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Polymorphism and phylogenetic species delimitation in filamentous fungi from predominant mycobiota in withered grapes

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ABSTRACT

Filamentous fungi are the main pathogens of withered grapes destined for passito wine production. Knowledge of which species inhabit these post-harvest fruits and their pathogenicity is essential in order to develop strategies to control infection, but is still scarce. This study investigated the predominant mycobiota of withered grapes through a cultivation-dependent approach. Strain and species heterogeneity was evidenced on examining isolates collected over three consecutive years. Colony morphology and PCR-restriction fragment length polymorphism (PCR-RFLP) analysis revealed the occurrence of several phenotypes and haplotypes, respectively. Strains were phylogenetically analyzed based on sequence typing of different genes or regions (e.g. calmodulin, β-tubulin and internal transcribed spacer region). Beside the most common necrotrophic-saprophytic species of *Penicillium*, *Aspergillus*, *Alternaria* and *Botrytis* species responsible for fruit rot, other saprobic species were identified (e.g. *Trichoderma atroviride*, *Sarocladium terricola*, *Arthrinium arundinis* and *Diaporthe eres*) generally not associated with post-harvest fruit diseases. Species such as *Penicillium ubiquetum*, *Cladosporium pseudocladosporioides*, *Lichtheimia ramosa, Sarocladium terricola*, *Diaporthe nobilis*, *Bipolaris secalis*, *Paraconiothyrium fuckelii* and *Galactomyces reessii* that had never previously been isolated from grapevine or grape were also identified. Moreover, it was not possible to assign a species to some isolates, while some members of Didymosphaeriaceae and Didymellaceae remained unclassified even at genus level. This study provides insights into the diversity of the epiphytic fungi inhabiting withered grapes and evidences the importance of their identification to understand the causes of fruit diseases. Finally, phylogenetic species delimitation furnished data of interest to fungal taxonomy.

1. Introduction

Filamentous fungi are the main pathogens of post-harvest fruits and can cause heavy economic losses. The type of fruit, maturity stage, pre-harvest and storage conditions strongly affect the level of fungal contamination and saprophytic growth to the detriment of fruit quality (Narayanasam, 2006).

The post-harvest contamination of fungi on grapes has mainly been investigated on table varieties under different storage conditions due to their high perishability and economic importance. Moreover, studies on fungal species present on raisins, with emphasis on the potential mycotoxin producers, have been undertaken (Magnoli et al., 2004; Romero et al., 2005; Valero et al., 2005).

Beside table grapes and raisins, wine grapes can be submitted to a post-harvest process to achieve partial dehydration, during which time they are vulnerable to fungal attack. Such grapes are used to produce special Italian passito wines. Off-vine dehydration (withering) generally occurs during the autumn-winter in a fruit-drying room (*fruttaio*), a special loft located close to the winery (Mencarelli and Tonutti, 2013). The saprophytic growth of filamentous fungi on withered grapes is the main concern for producers since it is detrimental to grape and wine quality.

Knowledge of the filamentous fungi inhabiting post-harvest fruits and their storage environments, and an understanding of their lifestyle and pathogenicity are essential to develop strategies to prevent and control infections (Narayanasam, 2006). Such information is even more important for issues that are as yet poorly studied, such as the fungal contamination of withered grapes. Our previous investigations indicated that fruit-drying rooms show interesting diversity, characterized by several fungal groups. Notably, intraspecific variability in the ability to infect grapes under different withering conditions was found (Lorenzini et al., 2015; Lorenzini and Zapparoli, 2014a, 2014b, 2015). Nevertheless, there is still little or no information on the occurrence of several other species such as those that include ochratoxin A-producing

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strains on withered grapes. Data on grape contamination by toxigenic species in vineyards (Rousseaux et al., 2014; Sage et al., 2004; Serra et al., 2005) and in drying vine fruit ecosystems (Magnoli et al., 2004; Romero et al., 2005; Valero et al., 2005) suggests it is vital to investigate their occurrence in fruit-drying rooms. Moreover, the finding of some isolates that could not be taxonomically defined (Lorenzini and Zapparoli, 2014b, 2016.) or belonging to species never previously associated with grapes (Lorenzini and Zapparoli, 2015) have revealed that fruit-drying rooms are interesting sources of fungal diversity which deserve further investigation.

With this aim, a collection of isolates representative of the predominant mycobiota on withered grapes sampled in fruit-drying rooms located in winemaking areas of Northern Italy was analyzed. This cultivation-dependent approach allowed us to investigate the heterogeneity among isolates and their species affiliation. Morphological and genetic polymorphism analysis was performed. Phylogenetic analysis of different gene sequences and the genetic relatedness of isolates to several currently recognized taxa was used to obtain a strict species delimitation.

2. Materials and methods

2.1. Grape sampling

The study site was a restricted geographic area of Northern Italy that includes three winemaking areas, i.e. Valpolicella, Soave and Valle dei Laghi, where the main cultivated varieties for the production of passito wine are Corvina, Garganega and Nosiola, respectively. Grape samples were collected during the 2012–14 vintages from eight fruit-drying rooms randomly selected within each winemaking area (three in Valpolicella, three in Soave and two in Valle dei Laghi). Each fruit-drying room is characterized by specific environment due to geographical position (i.e. altitude, exposure to sun and ventilation), vineyard and withering management (i.e. fungicide treatments, arrangement of racks and manual operations). Grape samples collected from all these fruit-drying rooms were considered as a single sampling. Comparison among different fruit-drying rooms, grape varieties, winemaking areas or vintages falls outside the confines of this study.

Samplings were carried out in the last phase of the natural withering process (from 20 to 5 days before the crushing). This post-harvest process generally lasts three–five months depending to the type of passito wine and season conditions, inducing berries weight loss approximately of 25 to 35% of initial fresh weight (0.96–0.98 a_w). The incidence of rotten grapes, estimated by visible inspection of 80–100 bunches of grapes selected randomly in each fruit-drying room, was approximately 7–10, 11–17 and 30–35% in vintages 2012, 2013 and 2014, respectively. Berries with and without visible disease symptoms were randomly selected in an amount proportional to the percentage of rotten grapes. Afterward, these berries were cut and aseptically transferred to the laboratory for analysis. A total of 75 out of 500 analyzed berries were rotten.

2.2. Isolation and phenotypic characterization of fungi and reference strains

Each whole berry was directly plated by rolling onto malt extract agar (MEA, 2% *w*/*v* malt extract, 0.1% w/v peptone, 2% w/v dextrose, 1.5% w/v agar) for 20 s and, after having removed it, plates were incubated at 25 °C in the dark. After two–four days, individual colonies were isolated and purified through repeated streaking on MEA.

About 600 isolates were cultivated on MEA and PDA (Difco, Laboratories, Detroit, MI) and after incubation for seven days at 25 °C were primarily classified based on gross morphology and microscopic features according to Pitt and Hocking (1997). Further characterization was carried out on representative isolates of each morphology that were selected approximately in the amount proportional to their incidence on berries.

Culture characteristics of 481 isolates were evaluated on different suitable media (i.e. Czapek yeast extract agar, CYA; Czapek agar, CzA; cornmeal agar, CMA; dichloran rose bengal yeast extract sucrose agar, DRYES; oatmeal agar, OA; potato dextrose agar, PCA; synthetic nutrient agar, SNA; yeast extract sucrose agar, YES) (Pitt and Hocking, 1997; Samson et al., 2004). Isolates were grouped into phenotypes based on their colony morphology (i.e. colour of mycelium, margin, texture, zonation and shape). Sporulation pattern of *Alternaria* isolates was examined as described by Lorenzini and Zapparoli (2014a).

The collection of strains included 21 strains described in our previous investigations (Lorenzini et al., 2015; Lorenzini and Zapparoli, 2014a, 2015). These strains, isolated from same fruit-drying rooms reported in the present work, were previously identified by morphological and molecular analysis and used as reference strains and, except for *Botrytis* sp. B83, are deposited at the Agro-Food Microbial Culture Collection — ITEM of Institute of Science of Food Production (ISPA–CNR), Bari (Italy) (Table S1).

2.3. DNA extraction and PCR-RFLP analysis

DNA was extracted from pure culture of each isolate as previously described (Lorenzini and Zapparoli, 2014b). The DNA solution of all isolates was used to amplify the internal transcribed spacer (ITS) region using ITS1 and ITS4 primers according to White et al. (1990). DNA of *Alternaria* and *Botrytis* isolates was also used to amplify the intergenic spacer (IGS) regions using the primer pairs 26S3111F/IGS27 and IGSnr1for/IGSnr1rev, respectively (Hong et al., 2005; Staats et al., 2005).

Restriction fragment length polymorphism (RFLP) of the ITS or IGS region was performed as described previously (Diguta et al., 2011; Hong et al., 2005). PCR products were digested with enzymes *Hinf*I, *Hae*III, *Taq*I, *Sdu*I, *Hind*III, *Mse*I, *Dra*I, *Xba*I, *Hinc*II, *Cfo*I, *Rsa*I, *Alu*I and *Msp*I (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA) and their restriction fragments were analyzed in 1.5–3.0% *w*/*v* agarose gels containing ethidium bromide. Sizes were estimated by comparison with DNA size markers (GeneRuler 100 bp and 1 Kb DNA Ladder, Fermentas).

2.4. DNA amplification and sequence analysis

The DNA solution was also used to amplify the partial gene or region sequences used for phylogenetical analysis such as the ITS and small subunit (SSU) (White et al., 1990), large subunit (LSU) (Rehner and Samuels, 1994; Vilgalys and Hester, 1990; Boekhout et al., 1995), actin (ACT) (Carbone and Kohn, 1999), β-tubulin (BT or BenA) (Glass and Donaldson, 1995), calmodulin (CAM or CaM) (Phan et al., 2004; Hong et al., 2006), glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Staats et al., 2005), heat shock protein 60 (HSP60) (Staats et al., 2005), DNA-directed RNA polymerase II subunit (RPB2) (Staats et al., 2005) and translation elongation factor 1-α (EF or Tef1) (Carbone and Kohn, 1999; O'Donnell et al., 1998; Kullnig-Gradinger et al., 2002).

The amplification products were visualized by agarose gel electrophoresis (1% *w*/*v*) and purified using the NucleoSpin gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The sequencing of these products was carried out in both directions using the same primers as for amplification (GATC Biotech, Köln, Germany).

The sequences were deposited in GenBank.

2.5. Phylogenetic analysis

Phylogenetic analysis was conducted using sequences from the Clustal Omega multiple alignment output using the Jukes–Cantor distance model and the neighbour-joining (NJ) method in the MEGA 6.0 interface. The phylogeny trees inferred from each sequence dataset were constructed by the NJ method and individually tested with a