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Characterization of Cultivated Fungi Isolated from Grape Marc Wastes Through the Use of Amplified rDNA Restriction Analysis and Sequencing

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Microbial assessment of grape marc wastes, the residual solid by-product of the wine-industry, was performed by identifying phylogenetically the fungal culturable diversity in order to evaluate environmental and disposal safety issues and to discuss ecological considerations of applications on agricultural land. Fungal spores in grape marc were estimated to 4.7×10^6 per g dry weight. Fifty six fungal isolates were classified into eight operational taxonomic units (OTUs) following amplified ribosomal DNA restriction analysis (ARDRA) and colony morphology. Based on 18S rRNA gene and 5.8S rRNA gene-ITS sequencing, the isolates representing OTUs #1, #2, #3, and #4, which comprised 44.6%, 26.8%, 12.5%, and 5.3%, respectively, of the number of the total isolates, were identified as *Aspergillus fumigatus*, *Bionectria ochroleuca*, *Haematonectria haematococca*, and *Trichosporon mycotoxinivorans*. The isolates of OTU#5 demonstrated high phylogenetic affinity with *Penicillium* spp., while members of OTUs #6 and #7 were closer linked with *Geotrichum candidum* var. *citri-aurantii* and *Mycocladus corymbifer*, respectively (95.4 and 97.9% similarities in respect to their 5.8S rRNA gene-ITS sequences). The OTU#8 with a single isolate was related with *Aspergillus* strains. It appears that most of the fungal isolates are associated with the initial raw material. Despite the fact that some of the species identified may potentially act as pathogens, measures such as the avoidance of maintaining large and unprocessed quantities of grape marc wastes in premises without adequate aeration, together with its suitable biological treatment (e.g., composting) prior to any agriculture-related application, could eliminate any pertinent health risks.

***Keywords*:** fungal diversity, agricultural residues, environmental quality, ITS, 18S rRNA gene, winery by-products

Grape marc (waste skins, seeds, and stalks) is a by-product of wineries resulting after crushing and pressing of grapes during the process of must production. The amount of marc produced varies with the grape variety and processing method, mostly ranging at 8-25% of the harvested weight. Hence, large grape marc quantities are generated in wine-producing countries; for example, in Greece ca. 90,000-130,000 tons are produced annually. Grape residues are mainly used for the production of traditional spirits (Silva *et al*., 2000), and to a lesser extent, of organic acids (Nurgel and Canbas, 1998), natural coloring agents (Malien-Aubert *et al*., 2001) and antioxidants (Spigno and Faveri, 2007). The use of grape marc has been rather recently introduced for agricultural use as soil conditioner and plant fertilizer (i.e., to increase water-holding capacity, to reduce temperature fluctuations and to supply nutrients), usually following a period of outdoor bulk storage and weathering, or composting (Flavel *et al*., 2005; Raviv *et al*., 2005; Moldes *et al*., 2007). Its suppressive effects against plant pathogens and nematodes have been also demonstrated (Nico *et al*., 2004; Kavroulakis *et al*., 2005, 2007; Ntougias *et al*., 2008). Less attention was drawn to various other potential applications related to its use as artificial topsoil for slate dump reclamation (Paradelo *et al*., 2009), energy feedstock and bedding for animals (Malossini *et al*., 1993), source of energy and protein for ruminants (Molina-Alcaide *et al*., 2008), mushroom growth substrate (Sanchez *et al*., 2002), and yeast production medium (Lo Curto and Tripodo, 2001).

Despite the fact that organic inputs originating from agro-industrial residues and by-products are essential for sustaining soil fertility and improving its structure, their large-scale application into agricultural crops may influence C-substrate and nutrient availability to soil microorganisms. They may also act as potent microbial-inoculum carriers capable of altering the functionality of indigenous soil microbiota and of introducing potential plant and human pathogens, hence affecting plant cultivation, crop production and human or animal health.

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Nevertheless, very limited information is available on the microbiological properties of the grape marc. A pertinent analysis during grape marc humification was performed by Streichsbier *et al*. (1982), which was based on the isolation of specific groups of microorganisms using semi-selective media; this material was initially colonized by a significant number of yeast species, succeeded by themophilic/thermotolerant bacteria, thereafter by filamentous fungi mainly belonging to *Aspergillus*, *Paecilomyces*, *Thermomyces*, and *Mucor* spp., before its final colonization by actinobacteria.

Ribotyping and molecular fingerprinting techniques, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and amplified ribosomal DNA restriction analysis (ARDRA), are essential tools in the assessment of microbial ecology (Dees and Ghiorse, 2001; Buchan *et al*., 2003; Malosso *et al*., 2006). ARDRA, which includes PCR amplification (using universal primers) of the rRNA gene followed by digestion with restriction endonucleases, clusters specimens under study into distinct groups, representatives of which are further analysed by sequencing of selected ribosomal DNA regions (Dees and Ghiorse, 2001; Chen and Song, 2002; Leeflang *et al*., 2002). Recently, Malosso *et al*. (2006) performed a fungal diversity and ecology study by applying ARDRA of SSU amplicons as a molecular fingerprinting tool.

In the present work, we investigated the diversity of cultivated fungi isolated from grape marc, by grouping them through ARDRA and then we studied their phylogeny by using 18S rRNA gene and 5.8S rRNA gene-ITS sequencing, in order to examine environmental and disposal safety issues and to discuss microbiological aspects of grape marc application into agricultural soils.

Materials and Methods

Materials and isolation of fungi

Grape marc was obtained from the "Oinomessiniaki Wineries" located in Kalamata (southern Peloponnese, Greece).

Analysis of total nitrogen content was performed by wet digestion of a 0.3 g sample in concentrated H_2SO_4, followed by steam distillation under alkaline conditions and titration of the ammonium-N collected in boric acid (Bremner and Mulvaney, 1982). Total organic matter content was estimated by weight loss during ashing, at 530ºC for 8 h. The ash was diluted in 0.8 N HNO_3 in which total K, P, Ca, Mg, Fe, and Mn were measured. Potassium was determined by flame emission photometry (Coring-410 photometer), and Ca, Mg, Fe, and Mn by atomic absorption spectrophotometry using a Varian Spectra-A300 analyser. Total phosphorus was determined colorimetrically as described by Anderson and Ingram (1993). Measurements of pH and electrical conductivity (EC) in grape marc were performed using a Scott Gerate TR156 pH-meter and a Wissenchaftlich-Technische Werkstätten LF530 conductivity meter respectively.

To isolate fungal population and to enumerate fungal spores from a grape marc pile, three sub-samples (100 g each) were analysed, by placing in 950 ml of a 8.5 g/L NaCl solution (Zuberer, 1994). The three independent mixtures were blended thoroughly, tenfold dilution series were prepared and 0.1 ml of the suspensions was aliquoted onto Petri dishes.

Grape marc extract solidified with agar (GMA) was autoclaved (121°C, 1.1 atm, 20 min) and used to isolate fungal strains and to estimate the number of fungal spores. Grape marc extract was obtained by adding 250 g of wet grape marc in 1 L of DW, agitated for 20 min, filtered, and the volume of the filtrate was adjusted back to 1 L. Isolation and enumeration media contained 60 μg/ml chlorotetracycline, 30 μg/ml streptomycin and 30 U penicillin G in order to avoid bacterial growth (Zuberer, 1994). All plates were incubated for 21 days at 25°C.

Extraction and PCR amplification of fungal DNA

DNA from fungi was extracted as described by Lee and Taylor (1990). A reaction mixture (50 μl) containing 1 μl (50 ng/μl) genomic DNA, 10× PCR buffer (Finnzymes OY, Finland), 2 mM $MgCl_2$, 200 μM each dATP, dTTP, dCTP, and dGTP, the appropriate set of primers, 0.5 μM each, and 1 U DNA polymerase (Dynazyme EXT-Finnzymes OY, Finland) was prepared. Primers nu-SSU-0817-5′ (5′-TTA GCA TGG AAT AAT (AG) (AG)A ATA GGA-3′) and nu-SSU-1536-3′ (5′-ATT GCA ATG C(CT)C TAT CCC CA-3′) (Borneman and Hartin, 2000) were used to amplify part of the 18S rRNA gene [817 to 1536 positions of the 18S rRNA gene in *S. cerevisiae* numbering, which contains the V4 (partial), V5, V7, and V8 (partial) variable regions]. Internal Transcribed Spacer 1 (ITS1) - 5.8S rRNA gene - Internal Transcribed Spacer 2 (ITS2) DNA region was amplified using primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′), which derived from the small (SSU) and large (LSU) subunits respectively (White *et al*., 1990). The 18S rRNA gene fragment was amplified using a denaturation step of 2 min at 94°C, followed by 35 cycles of 30 sec denaturation at 94°C, 30 sec primer annealing at 49°C and 1 min DNA chain extension at 72°C. A thermo-cycling amplification, which included an initial denaturation step of 4 min at 94°C, 35 cycles of 30 sec denaturation at 94°C, 30 sec primer annealing at 52°C and 1 min DNA chain extension at 72°C was performed for the amplification of the internal transcribed spacers on 5.8S rRNA gene. All amplifications were completed by 10 min DNA chain extension at 72°C. Polymerase chain reaction was performed using a PTC-200 thermocycler (MJ Research Inc., USA).

Amplified ribosomal DNA restriction analysis (ARDRA)

The amplified 18S rDNA fragments (817 to 1,536 position of the 18S rRNA gene in *S. cerevisiae* numbering) of all isolates were digested two times by adding 10 U of the restriction endonucleases *Sau*3AI and *Hinf*I (TaKaRa, Japan), in 20 μl reactions containing 10 μl of PCR product in 1× of the appropriate buffer (supplied by the manufacturer). The digests were analysed by agarose gel electrophoresis and visualised by ethidium bromide (0.5 mg/L) staining. Digital images were recorded and analysed using the Kodak Digital Science 1D image system (Kodak, USA) and band sizes in ARDRA patterns were estimated against a known standard (100 bp ladder, TaKaRa).

Sequencing of PCR products

For each operational taxonomic unit (OTU), at least two representative isolates (and up to 12 for the largest OTU) were selected for sequence analysis; three independent clones (per each isolate sequenced) were obtained by cloning the respective amplicons (partial 18S rRNA genes, and ITS1-5.8S rRNA gene-ITS2 regions) in the pGEM-T Easy Vector (Promega, USA) before insertion into DH5a competent cells. Plasmid DNA was purified using the NucleoSpin Plasmid Quick Pure kit (Macherey-Nagel, Germany). Sequencing reactions using the universal fluorescence-labelled primers SP6 and T7 primers were performed at the Institute of Molecular Biology and Biotechnology (IMBB), Heraklion, Greece and at Beckman Coulter Genomics, Takeley, UK.

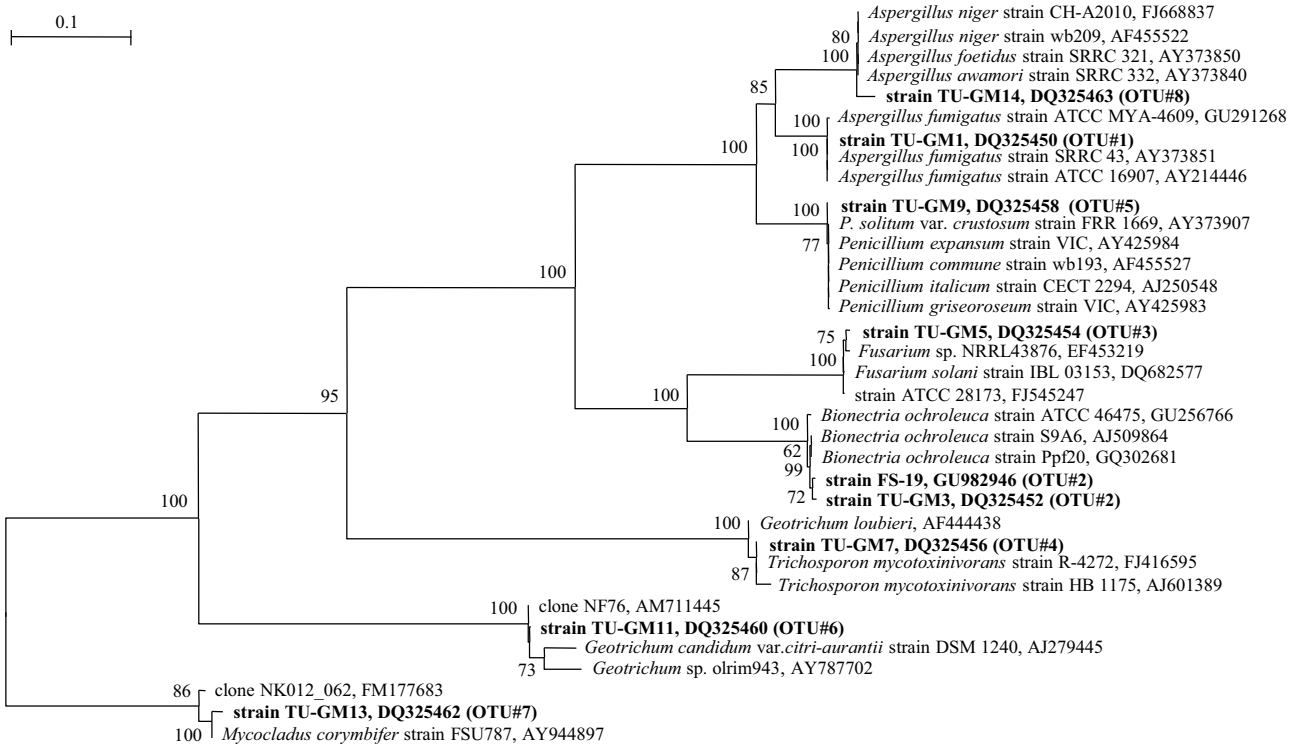

Fig. 1. Phylogenetic tree based on complete ITS1-5.8S rRNA gene-ITS2 sequencing (including parts of SSU and LSU) of fungi isolated from grape marc and previously classified in eight OTUs. For comparison purposes, closest associated species and strains from previous studies are also included (with their respective GenBank accession no. quoted). A representative isolate per each OTU is presented by being depicted in bold typeface, apart from OTU#2 where two representative isolates are included, since sequencing results revealed differences between them. Evolutionary distances were calculated using the method of Kimura (1980), taking into account insertions and deletions, and the topology was inferred using the "neighbor-joining" method (Saitou and Nei, 1987). Values on branches denote % bootstraps based on analysis of 1,000 trees. Bootstraps below 50% are not presented. Scale bar represents 0.1 inferred substitutions per nucleotide position.

Phylogenetic analysis

The sequences of the 18S rRNA gene part (817 to 1536 position of the 18S rRNA gene in *S. cerevisiae* numbering) and ITS1-5.8S rRNA gene-ITS2 (defined by the primers ITS1 and ITS4) were assembled using the CAP3 programme (http://pbil.univ-lyon1.fr/cap3.php) (Huang and Madan, 1999). Similarity searches against the NCBI database were carried out using the Basic Local Alignment Search Tool (BLAST) programme (http://www.ncbi.nlm.nih.gov/BLAST/). The closest relatives identified in both searches were included in further phylogenetic analyses. Alignment of the sequences was performed using the "CLUSTAL W Submission Form" (http://www.ebi.ac.uk/clustalw/). TREECON for Windows (Version 1.3b) was also used for constructing phylogenetic trees (Van de Peer and De Wachter, 1993). Evolutionary distances were calculated using the method of Kimura (1980) and the topology was inferred using the "neighbor-joining" method (Saitou and Nei, 1987). Bootstrap values were derived from a total of 1,000 replicates.

Table 1. Physicochemical characteristics of grape marc wastes examined in this study

	Grape marc
pH	5.7
EC (mS/cm)	2.4
Total organic matter (g/kg)	937 (12)
Nitrogen (g/kg dw)	24.1 (1.6)
P (g/kg dw)	1.7 (0.1)
K (g/kg dw)	15.3 (0.2)
Ca (g/kg dw)	6.4 (0.3)
Mg (mg/kg dw)	67.6 (3.8)
Mn (mg/kg dw)	63.8 (0.7)
Fe (mg/kg dw)	77.5 (4.7)

Values in parenthesis are Standard Errors of Means (n=3).

Results

The grape marc sample contained high amounts of nitrogen (24.1±1.6 g/kg dw) and organic matter (937±12 g/kg dw). The pH and electrical conductivity of grape marc were equal to 5.3 and 2.4 ms/cm, respectively, while the concentrations of other components are presented in Table 1. Fungal spores in grape marc were estimated at 4.7±1.1 (10^6) spores/g dw (CFU).

A total of 56 fungal isolates were obtained from the solidified culture medium (GMA) at the same suspension step. Using eukaryotic 18S rDNA-targeted PCR primers, a part of the 18S rRNA gene (817 to 1,536 positions in *S. cerevisiae* numbering) from the representative isolates analysed was sequenced. Amplified ribosomal DNA restriction analysis

(ARDRA) using the restriction endonucleases *Sau*3AI and *Hinf*I was performed in order to distinguish among the 56 fungal isolates. Restriction endonuclease *Sau*3AI grouped the isolates into three clusters, while restriction endonuclease *Hinf*I classified the fungal isolates into four other groups, distinct from those of endonuclease *Sau*3AI (Table 2). The combination of the results obtained from the use of the endonucleases *Sau*3AI and *Hinf*I placed the 56 fungal isolates into seven operational taxonomic units (OTUs), while examination of fungal colonies morphology led to the formation of an additional operational taxonomic unit (OTU#8), which was otherwise identical (based on ARDRA results) with OTU#1 (Table 2). Hence, eight OTUs were discriminated: OTU#1 consisted of 25 isolates, OTU#2 of 15 isolates, OTU#3 of 7 isolates, OTU#4 of 3 isolates, OTUs #5 and #6 included 2 isolates each, while OTUs #7 and #8 consisted of only 1 isolate each (Table 2). Representative isolates of each OTU (31 isolates in total) were identified by being further subjected to sequence analysis.

Based on complete ITS1-5.8S rRNA gene-ITS2 sequencing, a phylogenetic tree illustrating the relationships among the eight OTUs and other previously identified and affiliated strains was produced (Fig. 1). OTU#1 isolates showed 99.8-100% similarity with several *Aspergillus fumigatus* Fresen. strains, such as ATCC MYA-4609, WM 06.357, LZ2, CBMAI 1007, UOA/HCPF 10229, N40, xsd08062, WCH-AF001, NRRL 35204, 050994, a rice wine wheat isolate, IFM 54307, Ppf10, SRRC 43, ATCC 9197, UWFP 500, and ATCC 16907 (Table 2). The isolates representing OTU#2 presented 99.2-99.4% similarity with several *Bionectria ochroleuca* (Schwein.) Schroers & Samuels strains (Table 2). The members of OTU#3 showed 99.4-100% similarity with *Haematonectria haematococca* (Berk. & Broome) Samuels & Rossman (synonym: *Fusarium solani* (Mart.) Sacc.). Interestingly, strain ATCC 28173, one of the closest relative of members of OTU#3, was previously reported as member of the species *Acremonium falciforme* (Carrión) W. Gams 1971 [syn. *Fusarium falciforme* (G. Carrión) Summerb. & Schroers (2002)], although the taxonomic status of this deposited isolate is under identification within the frame of ATCC Mycology Authentication Project (direct submission FJ545247). The representatives of OTU#4 were identical with *Trichosporon mycotoxinivorans* Molnar, Schatzmayr & Prillinger, while their next closest phylogenetic relative was *Geotrichum loubieri* Morenz (99.4% similarity). The isolates of OTU#5 presented equal similarities (99.8%) with *Penicillium italicum* Wehmer, *P. commune* Thom, *P. expansum* Link, *P. solitum* var. *crustosum* (Thom) Bridge, D. Hawksw., Kozak., Onions, R.R.M. Paterson & Sackin and *P. griseoroseum* Dierckx. The members of the OTU#6 were associated, but not closely, with strain olrim943 (97.6% similarity), and their closest identified species (95.4% similarity) was *Geotrichum candidum* var. *citri-aurantii* (Ferraris) Cif. & F. Cif. However, the closest match (100% similarity) was an uncultured fungus (clone NF76-Table 2). The sole isolate of OTU#7 was related, but not closely, to environmental clone NK012_062 (98.1% similarity) and then to *Mycocladus corymbifer* (Cohn) Váňová (97.9% similarity). The only representative of OTU#8 showed equal phylogenetic distances (99.1% similarities) with *Aspergillus niger* Tiegh., *A. awamori* Nakaz., and *A. foetidus* Thom & Raper.

The partial 18S rRNA gene sequences produced the same identification results (at least) at the genus level when compared to the complete 5.8S rRNA gene-ITS sequences (Table 2). The only exception was the case of OTU#2 whose members were associated either with *Nectria curta* J. Webster (recent unpublished GenBank-sequence deposits have reported some close relatives as *Fusarium* spp.) or *B. ochroleuca* (Schwein.) Schroers & Samuels (Table 2). The isolates from each OTU sequenced were either identical or differed by only one base in the 18S rRNA gene, with the exception of members of OTU#2 whose 18S rRNA gene sequences showed up to 1.8% divergence. However, comparisons of 18S rRNA gene and 5.8S rRNA gene-ITS sequences should be attempted with care, due to the absence of relative sequences from the database, i.e. the lack of ITS1-5.8S rRNA gene-ITS2 sequence for *Nectria curta*, and the lack of 18S rRNA gene sequences for *T. mycotoxinivorans* (lack of equal size sequence), *Penicillium solitum* var. *crustosum*, and *P. griseoroseum*.

Discussion

Identification approach

In this study, the identity and phylogenetic relationships of cultivated fungi isolated from grape marc were established. After the grouping of 56 fungal isolates in eight OTUs based on ARDRA, identification of selected representative isolates for each OTU was performed by sequencing both part of the 18S rRNA gene (817 to 1,536 position in *S. cerevisiae* numbering) and the entire ITS1-5.8S rRNA gene-ITS2 fragment. This was judged necessary since similar or identical 5.8S rRNA gene-ITS patterns do not necessarily belong to related species (Esteve-Zarzoso *et al*., 1999); furthermore, one single mutation in the 5.8S rRNA gene-ITS region could lead to the loss or gain of a restriction site, which results in a completely different pattern and in potential misidentifications even at the genus level (taking also into the account the small size of conserved 5.8S rRNA gene) (Arias *et al*., 2002).

Characterization of fungal culturable diversity: Microbial assessment of grape marc waste and ecological considerations

Members of OTUs #1 and #8 were placed in the genus *Aspergillus*. The closest relatives of members of OTU#1 (99.8-100% similarity based on 5.8S rRNA gene-ITS sequencing) were *A. fumigatus* strains WM 06.357 (clinical pathogen-direct submission FM999061), UOA/HCPF 10229 (clinical pathogen, Greece-direct submission GQ461905), WCH-AF001 isolated from a patient with vocal cord infection (direct submission EU693451), SRRC 43 used in a fungal detection study of dust samples from different sources around the USA (Haugland *et al*., 2004), ATCC 9197 (a fungal pathogen involved in invasive mycosis) (Leinberger *et al*., 2005), and the pathogenic strains ATCC 16907 (type strain) and UWFP 500 (Rakeman *et al*., 2005). Based on partial 18S rRNA gene sequencing, the closest *A. fumigatus* relative of OTU#1 was strain ALI 57, a common airborne fungus (Wu *et al*., 2003). This species is widespread in soils and decaying organic matter, where it plays an essential role in carbon and nitrogen recycling. However, it is better known as the main causal agent of invasive aspergillosis in humans and animals by producing

Table 2. Molecular characterization of cultivated fungi isolated from grape marc wastes

OTUs	No of strains isolated (No of strains sequenced)	ARDRA (fragment lengths in bp)[a]		Accession numbers (18S rRNA gene)	Closest phylogenetic relatives[b]	Similarity (%)	Accession numbers (5.8S rRNA gene-ITS)	Closest phylogenetic relatives[b]	Similarity (%)
		*Hinf*I	*Sau*3AI						
1	25 (12)	400, 370	300, 210, 190	DQ325436-DQ325437 & GU982953-GU982962	*Aspergillus fumigatus*, *A. wentii*, *A. terreus* and *A. clavatus* (Nikkuni *et al*., 1998; Wu *et al*., 2003)	99.9-100%	DQ325450-DQ325451 & GU982936-GU982945	*A. fumigatus* strains ATCC MYA-4609 (GU291268), WM 06.357 (FM999061), LZ2 (GU258411), CBMAI 1007 (FJ986602), UOA/HCPF 10229 (GQ461905), N40 (GQ169480), xsd08062 (FJ478096), WCH-AF001 (EU693451), NRRL 35204 (EF634391), 050994 (EU664470), rice wine wheat isolate (EF136363), IFM 54307 (AB369897), Ppf10 (EF495242), SRRC 43 (Haugland *et al*., 2004), ATCC 9197 (Leinberger *et al*., 2005), UWFP 500, and ATCC 16907 (Rakeman *et al*., 2005) (other strains also included)	99.8-100%
2	15 (8)	370, 270, 120	510, 190	GU982963-GU982968	*Bionectria ochroleuca* strain WY-1 (GU112755); *B. pityrodes* strain CBS 246.78 (AY249900); *Nectria mariannaeae* (Yokoyama *et al.*, 2004)	99.9% 99.6% 99.0%	GU982946-GU982951	*Bionectria ochroleuca* strains ATCC 46475 (GU256766), ATCC 48395 (GU256754), and Ppf20 (GQ302681)	99.4%
				DQ325438-DQ325439	clone APf3_99 (Nakabachi *et al*., 2003); *Nectria curta* (Belliveau and Barlocher, 2005) *Bionectria ochroleuca* strain WY-1 (GU112755),	99.0% 98.9% 98.3%	DQ325452-DQ325453	*Bionectria ochroleuca* strains ATCC 46475 (GU256766), ATCC 48395 (GU256754), Ppf20 (GQ302681), C7.1 (FM998715), Vega618 (EF672316), BFM-L88 (AB369487), RS010 (Rodrigues *et al*., 2008), XSD-79 (EU326187), G14A (EF432268), GD507 (DQ810187), GCA-605-5 (DQ279793), GFI 11 (Sabev *et al*., 2006), 50-1 (Becerra-Lopez Lavalle, 2002), 4/97-110 (Wirsel *et al*., 2001), S9A6 (Barratt *et al*., 2003), and CBS 118525 (Ronhede *et al*., 2005) (other strains are also closely related, including clone A3_2_f_ITS1F- EU754945)	99.2%
3	7 (3)	390, 370	190, 510	DQ325440-DQ325441 & GU982969	*Fusarium solani* (synomyn: *Haematonectria haematococca*) (EF397944)	99.9-100%	DQ325454-DQ325455 & GU982952	*F. solani* f. sp. *pisi* strain HKU-543 (DQ535186) and strains TF4ATQ (Sen *et al.*, 2009), IBL 03153 (Posada *et al*., 2007), ATCC 28173[c] (FJ545247), NRRL 43876 (O'Donnell *et al*., 2007), and FMR 7985 (Azor *et al*., 2007)	99.2-100%
4	3 (2)	390, 300	120, 500	DQ325442-DQ325443	*Geotrichum loubieri*, *G. gracile*, *Trichosporon dulcitum* and *T. Multisporum* (Sugita *et al*., 1998); isolate BG02-6-6-2-5 (Suh *et al*., 2005)	99.7%	DQ325456-DQ325457	*T. mycotoxinivorans* (Molnar *et al*., 2004) and strain R-4272 (Hickey *et al.*, 2009); *G. loubieri* (Scorzetti *et al*., 2002)	100% 99.4%
5	2 (2)	370, 150, 110	300, 210, 190	DQ325444-DQ325445	*Penicillium expansum* (EU590663), *P. commune* (EU263609), *P. italicum* and *P. chrysogenum* (Wu *et al*., 2003)	99.7%	DQ325458-DQ325459	*P. italicum* (AJ250548), *P. commune* (Buzina *et al.*, 2003), *P. expansum* (Cardoso *et al*., 2007), *P. solitum* var. *crustosum* (Vega *et al*., 2006; Bakys *et al*., 2009) (EF634415), and *P. griseoroseum* (Cardoso *et al*., 2007)	99.8%
6	2 (2)	390, 300	190, 510	DQ325446-DQ325447	*Geotrichum candidum* var. *citri-aurantii* (AB162430)	99.7%	DQ325460-DQ325461	clone NF76 (Hultman, *et al.*, 2010); isolate olrim943 (Lygis *et al*., 2005); *G. candidum* var. *citri-aurantii* (Wirsel *et al*., 2001)	100% 97.6% 95.4%
7	1 (1)	390, 220, 170	500, 170	DQ325448	*Mycocladus corymbifer* (Voigt *et al*., 1999)	99.7%	DQ325462	clone NK012_062 (Hultman *et al*., 2008); *M. corymbifer* (Hoffmann *et al*., 2007)	98.1% 97.9%
8	1 (1)	400, 370	300, 210, 190	DQ325449	*Aspergillus niger* (Wu *et al*., 2003) and *A. awamori* (Nikkuni *et al*., 1996)	99.9%	DQ325463	*A. niger* (Buzina *et al*., 2003; Haugland *et al*., 2004; Huitron *et al*., 2008), *A. awamori* and *A. foetidus* (Haugland *et al*., 2004)	99.1%

[a] profiles did not consider restriction fragments under 70 bp.
[b] GenBank accession no. are quoted when no pertinent publications are available.
[c] The taxonomic status of strain ATCC 28173, previously deposited as *Acremonium falciforme* (syn. *Fusarium falciforme*) is under investigation in the frame of ATCC Mycology Authentication Project.

several toxic metabolites, which present potent immunosuppressive, genotoxic, cytotoxic, and apoptotic effects (Latge, 2001; Boudra and Morgavi, 2005). Although *A. terreus*, *A. wentii*, and *A. clavatus*, which also present 99.9-100% similarity in their 18S RNA gene sequences with OTU#1 isolates, are common soil fungi, the former is responsible for invasive infections and has rather recently emerged as an important human pathogen (Warnock, 2007) presenting a marked refractoriness to amphotericin B therapy (Walsh *et al*., 2003).

For the sole member of OTU#8, which was also associated with the genus *Aspergillus*, the closest matches (99.1%, 5.8S rRNA gene-ITS sequencing) were several *A. niger* strains, i.e., CH-A2010, ATCC 16888 and wb209 (Buzina *et al*., 2003; Haugland *et al*., 2004; Huitron *et al*., 2008), *A. awamori* SRRC 332 and *A. foetidus* SRRC 321 (used in a fungal detection study of dust samples from different sources around the USA) (Haugland *et al*., 2004). On the other hand, when analysis was based on 18S rRNA gene sequencing, *A. niger* strains HD86-9 (Zhao *et al.*, 2009), UPSC 1769 (Wu *et al*., 2003) and CBS 513.88, and *A. awamori* strain IFO4033 (Nikkuni *et al*., 1996) were the closest relatives (99.9% similarity) of OTU#8. *A. niger* is among the most common microorganisms colonizing grapes from setting to harvest (Bejaoui *et al*., 2006); it can produce mycotoxins such as ochratoxin A (Serra *et al*., 2005), which is a very dangerous fungal secondary metabolite exhibiting nephrotoxic, immunosuppressive, teratogenic, and carcinogenic properties (Smith and Moss, 1985). However, this fungus can only rarely cause problems through direct infections and only in patients already suffering from serious diseases (Fianchi *et al*., 2004). On the other hand, *A. awamori* is a potent cellulolytic enzyme producer used in several biorefinery-type applications including the production of xylanases, cellulases, and pectinases from grape pomace in solid state fermentation (Botella *et al*., 2005), whereas *A. foetidus* is a pectinase producing fungus commonly isolated from vineyards (Taskin *et al*., 2008).

The closest relatives (99.2-99.4%, 5.8S rRNA gene-ITS sequencing) of the members of OTU#2 were of environmental origin i.e. biocontrol agents, degraders, endophytes, marine fungi, and isolates from storage corm and common reed. *B. ochroleuca* isolate 50-1 originating from the stem tissue of *Gossypium hirsutum* L. was involved in disease resistance against Fusarium wilt of cotton (Becerra-Lopez Lavalle, 2002). *B. ochroleuca* was reported as an important species in the biocontrol of phytopathogenic fungi, e.g. *Botrytis cinerea* on strawberry, *Alternaria dauci* and *A. radicina* on carrot, and Fusarium wilts of raspberry and tomato (Schroers *et al*., 1999; Jensen *et al*., 2004; Lahoz *et al*., 2004). Many related taxa were also involved in the degradation of herbicides and soil-buried polymers (Barratt *et al*., 2003; Ronhede *et al*., 2005; Sabev *et al*., 2006).

The closest relatives (99.4-99.2%, 5.8S rRNA gene-ITS sequencing) of the members of OTU#3 were either beneficial endophytes or the cause of human infections. *Heamatonectria haematococca* (syn. *Fusarium solani*) strains NRRL 43876 (O'Donnell *et al*., 2007) and FMR 7985 (Azor *et al*., 2007), together with *F. solani* f. sp. *pisi* strain HKU-543 (direct submission DQ535186), were associated with human keratitis and corneal scrapping; on the other hand, the endophytic strain IBL 03153 (isolated from coffee plants) influenced negatively the establishment of the fungal entomopathogen *Beauveria bassiana* (Posada *et al*., 2007). *F. solani* is a fungus of wide distribution in nature, which often causes many plant diseases and occasionally severe human (usually eye-related) infections (Summerbell and Schroers, 2002; Azor *et al*., 2007), while it was also associated with the generation of potent mycotoxins on agricultural products (Park *et al*., 1999; Niessen, 2007). However, *F. solani* strains can also act as biocontrol agents, e.g. the recently described strain Fs-K isolated from grape marc-based compost (Kavroulakis *et al*., 2007), which demonstrated suppression of tomato plants pathogens.

The closest relative of OTU#4 isolates (100%, 5.8S rRNA gene-ITS sequencing), was *T. mycotoxinivorans*, a yeast species shown to be beneficial in the biological detoxification of several mycotoxins, such as ochratoxin A and zearalenone (Molnar *et al*., 2004). Both basidiomycetes, *T. mycotoxinivorans* (Molnar *et al*., 2004) and strain BG02-6-6-2-5 (Suh *et al*., 2005) closely related to the members of OTU#4 (Fig. 1), were isolated from the gut of a *Phrenapetes bennetti* larva (Tenebrionidae) and the hindgut of the lower termite *Mastotermes darwiniensis* (Mastotermitidae) respectively, indicating that insects might be held responsible for the transfer of microorganisms in the grape marc. Previous studies have reported the association of *Geotrichum loubieri* (syn. *Trichosporon loubieri*), the closest taxonomically relative (99.7% similarity, based on 18S rRNA gene sequencing) of the members of OTU#4 with trichosporonosis, which is a serious human disease caused by basidiomycetous yeasts of the genus *Trichosporon* Behrend (Sugita and Nakase, 1998). Interestingly, *T. mycotoxinivorans* strain R-4272 (100% sequence similarity in 5.8S rRNA gene-ITS with members of OTU#4) causing pneumonia in a patient with cystic fibrosis has been recently reported (Hickey *et al.*, 2009).

The closest matches (99.8% similarity) of members of OTU#5, examined by 5.8S rRNA gene-ITS sequencing, were *Penicillium crustosum* strains 147s-3 associated with necrotic shoots of declining common ash, *Fraxinus excelsior* (Bakys *et al*., 2009) and FRR 1669 used in a fungal detection study of dust samples from different sources around the USA (Haugland *et al*., 2004), *P. commune* wb193 isolated from nasal mucus (Buzina *et al*., 2003), *P. italicum* CECT 2294 (direct submission AJ250548), *P. griseoroseum* strain VIC and *P. expansum* strain VIC (Cardoso *et al*., 2007), and clone BF-OTU268 from indoor dust (Pitkäranta *et al*., 2008). Based on 18S rRNA gene sequencing, the common airborne fungi *P. commune* strain IBT 15141, *P. chrysogenum* ALI 229, *P. italicum* UPSC 1577 (Wu *et al*., 2003), and a *P. expansum* isolate from municipal wastewater sludge (Subramanian *et al*., 2008), were among the closest relatives (99.8% similarity) of OTU#5. *P. expansum* is a well recognized mycotoxin producer which is held responsible for the presence of patulin and citrinin toxins in wine-producing grapes and grape juice (Benkhemmar *et al*., 1993; Abrunhosa *et al*., 2001). Similarly, *P. chrysogenum*, *P. commune*, and *P. italicum* are among the numerous *Penicillium* species producing toxic secondary metabolites, including well-known antibiotics of significant value (Frisvad *et al*., 2004); on the other hand, *P. commune* and *P. solitum* are commonly found on processed foods, thus posing potential threats to allergic consumers (Asefa *et al*.,

2009).

The closest relative of the members of OTU#6 (based on 5.8S rRNA gene-ITS sequencing) were clone NF76 deriving from municipal compost (Hultman *et al.*, 2010) and strain olrim943 from the xylem in root collar of a declining *F. excelsior* tree (Lygis *et al*., 2005); *Geotrichum candidum* var. *citri-aurantii* DSM 1240 (an ascomycetous yeast-like fungus) was the closest taxonomically-described relative (95.4%, 5.8S rRNA gene-ITS sequencing) isolated from the common reed (Wirsel *et al*., 2001). Based on partial 18S rRNA gene sequencing, *G. candidum* var. *citri-aurantii*, was the closest match (99.7%) for the isolates of OTU#6.

The only member of OTU#7 was associated (97.9%, 5.8S rRNA gene-ITS sequencing) with *Mycocladus corymbifer* (syn. *Absidia corymbifera*), a clinically important zygomycete (Voigt *et al*., 1999; Hoffmann *et al*., 2007). *M. corymbifer* is a thermotolerant opportunistic animal and human pathogen causing mycotic infections, known as zygomycoses (Eucker *et al*., 2001; Hoffmann *et al*., 2007). Humans with previous medical record can be infected by *M. corymbifer*, while high populations of *M. corymbifer* have been found to be involved in farmer's lung disease causing chronic bronchitis (Reboux *et al*., 2001). However, the closest relative (98.1% sequence similarity in 5.8S rRNA gene-ITS) of OTU#7 was a clone obtained from a municipal waste compost (Hultman *et al*., 2008).

Common airborne fungi, such as members of the genera *Aspergillus*, *Penicillium*, *Mucor*, *Fusarium* (including *F. solani*), and *Cladosporium*, have been related with a large spectrum of human health threats, including allergies, asthma, infections, irritation, and toxic reactions (Shelton *et al*., 2002; Wu *et al*., 2003), which were all described by the term "occupational health" problems (Land *et al*., 1987); in addition, the abundance of *A. niger* and *A. fumigatus* spores in the air has been reported to be a significant criterion for the evaluation of air pollution and environmental quality (Lugauskas *et al*., 2003). In a study of air-borne fungi in wineries (Picco and Rodolfi, 2004), high populations of *A. niger*, *A. terreus*, *P. chrysogenum*, and *P. crustosum* were detected (all of them were highly associated with specimens recorded in this work), which is indicative of the potential health threats such fungi might pose for winery workers.

Previous studies demonstrated that mainly yeast species and members of the genera *Aspergillus*, *Penicillum*, *Alternaria*, *Cladosporium*, and *Botrytis* prevailed among fungi colonizing grapes (Battilani and Pietri, 2002; Tjamos *et al*., 2004; Serra *et al*., 2005). In addition, *Nectria haematococca* (synonyms: *Fusarium solani*, *H. haematococca*), *A. niger* and *A. awamori*, as well as *P. expansum*, *P. chrysogenum*, and *P. crustosum* were among the species isolated from grapes (Benkhemmar *et al*., 1993; Omer *et al*., 1995; Magnoli *et al*., 2004). These results are closely associated with the findings of the present study on grape marc, where cultivated isolates were linked with *F. solani* (12.5%), and with the genera *Aspergillus* (44.6% and 1.8% of total population were related with *A. fumigatus* and *A. niger*/*A. awamori* species respectively), *Penicillium* (3.6%), and *Mycocladus* (1.8%), as well as with yeasts of the genera *Trichosporon* (5.3%) and *Geotrichum* (3.6%). The relatively high occurrence of *Bionectria* isolates (26.8%) may be linked to the absence of other pertinent dominating fungal groups (notably, *Alternaria* and *Botrytis* spp.), since *B. ochroleuca* could play a role in the biocontrol of several plant-pathogenic fungi as previously stated.

Conclusion

The present study is the first attempt to identify cultivated fungi present in the grape marc by using molecular techniques and to evaluate microbiologically this agro-industrial by-product. It appears that most of the fungi isolated could be associated with the initial raw material. Any possible risks arising from the use of grape marc, which are associated with the occurrence of fungal strains acting as potential human and/or plant pathogens, are considered to be of rather low concern. Infections to humans may be caused under specific circumstances, e.g. when fungal spores are abundant, in covered/closed sites where people are active, and for individuals prone to such allergens or with previous medical record. Hence, it is recommended to avoid the storage of large and unprocessed piles of grape-marc in areas that are not adequately aerated. In addition, amendments of grape marc in soil for enhancing its structure and properties, and/or for supporting plant growth should be performed after its proper biological pre-treatment, i.e. composting, which ensures elimination of harmful pathogens.

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