A Metabarcoding Survey on the Fungal Microbiota Associated to the Olive Fruit Fly

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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 strongly affect quality and quantity of olives and oil [8, 9]. 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 [10-14]. Moreover, serious olive diseases can be caused by fungal plant

pathogens including *Spilocaea oleagina*, *Colletotrichum* spp. and *Pseudocercospora* spp. [15].

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 [16]. 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. [17]. Furthermore, relevant fungal pathogens including *Colletotrichum* spp. and *Pseucercospora cladosporioides* were also detected [16, 18, 19]. 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 [20-22]. 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 [16]. 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 [23, 24]. 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 [25, 26]. 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 [22, 27, 28]. 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 [22, 28].

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 [29]. 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 [25]. PCR reactions (total volume of 25 µl) were conducted using 1 µl of extracted DNA (≈50 µg), 1X Taq

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

144 ***HTS data processing***

145 Raw sequencing data were processed using QIIME 1.8.0 [32], setting the minimum
146 quality score to 25. Furthermore, mismatches in the primer sequence were not allowed
147 and sequences <150 bp, >1000bp, containing homopolymers >10 bp and with >6

148 ambiguous bases, were discarded. Reads were denoised using denoise wrapper [33],
149 and chimeric sequences were identified using USEARCH 6.1 algorithm [34] combining a
150 reference-based with a de novo detection. ITSx was used to extract ITS2 sequences [35].
151 Reads were then clustered into Operational Taxonomic Units (OTUs) using the BLAST
152 method, with 0.99 similarity threshold to the UNITE dynamic reference database [23]
153 accessed on January 2016 (http://unite.ut.ee/). When reads failed to hit to reference
154 database, sequences were clustered as de novo. Singletons were discarded from
155 analyses. The UNITE database was employed, using BLAST algorithm, for the taxonomic
156 identifications of OTU representative sequences.

157 The method described by Magurran and Anderson [36] was applied to decompose the
158 Species Abundance Distribution (SAD) and identify core and satellite OTUs, associated
159 to our samples. The threshold between the two categories was set as the number of
160 samples at which the SAD fitted a log-normal distribution. The core dataset was
161 employed for taxonomic and ecological aspects, while the whole community
162 composition was used in alpha and beta diversity analysis. Alpha diversity was estimated
163 through the Species Accumulation Curves (SAC) and a set of diversity indices (Shannon-
164 Weaver and Equitability). The beta diversity was tested through a Principal Coordinates
165 Analysis (PCoA) approach with 95% confidence ellipses, supporting these results with a
166 PERMANOVA non-parametric approach determined with 999 permutations. Analyses
167 were performed with QIIME 1.8.0 [32] and Microsoft® Excel® 2013.

168 ***Taxonomic identification of core taxa***

Since the UNITE database enabled a reliable identification of fungi only at the genus level [37], 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 [38] and phylogenetically analysed with MEGA6 [39] 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

191 sequencing was deep enough to detect most fungal diversity. Alpha diversity indices
192 revealed a high diversity and a low equitability of fungal taxa associated to the olive fruit
193 fly (Tab. 1). A slight higher diversity was revealed in males as compared to females (Table
194 1), but Beta diversity analyses did not show significant differences according to both
195 PCoA (Fig. S2) and PERMANOVA analyses (PseudoF = 1.13; *P* = 0.20).

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

205 The genus *Cladosporium* represented the 77.81±5.34% of core OTUs sequences, with a
206 total of 30,102 reads clustering into 6 OTUs. Among them, 3 OTUs were associated to
207 the *Cladosporium cladosporioides* and one to the *Cladosporium herbarum* species
208 complexes, respectively (Table 2; Fig. S1). The remaining 2 OTUs clustered within the
209 genus but did not match any currently known taxa (Fig. S1). A total of 4,344 sequences
210 (9.78±3.93%) clustering in a single OTU was associated to *Alternaria* sect. Alternata. Also
211 the genus *Aureobasidium* was well represented (4.64±1.34%), with a single OTU
212 identified as *Aureobasidium pullulans*. Finally, 1 OTU was identified as *Leptosphaerulina*

213 *chartarum* (0.57±0.21%) and another one was associated to the genus *Devriesia*
214 (0.61±0.07%) (Fig. S1).

215 Putative causal agents of plant diseases were also identified among core OTUs.
216 Specifically, we retrieved 2 OTUs belonging to the genus *Colletotrichum*. One of these
217 (COL1 – 0.72±0.41%) was associated with *C. acutatum sensu stricto* and *C. cosmi*, while
218 the other one (COL2 – 1.12±0.84%) clustered together with *C. gloeosporioides* (Fig. S1).
219 Another OTU accounting for the 0.36±0.16% of sequences was associated to *P.*
220 *cladosporioides*. Finally a group of sequences clustering in a single OTU were associated
221 to the genus *Botrytis* (0.51±0.17%). The remaining 15 OTUs were only analysed through
222 BLAST because of the lack of validated reference sequences (Tab. 3). According to this
223 analysis, they were associated to 4 species and 10 genera (Table 2).

224

225 **Discussion**

226 Olive is one of the most important crop around the world, and *B. oleae* represents the
227 most harmful pest, able to damage up to 100% of the production [8]. Our previous
228 survey investigated on the fungal microbiota of the olive fruit fly, providing preliminary
229 data based on a limited number of sequences [16]. Indeed, the use of a high-throughput
230 culture-independent sequencing approach provided a much high number of reads and,
231 as confirmed by rarefaction curves, enabled the analysis of the whole fungal diversity
232 associated to *B. oleae*. Furthermore, the lack of the cloning step needed in the Sanger
233 sequencing, allowed a more accurate quantitative analysis in term of relative abundance

234 of each detected taxon. In agreement with previous reports, coupling of QIIME analysis
235 together with the identification through BLAST and phylogenetic analysis, was useful to
236 identify taxa with the highest possible level of accuracy for the targeted fragment [22,
237 28]. According to these analyses, the 454 pyrosequencing confirmed all fungal genera
238 and species detected with the cloning/Sanger sequencing approach, along with new
239 previously undetected taxa. Considering both core and satellite OTUs a rich fungal
240 community was revealed and comprised fungi belonging to sooty moulds, plant
241 pathogens, and other mycetes with an undisclosed ecological role.

242 According to the earlier findings [16], the genus *Cladosporium* represented the wider
243 part of retrieved sequences, although its relative abundance (76%) was slightly lower as
244 compared to our previous study [16]. Within this genus, we identified OTUs belonging
245 to two species complexes (*C. cladosporioides* and *C. herbarum*), and other 2 OTUs that
246 may represent unknown species since they did not cluster with none of the currently
247 available sequences for this genus. The abundant presence of *Cladosporium* spp. was
248 expected, since it is one of the most common inhabitant of plants phylloplane and
249 carpoplane [40]. Sequences associated to *Alternaria* sect. Alternata were also widely
250 detected in our study (about 9% of sequences). This section of *Alternaria* represents not
251 only a widely known component of sooty moulds, but also a facultative pathogen of
252 both olive leaves and fruits [41, 42]. Moreover, we retrieved sequences that were
253 associated to *A. pullulans*, *Devriesia* sp. and *Epicoccum nigrum*, which together with
254 *Cladosporium* spp. and *Alternaria* spp. are the main representatives of sooty moulds
255 fungal communities [17, 40, 43].

A comparison of our results with those reported by Abdelfattah and colleagues [28], 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 since suggest 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 [44, 45]. *Colletotrichum acutatum* s.s. is an aggressive pathogen responsible for olive anthracnose, but it was considered absent in Italy until few years ago [18, 45]. 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 [45, 46]. 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 [47], 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 [17]. Similarly, *P. cladosporioides* is the agent of olive cercosporiosis and can affect leaves and fruits [19].

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 [16], 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 highlights 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 [28]. 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 represent 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.

Data accessibility

Raw data from 454 sequencing is available on NCBI SRA database under the Project Accession ID: SRP089714. Sequences used for phylogenetic analysis are available as Supplementary material 2.

Author contribution

All authors designed the research. LS and VP supervised and coordinated the work. AM, FL, SM and OC provided samples. AM performed lab work, data analysis and data interpretation. AM wrote the first draft of the manuscript. All authors contributed to, corrected and approved the final manuscript.

Acknowledgements

This work was partially funded by SAF@MED (PON a3_00016) – PON Ricerca e competitività 2007–2013, and PON PON03PE_00090_2 “Modelli sostenibili e nuove tecnologie per la valorizzazione delle olive e dell'olio extravergine di oliva prodotto in Calabria” – PON Ricerca e competitività 2007–2013.

Conflict of interest

The authors declare no conflicts of interest.

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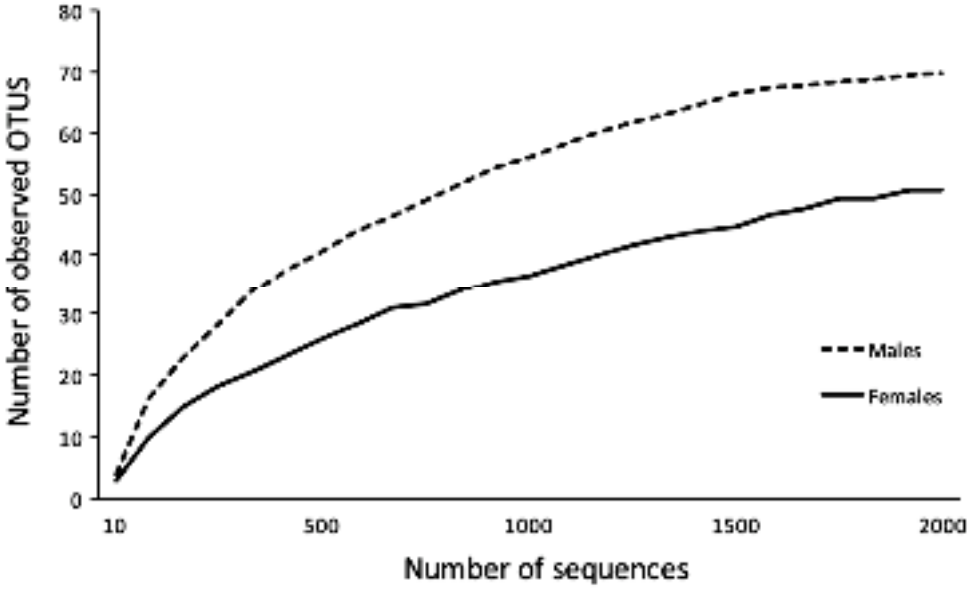

Fig. 1 Rarefied species accumulation curves (SACs) for both male and female specimens

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

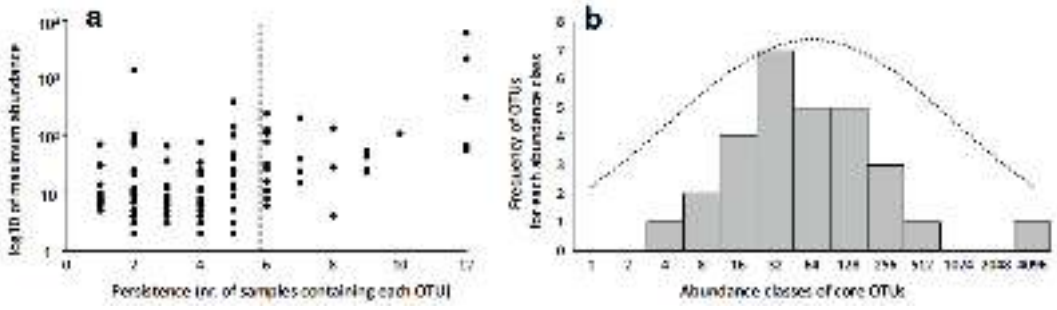

Fig. 2 Separation of core and satellite OTUs of the fungal community between core and

480 satellite OTUs set through process iterated until the *B. oleae*.associated toSpecies

481 abundance distribution (a) based on abundance class distribution and b fit a log-normal

482 distributiondashed linesabundance/persistence of OTUs, with on the threshold

Table 2 Fungal taxa associated to some OTUs detected in the present study and corresponding relative abundance (RA)

Taxa*	OTU	RA (% ± SE)
(P) *Cladosporium cladosporioides* s.s.	CLA1	76.28 ± 5.23
	CLA2	6.27 ± 0.32
	CLA3	0.15 ± 0.11
(P) *Alternaria* sect. *Alternata*	ALT1	9.78 ± 3.9%
(P) *Aureobasidium pullulans*	AUR1	4.64 ± 1.34
Epicoccum nigrum	EPI	1.24 ± 0.29
(P) *Colletotrichum gloeosporioides* sensu lat.	COL2	1.12 ± 0.34
Sarocladium palmivorum	SAR2	1.23 ± 0.48
(P) *Colletotrichum acutatum* sensu lat.	COL1	0.72 ± 0.41
(P) *Penicillium* sp.	PEN1	0.41 ± 0.20
(P) *Sporidesmium sclerotivorum*	SPO1	0.37 ± 0.21
(P) *Botrytis* sp.	BOT1	0.31 ± 0.15
Rhodotorula graminis sp.	RAT1	0.30 ± 0.23
(P) *Pseudocercospora cladosporioides*	PSE1	0.26 ± 0.16
(P) *Cladosporium herbarum* sp.	CLA5	0.25 ± 0.15
Stephanis sp.	TET1	0.20 ± 0.1
Mycosphaerella tassiana	MYC1	0.20 ± 0.09
Epicoccum sp.	EPI1	0.19 ± 0.11
Mycosphaerella sp.	MYC2	0.18 ± 0.06
Alternaria sp.	ALT2	0.16 ± 0.08
(P) *Cladosporium* sp.	CLA6	0.13 ± 0.13
(P) *Cladosporium* sp.	CLA4	0.13 ± 0.08
Aspergillus sp.	ASP1	0.09 ± 0.04
Penicillium sp.	PEN2	0.09 ± 0.05
Penicillium sp.	PEN3	0.08 ± 0.04
Penicillium sp.	PEN4	0.07 ± 0.03
Aspergillus tubingensis	ASP2	0.06 ± 0.02

*Taxa marked with (P) were identified according to both BLAST and phylogenetic analyses. Other taxa were only identified according to BLAST analyses

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