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Methodological Advances

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

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ABSTRACT

A molecular approach was used to investigate the fungal microbiome associated with *Bactrocera oleae* a major key pest of *Olea europea*, 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 into heir epidemiology.

1. 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 (Boucias et al., 2012). Fungi, with 99,000 described species, have been found in almost all habitats and are associated with a wide variety of organisms, and often essential to their survival (Blackwell, 2011). 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 (Janson et al., 2010; Kellner et al., 2013; Six, 2012). On the other hand, there are cases of antagonistic relationships between fungi and insects and fungi, as with entomopathogenic fungi such as *Beauveria* spp. and *Metarhizium* spp. Particular insects' behaviors may work as antifungal treatments, as in ants and termites that exploit self- and allo-grooming to clean themselves from fungal spores (Reber et al., 2011).

Bactrocera oleae, 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 on fruits, it is capable of strongly affecting quality and quantity of the olive production, despite numerous insecticidal treatments, commonly applied yearly. Control strategies rely on foliage spraying using chemical insecticides, or baiting using poisoned protein hydrolyzate. Attention has been devoted to the development of forecasting models that could help reduce environmental and economic impact, increasing the performance of treatments (Menesatti et al., 2013; Campolo et al., 2014). Recently, new control methods based on the use of symbionts as control factors are emerging (Zabalou et al., 2004; Alam et al., 2011; Apostolaki et al., 2011). This area could greatly benefit from a better understanding of the microbial communities associated with *B. oleae* (Kounatidis et al., 2009). Indeed a number of studies have been recently conducted to investigate bacterial communities, though very little information is available on fungi (Kounatidis et al., 2009).

Another relevant aspect is the possible interaction between the olive fruit fly and olive fungal pathogens of olive. Particularly interesting is the possible interaction of olive fruit fly with fungal pathogens responsible for significant damage to fruit. Among these,

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different *Colletotrichum* species are causal agents of olive anthracnose and may have a great economic impact, by severely affecting both fruit yield and quality of oil (Cacciola et al., 2012; Schena et al., 2014). 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 (Frisullo and Carlucci, 2011). 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. (Moral et al., 2008; Gomes et al., 2009). Iannotta and co-workers (Iannotta et al., 2008) revealed a correlation between *Botryosphaeria* olive rots, formerly associated with the fungus *Camarosporium dalmaticum*, and olive fly infestations, but did not provide any proof of 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.

2. Materials and methods

2.1. Ethics statement

No specific permits were required for the described field studies. This work did not involve endangered or protected species.

2.2. 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 (300 m a.s.l., southern exposition, 5–10% of slope and type of natural vegetation), age (50–70 yr 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 a low temperature (~5 °C) for a maximum of 4–5 h, before lyophilization was performed in laboratory.

2.3. 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 (2006). 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). DNA concentration and quality were 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, 1 × 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 (White et al., 1990; Bellemain et al., 2010). Amplifications were performed in a Mastercycler Ep Gradient S (Eppendorf, Germany) set at 94 °C for 3 min, 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, repeated 35 times, and ended with 10 min 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.

2.4. 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).

2.5. 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 were unreliable, poor in quality or with doubtful bases were sequenced again. Before analyses, sequences of primers were detected and trimmed with TAGCLEANER (Schmieder et al., 2010). 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. 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 (Koljalg et al., 2005) and the bioinformatic pipeline QIIME 1.8.0 (Caporaso et al., 2010). Since the UNITE database enabled a reliable identification of fungi only at the genus level (Knief, 2014), identified STs were also analyzed along with genetically closely related reference sequences 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* (Damm et al., 2012a), *Pseudocercospora* spp. (Crous et al., 2013), *Devriesia* spp. (Crous et al., 2009), *Cladosporium* spp. (Bensch et al., 2012), *Aureobasidium* spp. (Zalar et al., 2008), *Alternaria* spp. (Woudenberg et al., 2013), *Cochliobolus* spp. (Manamgoda et al., 2012), *Leptosphaerulina* spp. (Aveskamp et al., 2010) and *Lecanicillium* spp. (Zare and Gams, 2008). 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 (Edgar, 2004) and phylogenetically analyzed with RAxML 8.0.0 using a GTR + Γ model (Stamatakis, 2014). 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 with