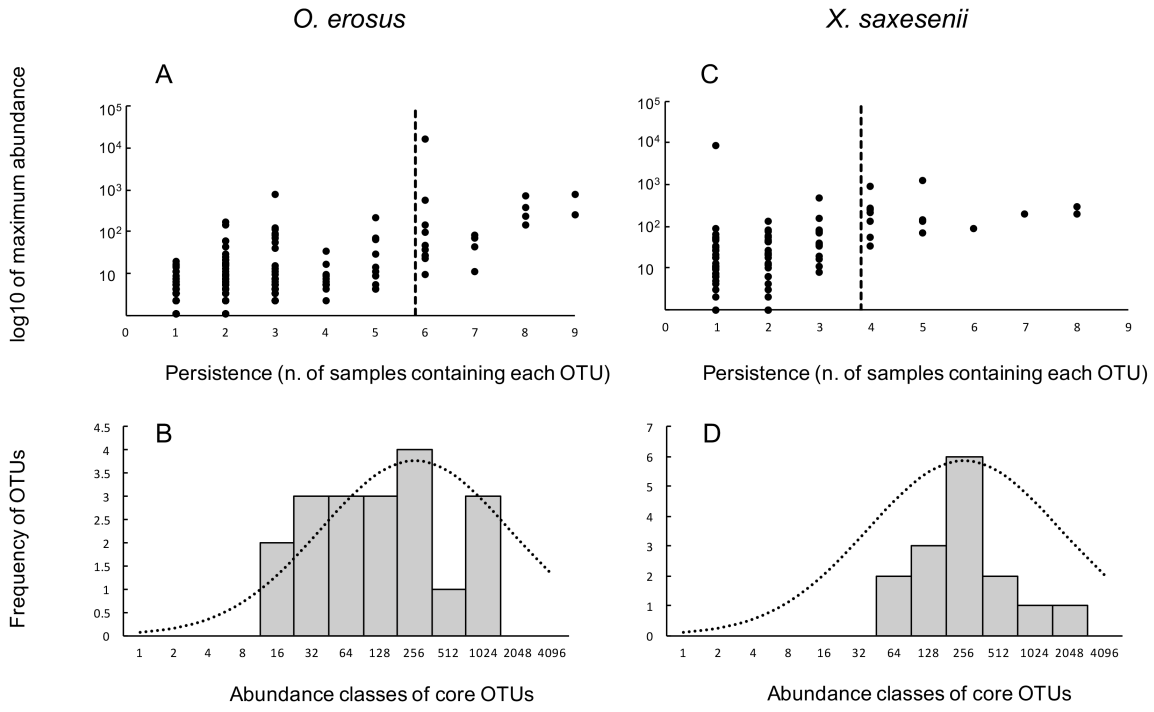

FIG. 3 SEPARATION OF CORE AND SATELLITE OTUS OF THE FUNGAL COMMUNITY FOR BOTH *ORTHOTOMICUS EROSUS* (LEFT) AND *XYLEBORINUS SAXESENII* (RIGHT). SPECIES ABUNDANCE DISTRIBUTION (A AND C) BASED ON ABUNDANCE/PERSISTENCE OF OTUS, WITH DASHED LINES ON THE THRESHOLD BETWEEN CORE AND SATELLITE OTUS SET THROUGH PROCESS ITERATED UNTIL THE ABUNDANCE CLASSES DISTRIBUTION (B AND D) FIT A LOG-NORMAL DISTRIBUTION.

For *X. saxesenii*, the core/satellite taxa approach divided the SAD in two parts, identifying as core OTUs that with a persistence ≥4 samples (Fig. 3 C-D). Of the total 96 OTUs, 15 were classified as the core part of SAD (goodness of fit χ^2=12.71; *P* = 0.47), including 60.79±9.39% of sequences associated to filamentous fungi, 2.78±1.53% to

different yeast species and 45.15±8.02% matched previous sequences from "Unknown fungi". The phylogenetic approach allowed us to identify the *CLA1* core OTU cluster as part of *Cladosporium herbarum* s.c. (Fig. S1C), while the cluster *AUR1* was identified as *Aureobasidium pullulans* (Fig. S1B). As indicated in Table 2, identified fungi belonged to *Alternaria* sp., *Aspergillus* spp., *Aureobasidium pullulans*, *Botrytis* sp. and *Cladosporium herbarum* s.c., while among core OTUs we found only one yeast OTU associated to *Candida* sp.

TABLE 2 FILAMENTOUS FUNGI CLASSIFIED AS CORE OTUS WITH THE SAD ANALYSIS (FIG. 3), THAT WERE CLASSIFIED AT LEAST AT GENUS LEVEL.

Beetle species	OTU	Fungal taxa	Abundance	Percentage (*k*±SE)
O. erosus	ASP1	*Aspergillus sp.*	15299	31.13±15.52
	DEV1	*Devriesia sp.*	229	7.46±2.45
	GEO1	*Geosmithia sp.*	518	2.83±2.69
	STE1	*Stemphylium sp.*	132	2.79±1.37
	FUS1	*Fusarium incarnatum-equiseti* s.c.	79	2.31±1.01
	OPH2	*Ophiostoma pulvinisporum*	65	1.90±0.63
	ASP1	*Aspergillus sp.*	88	1.15±0.6
	ALT1	*Alternaria sp.*	26	0.81±0.22
	BOT1	*Botrytis sp.*	22	0.42±0.14
	FUS2	*Fusarium incarnatum-equiseti* s.c	25	0.34±0.14
	BOE1	*Boeremia sp.*	9	0.25±0.13
X. saxesenii	BOT2	*Botryotinia sp.*	282	17.02±4.78
	CLA1	*Cladosporium herbarum* s.c.	190	9.59±3.41
	ASP2	*Aspergillus sp.*	55	8.52±8.48
	AUR1	*Aureobasidium pullulans*	861	6.50±3.29
	ASP3	*Aspergillus sp.*	215	4.16±2.36
	ASP4	*Aspergillus sp.*	241	3.31±1.48
	ALT1	*Alternaria sp.*	70	2.96±1.7

Analysis of the occurrence of fungal species

Considering the whole dataset, we can observe a slight overlapping of the fungal community (Fig. 2), mainly due to OTUs classified as "core", for one beetle species or both, and some satellite taxa. Generally, 52 OTUs were shared between *O. erosus* and *X. saxesenii* (Fig. S2A) and, among these, the shared core OTUs were identified as *Aspergillus* spp., *Aureobasidium pullulans*, *Botrytis* spp., *Devriesia* sp., *Cladosporium* sp., *Stemphylium* sp., *Alternaria* sp., and *Fusarium* sp.

Comparing the three harbours, we did not find major differences, indicating that the fungal microbiome associated with the two beetle species was rather similar. We found, however, one shared core OTU associated to the genus *Aspergillus* that was common to Marghera and Salerno only, whereas 4 shared core OTUs associated to the genera *Fusarium* (2 OTUs), *Stemphylium* and *Botrytis* were shared between Ravenna and Salerno. We did not find any shared core OTU between Ravenna and Marghera. Interestingly, we found OTUs associated to *Geosmithia*, *Graphium* and *Ophiostoma* shared between the samples collected in Salerno and Ravenna. On the other hand, 3 OTUs associated to the genera *Acremonium* and *Ophiostoma* were found only in Ravenna, and 2 OTUs (one *Geosmithia* and one *Ophiostoma*) were found only in Salerno (Fig. S2B).

Discussion

In this study, a HTS (High Throughput Sequencing) metabarcoding approach was utilized to investigate fungal communities associated to one bark and one ambrosia beetle species. The same approach was previously utilized to analyse symbionts of different ambrosia beetles [18, 61, 62] and, more recently, to assess the fungal diversity associated with three bark beetles [63]. Unlike previous studies, which mainly focused on nutritional symbionts, we characterized the fungal diversity of species of Scolytinae collected in international harbours, evaluating their role as potential carriers of novel and unknown fungal plants pathogens.

The ITS3 and ITS4 primers were used to amplify the ITS2 region of the rDNA, a widely accepted gene as official barcode for fungi [64, 65]. The use of the ITS2 region led to a series of advantages when it comes to HTS metabarcoding, mainly due to the low variability of its length compared to the ITS1 region (reduced sequencing bias), and the avoiding of the highly conserved 5.8S region that could lead to the formation of chimeric ITS1-ITS2 amplicons from different species [66]. On the other hand, the use of ITS2 region lead to problems associated to the study of fungal communities associated to bark and ambrosia beetles, in particular when dealing with fungi belonging to the order Ophiostomatales. First, amplification of the ITS2 region of these fungi is very difficult due to the formation of a secondary structure in the GC-rich region where 5' primers anneal [67]. In addition, the representation of *Ambrosiella* spp., *Raffaelea* spp., and other symbiotic fungi in public databases is low [18, 67], their nomenclature is not well defined, or the ITS2 region is not variable enough to discriminate closely related species [18, 68]. We decided to target the ITS2 region because we aimed to characterize the fungal community associated to these beetles, without focusing only to well-known symbionts [41, 42], but also to other fungi that could represent a serious threat to plants. This approach has been proven to be effective by Miller, Hopkins, Inward and Vogler [63], studying the fungal community associated to the bark

beetles *Hylastes ater* and *Tomicus piniperda*, and the ambrosia beetle *Trypodendron lineatum*).

The sampling procedure allowed us to collect beetles without using any preservative liquid, keeping the cross-contamination among trapped insects as low as possible. Furthermore, the core-satellite approach allowed us to focus our analysis on the fungal species that are more constantly associated to these beetles, and therefore unlikely representing an occasional environmental contamination. We did not surface sterilized samples in order to analyse the whole fungal community, including the fungi outside mycangia.

Overall, data obtained from this study are consistent with those available in literature and here we report novel important information, worthy of being explored further. Among Ophiostomatales, we obtained 3 OTUs that clustered with *O. pulvinisporum* (core OTU of *O. erosus*), *O. rectangulosporium*, and *O. saponiodorum* reference sequences, all known agents of blue-stain discoloration [69], and another OTU belonging to this genus but unresolved to species level. Although we did not retrieved sequences associated to *O. ips*, which is frequently associated with *O. erosus* [41], we identified 2 taxa, *O. pulvinisporum* and *O. saponiodorum*, which were never found to be associated with this bark beetle before, and *O. rectangulosporium* which was instead already retrieved in one study conducted in Spain [41]. Some ophiostomatoid fungi are known to be important pathogens of conifers and agents of bluestain on logs and freshly-cut wood [70], a discoloration mainly due to fungi belonging to *Ophiostoma* and *Ceratocystis* genera. Although many species of these fungi are not pathogenic, the bluestain can lead to the reduction of wood price up to 50% [71]. Microascales are also considered symbionts of bark and ambrosia beetles; *Graphium* species are reported associated to both bark and ambrosia beetles (Linnakoski *et al.* 2012). In our study, we retrieved one satellite OTU assigned to *Graphium* associated to *O. erosus*. Previous studies already reported the association between this bark beetle and fungi of this fungal genus [72].

Among Hypocreales, we obtained a total of 12 OTUs belonging to the genera *Geosmithia*, *Acremonium*, and *Fusarium*. We retrieved 3 OTUs that were associated to *Geosmithia* spp., of which one (*GEO1*) was comprised among the core OTUs of *O. Erosus*, while the other two were classified as satellite sequences of *X. saxesenii*. The phylogenetic analysis did not allow us to push the identification to species level, but it is known that *Geosmithia* spp. are regularly associated to many Scolytinae (about 30 species worldwide) including both *O. erosus* and *X. saxesenii* [73-75]. Although it is common to find *Geosmithia* spp. associated with insects, their relationship is still poorly understood [75]. These fungi lack of entomochory-related adaptations, such as sticky conidia or ascospores. Their phytopathogenic activity is poorly understood since confirmed only in one case (*Geosmithia morbida* on walnuts), while it has been

hypothesized that they may play an important role as nutrient suppliers for their vectors [74, 76].

Acremonium species were already reported associated with other bark and ambrosia beetles [77-79] but, to our knowledge, never with *O. erosus* and *X. saxesenii*. In our study we identified two satellite OTUs to the genus *Acremonium*, one associated only to *O. erosus*, and the other one shared between the two species. As reported by Belhoucine, Bouhraoua, Meijer, Houbraken, Harrak, Samson, Equihua-Martinez and Pujade-Villar [77], these fungi can support adults during galleries building, providing food supply.

We reported 7 *Fusarium* OTUs of which 2 (*FUS1* and *FUS2*) were identified as core OTUs of *O. erosus*, belonging to the *F. incarnatum-equiseti* species complex (s.c.). The OTU *FUS2* was also detected in *X. saxesenii* as satellite OTU. The satellite taxa clustered into *F. incarnatum-equiseti* s.c., *F. solani* s. c., and *F. lateritium* (shared between insect species), *F. oxysporum* s.c. and *F. brachygibbosum* (associated only to *O. erosus*). Fungi belonging to the genus *Fusarium* were reported associated to *O. erosus* [80], but never to *X. saxesenii*. Furthermore, *Fusarium* species have been reported as mutualistic symbionts of beetles of the *Euwallacea* genus, particularly *Fusarium solani* s.c. [81]. The same authors indicated that *Fusarium* may have allowed Scolytinae to exploit new food sources in non-native ecosystems. Indeed, it is worth noting that ambrosial *Fusarium* species belong to a specific monophyletic group within the *Fusarium solani* s.c., the Ambrosial *Fusarium* Clade [81].

A total of 16 OTUs were associated to the genus *Aspergillus*, of which one (*ASP1*) was part of the core OTUs of *O. erosus*, and *ASP2*, *ASP3,* and *ASP4* were associated to *X. saxesenii*. Taking into account the role of these fungi as ubiquitous, and widely distributed pathogen of plants and foodstuffs, as well as being known for their saprophytic behaviour, we suggest that in here they could not be considered as symbiont. Further researches could shed light on a stricter association with these beetles. Other studies reported the finding of *Aspergillus* together with bark and ambrosia beetles, but their strict association was never demonstrated [79, 82]. Furthermore, the analyses highlighted one OTU belonging to the *Alternaria* sect. Alternata (*ALT1*) shared between both *O. erosus* and *X. saxesenii*. As reported for *Aspergillus*, this genus is widely distributed so it should not be considered a symbiont, however other studies have reported its presence associated to bark and ambrosia beetles [79, 82].

We identified other core OTUs in our study associated to the genera *Devriesia*, *Stemphylium*, *Boeremia*, and *Botrytis*. These fungal genera comprise widely diffused species with a known saprophytic habit and, although available data does not enable supported speculations about their role, they are likely to be external contaminants of the insects. Moreover, we retrieved a high number of ITS sequences of yeasts taxa from our analysis: 35.7% for *O. erosus* and 2.8% for *X. saxesenii*. Our approach did not

allow us to identify these yeasts to species level, but they are known to be commonly associated with bark and ambrosia beetles, contributing to their development, reproduction, nutrition, defence and to be involved in other ecological relationships with plants and other microorganisms [83]. A part of our sequences (12.9% for *O. erosus* and 45.2% for *X. saxesenii*) was classified as "Unknown fungus." This can be due to a series of factors, including the high presence of sequences with unsettled nomenclature in public databases. Furthermore, many fungal species associated to insects are still unknown or their ITS barcode is still publicly unavailable, and this is particularly true for bark and ambrosia beetles.

These results provide novel information about the fungal community of two widely diffused Scolytinae trapped at international harbours, suggesting new associations with fungi that could represent agents of plant diseases, including species of the genera *Ophiostoma*, *Acremonium*, and *Fusarium*. Furthermore, for *O. erosus* we retrieved sequences that can be associated to generalist fungi, which might allow this bark beetle species to exploit food sources in non-native environments. Instead, for *X. saxesenii*, we got a high number of sequences for which the identification was not possible because of uninformative sequences or their lack in public databases. These sequences can be associated to unknown or unculturable fungi, which could be new symbionts or new pathogens, representing therefore potential new threats for plant health. Future endeavours could focus on their *in vitro* isolation, allowing their identification to species level. Although we cannot exclude that the trapped individuals that we analysed came from natural areas surrounding harbours, they might represent a sample of 'in-out travelling' populations, which are likely involved in horizontal transfer of fungal spores [39]. This kind of scenario is quite realistic, as the spreading of ophiostomatoid fungi was already reported through the movement of wood [39, 84-86]. This becomes particularly interesting if we consider the amount of unknown fungi and the wide spread, host, and climatic ranges of these beetles. They can adapt to new conditions and gain new fungal associations, which frequently lead to high environmental and economic losses. This approach could be successfully extended to the study of nutritional symbionts of these species, uncovering novel insights on their ecological relationships with microorganisms that can pose a threat to human economical activities, or which can be exploited to gain technological applications for pest control.

Given the threat posed by these bark and ambrosia beetles to forest, urban, and agricultural ecosystems, this study opens a new scenario that looks at these beetles not only as direct source of damage, but also as potential carriers of novel and unknown plants pathogens, suggesting that the surveillance at harbours should be extended also to fungi associated to wood-boring beetles.

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Chapter 5

Interactions between aphids and soil microbiota revealed through a community approach

Abstract

Plants are known to be able to shape their associated microbiota which, in turn, can influence plant growth and physiology. Across this reciprocal interaction, insect herbivores can influence their host, affecting also its relationship with the surrounding environment. Aim of the present study was the analysis of three-way interactions between plants, aboveground insects and soil microbial communities. To do this, we set up a microcosmos system using two *Solanum* species as hosts and *Macrosiphum euphorbiae* as herbivore. Then the microbial communities of aphids, leaves, roots and rhizosphere soil were analysed using a metabarcoding approach. The results suggest a strong influence of plant genotype on the microbial ecology of the entire system. Furthermore, the initial soil microbial community influenced the resulting biota. Interestingly, the microbial communities associated to aphids was affected by soil microrganisms, and in particular by arbuscular mycorrhizal (AM) fungi. According to our data the main drivers in modulating the plant-associated microbiota are plant genotype and soil communities, and this may have an important impact on the development of future generation of pest management strategies.

Keywords: High Throughput Sequencing, *Macrosiphum euphorbiae*, *Solanum tuberosum*, *Solanum vernei*, metabarcoding

Introduction

Microorganisms are known to have a cosmopolitan distribution, interacting with other organisms can structure and modulate several biotic interactions that can also influence the abiotic environment. Since their ecological and economical importance, the microbial communities associated to plants have received a great deal of attention. Furthermore, there is growing evidence that plants can play an active role on selecting their own microbiome, in particular during stress episodes, and microorganisms may be considered as an extension to form a second genome or collectively to form a pan-genome [1, 2]. This reciprocal interaction between plant and microbes can extend beyond the plant itself, influencing other organisms living on phyllosphere or rhizosphere, or even entire communities inhabiting aboveground and/or belowground [1, 3-5].

The rhizosphere is considered one of the most dynamic interfaces since the range of interactions with other organisms, both below- and aboveground, is very extended through the trophic ladder [4]. Within the rhizosphere, the microbial activity is very high and, compared to the surrounding non-rooted soil, it contains a much diverse and abundant population of microorganisms [3]. This compartment is directly influenced by the plants through the release of organic compounds (e.g. cells, mucilage, exudates, VOCs) that, in turn, modulate the community of microorganisms inhabiting this area [3, 6]. Herbivory, therefore, affecting the quality of organic compounds released at root interface, can alter the composition of rhizosphere microbial communities [7]. There is growing evidence that this modulation of the rhizosphere microbiota can be exploited by plants to recruit beneficial organisms in response to stresses, such as herbivory [7-11]. On the other side, phyllosphere can be considered as a more ephemeral environment, as annual plants complete their cycle within a single growing season and perennial plants, both deciduous and evergreen, shed their leaves every year [5]. For both compartments, plant genotype play an essential role in shaping the microbial community, which furthermore must cope with alterations due to biotic and abiotic factors and stochastic events [4, 5, 12].

Insect herbivory represents a serious limiting factor for plants all over the world, impacting on production with $400 billions of losses [13]. However, plant tissues by themselves do not represent a promising food for insects, since they include a wide range of indigestible and toxic compounds. Therefore, insects evolved a series of strategies to cope with these issues [14], including the association with bacterial and fungal symbionts to exploit different hosts [15, 16]. The interaction between insects and microorganisms extend at different levels of the trophic ladder, and can also comprise indirect interactions mediated by plants [17]. Tripartite and multipartite interaction represent a growing field of study in the last few years, leading to the discovery of important ecological associations. For example, there is evidence that fungal

endophytes can modify the spectrum of VOCs emitted by plants, also in response to herbivory [18], and it has been observed that mycelia networks formed by arbuscular mycorrhizal (AM) fungi, can mediate a signal transfer from plants infested by aphids to healthy ones [19].

Therefore, taking into account all these information, one can depict the following scenario. Plants can alter their microbiota on the basis of their needs, in particular for feeding and during stress. Even if the aboveground and belowground compartments are physically separated, the respective communities can influence each other, therefore the attack by an herbivore on leaves, can have effect on the microbial community at the root interface. Furthermore, microorganisms can have effects on the plant in different ways, e.g. modulating nutrients intake or secondary metabolites production. This bottom-up effect can travel up to the herbivores influencing, in turn, their own microbial community. As described above, these interactions have been widely studies in the past, however most studies focused on the microbial ecology of selected compartments of the system.

Therefore, our work aimed to disclose these aspects through a community approach on the microbial community in a microcosm system, using two *Solanum* species as hosts and *Macrosiphum euphorbiae* as herbivore. To characterize the microbiota of each compartment, we used a culture-independent approach based on High Throughout Sequencing (HTS) to investigate bacterial, fungal and mycorrhizal communities. We focused on three main question: (i) can aphid herbivory impact on soil microbial communities? (ii) can soil microbial communities alter the aphids' microbiota? (iii) which is the impact of plant and aphid genotype on these interactions?

Materials and methods

Experimental design

This study focused on the *Macrosiphum euphorbiae* – *Solanum* system. Two plant species were grown on 3 soils characterized by different microbial communities and were exposed to herbivory using 4 different aphid clones. Plants without aphids were used as a control. Each combination of plant species, soil microbial community, and aphid infestation was replicated 5 times, involving a total of 150 plants.

Plants and soil treatments

Seeds of *Solanum tuberosum* (genotype TBR-5642) and *Solanum vernei* (genotype VRN-7630), obtained from the Commonwealth Potato Collection at The James Hutton Institute (Dundee, Scotland, UK), were sowed on stem-sterilized coir, let to grow for 3 weeks and then outplanted. Plants were reared on three different types

of soil microbial communities: soil hosting original microbial community (WHS), sterilized soil inoculated with AM spores isolated from field soil (AMF) and sterilized soil inoculated with field soil bacterial community (MICROB). The soil used in this study was collected from an uncultivated field at the James Hutton Institute, and sieved (≈3 cm mesh) to remove rocks and large debris. A portion of the sieved soil was autoclaved at 121°C for 3 h, cooled for 24 h and then autoclaved at 121°C for further 3 h. Furthermore, a sterilized background soil was prepared by mixing Sterilized Loam (Keith Singleton, Cumbria, UK) and sand (ratio 1:1), and by autoclaving this mixture as explained above. This background substrate served to host the microbial communities and to provide a suitable environment for plants to growth.

Plants were outplanted on 1 L pots, filled with 100 mL of sterile background soil, 800 ml of inoculum and 100 ml of sterile background soil on the top (Fig. S1 – Supplementary material). The soil inoculum was alternatively constituted by: (i) a mixture of autoclaved and non-autoclaved soil in the ratio 4:1 (WHS treatment); (ii) sterilized soil inoculated with spores of AM fungi (n=49.7±1.5) previously isolated as described by Daniels & Skipper [20] (AMF treatment) or; (iii) sterilized soil added with original bacterial community isolated by vacuum filtration through a Whatman filter paper (MICROB treatment). For further details about the soil preparation, please refer to the Supplementary Material. Plants were left to grow in an insect-screened greenhouse with an average temperature of 25°C and 16:8 (L:D) photoperiod.

Aphids

After 5 weeks from outplanting, plants were inoculated with aphids, leaving a plot without aphids as control. In this study we used 4 different aphid clones of *M. euphorbiae* reared at the James Hutton Institute (AK13/08, AK14/02, AK13/18 and RB15/05). Plants were infested with two apterous adult aphids each. In order to avoid cross infestation and to keep the control plot clear from aphids, all plants were screened with a pierced plastic bag that allowed transpiration without allowing the aphids to escape.

Sampling

After 4 weeks from aphid infestation, we proceed with harvesting all the plants used in the experimental procedure. Plant material was separated into: leaves, stem, stolons, tubers and roots. Belowground parts were washed, and all organs were kept separated inside Kraft paper bags, immediately frozen at -20°C and then freeze-dried for 1 week. An even sample of leaves and roots from each plant was isolated for subsequent metabarcoding analyses. Furthermore, during the sampling we collected 15 adult apterous aphids from each infested plant and about 15 ml of rhizosphere soil.

DNA extraction, Illumina Miseq Libraries preparation and sequencing

Total DNA was extracted from aphids (*n*=5), leaves, roots and soil (≈50mg each), using the phenol/chloroform method through the procedure explained in the Supplementary Material S5.2. DNA was subsequently checked for quantity and quality with a Nanodrop 2000 (Thermo Fisher Scientific Inc., USA).

The metabarcoding analysis included the bacterial, fungal and AM fungal communities. Soil and roots were analysed for all three targets, while leaves and aphids were analysed only for bacterial and fungal communities. The bacterial community was characterized targeting the 16S gene. In order to limit the amplification of plant plastidial DNA from leaves and roots we used a nested-PCR approach in which a first round PCR with primers not amplifying from plants (Eub8F/984yR – [21]) was combined with a second round PCR with primers 515f/806rB reported by Caporaso *et al.* [22]. The general fungal community was analysed though the amplification of the fungal ITS2 region with primers ITS3-KYO/ITS4 [23]. However, a specific set of primers (AMV4.5NF/AMDGR) targeting the 18S region was utilized for AM fungi since they they are difficult to characterize using general fungal primers [24]. In order to include all the samples and targets within a single MiSeq run, all primers were modified to include a 8-bp GoLay barcode which, together with the Illumina double indexing approach, allowed us to distinguish each sample in the subsequent bioinformatics analyses (Supplementary Material S5.3). Furthermore, amplifications were carried out also on DNA extracted from sterilized soil, from the AM fungi inoculum, and from sterilized water as control groups.

PCR reactions were performed in a total volume of 25 µl, containing about 50ng of DNA, 0.5 µM of each primer, 1X of KAPA HiFi HotStart ReadyMix (KAPA Biosystems, USA) and nuclease-free water. Amplifications were performed in a Mastercycler Ep Gradient S (Eppendorf, Germany) set at 95°C for 3 minutes, 98°C for 30s, 55°C for 30s and 72°C for 30s, repeated 35 times, and ended with 10 minutes of extension at 72°C. Reactions were carried out in triplicate, in order to reduce the stochastic variability during amplification (Schmidt et al., 2013), and a non-template control in which nuclease-free water replaced target DNA was utilized in all PCR reactions. Libraries were checked on agarose gel for successful amplification, and purified with Agencourt AMPure XP kit (Beckman and Coulter) using producer's instruction. A second short-run PCR was performed in order to ligate the Illumina i7 and i5 indexes following producer's protocol, and amplicons were purified again with Agencourt AMPure XP kit. Libraries were then quantified through Qubit spectrophotometer (Thermo Fisher Scientific Inc., USA), normalized using nuclease-free water, pooled together and sequenced with the Illumina MiSeq NGS sequencer, using the MiSeq Reagent Kit v3 600-cycles chemistry following producer's protocol.

Data analysis

Data obtained from DNA targeted sequencing was analysed by a bioinformatics pipeline that included OBITOOLS[25] and QIIME [26], and microbial communities were characterized and compared as described in Supplementary Material S5.4, calculating the impact on the microbiota of plant genotype, aphid presence, aphid line and soil inoculum.

Results

Sequencing results

Considering the whole community, the output of this analysis produced a total of 5,154,181 sequences, which generated 16,584 OTUs at 97% of similarity after quality filtering, deletion of chimeric sequences and discarding singletons. The analysis of species accumulation curves indicated that the sequencing depth was enough to analyse most of the species diversity within all target organisms (bacteria, fungi and AM fungi) and samples (Supplementary Material S5.5).

Soil microbial community

Considering the whole microbial community (bacteria, fungi and AM fungi), results revealed a significant impact of soil inoculum (PseudoF=23.97, $P<0.01$) and plant species (PseudoF=18.10, $P<0.01$) on the soil microbiota (Fig. 2). At this level of detail, herbivory did not seem to affect microbial populations ($P>0.05$). Focusing the analysis on the soil bacterial community, it is possible to highlight a strong effect of soil inoculum (PseudoF=41.66, $P<0.01$) on the resulting microbial asset.

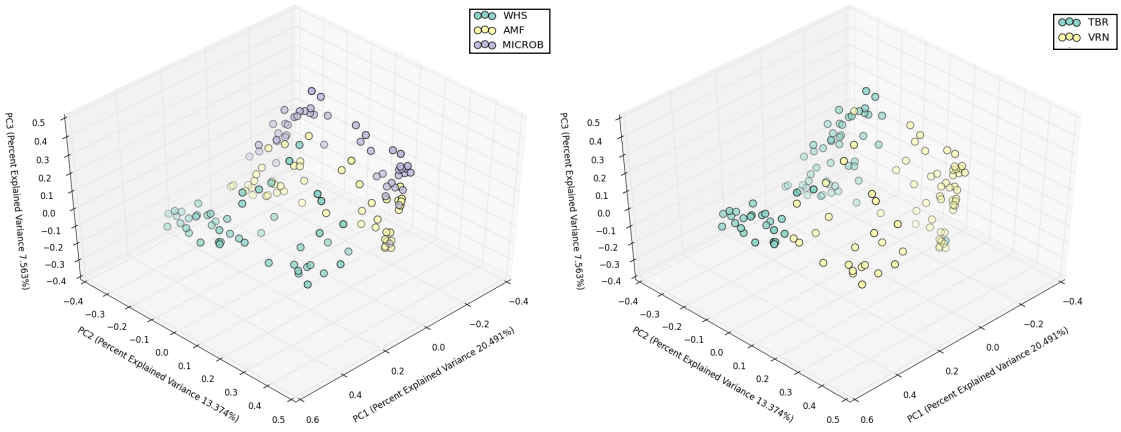

FIG. 2 PCOA PLOTS OF WHOLE SOIL MICROBIAL COMMUNITIES, COLOURED BY SOIL INOCULUM (LEFT) AND PLANT SPECIES (RIGHT). (TBR = *SOLANUM TUBEROSUM*; VRN = *SOLANUM VERNEI*; WHS = SOIL FROM FIELD; AMF = AM FUNGI SPORES ISOLATED AND INOCULATED; MICROB = ORGINAL BACTERIAL COMMUNITY).

On the contrary plant genotype and aphid presence did not have a significant effect on soil microbiota (P>0.05). The soil bacterial community was mainly represented by Proteobacteria (51.80±0.77%) and Bacteroidetes (12.99±0.51%) but Saprospirae (6.99±0.32%), Acidobacteria (7.23±0.55%) and Verrucomicrobiae (5.38±0.21%) were also well represented in these samples. Interestingly, compared to other samples, the pots inoculated with soil from the field (WHS) showed a higher abundance of Acidobacteria compared to the other groups ($F_{2, 87}$=215.43, P<0.001), as well as Pseudomonales ($F_{2, 87}$=51.63, P<0.001). On the other hand, the presence of Rhizobiales was higher in AMF and MICROB treatments ($F_{2, 87}$=50.09, P<0.001) and the amount Saprospirae was different among all three groups ($F_{2, 87}$=57.34, P<0.001).

The fungal community was shaped by both soil inoculum (PseudoF=17.74, P<0.01) and plant species (PseudoF=36.79, P<0.01). Aphid genotype and aphid presence had no effects on the composition of this community (P>0.05). In general terms it was composed by fungi belonging to the phyla Ascomycota (35.66±1.28%), Basidiomycota (0.75±0.08%), Zygomycota (4.76±0.37%), Chytridiomycota (0.41±0.06%), Rozellomycota (0.06±0.01%), and a large portion of unidentified taxa (52.59±1.44%). For WHS treatment, results highlighted a higher presence of Basidiomycota, Chytridiomycota and Rozellomycota compared to AMF and MICROB, while both showed a higher abundance of Ascomycota (Tukey's MCT P<0.05). Mycorrhizal community was also influenced by soil (PseudoF=33.08, P<0.01) and plant species (PseudoF=5.26, P<0.01).

Roots microbial community

Roots microbial community was influenced by soil inoculum (PseudoF=24.33, P<0.001) and plant species (PseudoF=28.20, P<0.001) (Fig. 3), however aphid clone and aphid presence herbivory did not seem to have a significant effect (P>0.05).

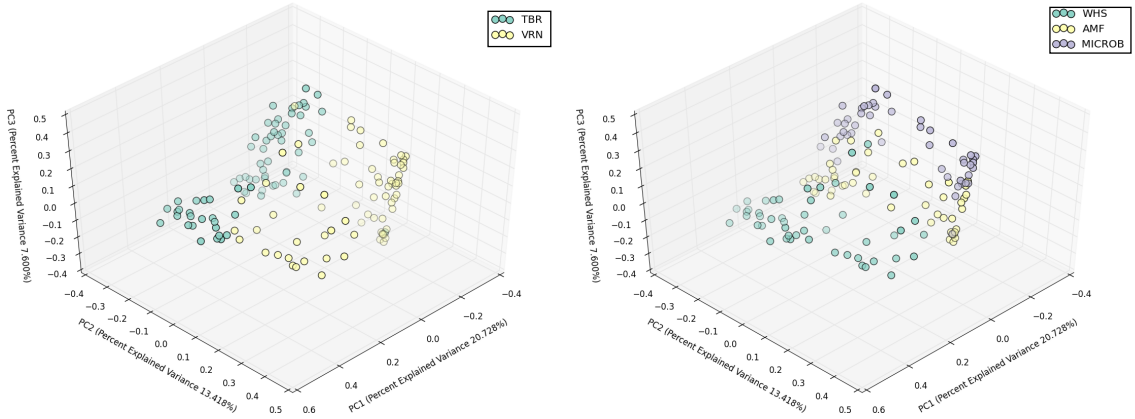

FIG. 3 PCOA PLOTS OF WHOLE SOIL MICROBIAL COMMUNITY, COLOURED BY PLANT SPECIES (LEFT) AND SOIL INOCULUM (RIGHT). (TBR = *SOLANUM TUBEROSUM*; VRN = *SOLANUM VERNEI*; WHS = SOIL FROM FIELD; AMF = AM FUNGI SPORES ISOLATED AND INOCULATED; MICROB = ORGINAL BACTERIAL COMMUNITY).

The microbiota of roots was dominated by Bacteroidetes (82.27±0.82%) which were mainly represented by Flavobacteriales (32.44±0.082%), Saprospirales (26.95±0.55%), Cytophagales (15.48±0.63%), Sphingobacteriales (7.2±0.25%), and Actinomycetales (4.15±0.19%). Multivariate analysis highlighted differences in the composition of this community according to soil inoculum (PseudoF=25.88, *P*<0.001) and plant genotype (PseudoF=2.21, *P*=0.03). Aphid clone and aphid presence had no a significant effect (*P*>0.05).

The fungal community, among the factor analysed, was influenced only by the plant species (PseudoF=241.78, *P*<0.001). On the other hand, the community of mycorrhizal fungi was shaped by both soil inoculum (PseudoF=39.10, *P*<0.001) and plant species (PseudoF=8.39, *P*<0.001).

Leaves microbial community

Differently from the other analysed compartments, leaves microbial communities were differentiated according to plant species (PseudoF=12.77, *P*<0.01 – Fig. 4), aphid presence (PseudoF=2.99, *P*=0.004) and aphid genotype (PseudoF=12.77, *P*<0.01). Soil inoculum did not influence this microbial community (*P*>0.05).

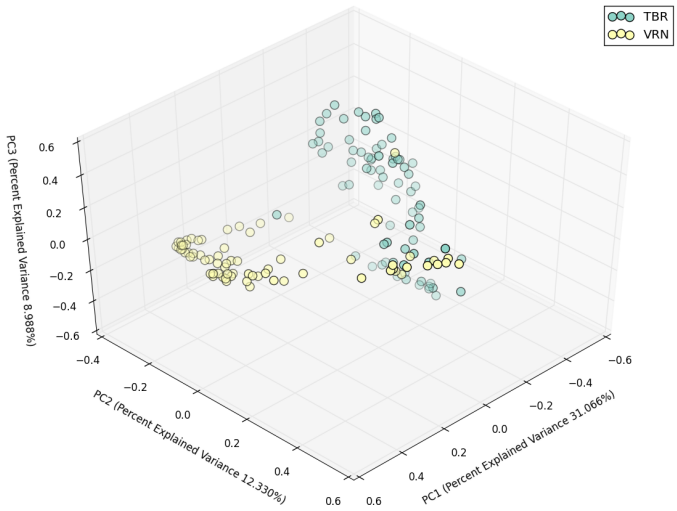

FIG. 4 PCOA PLOTS OF THE WHOLE LEAVES MICROBIAL COMMUNITY, COLOURED BY PLANT SPECIES (TBR = *SOLANUM TUBEROSUM* – VRN = *SOLANUM VERNEI*).

The leaves bacterial community was dominated by Proteobacteria (61.31±2.27%) and in particular by α-Proteobacteria (30.67±2.68%) and γ-Proteobacteria (29.21±2.56%). The main members to this group, retrieved in our analysis, were assigned to Rickettsiales (29.67±2.69%), *Hamiltonella* (10.9±1.99%) and *Stenotrophomonas* (13.29±2.01%).

Interestingly, their abundance pattern changes in response of the aphid clone (PseudoF=7.26, *P*<0.01), and specifically *Hamiltonella* is present only on plants with

aphid AK13/18 feeding on them ($F_{4, 145}$=67.97, *P*<0.001) while Rickettsiales were more abundant on plants reared without aphids ($F_{4, 145}$=9.83, *P*<0.001), less on plants with aphids AK13/18 (Tukey's MCT *P*<0.05). Furthermore, the relative abundance of *Stenotrophomonas* was different among plants ($F_{4, 145}$=3.9, *P*=0.005), with higher presence on plants infested with AK13/08 compared to uninfested plants (Tukey's MCT *P*<0.05). Plant genotype affected the bacterial community (PseudoF=2.86, *P*=0.01), while soil inoculum had no effects (*P*>0.05). Also the plant species significantly affected leaves fungal community (PseudoF=171.54, *P*<0.01).

Aphid microbial community

In general, Aphids microbiota was dominated by Proteobacteria (84.59±1.45%), followed by Bacteroidetes (6.24±0.97%) and Firmicutes (3.98±0.56%). Within Proteobacteria, γ-Proteobacteria represented the widest part of the whole microbiota (81.26±1.79%) together with α-Proteobacteria (2.31±0.32%). Deepening the taxonomic analysis, the genus *Buchnera* accounted for the higher part of sequences (59.44±3.06%) together with *Hamiltonella* (20.23±3.3% - retrieved in all AK13/18 clones). Among the others, it is interesting the presence of Rickettsiales (1.38±0.21%) and bacteria belonging the genus *Pseudomonas* (0.46±0.09%).

Giving a look to the entire community (Fig. 5), it clearly clusterize by Aphid genotype (PseudoF=22.18, *P*<0.001), since AK13/18 hosted the facultative endosymbiont *H. defensa*. Interesting, soil inoculum showed a slight effect on the composition of aphid microbial community (PseudoF=3.06, *P*=0.04). Plant genotype had no a significant effect (*P*>0.05).

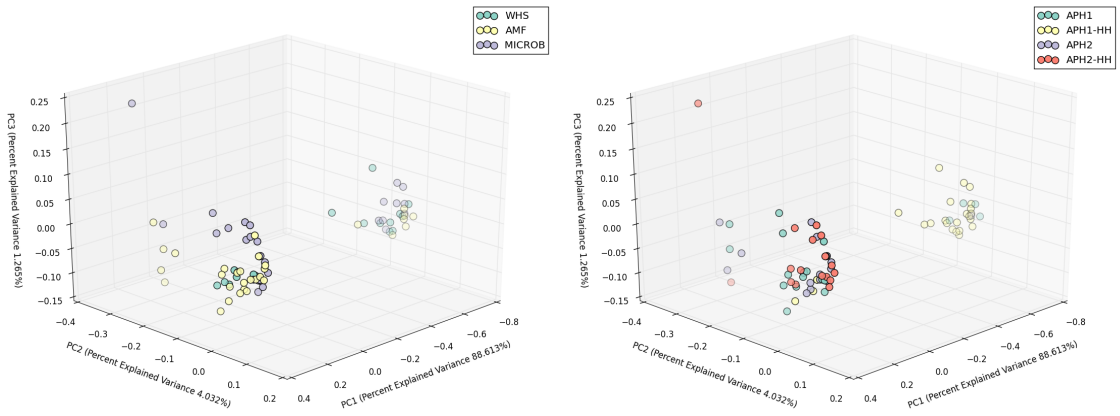

FIG. 5 PCOA PLOT OF APHIDS BACTERIAL COMMUNITY COLOURED BY (LEFT) SOIL INOCULUM AND (RIGHT) APHID GENOTYPE. (WHS = SOIL FROM FIELD; AMF = AM FUNGI SPORES ISOLATED AND INOCULATED; MICROB = ORGINAL BACTERIAL COMMUNITY)

Discussion and conclusions

In this work we used a microcosm system, coupled with a metabarcoding approach, to investigate how the microbial community of different compartments can respond to aphids, plant species and soil inoculum.

The novel outcome of this study regards the analysis of effects of soil microbial communities on aphid microbiota. The effects of belowground communities on the aboveground biota are widely studied all over the world [27-29]. Previous studies demonstrated that belowground microbial communities could affect plant growth and quality, with effects also on the aboveground herbivores and other organisms [30-32]. It has been also observed that the composition of belowground microbial communities can influence herbivore fitness [33]. The effect observed in our study could be related to the presence of AM fungi that, interacting with the plant, can influence aphid fitness [34, 35]. Therefore, soil microbial community, through these effects on the plant, can have an impact on aboveground community [10]. In a more holistic vision, there is evidence that soil microbiota can influence the entire relationship between plant and insects, and within insects, between the herbivores and their parasitoids [36].

Besides this unexpected effect, and no modulation by plant genotype, aphid microbiota showed differences across the clonal lines used in this study. Generally, insects harbouring different symbiont bacteria, can improve their performance in exploiting the host plant. However, currently we have limited knowledge of how these symbionts can modulate the interactions between above- and belowground community, through the different trophic levels. In our study, the clone AK13/18 demonstrated to host the facultative endosymbiont *Hamiltonella defensa*. This is a defensive bacterium, sporadically diffused among different families of sap-feeding insects, known to be able to protect pea aphids from the parasitoid *Aphidius ervi*, blocking its larval development (reviewed in [37]). Unlike precedent studies [38], no effect of *H. defensa* were highlighted on plants and on microbiome. Therefore, further studies could focus on this aspect in the future, helping to figure out which are the effects of this bacterium on plants and on belowground community. Among the abundant taxa we identified sequences of *Buchnera*, an obligate nutritional symbiont occurring in several species of aphids which provide essential nutrients to its host [39], and Rickettsiales which role is still matter of discussion [40].

Analysing the belowground community, in our study both plant species and soil inoculum shaped rhizosphere microbiota. To our knowledge, few studies covered this aspect using HTS technologies, and this is the first one in which three different markers, covering three microbial targets, are used together. The effects of soil inoculum and plant species on resulting soil microbiota were quite expected. Indeed, it is widely known that both soil history and plant species can structure the soil microbiota [41-43]. Peiffer et al. [44] analysed the bacterial community of maize rhizosphere through 16S-

targeted pyrosequencing, reporting genotype effects on the respective microbiota. Different plant species impact differently on soil, modifying pH, C and N levels, and releasing different assets of exudates [45, 46]. How this interaction is phylogenetically related among plants is still matter of discussion [47, 48], although there is evidence that in particular the fungal fraction of soil microbiota is more strongly related to plant species [49]. Indeed, in our study we observed that even though the whole microbial community is affected by plant species, this effect is due to the interaction with the fungal community of soil. These effects, as reviewed by Philippot et al. [4], can be the result of a cascade of events since the geographical area determines the indigenous microbial community, the soil characteristics influence the structure of this biota, and the plant genotype selects the inhabitants of the rhizosphere.

Results indicated that the dominant bacterial taxa in the rhizosphere were Proteobacteria. These are reported as the most abundant bacterial group in the rhizosphere, because of their responsiveness to different carbon sources [50, 51]. Furthermore, we observed a higher abundance of Acidobacteria and Pseudomonales in pots inoculated with field soil compared to the others that, in turn, selected for a higher abundance of Rhizobiales. Acidobacteria are also known as dominant taxa in soil microbiota, and since they are unculturable like most soil microorganisms, their ecological role is still unclear [52]. On the other hand, Pseudomonales and, in particular, various species of the genus *Pseudomonas* are reported to provide plant-beneficial effects and to be part of a group of so-called Plant-Growth Promoting Rhizobacteria [53], as well as Rhizobiales can do [54]. The fungal community was represented by taxa belonging to Ascomycota, Basidiomycota, Chytridiomycota and Rozellomycota, already known components of soil microbial communities [55, 56]. Previous studies dealing on the impact of insect herbivory on soil microbes are limited, and this relationship has been previously highlighted, using pyrosequecing, on *Bemisia tabaci* feeding on pepper plants [57]. This different feedback on rhizosphere microbial community by aphid infestation, compared to the study on *B. tabaci*, could be indicative of a different response of potato plants compared to peppers, or a different effects of aphids compared whiteflies. We are still in the beginning phase to understand these relationships, therefore further studies are needed to shed light on this interesting effects.

Moving to the plant side, we analysed both above- and belowground compartments. As reported for the rhizosphere, the roots microbiome was mainly shaped by soil inoculum and plant genotype. However, in this compartment, the bacterial community was mainly composed by Bacteroidetes. This is consistent with the finding that Hacquard et al. [58] observed analysing the root bacterial community of different plant species. On the other hand, leaves microbiota was both shaped by plant genotype and aphid clone. In particular, plant infested by AK13/18 aphids (hosting *H.*

defensa) bear a distinct bacterial community, while the fungal one was not involved in this interaction.

Summarizing, we found that plant genotype influenced most of the microbial communities in our system, apart of soil bacterial biota. Furthermore, soil inoculum shaped the resulting microbiota of the belowground compartment, and unexpectedly also the microorganisms associated to aphids. Insects influenced also the microbial community inhabiting leaves. Our results, together with other few studies, represent the first steps leading to a more comprehensive understanding of plant-microbe-insect interactions, and more widely to above- belowground interactions. Unfortunately, these effects remain poorly understood and few studies dealt with them from a microbial ecology point of view. However, technologies like HTS are becoming more widely available, and in the near future likely we will able to have a more comprehensive vision of the effects of soil community on plant and insects, and vice versa. The implications of these interactions can be extended beyond the pure ecological meaning, with a great potential to be applied on field, and helping to shape the future generation of pest management strategies.

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Chapter 6

Conclusions

In the present PhD thesis, we investigated interactions between microrganisms and insects taking advantage of advanced High Throughput Sequencing (HTS) technologies to perform metabarcoding analyses. Our researches provided relevant answers to numerous ecological questions, and new important results of basic and applied interest. Since the field of insect/microorganism interactions is huge and hardly unexplored we focused our attention on some specific topics considered of relevant interest.

Firstly, we analysed the composition of the fungal communities associated to the olive fruit fly, *Bactrocera oleae* using two different approaches based on cloning/Sanger sequencing and on 454 pyrosequencing technology, respectively (Chapters 2 and 3). The two methods provided similar results but the use the high-throughput culture-independent sequencing approach provided a much high number of reads and enabled the analysis of the whole fungal diversity. Overall, results of both investigations opened a window on the fungal community associated with *B. oleae* and more generally to insects, highlighting the need to further investigate the ecological role of identified taxa. Some of them seemed to have a clear interaction with the fly, opening therefore new ways for the definition of the ecological role of the olive fruit fly, not only as direct source of damage, but also as a major component of olive agroecosystem and as a vector of plant pathogenic fungi. The knowledge of the composition and the dynamics of those fungal communities, could be pivotal in shaping the future generation of pest management and control strategies.

Another study (Chapter 4) touched another problem: can international harbours be point of entry of known and unknown plant pathogens through the introduction of insects? We used metabarcoding to survey the microbial community of introduced bark and ambrosia beetles from three Italian international harbours. We got novel information on the fungal community of *Orthotomicus erosus* and *Xyleborinus saxesenii*, two Scolytinae widely trapped at international harbours. We suggested new associations with fungi that could represent agents of plant diseases, including species of the genera *Ophiostoma*, *Acremonium*, and *Fusarium*. We retrieved a high number of sequences associated to unknown or unculturable fungi, which could be new symbionts or new pathogens, representing therefore potential new threats for plant health. These beetles might represent a sample of 'in-out travelling' populations, which are likely involved in horizontal transfer of fungal spores. They may adapt to new conditions and gain new fungal associations, which frequently lead to high environmental and economic losses. Given the threat posed by these bark and ambrosia beetles to forest, urban, and agricultural ecosystems, this study opens a new scenario that looks at these

beetles not only as direct source of damage, but also as potential carriers of novel and unknown plants pathogens, suggesting that the surveillance at harbours should be extended also to fungi associated to wood-boring beetles.

Finally, we focused on the fascinating field of tritrophic interactions by studying the mutual interaction between insect microbiota and soil microbial communities in a microcosmos system (Chapter 5). We found that the plant genotype can influence most of the microbial communities in the system, apart of soil bacteria. Furthermore, soil microrganisms and, in particular, mycorrhizal fungi, shaped the resulting microbiota of the belowground compartment, and unexpectedly also the microorganisms associated to aphids. Insects influenced also the microbial community inhabiting leaves. The implications of these interactions can be extended beyond the pure ecological meaning, with a great potential to be applied on field, and helping to shape the future generation of pest management strategies.

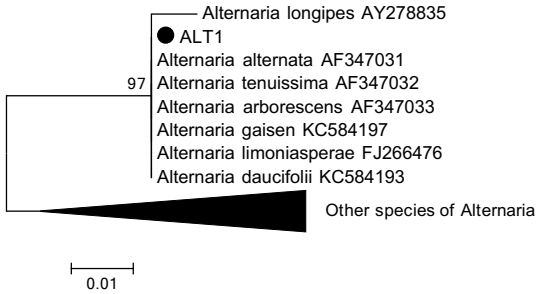
Concluding, this work provides a wide overview on the importance of the interactions between insects and microrganisms, particularly fungi, in a various set of contexts, highlighting that further studies in this direction could provide novel and unexpected answers to this ecological topic still poorly understood.

Supplementary material

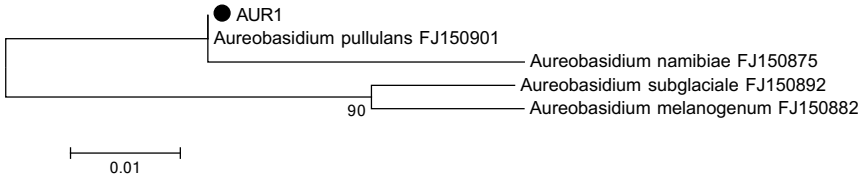
Supplementary material Chapter 3

FIGURE S1. PHYLOGENETIC TREES BUILT USING FUNGAL ITS2 SEQUENCES RETRIEVED FROM *BACTROCERA OLEAE* (●) AND REFERENCE SEQUENCES OF THE GENERA *ALTERNARIA* (WOUDENBERG *ET AL.* 2013), *AUREOBASIDIUM* (ZALAR *ET AL.* 2008), *BOTRYTIS* (STAATS *ET AL.* 2005), *CLADOSPORIUM* (BENSCH *ET AL.* 2012), *COLLETOTRICHUM* (DAMM ET AL. 2012), *DEVRIESIA* (LI *ET AL.* 2013), *LEPTOSPHAERULINA* (AVESKAMP ET AL, 2010), *AND PSEUDOCERCOSPORA* (CROUS ET AL, 2013).

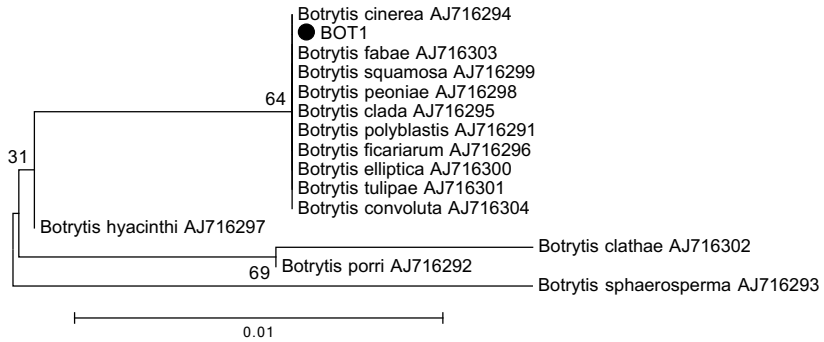
Alternaria



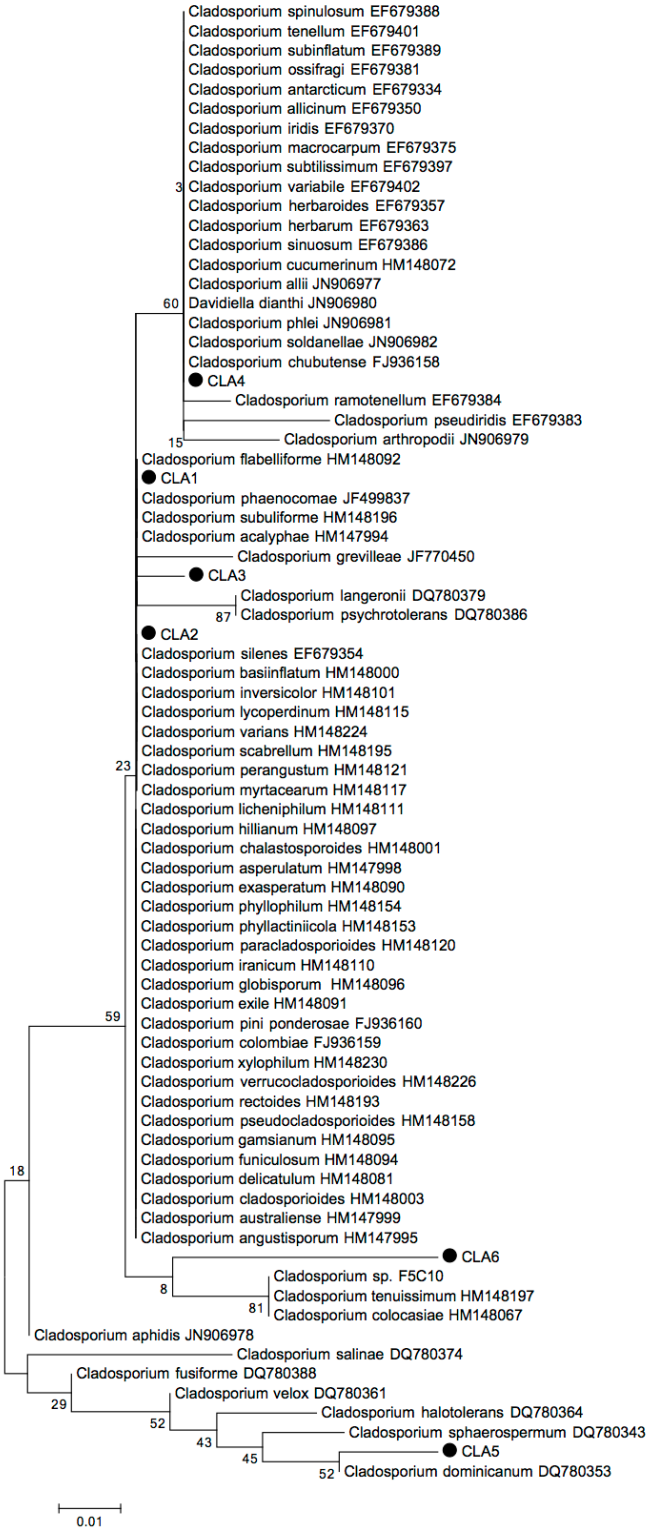
Aureobasidium



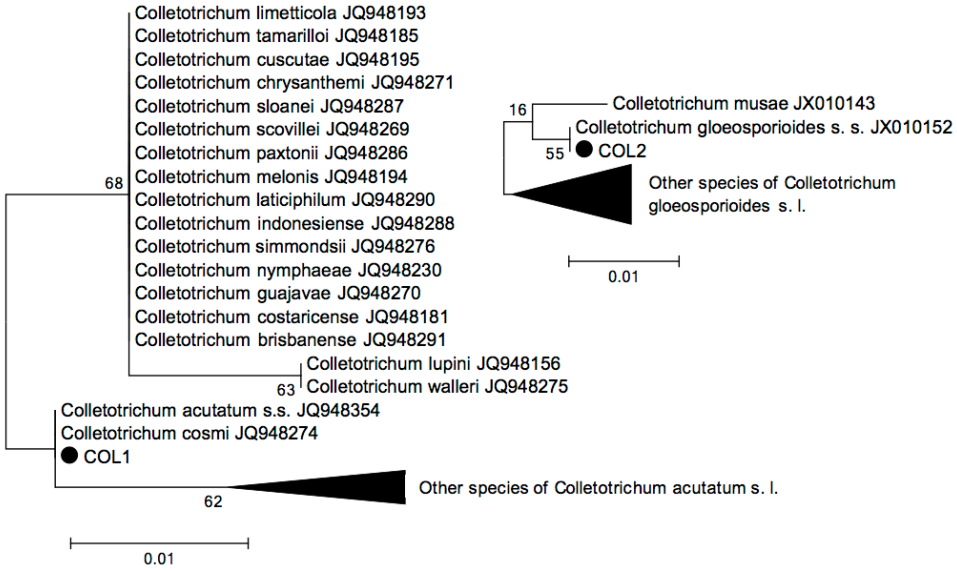
Botrytis



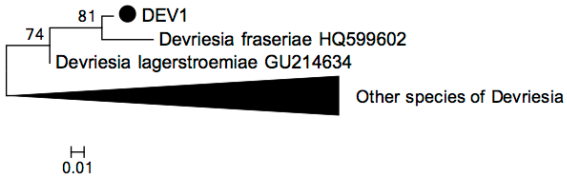
Cladosporium



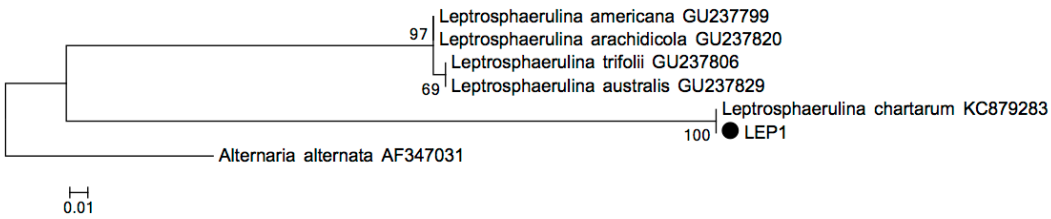
Colletotrichum



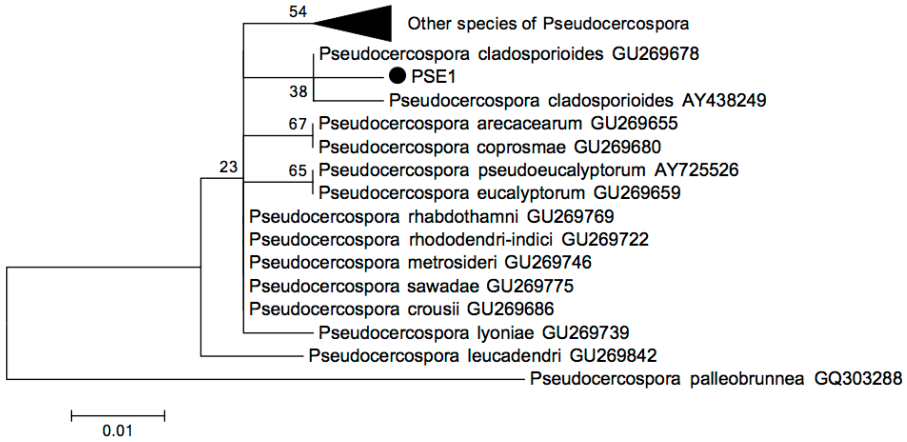
Devriesia



Leptosphaerulina



Pseudocercospora



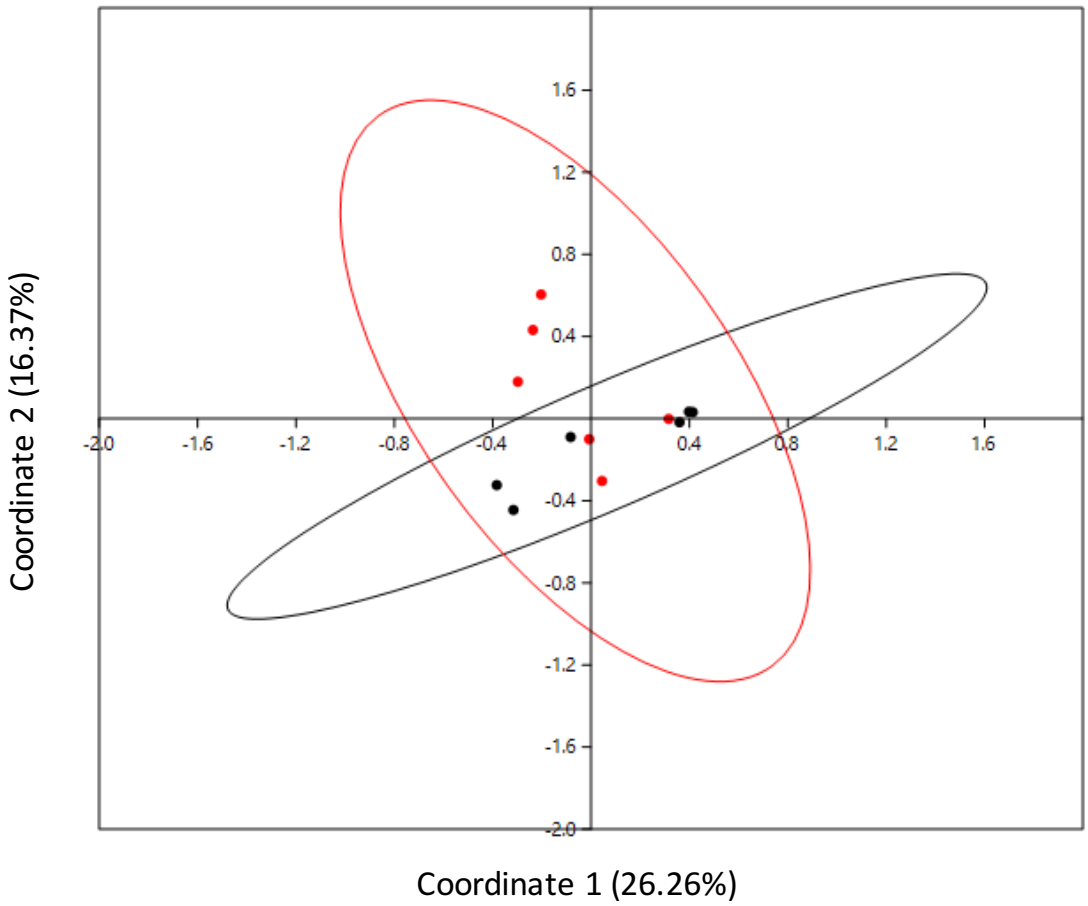


FIGURE S2. PRINCIPAL COORDINATES ANALYSIS (PCOA) RESULTS OF FUNGAL COMMUNITY DIVERSITY ANALYSIS OF MALE (BLACK) AND FEMALE S (RED) SPECIMENS OF *BACTROCERA OLEAE*.

TAB. S1 – FUNGAL TAXA ASSOCIATED TO SATELLITE OTUS DETECTED IN THE PRESENT STUDY AND CORRESPONDING RELATIVE ABUNDANCE (%±SE) AND NUMBER OF OTUS DETECTED.

Taxa	Relative abundance (%±SE)			Number of OTUs
	MALES	FEMALES	TOTAL	
Hannaella oryzae	6.19±4.44	0±0	3.48±3.36	1
Uncultured fungus	4.14±1.72	1.62±0.87	3.04±1.45	45
Penicillium sp.	1.17±0.47	0.12±0.08	0.71±0.39	3
Talaromyces funiculosus	0.51±0.32	0.05±0.06	0.31±0.25	1
Geosmithia sp.	0.44±0.25	0.12±0.04	0.3±0.18	2
Alternaria sp.	0.18±0.08	0.28±0.21	0.23±0.16	5
Fusarium sp.	0.14±0.13	0.27±0.29	0.19±0.23	3
Gibellulopsis nigrescens	0.33±0.23	0±0	0.19±0.18	1
Candida sp.	0.31±0.09	0.02±0.02	0.18±0.08	1
Cladosporium sp.	0.13±0.13	0.06±0.02	0.1±0.1	3
Nigrospora sp.	0.13±0.11	0.01±0	0.08±0.08	1
Aspergillus sp.	0.13±0.07	0.02±0.01	0.08±0.05	1
Neofusicoccum sp.	0.14±0.08	0±0	0.08±0.06	1
Clonostachys rosea	0.13±0.1	0±0	0.07±0.08	1
Podosphaera xanthii	0.13±0.1	0±0	0.07±0.07	1
Schizophyllum sp.	0.12±0.13	0±0	0.07±0.1	1
Peniophora sp.	0.08±0.04	0.01±0.01	0.05±0.03	1
Trichomonascus ciferrii	0.08±0.04	0.01±0	0.04±0.03	1
Capnobotryella sp.	0.07±0.08	0±0	0.04±0.06	1
Blumeria graminis	0.06±0.09	0±0	0.04±0.07	1
Dioszegia sp.	0.01±0.01	0.06±0.05	0.03±0.04	1
Devriesia sp.	0.05±0.03	0.01±0	0.03±0.02	1
Lyomyces sp.	0.02±0.02	0.04±0.05	0.03±0.04	1
Colletotrichum sp.	0.05±0.04	0±0	0.03±0.03	1
Cheiromoniliophora elegans	0.03±0.04	0.02±0.02	0.03±0.03	1
Thecaphora thlaspeos	0.04±0.02	0.01±0	0.02±0.01	1
Acremonium sp.	0.04±0.04	0±0	0.02±0.03	1
Aureobasidium sp.	0.004±0.003	0.04±0.04	0.02±0.03	1
Lecanicillium sp.	0.01±0.01	0.04±0.04	0.02±0.03	1
Leptoxyphium kurandae	0.03±0.03	0.01±0.01	0.02±0.03	1

Taxa	Relative abundance (%±SE)			Number of OTUs
	MALES	FEMALES	TOTAL	
Macrophoma sp.	0.04±0.05	0±0	0.02±0.04	1
Mucor sp.	0.02±0.02	0.02±0.01	0.02±0.02	1
Bipolaris sp.	0.01±0.01	0.03±0.02	0.02±0.02	1
Sporobolomyces odoratus	0.03±0.02	0±0	0.02±0.02	1
Caratobasidium sp.	0±0	0.04±0.06	0.02±0.04	1
Fusicladium sp.	0.02±0.01	0.02±0.01	0.02±0.01	1
Toxicocladosporium sp.	0.02±0.01	0.02±0.01	0.02±0.01	1
Phaeosphaeriopsis sp.	0.03±0.02	0±0	0.01±0.01	1
Ampelomyces sp.	0.02±0.02	0±0	0.01±0.01	1
Diaporthe ambigua	0.02±0.02	0±0	0.01±0.01	1
Emericellopsis terricola	0.02±0.03	0±0	0.01±0.02	1
Vuilleminia coryli	0.02±0.02	0±0	0.01±0.01	1
Xylaria palmicola	0±0	0.03±0.02	0.01±0.01	1
Rhizopus oryzae	0.01±0.01	0±0	0.01±0.01	1

Supplementary material
Chapter 4

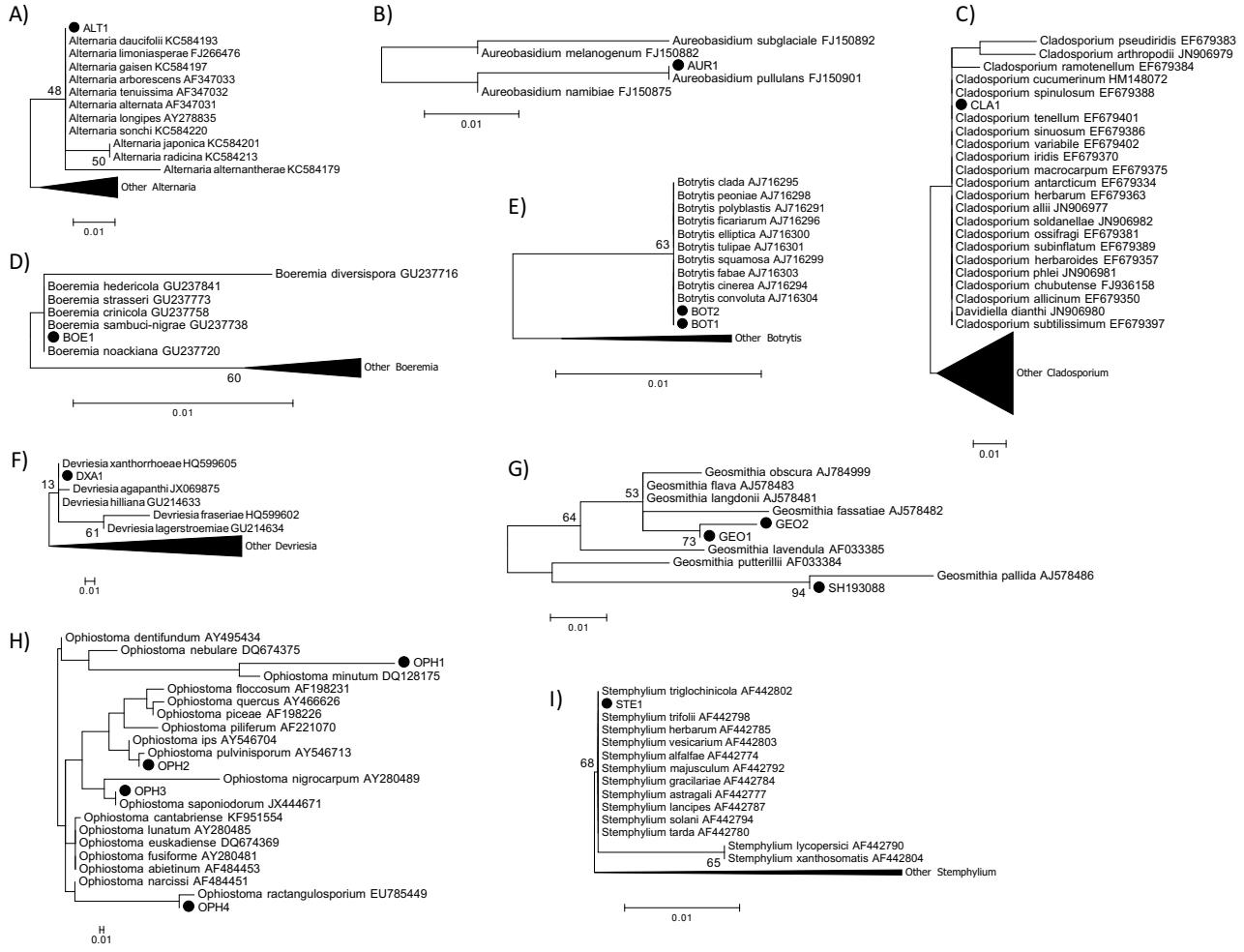


FIGURE S1. PHYLOGENETIC IDENTIFICATION OF DETECTED SEQUENCE TYPES (STS). TREES WERE BUILT USING UNIQUE SEQUENCES REPRESENTATIVE OF STS OF THE MOST RELEVANT FUNGAL GENERA DETECTED IN THE PRESENT STUDY AND ITS2 SEQUENCES RETRIEVED FROM *ORTHOTOMICUS EROSUS* AND *XYLEBORINUS SAXESENII* (●) ALONG WITH VALIDATED REFERENCE SEQUENCES FROM *ALTERNARIA* SPP. (A – WOUDENBERG *ET AL.* 2013), *AUREOBASIDIUM* SPP. (B – ZALAR *ET AL.* 2008), *BOEREMIA* SPP. (C – AVESKAMP *ET AL.* 2010), *BOTRYTIS* SPP. (D – STAATS *ET AL.* 2005), *CLADOSPORIUM* SPP. (E – BENSCH *ET AL.* 2012), *DEVRIESIA* SPP. (F – LI *ET AL.* 2013), *GEOSMITIA* SPP. (G – KOLAŘÍK *ET AL.* 2008), *OPHIOSTOMA* SPP. (H – ZHOU *ET AL.* 2006; LEE *ET AL.* 2008; LU *ET AL.* 2009; JANKOWIAK *ET AL.* 2013; ROMON *ET AL.* 2014A; ROMON *ET AL.* 2014B), AND *STEMPHYLIUM* (I – CÂMARA & O'NEILL 2002). NUMBERS ON NODES REPRESENT THE POSTERIOR PROBABILITIES FOR THE RANDOMIZED AXELERATED MAXIMUM LIKELIHOOD (RAXML) METHOD.

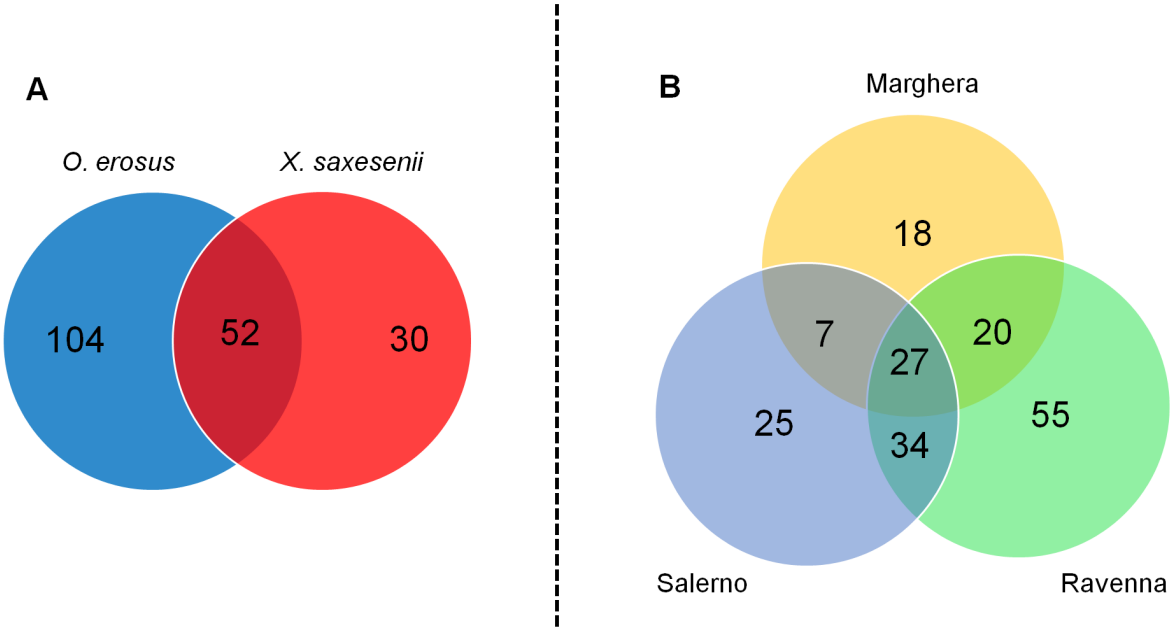


FIGURE S2. VENN DIAGRAMS SHOWING THE SHARED OTUS (A) BETWEEN THE TWO BEETLE SPECIES AND (B) AMONG THE SAMPLING SITES.

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Supplementary material Chapter 5

S5.1 – Soil preparation

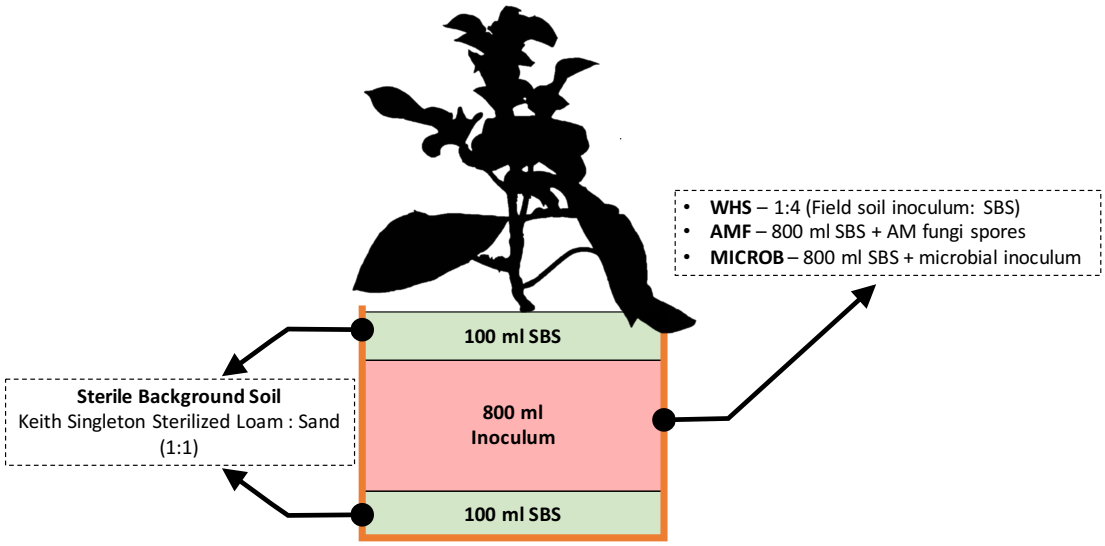


FIG S1. SOIL SETUP

S5.2 – DNA extraction procedure

Reagents

1. Breaking buffer (50mM NaCl, 25mM Tris-HCl, 10mM EDTA, 1% v/v SDS)
2. Phenol:Chloroform:Isoamil alcohol mixture 25:24:1
3. Chloroform:isoamil alcohol mixture 24:1
4. Proteinase K solution (1 mg/ml)
5. Lysozyme solution (50 mg/ml in KH_2PO_4)
6. Sodium acetate 0.3 M
7. Bleach 5%
8. Ultrapure H_2O
9. Ethanol 100%
10. Ethanol 70%

Procedure

1. Add the matrix to be extracted inside a 2 ml Eppendorf tube
2. *In case of insects* – sterilize insect body surface with **1 ml of 5% Bleach** for 3 minutes, then rinse them once with **1 ml of Ultrapure H_2O** and once with **1 ml of Breaking buffer**.
3. Add 2 sterilized stainless steel beads (∅ 2 mm) to each tube
4. Disrupt tissues with a bead mill homogenizer (e.g. QIAGEN TissueLyser II) at maximum speed for 5 minutes.
5. Add **300 µl of Breaking buffer** to each tube
6. Mill the matrix again with the bead mill homogenizer at maximum speed for 5 minutes.
7. Centrifuge briefly (≈5 sec.) at 10,000 x *g*
8. Add **50 µl of Proteinase K solution** to each tube
9. Incubate samples at 40°C for 30 minutes
10. Add **50 µl of Lysozime solution** to each tube
11. Incubate samples at 50°C for 30 minutes
12. Add **500 µl of Phenol solution** to each tube
13. Vortex each sample (≈10 sec.)
14. Incubate samples at room temperature for 5 minutes
15. Add **500 µl of Chloroform:Isoamil alcohol solution** to each tube
16. Vortex each sample (≈10 sec.)
17. Incubate samples at room temperature for 3 minutes
18. Centrifuge at 10,000 x *g* for 15 minutes at 4°C
19. Extract surnatant and transfer it to a new tube
20. Add **100% Ethanol as 2.5X the extracted volume** to each tube
21. Add **Sodium acetate solution as 1/10 the extracted volume** to each tube
22. Mix gently by inverting tubes
23. Incubate samples on ice for 60 minutes
24. Centrifuge at 10,000 x *g* for 30 minutes at 4°C
25. Discard ethanol from each tube
26. Add **500 µl of Ethanol 70%** to each tube
27. Mix gently by inverting tubes

28. Centrifuge at 10,000 x *g* for 15 minutes at 4°C
29. Incubate samples at room temperature for 15 minutes
30. Centrifuge at 10,000 x *g* for 5 minutes at 4°C
31. Discard ethanol from each tube
32. Dry out pellet by means of a vacuum evaporator or leaving tubes open overnight and covered with paper towel
33. Add **200 µl of Ultrapure water** to each tube
34. Measure DNA concentration and purity
35. Proceed with DNA purification if needed

S5.3 – Multiplexing strategy

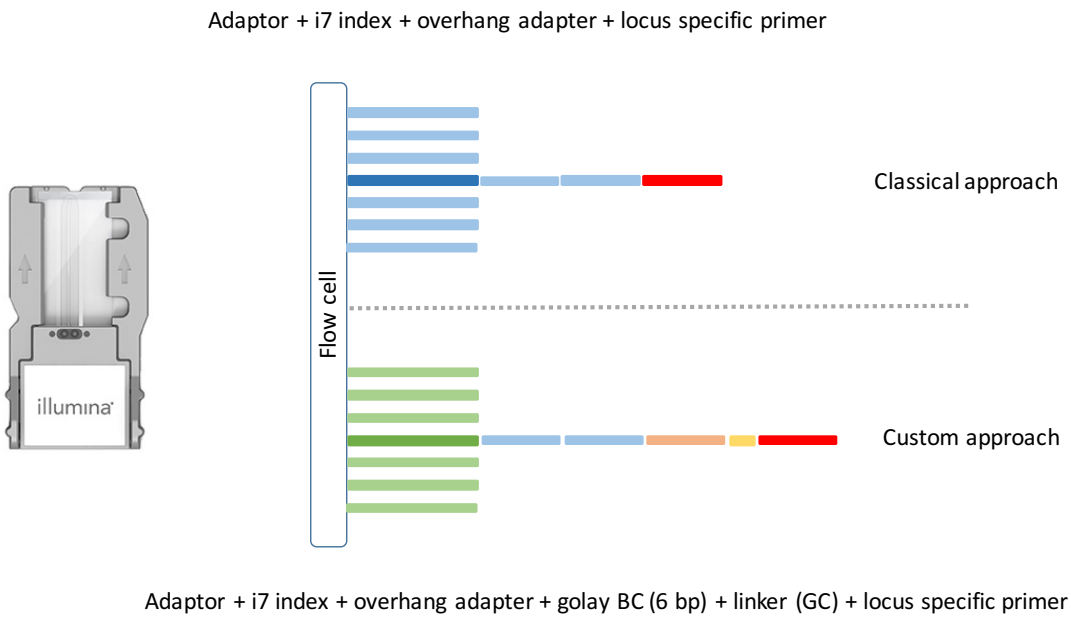


FIG S2. MULTIPLEXING STRATEGY DEVELOPED FOR THIS STUDY. BASICALLY AN ADDITIONAL GOLAY BARCODE (6BP) WAS INCLUDED INSIDE THE FORWARD PRIMER USED IN THE FIRST PCR RUN, BETWEEN THE OVERHANG ADAPTER PORTION AND THE LOCUS SPECIFIC PRIMER.

S5.4 – Bioinformatic analysis

PEAR

1. `PEAR` (Paired-End reAd merger) with default settings was used to merge forward and reverse reads from MiSeq run.

OBITOOLS

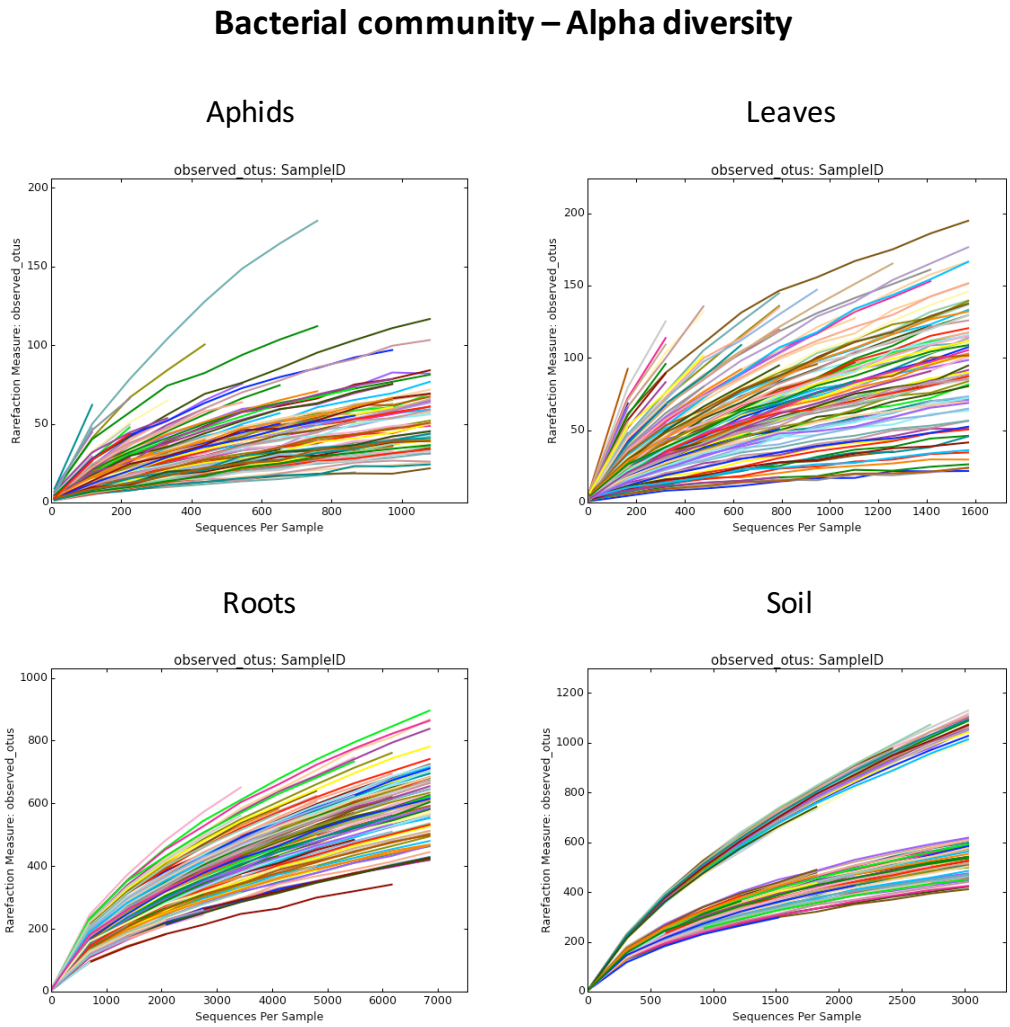
2. This software was used to demultiplex reads, on the basis of the supplementary barcode integrated with the first PCR run, through the scripts `ngsfilter` and `obisplit`.

QIIME

3. QIIME was used to carry out the downstream analyses, using the following integrated scripts
4. `multiple_split_libraries_fastq.py` in order to filter by quality the demultiplexed files. Sequences with a Q score < 20 on a sliding windows of 50bp and those < 150bp were discarded.
5. `identify_chimeric_seqs.py` using USEARCH6.1 algorithm to delete chimeric sequences
6. ITS sequences were further processed with `ITSx` to isolate the ITS2 region from reads and to discard non-fungal sequences
7. `parallel_pick_otus_usearch61_ref.py` to clusterize sequences using the USEARCH6.1 algorithm using 97% of similarity threshold
8. `pick_rep_set.py` to pick a representative set of sequences to perform taxonomy assignment using the `most_abundant` method
9. `parallel_assign_taxonomy_blast.py` to perform the taxonomy assignment using the BLAST algorithm using default settings and the databases greengenes, UNITE and SILVA respectively for bacteria, fungi and mycorrhizal communities.
10. `make_otu_table.py` to build the OTU table on the basis of the previous outputs
11. `filter_otus_from_otu_table.py` to discard singletons and OTUs retrieved in less than 5 samples.
12. Data analysis steps, through the following scripts:
    a. `alpha_rarefaction.py`
    b. `jackknifed_beta_diversity.py` using an even depth of 1000 sequences, the PCoA method of multivariate analysis on a bray-curtis distance matrix.
    c. `compare_categories.py` using PERMANOVA method
    d. `summarize_taxa.py`

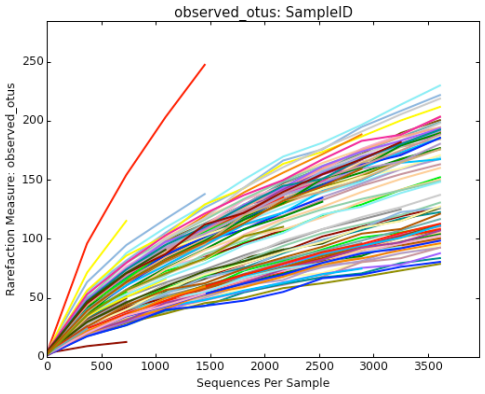
S5.5 – Species accumulation curves

FIG S2. SPECIES ACCUMULATION CURVES FOR THE THREE MICROBIAL COMMUNITIES SURVEYED.

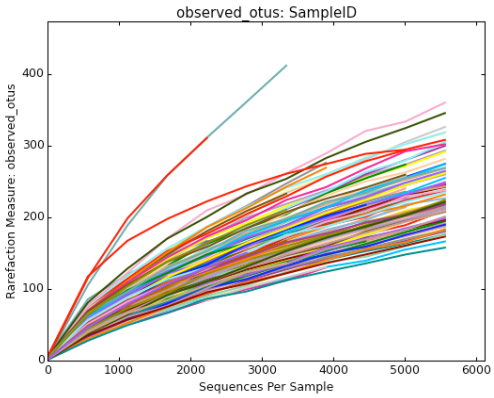


Fungal community – Alpha diversity

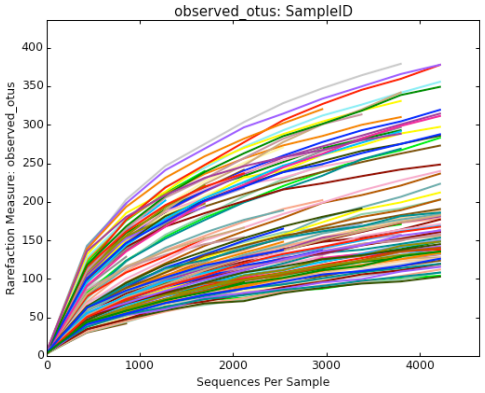
Leaves



Roots

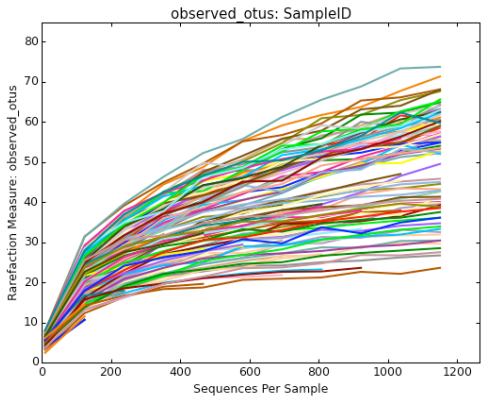


Soil



Mycorrhizal community – Alpha diversity

Roots



Soil