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DETECTION OF OCHRATOXIN A–PRODUCING FUNGI IN GRAPES FROM MARCHE VINEYARDS

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ABSTRACT

Species belonging to *Aspergillus* section *Nigri* (black aspergilli) are the main fungal contaminants of table grapes. Besides their ability to cause black rot, they can produce ochratoxin A (OTA), which is a mycotoxin with nephrotoxic, immunotoxic, and carcinogenic effects in grape derivative products. In order to evaluate the risk of contamination of grape and grape derivative products, the mycoflora from grapes, Verdicchio cultivar, harvested in September 2018 in the province of Ancona, central-eastern Italy, were isolated and fungi were identified morphologically and by applying molecular tools. Using High-Resolution Melting Polymerase Chain Reaction (HRM-PCR) approach, a rapid method for the identification of fungal species *Aspergillus* spp. section *Nigri* was set-up. The primer pairs selected on β-tubulin genes including several specific nucleotide variants which belong to *A. tubingensis*, *A. niger*, *A. welwitschiae*, *A. luchuensis*, *A. brasisliensis*, *A. awamori*, and *A. neoniger* species were selected using Primer3web version 4.1.0. (http://bioinfo.ut.ee/primer3- 0.4.0/). Melt curve analysis confirms the all isolates selected in this work, are clustered in the same group. The Basic Local Alignment Search Tool (BLAST), analysis of amplicon sequences generated by HRM-PCR confirms that all isolates selected in this work were homologous to *A. tubingensis* species of 98.88%. This suggests a possible recombination among *A. tubingensis* and *A. niger* or *A. welwitschiae* sequence associated to β-tubulin genes. In addition, the PCR assays based on polyketide synthases (PKS) involved in OTA biosynthesis suggest that this isolate could produce OTA, but future investigation must be performed for gene sequence prediction.

RIASSUNTO

Le specie appartenenti alla sezione *Aspergillus Nigri* (black *aspergilli*) sono i principali contaminanti fungini delle uve. Oltre alla loro capacità di causare marciume nero, possono produrre ocratossina A (OTA), una micotossina con effetti nefrotossici, immunotossici e cancerogeni nell'uva e nei prodotti derivati rappresentando un rischio elevato per la salute pubblica. Al fine di valutare il rischio di contaminazione dell'uva, è stata raccolta nel Settembre 2018 la microflora da grappoli di uva appartenenti alla varietà 'Verdicchio' coltivata in un vigneto commerciale collocato nella provincia di Ancona, Italia Centro Orientale. Il micelio fungino è stato isolato e l'agente identificato morfologicamente e con tecniche molecolari. Utilizzando il saggio PCR di *High Resolution Melting* (PCR-HRM) è stato messo a punto un metodo rapido per identificare alcune tra le specie fungine incluse in *Aspergillus* spp.. Primers relativi al gene della β-tubulina sono stati selezionati utilizzando la versione Primer3web 4.1.0. (http://bioinfo.ut.ee/primer3-0.4.0/), in grado di includere specifiche varianti nucleotidiche relative alle specie *A. tubingensis*, *A. niger*, *A. welwitschiae*, *A. luchuensis*, *A. brasisliensis*, *A. awamori* e *A. neoniger*. L'indagine HRM della curva di melting dei frammenti PCR amplificati ha evidenziato che tutti gli isolati selezionati in questo lavoro avevano la stessa sequenza nucleotidica che si univa nel medesimo gruppo. L'analisi dei frammenti PCR sequenziati effettuata mediante *Basic Local Alignment Search Tool* (BLAST), ha confermato che tutti gli isolati analizzati in questo lavoro erano omologhi delle specie *A. tubingensis* del 98,88% suggerendo una possibile ricombinazione tra *A. tubingensis*, *A. niger* e/o *A. welwitschiae* associata ai geni della β-tubulina. Inoltre, i test PCR basati sul gene polichetidiche sintetasi (PKS) coinvolto nella biosintesi delle ocratossine, suggeriscono che questi isolati potrebbe produrre OTA. Tuttavia, ulteriori indagini devono essere condotte per verificare la corretta sequenza genica PKS associata a questi isolati da uva.

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LIST OF ABBREVIATIONS

AFB- aflatoxin
BEN- Balkan endemic nephrophaty
BLAST- Basic Local Alignment Search Tool
CTAB- cetxy trimethyl ammonium bromide
D1/D2- domain 1/ domain 2 regions
DNA- deoxyribonucleic acid
DON- deoxynivalenol
dsDNA- double-stranded deoxyribonucleic acid
EDTA- ethylenediaminetetraacetic acid
FAO- Food and Agriculture Organization of the United Nations
FB1- fumonsim B1
HAL- halogenase
HRM- high resolution melting analysis
ITS- internal transcribed spacer
IUPAC- International Union of Pure Applied Chemistry
JECFA- Join FAO/WHO Expert Committee on Food Additives
MEGA- molecular evolutionary genetic analysis
MW- molecular weight
NaCl- sodium chloride
NCBI- National Center for Biotechnology Information
NRPS- non-ribosomal peptide synthetase
OTA- ochratoxin A
OTB- ochratoxin B
OTC- ochratoxin C
P450- cytochtome p450 monoxigenase
PCR- polymerase chain reaction
PKS- polyketide synthase
PKS- polyketide synthase
qPCR- real time polymerase chain reaction
RH- relative humidity
RNA- ribonucleic acid
SCF- Scientific Committee on Food

SNP- single-nucleotide polymorphism

T2 – trichothecene

Tm- melting temperature

WHO- World Health Organization

ZEA- zearalenone

1 INTRODUCTION

1.1 MYCOTOXINS

The term mycotoxin was first used in the 1960s to describe the toxin associated with contaminated peanuts in animal feed and the loss of turkeys in England (Turkey-X-disease). This mycotoxin was later identified as the *Aspergillus flavus* toxin aflatoxin B1. Bennett (1987), defined mycotoxins as "natural products produced by fungi that evoke a toxic response when introduced in low concentrations to higher vertebrates and other animals by a natural route". Many mycotoxins display overlapping toxicities to invertebrates, plants, and microorganisms (Atanda *et al.*, 2013). They are secondary metabolites with a low molecular mass (MW ~700 Da) produced by many filamentous fungi belonging to the phylum Ascomycota (Alshannaq and Yu, 2017; Liew and Mohod-Redzwan, 2018). Mycotoxins are classed as 'secondary' metabolites, because they are not considered essential for the 'primary' purpose of growth and reproduction in fungi. Secondary metabolites have important roles, such as helping the fungus to invade plant tissue and as defense against insect predators or competing fungi. Of a vast range of secondary metabolites produced by fungi, only few are regarded as mycotoxins, and these can affect an array of food and feed, especially cereals, forages, grain, fruits, and manufactured products. These compounds when ingested, inhaled, or absorbed through the skin can cause disease or death in humans and domestic animals, including birds. By general agreement this definition excludes the toxins produced by macrofungi (the mushrooms), and compounds that cause disease only in plants or lower animals such as insects.

Development of mycotoxins may occur in almost any food and feed stuff during the growing season, at harvest, or during storage. Their biosynthesis depends on environmental conditions and physicochemical parameters (amount of water activity, temperature, oxygen content, chemical composition and pH of the substrate), but also about the genetic predisposition of the strain molds to form secondary metabolites on a favorable substrate. The optimum temperature for the production of most mycotoxins is between 20°C and 30°C and water activity should be above $0.7a_w$. Molds can withstand a wide range of pH, although extremely acidic or basic substrate does not allow their growth (Bennett and Klich, 2003).

Mycotoxins are chemically very different structures with diverse biological effects. In the case of direct contamination, the nutritional material is contaminated with mycotoxigenic molds, while indirect contamination occurs most often through the consumption of meat, milk or eggs of animals that have consumed mycotoxin-contaminated food. Since molds produce

mycotoxins, the only way they can get into food is by contaminating the food with mold. If mold growth is successfully prevented at all steps of food production, the problem of mycotoxin contamination would disappear (Murphy *et al*., 2006).

1.2 MYCOTOXIGENIC FUNGI

The major fungi causing contamination of foods and feeds with mycotoxins are members of the fungal genera *Aspergillus*, *Fusarium*, and *Penicillium* (Alshannaq and Yu, 2017). Traditionally, toxigenic fungi contaminating agricultural grains have been conventionally divided into two groups: those invade seed crops have been described as "field" fungi (e.g., *Fusarium, Alternaria* spp.), which gain access to seeds during plant development, and "storage" fungi (e.g., *Aspergillus* spp., *Penicillium* spp.), which proliferate during storage (Legan, 2000). Currently, this division is not so strict because according to Miller (1995) four types of toxigenic fungi can be distinguished: first group (Bennet, 1987) consists of plant pathogens such as *Fusarium graminearum* and *Alternaria alternata*; second group (Bennet, 2003) includes fungi that grow and produce mycotoxins on senescent or stressed plants, e.g., *F. moniliforme* and *Aspergillus flavus*; third type (Bennet, 2003) includes fungi which initially colonize the plant and increase the feedstock's susceptibility to contamination after harvesting, e.g., *A. flavus*; and the fourth category (Legan, 2000) refers to fungi which can be found on the soil or decaying plant material; they initially appear on the developing kernels in the field and later on proliferate in the storage if conditions permit, e.g., *P. verrucosum* and *A. ochraceus*.

1.3 A MAJOR MYCOTOXIN GROUPS

To date, about four hundred species of mycotoxins have been detected (Cinar and Onbaşı, 2019; Tola and Kebede, 2016; Escrivá *et al.*, 2017). Aflatoxin (AFB), ochratoxin (OTA), zearalenone (ZEA), deoxynivalenol (DON), fumonisin B1 (FB1) and trichothecene (T2) toxins are the key contaminants that have been investigated extensively (Gruber-Dorninger *et al.*, 2019; Alshannaq and Yu, 2017). These mycotoxins can produce nephrotoxic, hepatotoxic, carcinogenic, mutagenic, teratogenic and immunosuppressive effects leading to both acute and chronic disease, liver and kidney damage, cancer or immune suppression (Zain, 2011; Zhu *et al.*, 2017) (Table 1). Cool, wet weather favors *Fusarium* toxins, while hot, humid weather encourages aflatoxin formation (Atanda *et al.*, 2013). The numbers of reports about different mycotoxins strongly suggest that growth of fungi depends on location, climate and conditions which are conducive for fungal growth.

Table 1 - A list of mycotoxins produced by different fungal species found in various food matrices (Cinar and Onbaşı, 2019).

Mycotoxin	Matrix	Toxic effect	Fungal species
Aflatoxin	Peanuts, maize, tree nuts, cottonseed, milk	Hepatotoxicity, cancer, probable immune suppression and childhood stunting reduced growth	*A. flavus* *A. parasiticus*
Ochratoxins	Cereals, coffee, cocoa, wine, beer, grapes, dried fruit	Nephrotoxicity, hepatotoxicity, neurotoxicity, teratogenecity, immunotoxicity	*A. ochraceus* *A. carbonarius* *Penicillum verrucosum*
Fumonisins	Maize	Neurotoxicity, genotoxicity, immunotoxicity, cancer	*Fusarium verticillioides* *F. proliferatum*
Thrichothecens	Grains	Inhibition of protein synthesis, human intestinal upset	*F. graminearum*
Patulin	Apples	Genotoxicity, teratogenicity, cancer	*Penicillum expansum*
Zearalenone	Corn, oats	Hepatotoxicity, genotoxicity, immunotoxicity	*F. graminearum*

1.4 MYCOTOXIGENIC FUNGAL SPECIES IN GRAPES IN THE MEDITERRANEAN AREA

Mycotoxin contamination of grapes and their derived products is an adverse aspect that usually begins in the vineyard. Several fungal species are pathogenic to grapes and are able to infect berries. A number of mycotoxigenic fungal species that are able to biosynthesize mycotoxins can be found in grape berries: *Aspergillus* spp., *Penicillium* spp., *Alternaria* spp., and *Trichothecium roseum* (Hocking *et al.*, 2007). The main mycotoxigenic species and mycotoxins found in grapes in the Mediterranean area have been reported from several studies in the past years and are summarized in Table 2. All the above-mentioned species can be directly or indirectly considered as the casual agents of different types of bunch rots, diseases related to mycotoxin contamination of grapes and grape-derived products.

Table 2 - Mycotoxigenic Fungal Species Occurring in Grapes in the Mediterranean area (Covarelli *et al*, 2015).

Fungal Species	**Main Mycotoxins Detected**	**Grape Products**	**Distribution in the Mediterranean Area**
A. carbonarius	Ochratoxin A (OTA+)	Grapes, wine, raisins	France, Greece, Israel, Italy, Portugal, Tunisia
A. niger	Ochratoxin A (OTA+), fumonisins (B2, B4)	Grapes, wine, raisins	France, Greece, Israel, Italy, Portugal, Spain
A. ochraceus	Ochratoxin A (OTA+)	Grapes	Italy, Portugal, Spain
A. brasiliensis	Ochratoxin A (OTA+)	Grapes	Portugal, Spain
A. ibericus	(OTA-)	Grapes, dried vine, fruit	Portugal, Spain
A. awamori	Ochratoxin A (OTA+ and OTA-), fumonisins (B2, B4)	Grapes	Greece, Israel, Italy, Portugal, Spain
A. tubingensis	Ochratoxin A (OTA+)	Grapes	Italy, Spain
A. aculeatus	Ochratoxin A (OTA+)	Grapes	Italy, Spain
A. japonicus	Ochratoxin A (OTA+),	Grapes	France, Greece, Israel, Italy, Portugal, Spain
A. uvarum	(OTA-)	Grapes	France, Greece, Israel, Italy, Portugal, Spain
A. flavus,	Ochratoxin A (OTA+), aflatoxin (B1),	Grapes, must	Lebanon, Tunisia
Penicillium spp.[a]	OTA, patulin, citrinin	Grapes, wine, grape juice	France, Italy, Portugal, Spain
Alternaria spp.[a]	Various compounds	Grapes	Spain, Tunisia
Trichothecium Roseumb	Trichothecium	Grapes, wine	Portugal
OTA+: OTA producing strains; OTA–: OTA non-producing strains; [a]low fungal occurrence and low mycotoxin risk.			

Most mycotoxins are chemically stable and as a consequence, wines and dried vine fruits produced with contaminated berries pose a serious hazard to human health. Toxigenic fungal species are able to produce different types of mycotoxins. However, the presence of a certain fungus is not always an indicator of a particular mycotoxin, and the absence of visible rot symptoms does not guarantee the absence of contamination.

1.5 OCHRATOXINS IN GRAPE AND GRAPE DERIVATIVES

Several surveys in different European countries, in Morocco, Japan and Australia confirmed the frequent presence of OTA on grape products and wine (Ueno, 1998; Battilani and Pietri 2002).

European countries are divided into Grape Growing Zones depending on their climatic conditions. Zone A covers growing areas in Germany, Luxembourg, and the United Kingdom, whereas zone B includes southern Germany and the north of France. Zone C represents the most southern growing zones in Europe including Italy, Spain, and Greece. Gil-Serna *et al.* (2018) reported that wines produced in northern regions (zones A and B) were significantly less contaminated with OTA than those produced in southern Europe (zone C). This state could be extrapolated to northern and southern regions even within the same country. Pietri *et al.* (2001) found that contamination of OTA significantly increases from north to south Italy, which can be attributed to considerable climatic differences occurring in those latitudes. Similar situation was detected in Greece (Stefanaki *et al*., 2003) possibly due to high humidity and temperature in southern regions of country.

Wine is the second major source of OTA consumption in the EU (10%) after cereals. However it is an important beverage in the world trade in France, Italy and Spain which are known as the main exporters (Mateo *et al.*, 2007). OTA was discovered, isolated and chemically characterized in South Africa in 1965 (Malir *et al*., 2016). The presence of OTA in grape juices and wines was reported for the first time by Zimmerli and Dick (1996) which analyzed 18 wine samples (two white, 10 red and 6 rosè). The highest percentage of positive samples were in red wine, which brought them to a conclusion that red and rosè wine consumption contributes more to the overall intake of OTA than consumption of white wine. Visconti *et al*. (1999) have explored OTAs in commercial wines and wines produced in family wineries (38 red, 8 rosè, 9 white and one dessert) and found that all wines produced in family wineries contained OTA. Other studies have also found that white wine typically contains less OTA than rosé and red and that the OTA concentration depends on the geographical origin of the grapes. The wines from the southern regions are more

contaminated with OTA than those from the northern ones (Ottender and Majerus, 2000; Dachery *et al.*, 2016). Examples of OTA occurrences in three types of wine (red, white, rose) produced in different places are shown in Table 3 (Dachery *et al.*, 2016). Since then, the occurrence of this toxin in wines has been defined in many countries and different types of wine (Gil-Serna *et al.*, 2018).

Table 3 - Occurrence of OTA in wines produced in different places and estimated exposure to this toxin (Dachery *et al.*, 2016).

Contry	**Type of wine**	**Estimated exposure (ng-kg^{-1})**
Brazil	Red	< 0.04 - 11.25
	White	< 0.07
China	Red	< 0.07 - 14.12
	White	< 0.07 - 0.17
	Rose	< 0.07- 0.55
Spain	Red	0.001 - 0.23
	Rose	0.017 - 0.22
Italy	Red	< 0.18 - 2.35
	White	0.025 - 2.42
	Rose	< 0.025 - 2.88
France	Red	< 0.025 - 0.6
Portugal	Red	2.50 – 6.00
	White	2.50- 3.08
	Rose	0.47-6.30
Greece	Red	< 0.025- 1.78

1.6 OCHRATOXIN A

1.6.1 Chemical characteristics of OTA

The family of ochratoxins consists of three members, ochratoxin A (OTA), ochratoxin B (OTB, non-chlorinated form of OTA) and ochratoxin C (OTC, an ethyl ester of OTA). However, OTA, carbonyl phenylalanine, is the most abundant and hence the most commonly detected member but is also the most toxic of the three (Mine *et al.*, 2008; Heussner and Bingle, 2015). According to International Union of Pure and Applied Chemistry (IUPAC), the

name OTA is L-phenylalanine-N - ((5-chloro-3, 4-dihydro-8-hydroxy-3-methyl-1-oxoH-2-benzopyran-7-yl) carbonyl) - (R) – isocoumarin (Fig. 1). Chemically, OTA is composed of an isocoumarin polyketide and the (modified) amino acid phenylalanine, which are linked via a peptide bond. Fungal polyketide synthases (PKSs) have been demonstrated recently to be involved in OTA biosynthesis in both *Penicillium* and *Aspergillus* species (El Khoury and Atoui, 2010).

OTA is a colorless crystalline compound soluble in polar organic solvent i.e. alcohols, chloroform and ketones, but slightly soluble in H_2O, and insoluble in petroleum ethers and saturated hydrocarbons. Under UV light it gives intense green fluorescence in acidic medium and blue fluorescence in alkaline conditions. Moreover, it is a moderately stable molecule which is able to endure acidic environments and remains unchanged in most nutritional processes such as roasting, heating, salting, baking, and cooking (Varga *et al*., 2010; Alshannaq and Yu, 2017; Hussein and Brasel, 2001; El Khoury and Atoui, 2010).

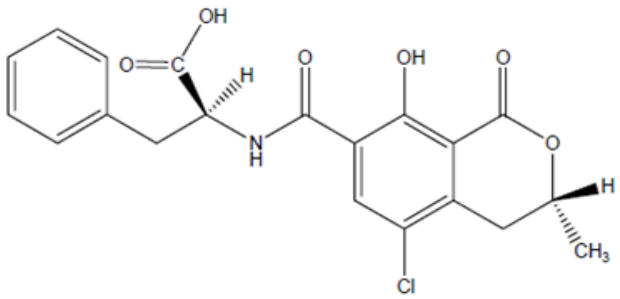


Figure 1 - Chemical structure of OTA (Khoury and Atoui, 2010)

1.6.2 Biosynthesis

The production of OTA depends on the genetic traits and physiology of the mold, which is influenced by different factors such as temperature, water activity and composition of the nutrient medium. OTA has been found in wide variety of agricultural commodities such as corn, wheat, barley, flour, coffee, rice, oats, rye, beans, peas and are notably present in wine, grape juice and dried vine fruits (Alshannaq and Yu, 2017). It is generally assumed that *P. verrucosum* and *A. westerdijkiae* are the major producers of OTA in cereals and stored foods, respectively, while the source of OTA in wine, grapes, and dried vine fruits are some species from *Aspergillus* section *Nigri*, commonly known as black *aspergilli* (Castellá and Cabañes, 2011).

There is limited information about the genes involved in the OTA biosynthesis, but it is known that a polyketide synthase (PKS) is required in the ochratoxin production (Castellá and

Cabañes, 2011). At this regard, several theories have tried to explain OTA biosynthesis. Huff and Hamilton (1979) hypothesized that mullein catalyzed by PKS was oxidized to OTβ and then transformed to OTα by a halogenase/chloroperoxidase. Subsequently, OTα was esterified to ochratoxin C via link with the ethyl ester, and finally biosynthesized to OTA by a desertification reaction.

Harris and Mantle (2001) suggested that one, possibly dominant, biosynthetic pathway involves the passage from OTβ to OTα and then to OTA, with a chlorinating step prior to the ligation of the polyketide (OTα) to phenylalanine, without OTC and phenylalanine ethyl ester intermediates. Since they could not rule out the role of OTB, they also maintain a possible alternative pathway in which the formation of OTA goes through the synthesis from OTβ to OTB, but in this case, a biosynthetic role for OTα, which occurs naturally, could not be explained. The hypothetical pathways for OTA biosynthesis are summarized in Fig. 2.

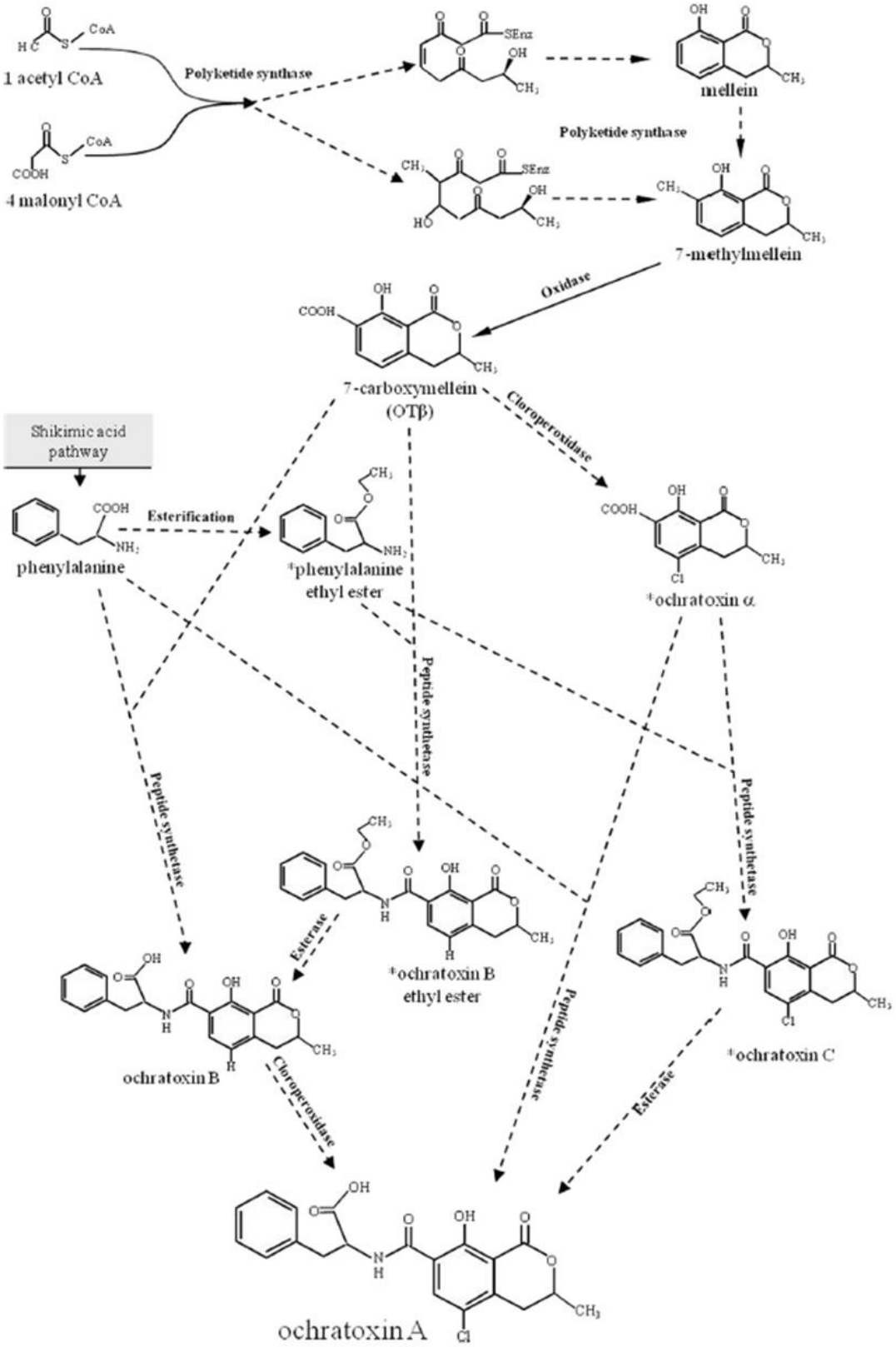


Figure 2 - Scheme showing all the different hypotheses of the OTA biosynthesis pathway (Gallo *et al.*, 2012).

1.6.3 Health aspects

Ochratoxin A is a fungal metabolite which has been cited numerous times for its deleterious effects on both human and animal kidneys, immune system and has also been tragically involved in teratogenesis and carcinogenesis (Battilani and Camardo, 2015). It is considered to play a major role in Balkan endemic nephropathy (BEN) and chronic interstitial nephropathy (CIN) (Abid *et al*., 2003). BEN is a chronic tubulointerstitial disease which slowly progress into terminal renal failure. At the beginning, the BEN disease is characterized by a modification to epithelial cells without any change in the size of the organ. After chronic exposure, kidneys are reduced and interstitial fibrosis is the most important picture. At the end stage, impairment of renal function leads to enzymuria (e.g., gamma glutamyl transferase, alkaline phosphatase, lactate dehydrogenase), polyuria accompanied with red tongue, thirst, and bitter taste. Other symptoms such as headaches, lumbar pain, asthenia, and anemia (iron deficiency) were recorded. Indeed, a 15 years study confirmed that BEN is correlated with upper urothelial tract cancer. It is classified by the International Agency for Research on Cancer in group 2B, possible human carcinogen (Battilani and Camardo, 2015; Marin *et al.*, 2013; Liew and Mohd-Redzwan, 2018; Malir *et al*., 2016).

New information regarding genotoxicity of OTA (formation of OTA-DNA adducts), its role in oxidative stress, and the identification of epigenetic factors involved in OTA carcinogenesis, should they indeed provide strong evidence that OTA carcinogenicity is mediated by a mechanism that also occurs in humans, could lead to another reclassification of OTA. In the light of recently available data, it does not seem inappropriate to upgrade its carcinogenicity from Group 2B (possibly carcinogenic to humans) to at least Group 2A (probably carcinogenic to humans) or even to Group 1 (carcinogenic to humans) (Malir *et al*., 2016).

The toxicology and human health risks of OTA have been assessed at both European and international levels by the European Commission Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) who have established tolerable intakes of OTA from food (Bui-Klimke and Wu, 2015).

Gil-Serna *et al.* (2018) showed that after cereals, wine and grape juices are the most important sources of OTA in human diet. The contribution from wine is estimated to be 21% of the overall exposure (8.9 ng/kg body weight per week) based on a daily consumption of 240 g, whereas the contribution from grape juice constitutes the 7% of the exposure (3.1 ng/kg body weight per week) based on a daily consumption of 69 g.

Commission Regulation (CE) No. 1881/2006 has set the maximum allowed levels for OTA in wine, whereas the sampling methods have been regulated by Commission Regulation (CE) No. 401/2006. These EU standards were adopted based on the opinion of the Scientific Panel on Contaminants in Food of the European Food Safety Authority. According to this regulation, the maximum tolerable levels of OTA are established at 2 µg/L in wine (including sparkling wine, aromatized wine, aromatized wine-based drinks, and aromatized wine-product cocktails), as well as in grape juice and derivatives that are intended for direct human consumption and 10µg/ kg for dried vine fruits (Gil-Serna *et al.*, 2018; Patelides *et al.*, 2017).

1.6.4 Production of OTA by different *Aspergillus* species

Different *Aspergillus* species have the ability to produce OTA. Some *A. niger* aggregate species have been traditionally considered as important OTA A producers in foodstuffs, mainly in grapes and grape products (Gil-Serna *et al*., 2019). However, their ability to produce this toxin seems to be limited to a small number of strains. Recently, the complete cluster of genes involved in OTA production has been described in *A. niger* aggregate species (Susca *et al.*, 2016; Gil-Serna *et al.*, 2018). This region is formed by five genes encoding a halogenase (HAL), a bZIP transcription factor (bZIP), a cytochrome p450 monooxygenase (P450), a non-ribosomal peptide synthetase (NRPS) and a polyketide synthase (PKS). Several molecular studies reported that many *A. niger* aggregate isolates present a deletion of most of the OTA biosynthetic genes remaining only a small non-functional part of the polyketide synthase encoding one. The loss of OTA production seems to be related to the presence of that deletion in fungal genomes (Susca *et al.*, 2016; Gil-Serna *et al*., 2018). The following table list presents the results of *Aspergillus* species producing OTA.

Table 4 - *Aspergillus* species producing OTA (Garcia Cela, 2014).

N°	**Species**	**OTA**	**N°**	**Species**	**OTA**
1.	*A. auricomus*	-	21.	*A. aculeatus*	-
2.	*A. bridgeri*	-	22.	*A. awamori*	+
3.	*A. cretensis*	+	23.	*A. brasiliensis*	-
4.	*A. elegans*	-	24.	*A. carbonarius*	+/-
5.	*A. flocculosus*	+	25.	*A. costaricaensis*	-
6.	*A. insulicola*	-	26.	*A. ellipticus*	-
7.	*A. melleus*	-*	27.	*A. foetidus*	-
8.	*A. neobridgeri*	-	28.	*A. heteromorphus*	-
9.	*A. ochraceus*	+/-	29.	*A. homomorphus*	-
10.	*A. ostianus*	-*	30.	*A. ibericus*	-
11.	*A. perseii*	-*	31.	*A. japonicas*	-
12.	*A. petrakii*	-*	32.	*A. lactoffeatus*	+
13.	*A. pseudoelegans*	+	33.	*A. niger*	+/-
14.	*A. roseoglobulosus*	+	34.	*A. piperis*	-
15.	*A. sclerotium*	+/-	35.	*A. scleroticarbonarius*	-
16.	*A. steynii*	+	36.	*A. sclerotiniger*	+
17.	*A. sulphurous*	+	37.	*A. tubingensis*	+/-
18.	*A. westerdijkiae*	+	38.	*A. uvarum*	-
19.	*A. acidus*	-	39	*A. vadensis*	-
20.	*A. aculeantinus*	-			

+ = production; - = no production; * = produce trace amounts

1.6.5 Molecular tools for *Aspergillus* spp. identification

To accurately identify *Aspergillus* spp. to species level, both morphological and molecular identification are applied. Morphological characteristics may not be sufficient as the microscopic and macroscopic characteristics for some species are similar within the black *aspergilli* species. Several genes were analyzed for *Aspergillus* species identification based on universal barcode of fungi. Currently, molecular-based species identification methods are considered reliable for fungal identification, and the internal transcribed spacer (ITS) and Domain 1/Domain 2 (D1/D2) regions of the ribosomal RNA large subunit gene are widely

used as the target sequences for identifying *Aspergillus* species. The *Aspergillus* genus is composed of various closely related species, and sequence analyses of β-tubulin and calmodulin genes are used to identify species in this genus (Samson *et al.*, 2014; Hagiwara *et al.*, 2019). An extensive number of endpoint and real-time PCR assays have been established during the last two decades in order to detect and quantify these species in different food commodities and predominantly in table and wine grapes (Xanthopoulou *et al.*, 2019). A considerable number of endpoint and real-time PCR assays, either simplex or multiplex, have been developed able to detect *Aspergillus* spp. isolated from several crops according to genes involved in the ochratoxin pathway as Polyketide synthase, cytochrome P450, and fumonisins (Atoui *et al.*, 2007; Castellá and Cabañes, 2011; Ferracin *et al*, 2012; Kizis *et al*, 2015; Susca *et al*, 2016).

Over the last few years, there have been intensive researches aimed at developing molecular tools. Among these a rapid and highly accurate molecular method for species identification, high-resolution melting (HRM) analysis is extremely effective. HRM has significant advantages over conventional methods, as it is carried out in a closed tube and represents a very rapid and cost-effective gene-scanning method, capable of detecting genetic variation in DNA sequences by measuring changes in the fluorescence level of a melting DNA amplicon (Landi *et al.*, 2019). The method is specifically able to genotype and discriminate species, based on even single-nucleotide polymorphisms (SNPs) and small insertion/deletions, through the analysis of the melting behaviors of double-stranded DNA (dsDNA). HRM technique was already utilized for DNA genotyping of fungal genera and species (Sanzani *et al.*, 2013; Garganese *et al.*, 2018) included *Aspergillus* spp. from pomegranate (Mincuzzi *et al.*, 2020) and grape (Xanthopoulou *et al.*, 2019).

1.7 AIM OF THE WORK

Grapes and their derivatives (grape juice, raisin and wine) are important foodstuff for the human diet, while wine is considered an income commodity. Several diseases can affect grapes during the growing season, causing quantitative and qualitative yield losses, but the menace to human health is actually only associated with fungi belonging to *Aspergillus* section *Nigri* (commonly called black *aspergilli*) and their possible ochratoxin A (OTA) production. These fungi colonize grape berries from setting, with increasing incidence moving towards ripening. Among the species isolated in grapes, those belonging to *A. niger* aggregate (i.e., *A. niger* and *A. tubingensis*) dominate but *A. carbonarius* is confirmed as the key one responsible for OTA contamination. The microscopic and macroscopic characteristics of black *Aspergillus* are indistinguishable and morphological identification of *Aspergillus* species is not sufficient. The aim of this study was to develop a molecular method able to identify *Aspergillus* spp. commonly found in grapes, and to examine their possibility for producing OTA. We have used PCR-HRM analysis to accomplish our aim of identification, since it is a very rapid and cost-effective gene-scanning method and it has been applied successfully to fungal genotyping.

2 MATERIALS AND METHODS

2.1 SAMPLES COLLECTION

With the aim to identify the *Aspergillus* spp. able to produce OTA, fungal samples from grape cultivar 'Verdicchio' were collected in the 2018 year from the vineyard located in Castelplanio (AN), in the central Italy Marche region 43°30'10''N - 13°05'42''E,

Figure 3 - Castelplanio (AN) vineyard, Marche Region

2.2 FUNGAL ISOLATION AND CHARACTERIZATION

From each cluster, 2 berries showing damage around the petiole were selected. Each of these were separated by sterile tweezers and placed on Petri dish containing Potato Dextrose Agar (PDA). Petri dishes were incubated in a thermostat at 28° C and 95% humidity and examined after 48 hours. The newly formed colonies of fungi were transplanted into PDA culture media and incubated under the same conditions to isolate pure culture. Pure fungal cultures were incubated for 14 days in a thermostat at 28° C and 95% RH. Each fungal isolate was stored at 4° C.

After seven days of incubation, plates were observed for both, macroscopic and microscopic characteristics. According to Samson *et al.*, (2007) were examined colony characteristics, conidiophore and conidia, using Nikon System P101S photometer (Nikon).

2.3 DNA EXTRACTION

From each fungal isolate the DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1990) modified by Landi *et al.* (2019). In detail, from each sample, at 14 day grown PDA culture, 100 mg of mycelium was collected and put in the 2-mL micro centrifuge tubes. The cold nitrogen was used with the aim to grind the mycelium. After this phase, 1 mL extraction buffer (3% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% [w/v] soluble PVP-40), and 1% (w/v) metabisulphite was added. The tubes were mixed and incubated at 65°C for 30 min. After incubation one volume of chloroform/ isoamyl alcohol (24:1), was added and mixed for 2 min. The DNA was separated from the chloroform solution using the centrifuge at 8,000 rpm for 10 min. The supernatant was collected and put in the new tube. After this the precipitation phase was performed and 0.6% isopropanol was added to the solutions. Later the DNA precipitation was obtained using the centrifuge at 14,800 rpm for 20 min. The DNA pellet obtained was washed adding 70% of cold ethanol and centrifuged for 5 min. The DNA was drayed at room temperature for eliminate the alcohol, and it was dissolved in 50 µL of ultrapure water. The DNA purity and quantity were determined (BioPhotometer plus; Eppendorf Inc., Westbury, NY, USA). For the samples the quantity ranged from 50 to 100 ng/µL DNA while the absorption ratios at 280/260 ranged from 1.6-1.8, and at 260/230 from 1.3-2.0. The DNA was extracted two times from two sub-cultured.

2.4 PRIMER SELECTIONS AND VALIDATION

For the *Aspergillus* spp. analysis specific primers were selected according to β-tubulin gene and several genes included in the OTA metabolic pathway as the Polyketide Synthase (PKS). The gene sequences related to these proteins were selected from the National Center for Biotechnology Information (NCBI). The primers were designated using Primer3web version 4.1.0. (http://bioinfo.ut.ee/primer3-0.4.0/). The species specificity was verified *in silico* using primer BLAST software (http://www.ncbi.nlm.nih.gov/Blast.cgi). Primer-BLAST was designed to make primers that are specific to an input PCR.

2.5 QPCR-HIGH RESOLUTION MELTING (HRM)

The best quantitative Real time PCR (qPCR) conditions were set-up. The primers were validated to their ability to amplify fungal DNA species using qPCR. Two µL of DNA from each sample was mixed and a serial dilution from 100 ng/µL to 1 ng/µL of total DNA were analyzed according the thermal conditions described below. Both melting profile and

sequencing analysis were performed for the positive samples. In addition, a blank control sample, obtained by inserting the water instead of the DNA, was added.

The final step included the melting curve analyses (0.2 °C step increments; 10s hold before each acquisition), which were analyzed from 70 °C to 95 °C. The qPCR-HRM and nested-qPCR-HRM amplifications were both performed using the CFX real-time PCR detection system and analyzed using the 'High-Resolution Melting analysis software' (Bio-Rad Laboratories). This software automatically clusters the samples according to their melting profiles and assigns confidence scores to each of the samples. The confidence level threshold for a sample to be included in a cluster was 99.0%. All experiments were performed in duplicate and repeated three times.

2.7 SEQUENCING

The specific amplicons obtained using *ANWT* primers related to isolates 4CMa, 7CMa, 2BMa, 3AM, 11DM, 1AMa, 6BMa, 3BMb, and amplicons obtained according to *ANPKS* primers from the isolates 3BMb, 6BMa, were before validated according the melting curve analysis and then were sequenced by Genewiz (Hope End, Takeley, UK) and subjected to bioinformatic analysis. Sequence similarity searches were performed using BLAST analysis in NCBI.

2.8 PHYLOGENETIC ANALYSIS OF QPCR AMPLICONS

The sequences with the highest homology with qPCR amplicons were downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were achieved using Clustal X (Thompson *et al.*, 1997). Phylogenetic trees were constructed using the molecular evolutionary genetics analysis (MEGA) program, version 5.2 (Tamura *et al.*, 2011), according to the neighbor-joining method (Saitou and Nei, 1987), with pairwise deletion with 1,000 bootstrap replicates. The sequence pairs within and among the *Aspergillus* spp. profiles were carried out using the maximum composite likelihood model for the β-tubulin sequences. The average genetic distances among the clades inferred by the phylogenetic analyses were computed according, to the NCBI Blast analysis of the sequences.

2.9 QUALITATIVE END-POINT PCR

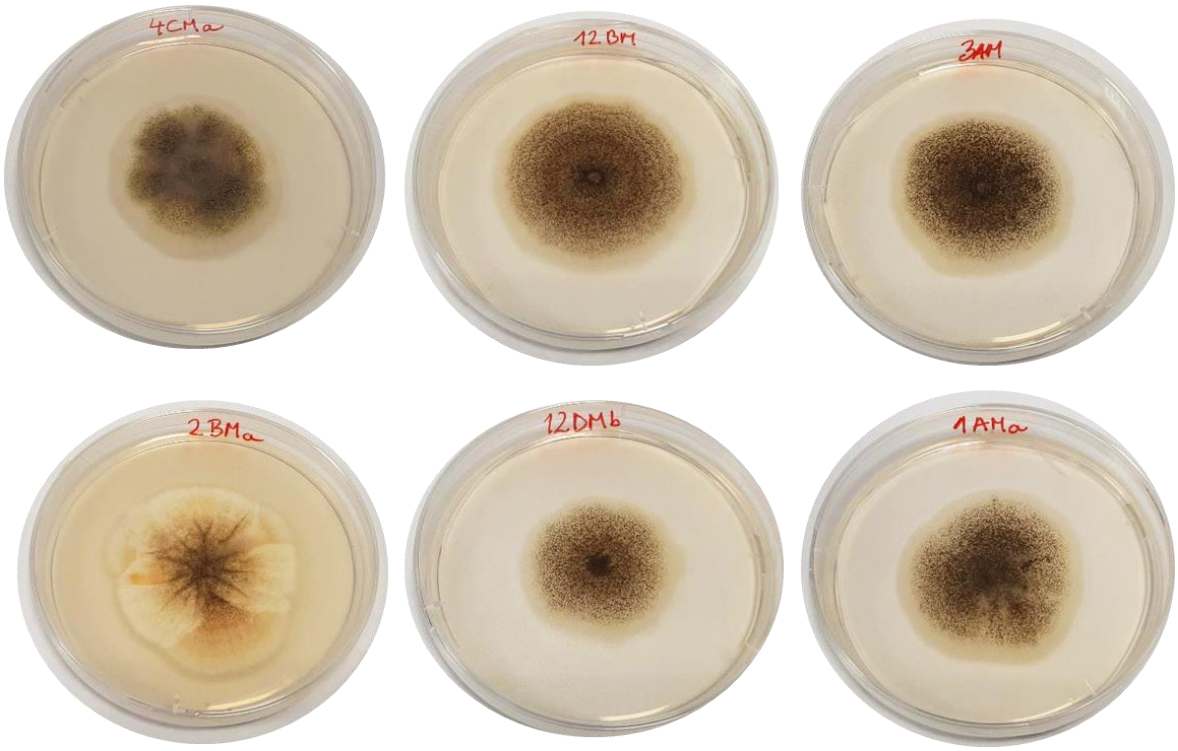
Using the end-point PCR, the DNA extracted from pure colony were tested according the primers OTA1 related to *Aspergillus* spp. (Susca *et al.*, 2016). For the PCR mix, 10-20 ng DNA was included in each 25 µL PCR reaction, with 1 mM of each primer, 15 µL 2× EconoTaq Plus Green Master Mix (Lucigen; Tema Ricerca S.r.l., Castenaso, Bologna, Italy). PCR amplicons were analyzed by electrophoresis in 2 % (w/v) agarose gel.

3 RESULTS

3.1 MORPHOLOGICAL CHARACTERIZATION

Based on morphological characteristics, according to Samson *et al.* (2007), after 7 days of incubation, the isolates were identified as *Aspergillus* spp.

a



b **c**

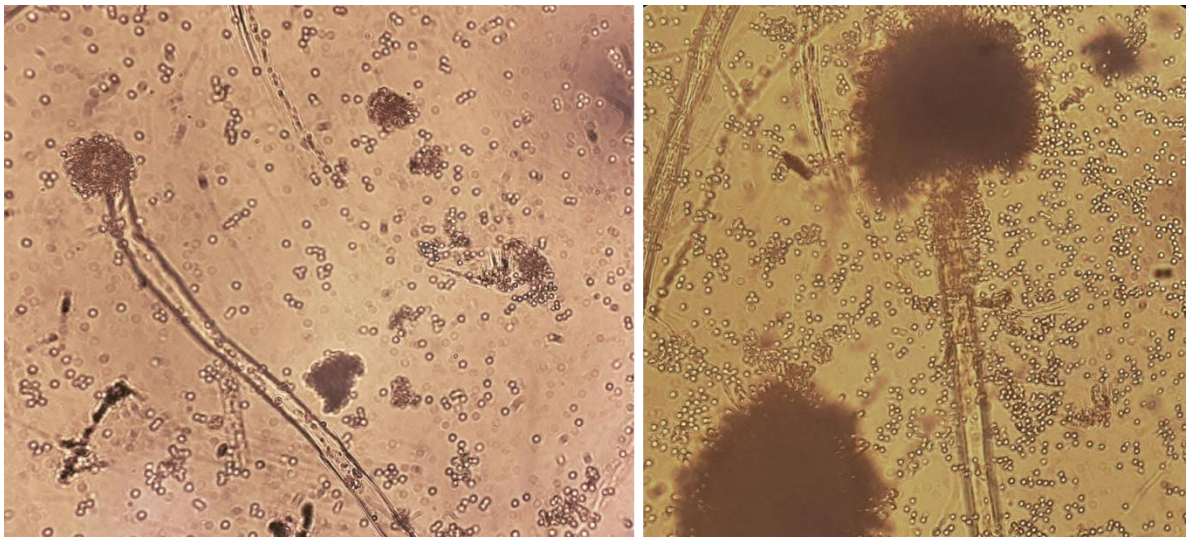


Figure 4 - Fungal feature after 7 days growing on PDA. (**a**) Colony morphology. (**b**) Microscopic characteristics of conidiophores, stipe and conidia of 4CMa isolate and (**c**) 12DMa isolate (×40 objective).

3.2 PRIMERS SELECTIONS

For the qPCR analysis of DNA from fungal isolates, we have selected specific primers for the main *Aspergillus* spp. producing OTA and the primers able to amplify for several *Aspergillus* spp. The list and features of the primers tested in this work were described in table 5. The last NCBI database research was performed in September 2019. Based on this, the primers *ANWT* were able to identify 1,439 sequences related to the part of β-tubulin genes, which belong to the isolates of several *Aspergillus* spp. according to the sequences described in figure 5. In more detail, the same sequence for *A. tubingensis* was detected on 678 isolates, while for *A. niger* 470, for *A. welwitschiae* 138, for *A. luchuensis* 30, for *A. brasilisensis* 66, for *A. awamori* 33 and for *A. neoniger* 24 isolates (Fig. 5).

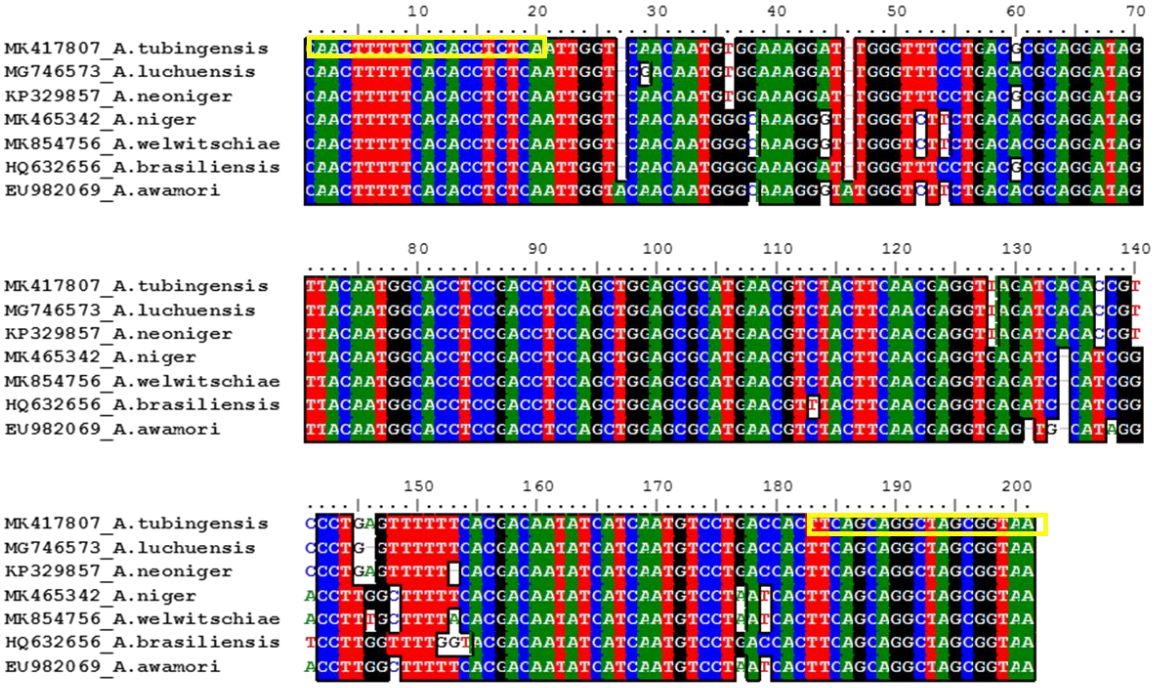


Figure 5 - Multiple sequence alignment of β-tubulin sequences of *Aspergillus* spp. according to *in silico* NCBI analysis. Selected primers *ANWT* for use in the HRM analysis are marked in boxes.

In addition, specific primers related to β-tubulin and PKS genes were selected for the *A. ochraceous, A. carbonarius* and *A. niger* (data not shown).

3.3 qPCR-High Resolution Melting (PCR-HRM)

3.3.1 qPCR validation: melt curve analysis

Among the tested primers, the primers set *ANWT* and *ANPKS* showed specific single peak melting curves, which confirmed the homogeneity and specificity of the amplicons produced in the qPCR for these primers. No amplification was observed in any of the control assays, which confirmed that the samples were free of contamination with other genomic DNA (data not shown). Unspecific melt curves were detected for the other primers tested. The thermic cycle tested the specific melt curve for the primer's pairs *ANWT* at 83.5 °C (Fig. 6a and b) and for the primers *ANPKS* at 84 °C (Fig. 7a and b). The standard curve performed according to 10-fold serial dilution of pooled DNA evidenced for the *ANWT* primers the amplification efficiency of 101.2%, with Threshold cycle (Tc) ranging from 24.8 to 32.1 value (Fig. 6c and d). The standard curve related to *ANPKS* has shown the amplification efficiency of 100.8%, with Tc ranging from 24.8 to 32.1 value (Fig. 7c and d).

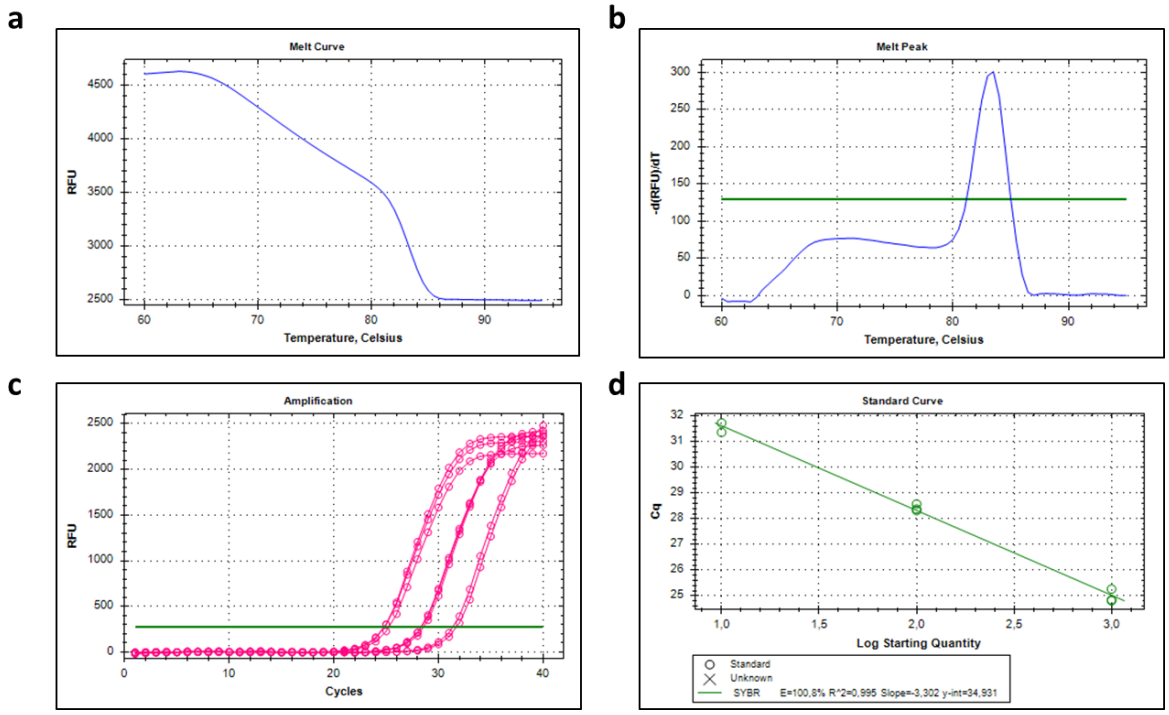


Figure 6 - Specificity of the primers *ANWT* used in HRM-qPCR amplification. (**a**) Melting curves have shown a single peak at 83 °C (**b**). Threshold cycle number (**c**) and standard curve (**d**) obtained from the amplification of a 10-fold serial dilution of a pooled DNA template (from 10 ng to10^{-1}ng). Data represent two replicates of each dilution.

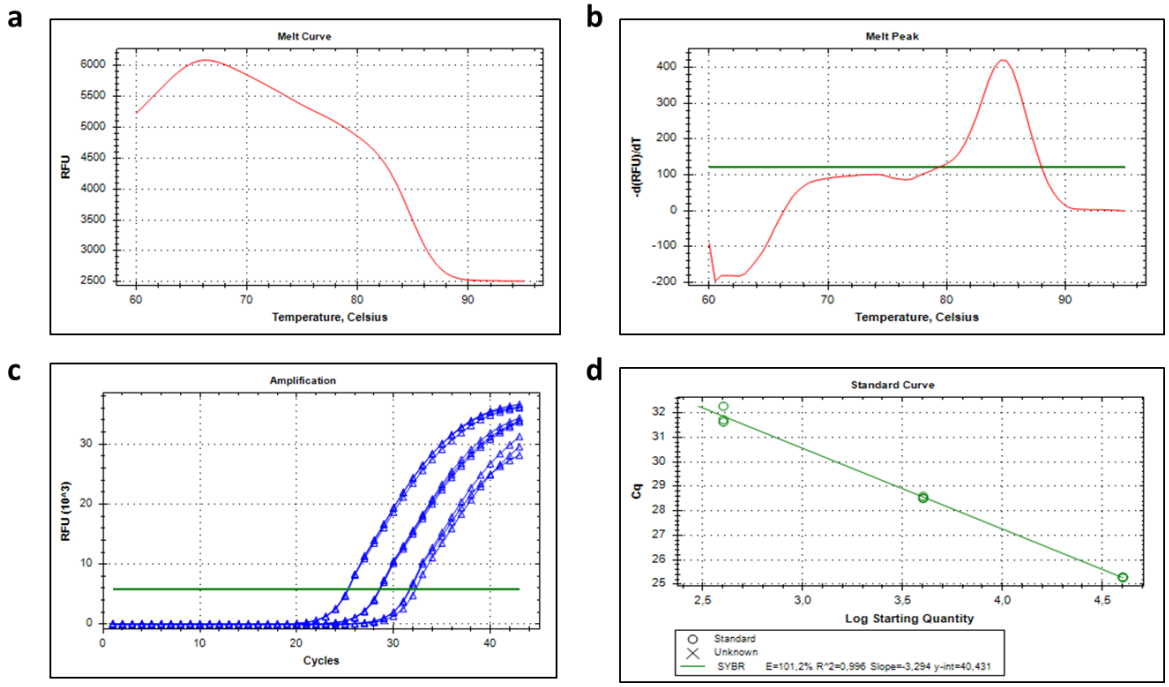


Figure 7 - Specificity of the primers *ANPKS* used in HRM-qPCR amplification. (**a**) Melting curves have shown a single peak at 84.5 °C (**b**). Threshold cycle number (**c**) and standard curve (**d**) obtained from the amplification of a 10-fold serial dilution of a pooled DNA template (from 10 ng to10^{-1} ng). Data represent two replicates of each dilution.

3.3.2 High Resolution Melting (HRM) analysis

The PCR HRM analysis performed at the end of qPCR cycles show that there are no differences within the group in relation to the *ANWT* primers tested (Fig. 8a and b). This demonstrates that all the isolates from grapes analyzed in this work have the same nucleotide sequence.

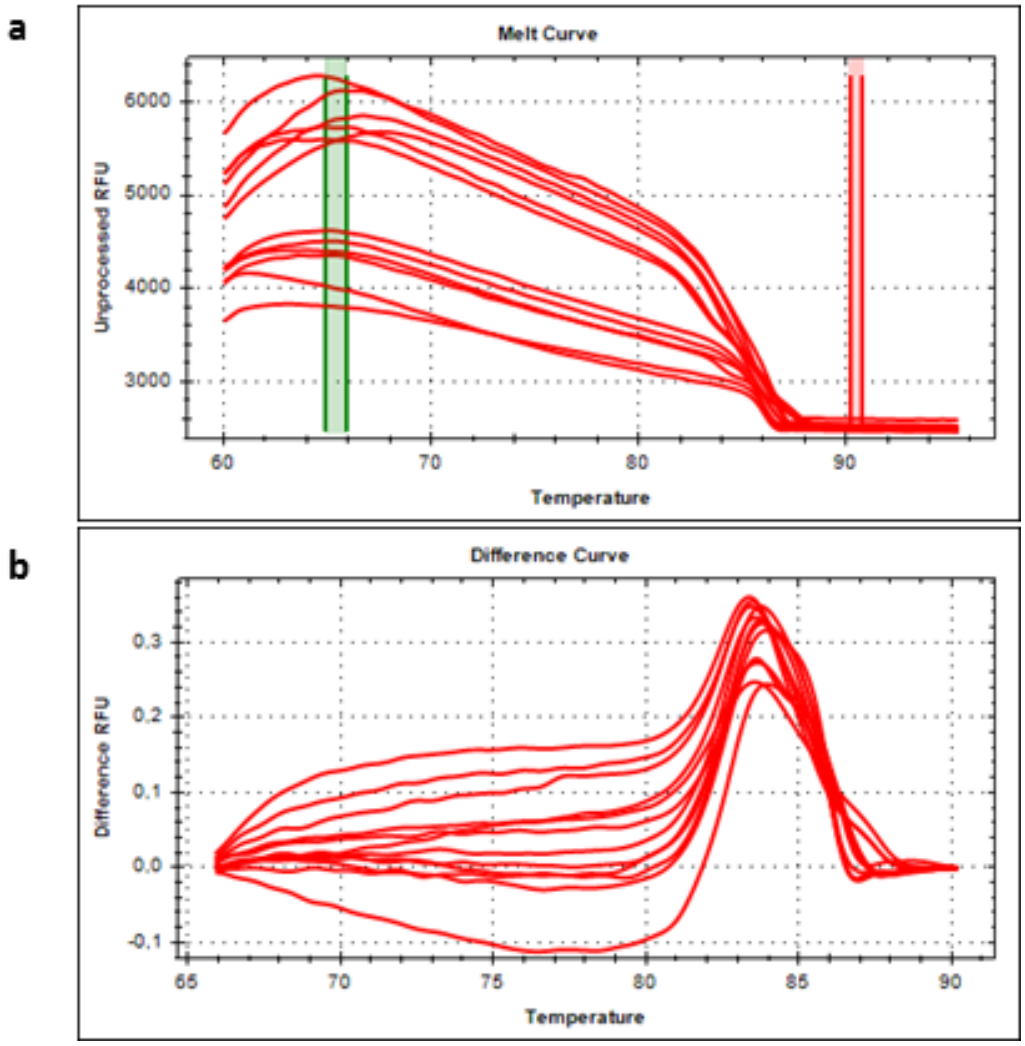


Figure 8 - qPCR-high-resolution melting (HRM) analysis to discriminate between *ANWT*, primers results in the *Aspergillus* spp. isolates. Typical genotyping patterns as normalized melting curves (**a**) and normalized difference plots (**b**) are shown. RFU: relative fluorescence units.

3.4 SEQUENCE ANALYSIS OF THE PCR AMPLICONS

A phylogenetic analysis of the 200 pb amplicons obtained with *ANWT* primers cluster with *A. tubingensis* isolates according to β-tubulin genes (Fig. 9). Sequence similarity searches performed using the BLAST analysis has shown the highest homology of 98.99% with 464 *A. tubingensis* isolates. This analysis has detected that the *Aspergillus* spp. isolates from grapes have TGGGC sequence from nucleotide (nt) 33 to nt 37 position, which corresponds to *A. niger* and *A. welwitschiae*. This is in contrast with the typical *A. tubingensis* sequence, TGTGG from nt 33 to nt 37 position. Starting from nt 38 the isolates form grapes shown *A. tubingensis* nucleotide sequence (Fig.10).

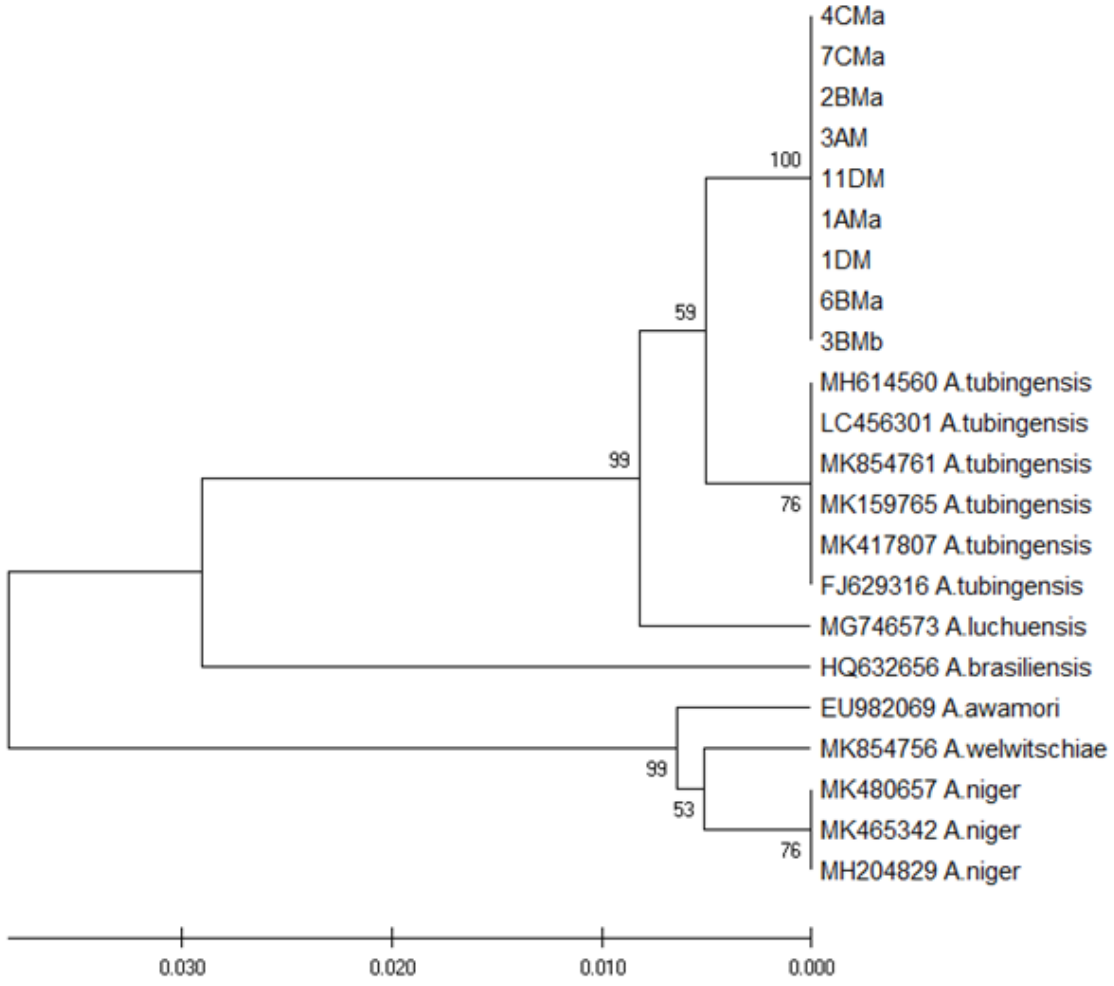


Figure 9 - Phylogenetic tree based on β-tubulin sequence of *Aspergillus* spp. Numbers next to nodes are bootstrap values. The *Aspergillus* spp. isolates collected from grapes, show the relationships with the NCBI β-tubulin sequences selected as references.

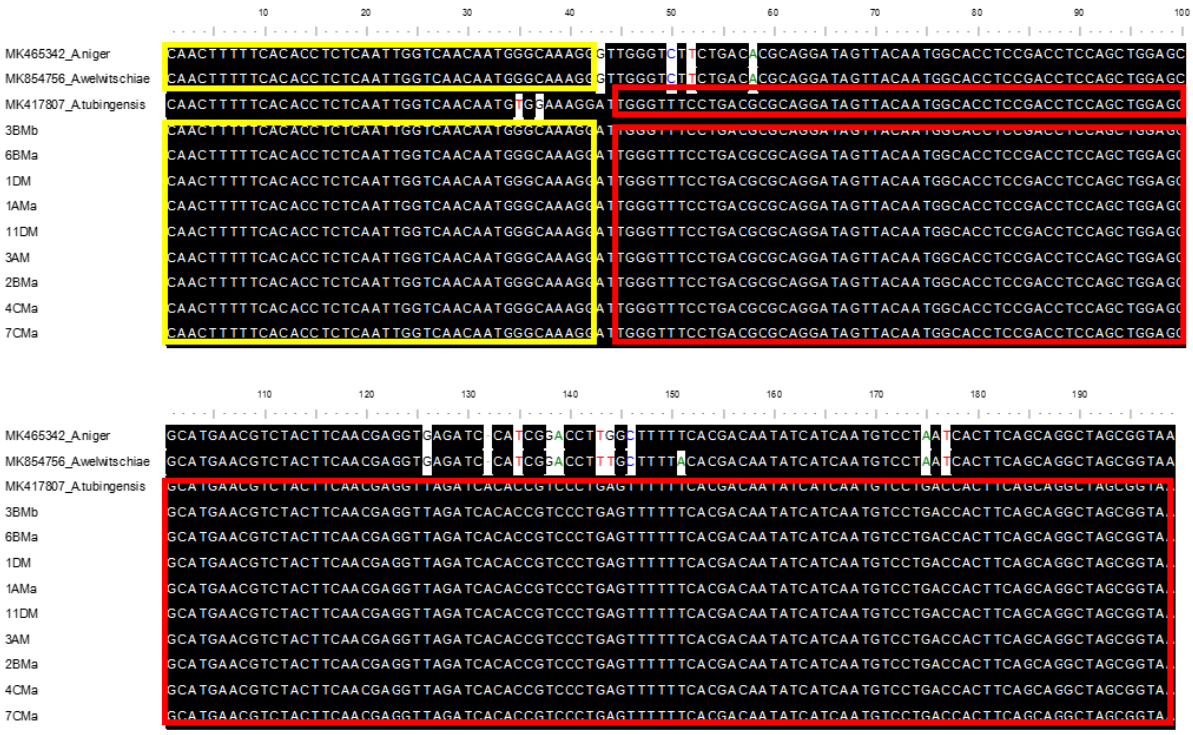


Figure 10 - The alignment of *Aspergillus* spp. isolates from grapes with *A. niger*, *A. welwitschiae* and *A tubingensis.* Part of β-tubulin sequence was analyzed in this work.

The amplicons obtained with *ANPKS* primers have shown highest homology of 97,60% with gene related to hypothetical proteins of *A. niger* isolates XM025678849 and XM025625364. Insignificant homology was detected with the NCBI N° HM011537 used for primers draw and related to gene Polyketide synthase gene (Fig. 11).

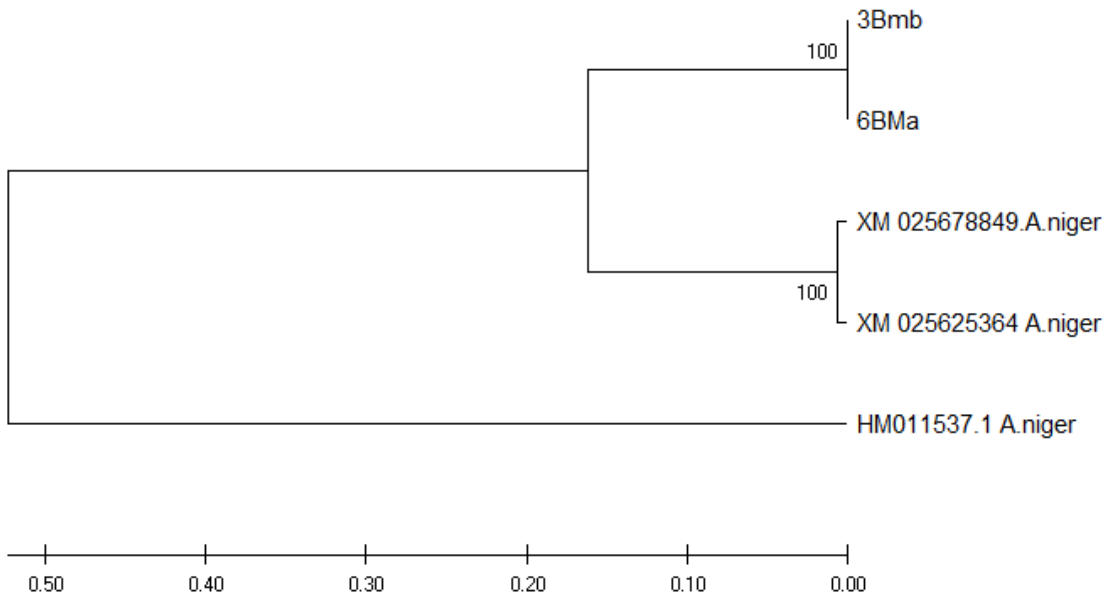


Figure 11 - Phylogenetic tree of specific qPCR amplicons obtained with *ANPKS* primers correlated with NCBI *Aspergillus* isolates.

3.6 *ASPERGILLUS* SPP. IDENTIFICATION

The molecular investigation using qPCR technology has evidenced a positive result for 18 isolates analyzed with *ANWT* primers (Table 5). In addition, the HRM analysis performed on qPCR amplicons confirm that they have the same nucleotide sequence. The preliminary analysis performed according to degenerate primers related to several OTA cluster genes, OTA1 genes, have shown a positive PCR results according to OTA 1 for the isolates (3BMb, 2BMa, 4CMa, 7CMa, 1DMa, 1AMa, 11DM, 3AM, 12BM) with the expected size of 776 bp amplicon.

Table 5 - Molecular investigation of *Aspergillus* spp. isolates from grape collected in the vineyard located in Castelplanio (AN), Italy. The table show the qPCR investigation performed using *ANWT* primers related to β-tubulin genes and ocratoxin A investigation performed using the OTA 1 primers (Susca *et al.,* 2016) using end-point PCR method.
qPCR Data are means ± standard deviation. *Cq* = quantification cycle.

Samples	ANWT	OTA 1
3BMb	27.4 ± 0.5	+
2BMa	29.6 ± 0.2	+
6BMa	31.4 ± 0.9	
9DMa	28.1 ± 1.5	
4AMa	32.1 ± 0.3	
10CMb	29.6 ± 0.5	
7AMa	33.1 ± 0.9	
5AMb	26.9 ± 1.1	
4CMa	29.3 ± 0.4	+
8BMa	31.1 ± 0.3	
7CMa	32.4 ± 0.7	+
8AMa	28.4 ± 0.5	
1DMa	26.2 ± 0.3	+
9CMa		
12DMb	32.8 ± 0.9	
1AMa	29.5 ± 0.8	+
11DM	29.4 ± 0.7	+
3AM	31.3 ± 0.4	+
12BM	32.4 ± 0.6	+

4 DISCUSSION AND CONCLUSIONS

Grape (*Vitis vinifera* L.) is one of the economically most important fruit commodities (Jiang *et al*., 2014). Several diseases can affect grapes during the growing season causing quantitative and qualitative yield losses (Prusky, 2011; Abeer *et al.*, 2013), but the menace to human health is actually only associated with fungi belonging to *Aspergillus* section *Nigri* (commonly called black *aspergilli*) and their possible OTA production.

With the aim to estimate the occurrence of OTA in grapes, a preliminary study was performed using grape barries collected from cultivar Verdicchio in the vineyard of the Marche region. In this work, based on morphological and conidia characteristics, black *Aspergillus* spp. was identified. However, this has proven to be difficult (Mirhendi *et al*., 2016). Subsequently we performed molecular identification, so a set of primers specific for β-tubulin and PKSs genes related to the main species involved in the grape OTA production of *Aspergillus* section *nigri* (Wang *et al*., 2013) were tested.

Many similar previous investigations have shown the domination of the *A. niger* aggregate (*i.e., A. niger* and *A. tubingensis*) in the vineyard (Battilani and Silva, 2010; Barberis *et al*., 2014), while the *A. carbonarius,* which is considered the main source of OTA in grapes, was found mostly in southern Italy (Lucchetta *et al*., 2010).

The genes which are most commonly used for the purpose of the identification of *Aspergillus* spp. are β-tubulin and calmodulin (Geiser *et al*., 2007; Kizis *et al*., 2014) and they were used. In addition, β-tubulin is also recommended as secondary identification marker for *Aspergillus* and used mainly for phylogenetic analysis for species confirmation (Samson *et al.*, 2014).

In this work, a new approach involving HRM investigation of PCR fragments was set up according the β- tubulin analysis. PCR-HRM can be used for detection of genetic variants (Landi *et al.*, 2019). The assay characterizes amplified PCR products according to their dissociation behavior without requiring additional instrumentation. A fluorescently labeled dye was bound to double stranded DNA. When increasing the temperature, the double stranded DNA dissociated into single strands leading to decrease in fluorescence intensity. The melting temperature depends on GC content, length and nucleotide sequence. HRM method is easy to perform and it yields the results within the two hours. PCR-HRM technology was used in previous works for the identification of *A. versicolor*, *A. creber* and *A. sydowii* (Libert *et al.*, 2017) or clinical isolates of several *Aspergillus* spp. (Fidler *et al*., 2017). Recently a similar approach was suggested for *A. carbonarius, A. tubingensis, A.*

niger, *A. ibericus* and *A. japonicus* discrimination in grape samples artificially inoculated with conidia of these fungi according to ITS2 barcode gene (Xanthopoulou *et al*., 2019). This method was also used for *Penicillium sensu strictu* and *Aspergillus* sect. *nigri* population discrimination from pomegranate fruit using β- tubulin gene (Mincuzzi *et al.*, 2020).

In our work, this technology has provided useful information about *Aspergillus* spp. identification. These universal primers were selected according to β-tubulin genes, including specific genomic variants related to several *Aspergillus* spp. as *A. tubingensis, A. niger, A. welwitschiae, A. luchuensis, A. awamori, A. brasieliensis,* and *A. neoniger*. Each of these species shows complete homology with the sequences described by the *ANWT* primers. In particular, 670 isolates belong to *A. tubingensis*, 470 isolates correspond to *A. niger* and 138 associates with *A. welwitschiae*. Only few isolates show small variations within the sequences observed (2 isolates for *A. tubingensis* and 1 for *A. niger*).

We have tested these pair primers in 19 *Aspergillus* spp. isolates from grapes. According to PCR-HRM analysis, all positive PCR amplicons were grouped in the same cluster suggesting a similar nucleotide sequence for all DNA amplified samples. The sequence analysis performed in order to verify the nucleotide sequence of qPCR amplicons, have showed that all isolates had had the same identical nucleotide sequence. BLAST and phylogenetic analysis displayed that our isolates are close to *A. tubingensis*. However, the homology with the *A. tubingensis* was not 100%. Interestingly, it was observed that all isolates from grape had a typical sequence at position 5', nt 33 to nt 37 (TGGGC instead of TGTGG typical of *A. tubingensis*) which is typical of all isolates in NCBI classified as *A. niger* and *A. welwitschiae*, while the rest of the sequence is homologous to *A. tubingensis*. It is known that *A. tubingensis*, *A. niger* and *A. welwitschiae* belong to *Aspergillus* section *Nigri* and that due to their similar morphologic features they are called *A. niger* aggregate (Parenicova *et al*., 2001). Nevertheless, these are not the only species within the *Aspergillus* section *nigri*, since this group also contains many other biseriate species. To date, this group encompasses 15 morphologically indistinguishable species including *A. tubingensis, A. niger* and the recently described *A. welwitschiae* together with *A. acidus, A. brasiliensis, A. coreanus, A. costaricaensis, A. eucalypticola, A. foetidus, A. kawachii, A. lacticoffeatus, A. luchuensis, A. neoniger, A. piperis* and *A. vadensis* (Perrone *et al.*, 2011; Varga *et al*., 2011; Hong *et al.*, 2013). Our investigation highlights this close relationship among *A. tubingensis A. niger* and *A. welwitschiae* species. This observation was confirmed in several investigations (Battilani and Silva, 2015). The nucleotide modification detected of β-tubulin sequence could be linked to genomic cross recombination among close species involving *A.*

tubingensis and *A. niger* or *A. welwitschiae* species. Biological implications of this observation should be investigated. However, for the accurate species identification, a MultiLocus Sequences analyses could be also suggested (López-Hermoso *et al*., 2017).

Regarding the OTA production, different genes were noticed among the *Aspergillus* aggregate species (Gil-Serna *et al*., 2019). Among these PKS is polyketide important for plant pathogenicity, and it has been clearly demonstrated to be involved in OTA biosynthesis in both *Penicillium* and *Aspergillus* species (El Khoury and Atoui, 2010). Several studies have shown the ability of *A. tubingensis* to produce less toxic ochratoxin α (Cho *et al*., 2016). Recently a lack of OTA cluster in *A. tubingensis* isolates was observed (Gil-Serna *et al*., 2019). Currently our study has not considered the analysis of PKS specific genes related to *A. tubingensis*, but we have not observed PCR amplifications according to specific primers for PKS genes selected for *A. carbonarius* and *A. ochraceus*, while the primers for the PKS related to *A. niger* species amplified for qPCR specific amplicons. However, the sequence analysis was not able to identify PKS genes, but it managed to identify hypothetical proteins close to *A. niger*. This could suggest high heterogeneity of polyketides important for plant pathogenicity. Polyketides are structurally diverse group of secondary metabolites with several functions important for ecological and evolutionary adaptation of fungi (Fatema *et al*., 2018). Regarding *A.niger*, it was reported that out of the 14,165 open reading frames predicted in the *A. niger* genome only 2% have been experimentally verified and over 6,000 are hypothetical (Schäpe *et al*., 2018). So, although it is not excluded that the amplified sequence has a relationship with PKS genes, different approach must be used for the PKS investigation according to qPCR analysis.

The preliminary investigation related to other primers set-up to OTA genes (Susca *et al*., 2016) has shown a positive PCR only for some isolates. Several works have already detected variations in the presence of genomic clusters associated with the isolates of the same species which produce ochratoxin. Climatic conditions and geographical location are important factors favoring OTA accumulation in grape berries (Spadaro *et al*., 2012; Battilani and Silva, 2015; Gil-Serna *et al*., 2019). Further specific investigations are needed to clarify which and how many OTAs are produced by these isolates.

Our investigation has identified *A. tubingensis* in the vineyards of Verdicchio cultivar in the Marche region. The discovery of cross mutation within the β-tubulin gene among *Aspergillus* section *Nigri* species could be the focus of the future investigations on this topic, so that further clarifications of this scientific observation can be pursued. The HRM method used in this work can be recommended as a rapid method for screening *Aspergillus* section

Nigri occurring in grapes. It is highly reliable and easy to use, so it can be suggested as a 'gold standard' in the understanding of epidemiology and distribution of OTA producing *Aspergilli* in grapes, since it can screen a large amount of isolates in a short time.

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