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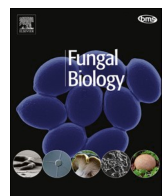

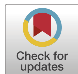

Monitoring fungi and mycotoxin potential in pistachio nuts of Turkish origin: A snap-shot for climate change scenario

Pamela Anelli [a,1], Miriam Haidukowski [a,1], Massimo Ferrara [a], Asli Kisikkaya [b], Ceyda Pembeci [b], Hayrettin Ozer [b], Giuseppina Mulè [a], Martina Loi [a], Antonio Moretti [a], Antonia Susca [a,*]

[a] *Institute of Sciences of Food Production, National Research Council, via Amendola, 122/O – 70126 Bari, Italy*
[b] *TUBITAK MRC Life Sciences, Barış Mah. Dr. Zeki Acar Cad. No:1 P.K. 21, 41470, Gebze Kocaeli, Turkey*

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A B S T R A C T

Pistachio (*Pistacia vera* L.) is an economically important tree nut. Due to its nutritional properties and health benefits, it is considered a healthy food and thus widely consumed worldwide. However, fungal contamination of the commodities has received considerable attention because of possible contamination by toxigenic fungi, important source of mycotoxins, resulting from secondary metabolism and hazards to health consumer. Members of the genus *Aspergillus*, mainly *Aspergillus flavus* and *Aspergillus niger*, are reported as occurring most frequently on pistachio nuts, because able to grow in the presence of low amounts of water and to produce mycotoxins (aflatoxins and ochratoxins), that are well known for their harmful health effects on humans.

Monitoring the contaminating fungal species is particularly worthy of note also in climate change scenario, allowing to notice changes in fungal population composition through the time.

This study aimed to contribute to collect data about fungal population and mycotoxins occurred in pistachio samples collected in Turkey: prevalence of 2 species, *A. flavus* and *Aspergillus tubingensis,* was assessed. The *A. flavus* strains consisted of a mixed population of aflatoxin producers and non-producing strains *in vitro,* with evidence of a new genotype in gene cluster within strains of aflatoxin non-producing chemotype.

1. Introduction

Pistachio (*Pistacia vera* L.) is a member of the Anacardiaceae family and is an important tree nut consumed worldwide. Iran, USA, Turkey, China, and Syria are the main pistachio growing countries. According to FAO data, the world's annual pistachio production amounts were about $9 \bullet 10^8$ kg in 2019 and $1.1 \bullet 10^9$ kg in 2020. Considering the shares of countries in production in 2019 and 2020, the USA is the main producer (38 %, 42 %), followed by Turkey (10 %, 26 %), Iran (38 %, 17 %), and China (9 %, 7 %) (FAOSTAT, 2022). Turkey has been in the top three rankings in world pistachio production in recent years. Italy is one of the countries to which Turkey exports the most pistachios (33 % of annual production), followed by Germany and İsrael in 2020/2021 (Bars, 2022). Pistachio is generally consumed as a snack (raw, roasted, salted or flavoured) and also used extensively in ice cream and bakery products.

Contamination of pistachio nuts by fungi and their mycotoxins is the most important problem for production, consumption, and export of pistachio. In Turkish pistachio, aflatoxins (AFs) levels were found to have higher distribution in comparison to other groundnuts (Hepsag et al., 2014), while ochratoxin A (OTA) levels vary from year to year, depending on climate changes, suggesting that it could be an health risk in Turkish and worldwide consumers (Kulahi et al., 2020).

Previous studies in different countries have shown that the dominant mycobiota associated with pistachio nuts is composed by *Aspergillus* spp. and the most significant associated mycotoxins are AFs and OTA (Varga et al., 2008; Fernane et al., 2010; Sedefoğlu, 2013; Soares Mateus et al., 2021). Particularly, studies from Spain (Fernane et al., 2010), Algeria (Amar et al., 2013), Turkey (Denizel et al., 1976), Iran (Sedaghati et al., 2011) and California (Bayman et al., 2002) have reported that *Aspergillus* Section *Nigri* (Black Aspergilli) and *Aspergillus flavus* were the most common *Aspergillus* species isolated from pistachio nuts.

The presence of *A. flavus* is associated with AFs contamination, especially aflatoxin B_1 (AFB_1) (Klich, 2007). It is known that AFB_1

* Corresponding author.
E-mail address: antonella.susca@ispa.cnr.it (A. Susca).
[1] Authors equally contributing to the work.

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contamination is an important public health problem in tropical and subtropical regions and causes economic losses worldwide. Because of these effects, it has become important to clarify the biosynthesis, in order to prevent its production in food and feed commodities, as well as to identify molecular markers for early detection of AFs risk. The AFs biosynthetic pathway involves approximately 25 genes clustered together in a 70 kb DNA region (Yu et al., 2004), which are highly conserved within *Aspergillus* Section *Flavi* and regulated by specific transcription factors, including *aflR*, *aflS*, and some general transcription factors (Georgianna and Payne, 2009). In recent years, PCR detection of the presence or expression of *afl* biosynthetic genes was attempted to find a diagnostic tool for rapid characterization and monitoring of indels within aflatoxigenic fungi in selected food commodities (Geisen, 2007; Gallo, 2012; Callicott and Cotty, 2015; Niessen et al., 2018). However, the high level of nucleotide variability within the AFs gene cluster makes difficult the identification of a single and universal DNA marker for all *A. flavus* populations examined globally. The still current challenge is finding a molecular tool able to point out AFs alert. The multiplex PCR, targeting different variabilities in AFs gene cluster, related to non-producing chemotype, is a rapid, already proposed and still effective, method to detect at least one defective trait indicating strains safe, in terms of aflatoxins producton

The *Aspergillus* Section *Nigri* is a common food spoilage fungus which is distributed worldwide, including some species difficult to recognize based solely on their phenotypic characters (Cabañes and Bragulat, 2018). Among them, *Aspergillus tubingensis,* has been controversially reported through the time as potentially responsible for OTA contamination (Medina et al., 2005; Perrone et al., 2006; Oliveri et al., 2008; López-Mendoza et al., 2009; Lahouar et al., 2017), and rarely reported as isolated from pistachio (Singh et al., 2022). As *A. tubingensis* could be considered as a species complex, with possible genetic variability related to OTA cluster inside the species, suggested by evidence of remnant incomplete biosynthetic gene cluster (Storari et al., 2012; Gil-Serna et al., 2019), further characterization of *A. tubingensis* strains and evaluation of OTA production ability, remain still an informative aspect to increase knowledge for the species.

Therefore, the present work aims to add a further check-point in monitoring fungal contamination of pistachio nuts in time and Countries, contributing to produce information useful for mapping climate change-driven emergence and spread of mycotoxigenic fungi, to characterize related potential mycotoxin impact on pistachio nut in Turkey, to deeply investigate genetic biodiversity in AFs gene cluster of *A. flavus* occurring worldwide, and to add further data about potential ochratoxigenicity of *A. tubingensis* species, still controversial.

2. Material and methods

2.1. Sample collection

A total of 48 *Aspergillus* isolates were retrieved from the ITEM culture Collection (CNR-ISPA, Bari, Italy). The isolates were previously collected from Turkish pistachio nuts (Table S1). Supplemental information about isolates drawn from culture collection can be retrieved from the ITEM online catalogue (http://www.ispa.cnr.it/Collection).

2.2. DNA isolation

Pure fungal cultures were grown in Potato Dextrose Agar (PDA, Conda, Madrid, Spain) for 5–7 days; the mycelia were collected in 2.0 ml tubes, then stored at −20 °C, for DNA-based identification (Visagie et al., 2014). DNA isolation was performed using the Wizard® Magnetic Purification System for Food kit (Promega, Madison, WI, USA). Small protocol modifications were applied, such as starting from 50 to 70 mg of fresh mycelia, added with lysis buffer (Buffer A, Promega, Madison, WI, USA) and stainless-steel ball and frozen at - 20 °C. In the first extraction phases, before supernatant collection after centrifugation, vortex was replaced by mixer-mill.

The quality and the amount of the isolated DNA were evaluated by electrophoresis in 0.8 % agarose gel stained with GelRed (Biotium, Hayward, CA, USA) and sample observation was set up under UV lamp, by comparison with the GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Santa Clara, CA, USA).

2.3. DNA-based identification of fungi and phylogenetic analysis

Four-loci were amplified by singleplex PCRs, using DreamTaq™ polymerase (Thermo Fisher Scientific, Santa Clara, CA, USA) and following primer pairs, according to the manufacturer recommendations and published amplification conditions: ITS4 and ITS5 for internal transcribed spacer regions (ITS*,* White et al., 1990); Bt2a and Bt2b for partial beta-tubulin gene (*BenA*, Glass and Donaldson, 1995); CL1 and CL2A for calmodulin gene (*CaM,* O'Donnell et al., 2000); 5F and 7CR for RNA polymerase II gene (*RPB2*, Liu et al., 1999).

The amplification products were evaluated by 1.5 % agarose gel electrophoresis. The PCR products were purified with the ExoSAP-IT™ PCR Product Cleanup reagent (Thermo Fisher Scientific, Santa Clara, CA, USA). The PCR fragments were subjected to direct sequencing in both directions using a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence alignments of the two strands for each locus were performed using the BioNumerics v8.1 (Applied Maths, Biomérieux, Belgium) software package. Nucleotide sequences for regions of ITS (OR528955-OR529002), BenA (OR909921-OR909968), CaM (OR947216-OR947263), and RPB2 (OR947264-OR947311) genes were deposited in GenBank, through BankIt platform.

After sequences cleaning and trimming, the taxonomical identification was performed by sequence alignment using the BLAST (Basic Local Alignment Search Tool) algorithm (http://www.ncbi.nlm.nih.gov) against the non-redundant nucleotide collection (nt) database managed by the National Center for Biotechnology Information (NCBI) (Altschul et al., 1990).

Phylogenetic analysis was performed for ITS, BenA, CaM, and RPB2 loci, separately and concatenated in a combined dataset, for 82 isolates (48 isolates and 33 species reference isolates retrieved by GenBank). MUSCLE (Edgar, 2004) was employed to align the sequences and investigate evolutionary relationships using Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 software (Tamura et al., 2011). Furthermore, the evolutionary history was inferred by using the Maximum Likelihood method (ML), based on the Kimura 2-parameter model (Kimura, 1980), using bootstrap analysis (1000 replications, Felsenstein, 1985). The obtained dendrogram was drawn to scale, with branch lengths measured in the number of substitutions per site. The tree with the highest log likelihood (−16415.34) was chosen. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. All ambiguous positions were removed for each sequence pair. There were a total of 2761 positions in the final dataset. *Penicillium roqueforti* CBS 221.30 was included as outgroup.

2.4. Determination of mycotoxin production "in vitro"

All 48 isolates were analysed for their capability to produce mycotoxins *in vitro*, according to species capability reported in literature: *A. tubingensis* for OTA, *A. flavus* for aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2) and sterigmatocystin (STC), *Aspergillus clavatus* for all above mentioned mycotoxins, as negative control, because it is known as non-producing for all of them.

Isolates were previously revitalized on PDA, at 25 ± 0.5 °C, for 7 days, and a spore suspension of each was spread on plates containing a

polypropylene cellophane disc on Yeast Extract Sucrose agar (YES) medium (Frisvad and Samson, 2004), at 25 ± 0.5 °C, for 14 days, in darkness. Acetonitrile, methanol (HPLC-grade), toluene and glacial acetic acid were purchased from Mallinckrodt Baker (Milan, Italy), phosphate saline buffer (PBS) was purchased from Sigma–Aldrich (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Standards of OTA, AFB_1, AFB_2, AFG_1, and AFG_2, and STC were purchased from Sigma–Aldrich. The OTA standard solutions for calibration curve purposes (range of 0.15–100.00 ng/mL) were prepared in the HPLC mobile phase acetonitrile/water/-acetic acid (99:99:2, v/v/v).

AFB_1, AFB_2, AFG_1, and AFG_2 Standard solutions for the calibration curve ranged from 0.4 to 10.0 ng/mL of AFB_1 and AFG_1, from 0.2 to 5.0 ng/mL of AFB_2 and AFG_2 in water/methanol (60:40, v/v).

The STC standard solutions for calibration curve purposes (range of 0.5–100.0 μg/mL) were in the HPLC mobile phase with water/methanol (40:60, v/v). Standards solutions were stored at −20 °C and warmed to room temperature before use.

2.4.1. Determination of OTA in Aspergillus tubingensis

Analysis of OTA was carried out based on DIN EN 14132:2009. Culture samples (approximately 2 g) were extracted with 10 mL acetonitrile/water (60:40, v/v) on an orbital shaker at 200 rpm for 60 min. The extracts were diluted with PBS (2 mL extract +22 mL PBS), then the diluted extract was loaded on the immunoaffinity column OchraTest WB™ (Vicam, Watertown, MA, USA) (IMA), after washing with 10 mL of water, the OTA was eluted with 2 mL of methanol. The eluate was evaporated and solubilized with 0.5 mL of mobile phase. One hundred microliter of extract was injected into the HPLC apparatus. The HPLC apparatus consisted of Agilent, technology series 1100, (Agilent, Waldbronn, Germany) with a binary LC system. The analytical column was a Zorbax SB-C18 (4.6 mm by 150 mm, 5 μm, Agilent, USA) (Phenomenex, USA). The mobile phase was an isocratic mixture of acetonitrile/water/acetic acid (99:99:2, v/v/v) eluted at a flow rate of 1.0 mL/min. The excitation and emission wavelengths of the fluorometric detector were set at 340 and 460 nm, respectively.

OTA was measured by comparing peak areas with a calibration curve; in these experimental conditions the limit of quantification was 2 μg/kg based on a signal-to-noise ratio of 10:1.

2.4.2. Determination of AFB_1, AFB_2, AFG_1, AFG_2 and STC in A. flavus

For AFB_1, AFB_2, AFG_1, AFG_2 and STC determination, culture samples were extracted with methanol/water (80:20, v/v) in a ratio 1 to 5 on an orbital shaker at 200 rpm for 60 min. The extracts were diluted with ultrapure water (1:1, v/v), then filtered using regenerated cellulose (RC) 0.2 μm filters (Phenomenex, USA).

After filtration, for AFs quantification, 100 μL of extract were injected into the HPLC apparatus with post-column photochemical derivatization (UVE™, LCTech GmbH, Dorfen, Germany). The analytical column, a Luna PFP (150 × 4.6 mm, 3 μm) (Phenomenex, Torrance, CA), was thermostated at 30 °C. The mobile phase consisted of a mixture of water/acetonitrile (70:30, v/v) eluted at a 1.0 mL/min flow rate. The excitation and emission wavelengths of the fluorometric detector were set at 365 and 435 nm, respectively. AFs were measured by comparing peak areas with calibration curves. In these analytical conditions, the limit of quantification was 1 μg/kg for AFB_1, AFG_1 and 0.5 μg/kg for AFB_2 and AFG_2 based on a signal-to-noise ratio of 10:1.

After filtration, for STC quantification, 100 μL of extract were injected into the HPLC. The analytical column was a Luna-C18 (4.6 × 150 mm, 5 μm) (Phenomenex, Torrance, CA) and the column was thermostated at 30 °C. The mobile phase consisted of a mixture of water/methanol (30:70, v/v) at a flow rate of 1 mL/min. The array detector (DAD) was set at wavelengths 245 and 325 nm. STC was measured by comparing peak areas with a calibration curve; in these analytical conditions the limit of quantification was 0.5 μg/kg based on a signal-to-noise ratio of 10:1.

2.5. Detection of afl biosynthetic genes

DNA extracted from *A. flavus* isolates was also used to analyse the AFs biosynthetic genes. The presence of 13 genetic markers for AFs biosynthetic genes was investigated by multiplex PCR amplification according to the method reported by Callicott and Cotty (2015). Amplification was performed in 20 μL reaction volume containing 1 × Platinum™ SuperFi II PCR Master Mix (Thermo Fisher Scientific, Santa Clara, CA, USA), 200 nM of each primer, and 10 ng of genomic DNA. Amplification parameters were initial denaturation at 98 °C (30 s), 30 cycles of denaturation at 98 °C (15 s), annealing at 60 °C (30 s), extension at 72 °C (45 s), and a final extension at 72 °C (5 min). Amplification products were visualised by 1.5 % agarose gel electrophoresis (data not shown).

3. Results

The study investigated molecular biodiversity of *Aspergillus* spp. isolates from 5 samples of pistachio bulk nuts from Turkey, through the analysis of 4 taxonomically informative loci (calmodulin, β-tubulin, the second largest subunit of RNA polymerase II and internal transcribed spacer regions), and measurement of AFs and OTA production abilities *in vitro*. Furthermore, *A. flavus* genotypes present in Turkey were investigated by a rapid multiplex PCR method for identifying and monitoring indels associated with atoxigenicity in *A. flavus*, identifying a DNA marker possibly associated mycotoxin risk.

3.1. Fungal species and mycotoxigenic potential occurring on Turkish pistachios

The set of 48 isolates included in the study were preliminary identified by observation of morphological characters and assigned to 2 *Aspergillus* taxa: *Aspergillus* Sect. *Nigri* (30) and *Aspergillus* Sect. *Flavi* (18).

The preliminary BLAST analysis in GenBank, performed with the sequences of the DNA barcode for fungi (the nuclear ribosomal internal transcribed spacer, ITS), confirmed 17 isolates belonging to *Aspergillus* Sect. *Flavi* (99.65 % sequence similarity with *A. flavus* - AF027863 and 99.31 % sequence similarity with *Aspergillus oryzae* - EF661560), 30 isolates belonging to *Aspergillus* Sect. *Nigri* (100 % sequence similarity with multiple *Aspergillus* spp. included in Sect. *Nigri*: e.g. *A. tubingensis* – EF661193, *Aspergillus niger* - EF661186, *Aspergillus neoniger* - FJ491682) and revealed 1 isolates belonging to *Aspergillus* Sect. *Clavati* (99.82 % sequence similarity with *A. clavatus* - EF669942).

The Genealogical Concordance Phylogenetic Species Recognition (GCPSR) approach (Taylor et al., 2000; Chethana et al., 2021) was also used to identify at species level all the 48 isolates from pistachio nuts of Turkish origin. The relationships among *Aspergillus* spp. isolates from pistachios and members of the *Aspergillus* Sect. *Flavi, Aspergillus* Sect. *Nigri* and *Aspergillus* Sect. *Clavati* (33 related species reference strains) were reconstructed by combining the *ITS, BenA, CaM, and RPB2* sequences, including in the analysis the sequences of *P. roqueforti* CBS 221.30 as outgroup (Fig. 1).

The evolutionary history was inferred using the Maximum Likelihood method with bootstrap test (1000 replicates) in MEGA 7 software, covering 2761 positions and comprising 328 sequences in the final dataset. The phylogeny showed that 30 isolates belong to *Aspergillus* Sect. *Nigri* (Fig. 1A)*,* 17 isolates belong to *Aspergillus* Sect. *Flavi* and 1 isolate belongs to *Aspergillus* Sect. *Clavati* (Fig. 1B). Most of the isolates included in the *Aspergillus* Sect. *Flavi* were closely related to genotype of *A. flavus*, reference strains CBS 310.38, and *Aspergillus oryze*, reference strain CBS 100925, not properly distinguishable molecularly (Hedayati et al., 2007; Frisvad et al., 2019). The 30 isolates clustering in the *Aspergillus* Sect. *Nigri* were closely related to *A. tubingensis,* reference strains CBS 133056, showing intra-species variability. The single isolate belonging to the *Aspergillus* Sect. *Clavati* resulted to be identical to the

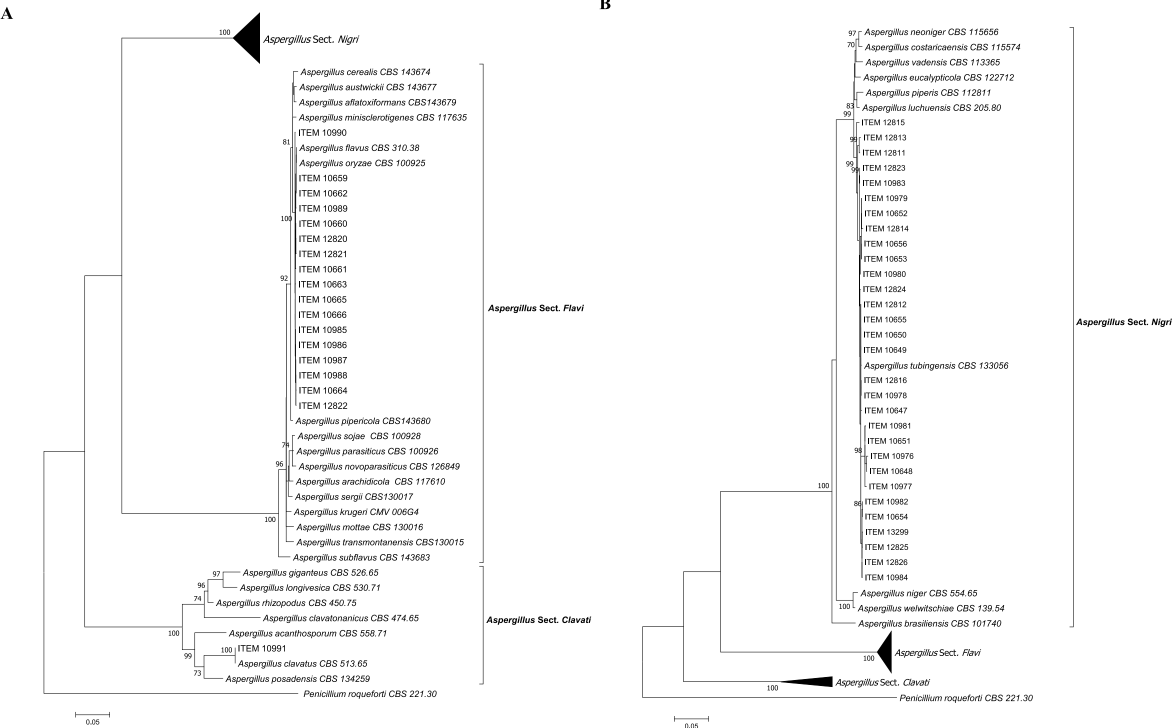

Fig. 1. Molecular phylogenetic analysis of 3 *Aspergilli* sections, by Maximum Likelihood method. **A.** *Aspergillus* Sect. *Flavi*, Sect. *Clavati*, collapsing *Aspergillus* Sect. *Nigri*; **B.** *Aspergillus* Sect. *Nigri*, collapsing *Aspergillus* Sect. *Flavi* and Sect. *Clavati*. The percentage of trees in which the associated taxa clustered together is shown next to the branches (values $\geq$ 70 %).

genotype of *A. clavatus* species, reference strain CBS 513.65.

OTA production analyses, set up for 30 isolates of *A. tubingensis* and 1 *A. clavatus,* resulted in negative OTA production for all tested isolates (LOD, detection limit, OTA = 2 μg/kg).

AFs production capability was evaluated in 17 of *A. flavus* and in 1 of the single *A. clavatus* isolate. AFG_1, AFG_2 and STC were not detected in any isolate (LOD, detection limit, AFB_2 and AFG_2 = 0.5 μg/kg; LOD, detection limit, STC = 0.5 μg/kg). Among the 17 *A. flavus* isolates, 7 produced both AFB_1 and AFB_2 in a range 13–2076 μg/kg and 1.5–633 μg/kg, respectively. A single isolate (ITEM10660) produced only AFB_1 (13 μg/kg) (Table 1).

3.2. Nucleotide variability in AF biosynthetic gene cluster

A. flavus isolates were tested by multiplex PCR for checking the presence of thirteen genetic markers specific for AFs biosynthetic gene cluster (AC01-AC13).

As shown in Table 2, most of the screened isolates were positive for the presence of all the genetic markers, excepting for isolates ITEM10659, ITEM10662, and ITEM10989, which lack makers AC01, AC06-AC13. Moreover, isolate ITEM10990 resulted in a deletion of AC01 genetic marker. Results obtained highlighted natural occurrence of 3 genotypes, among which genotype I is associated to both AFs producing and non-producing chemotypes, and two genotypes (II and III) are associated specifically to AFs non-producing chemotype. Genotype I, showing the presence of all tested DNA regions, was detected in 13 strains, 8 AFs producing and 5 non-producing chemotype strains, genotype II, showing the lack of genetic marker AC01, was detected in 3 strains, and genotype III, showing the lack of multiple genetic markers (AC01 and from AC06 to AC13), was detected in only 1 strain.

4. Discussion

Turkey is one of the homelands of pistachio nuts, where they have been cultivated for more than a thousand years. Their production has played an important role in Turkish culture, and they have become an integral part of the history and identity of the region in which they grow. Pistachios are often used in traditional Turkish food such as baklava, lokum and kebab, and are also a popular snack food. 60–70 % of the pistachios produced in Turkey are consumed as salted, roasted nuts,

Table 1
Aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2) and sterigmatocystin (STC) production *in vitro* on agar medium (n.d. = not detected, LOD (detection limit) $AFB_1$and AFG_1 = 1 μg/kg; LOD (detection limit) AFB_2 and AFG_2 = 0.5 μg/kg; LOD (detection limit) STC = 0.5 μg/kg).

ID	SPECIES	AFB_1 (μg/kg)	AFB_2 (μg/kg)	AFG_1 (μg/kg)	AFG_2 (μg/kg)	STC (μg/kg)
ITEM10991	*A. clavatus*	n.d.	n.d.	n.d.	n.d.	n.d.
ITEM10659, ITEM10662, ITEM10989, ITEM10663, ITEM10665, ITEM10666, ITEM10985, ITEM12821, ITEM10990	*A. flavus*	n.d.	n.d.	n.d.	n.d.	n.d.
ITEM10660	*A. flavus*	13	n.d.	n.d.	n.d.	n.d.
ITEM10661	*A. flavus*	2076	27.0	n.d.	n.d.	n.d.
ITEM10664	*A. flavus*	633	633	n.d.	n.d.	n.d.
ITEM10986	*A. flavus*	8.5	8.5	n.d.	n.d.	n.d.
ITEM10987	*A. flavus*	145	1.5	n.d.	n.d.	n.d.
ITEM10988	*A. flavus*	73	1.5	n.d.	n.d.	n.d.
ITEM12820	*A. flavus*	234	234	n.d.	n.d.	n.d.
ITEM12822	*A. flavus*	2.5	2.5	n.d.	n.d.	n.d.

n.d. = not detected.
LOD (detection limit) AFB_1 and AFG_1 = 1 μg/kg.
LOD (detection limit) AFB_2 and AFG_2 = 0.5 μg/kg.
LOD (detection limit) STC = 0.5 μg/kg.

Table 2
Multiplex PCR amplification patterns obtained analysing thirteen genetic markers within AFs gene cluster of *A. flavus* isolates.

	Mycotoxin production*	AFs cluster genes	AFs genes presence°												
ITEM culture collection ID	AFB_1/AFB_2	Genotype	*AC01*	*AC02*	*AC03*	*AC04*	*AC05*	*AC06*	*AC07*	*AC08*	*AC09*	*AC10*	*AC11*	*AC12*	*AC13*
ITEM10659	–	II	–	+	+	+	+	–	–	–	–	–	–	–	–
ITEM10660	+	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM10661	+	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM10662	–	II	–	+	+	+	+	–	–	–	–	–	–	–	–
ITEM10663	–	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM10664	+	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM10665	–	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM10666	–	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM10985	–	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM10986	+	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM10987	+	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM10988	+	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM10989	–	II	–	+	+	+	+	–	–	–	–	–	–	–	–
ITEM10990	–	III	–	+	+	+	+	+	+	+	+	+	+	+	+
ITEM12820	+	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM12821	–	I	+	+	+	+	+	+	+	+	+	+	+	+	+
ITEM12822	+	I	+	+	+	+	+	+	+	+	+	+	+	+	+

*: detected (+) or not detected (–).

30–40 % are consumed in the dessert and pastry (ice cream and baklava) industry, and among them 90 % are consumed as snacks in the USA and Europe. Especially in places where used as green kernel, Turkish pistachios are preferred due to their colour and taste. There is always a balance between the worldwide supply of and demand for pistachios. Remarkably, the producer countries are usually the main consumers at the same time. Iran, Turkey, United States, and Syria are the worldwide leading consumers. On the other hand, Italy has the highest consumption among European countries (Ak et al., 2016). Today, pistachio is produced in more than 40 provinces in Turkey. Among them, the Southeastern Anatolia Region (GAP) offers best climate for the growth of pistachio. Approximately 95 % of the total production in Turkey is met by the provinces of Şanlıurfa, Gaziantep, Adıyaman and Siirt in the GAP Region (Aydogdu, 2020).

Similarly to other crops, pistachio is susceptible to fungal colonization depending on the weather conditions during different steps in food chain, causing pistachio decay when it occurs during maturation and harvest. The event is also related to food safety when contamination is due to mycotoxigenic fungi. Therefore, evaluation of mycotoxigenic potential of fungi colonizing crops, and specifically pistachios is relevant, but its monitoring through the time, in view of climate change scenario, gives added value to the study, reporting data useful for scientific community, and especially for research groups working on development of previsional models. The present study on aspergilli isolated from pistachio sampled in Turkey, revealed presence of two principal species: *A. flavus*, including 17 % AFB_1 and AFB_2 producing isolates, and *A. tubingensis*, whose tested isolates resulted 100 % OTA non-producers.

Aspergillus species detected in Turkish pistachio nut samples, belong to *Aspergillus* Sections *Flavi*, *Nigri*, and *Clavati,* which include relevant and cryptic species differing significantly in mycotoxin profile.

A. flavus has similar morphological features than *A. oryzae*, considered as ecotypes of the same species (Payne et al., 2006; Rokas et al., 2007), generally distinguished according to their specific ecophysiology, and their ability to produce aflatoxins (Payne et al., 2006; Houbraken et al., 2014; Nugraha et al., 2018; Akinola et al., 2019).

Recent studies showed homogeneity in the whole-genome size of *A. flavus* and *A. oryzae* and revealed in both species the presence of genes encoding the enzymes for AFs biosynthesis, clustering in a 75 Kb DNA region, even if they appear to be not functional in *A. oryzae*, therefore Regarded as safe. Despite the similarities of the 2 species, due to the well-known presence of Afs producer and non-producing isolates in *A. flavus*, as well as the common ecological niche where they were isolated from, all the 17 isolates in the present study were identified as *A. flavus*.

The 17 *A. flavus* isolates isolated from Turkish pistachio nuts showed variable ability to produce Afs *in vitro*: for AFB_1, 9 were non-producing and 8 were producing; for AFB_2, 10 were non-producing and 7 producing. All AFB_2 producer isolates were able to produce also AFB_1. A single isolate (ITEM10660) produced only AFB_1. None of them produced AFG_1, and AFG_2 or STC.

The analysis of AFs biosynthetic gene cluster in the 17 strains, conducted by multiplex PCR, detected three different genotypes (I, II, III). Based on Callicott and Cotty (2015) study, genotype I is consistent with pattern B, described in AFs producing strains, and with multiple patterns (A, C, D) described in AFs non-producing strains, and differing among them for other markers, here not investigated. Genotype II is consistent with pattern E, and genotype III has not correspondence with any pattern described by Callicott and Cotty (2015). Furthermore, genotypes I and II were reported by other studies (Camiletti et al., 2018; Dadzie et al., 2019; Singh et al., 2022) in strains collected from other food commodities, such as maize and dried chili, in South America and Africa. Genotype III is described here for the first time in single *A. flavus* strain, isolated from Turkish pistachio nuts.

The AC01 marker region corresponds to the genomic region including the *aflW* (*moxY*) gene which codes for a cytosolic monooxygenase involved in transformation of hydroxyversicolorone to versiconal hemiacetal acetate by a Baeyer–Villiger reaction, in *Aspergillus parasiticus* (Wen et al., 2005). The entire chromosomic region from AC06 to AC13 markers includes the backbone enzyme *aflC* (*pksA*) and the two AFs biosynthesis regulators, *aflR* and *aflS*. Polymorphisms and single nucleotide polymorphism (SNPs) within one or several of the coding gene sequences, affecting functionality of those genetic markers, can inactivate the whole biosynthetic pathway (Chang et al., 2005, 2012), explaining assessed lack of ability to produce AFs for these isolates.

Negative results for investigated markers by multiplex PCRs give information about the possible occurrence of polymorphisms in primers target of DNA regions, possibly responsible of lack AFs production. On the other hand, positive results exclude lesions in DNA regions investigated, but not exclude them in other genomic regions, potentially involved in the regulation of AFs biosynthesis. Those investigations confirm that there is not a unique DNA marker related to non-toxigenicity. However, characterization of non-toxigenic strains is important to identify good candidates, adapted to pistachio nuts host, potentially applicable as antagonistic isolates against natural aflatoxigenic isolates (Moral et al., 2020; Maxwell et al., 2021).

A. tubingensis is a black *Aspergillus species* associated with different agricultural products, including pistachios (Singh et al., 2022; Mellikeche et al., 2024).Ecophysiological data reported *A. tubingensis* able to grow in a wide range of water activity (>0.86–0.88) and temperature (15–35 °C), highlighting its adaptation to extremely temperatures and drier conditions (García-Cela et al., 2014). Whereas, OTA production by *A. tubingensis* is still controversial. Some authors report OTA production (Storari et al., 2012; Gil-Serna et al., 2019; Singh et al., 2024), favoured at water activity of 0.950–0.965 and 20 °C (Chiotta et al., 2014), others hadn't evidence for OTA production, in concordance with absence of OTA gene cluster, or with detection of a truncated cluster (Susca et al., 2016; Gil-Serna et al., 2020). *A. tubingensis* belongs to a complex group in terms of classification and identification, whose taxonomy at species level has been continuously revised over recent years. Mosseray (1934) included 35 species in this section, but later Raper and Fennell (1965) reduced it to 12 species and two varieties. Al-Musallam (1980) suggested at least 7 species using morphological and cultural features; Kozakiewicz (1989) distinguished 16 taxa. New molecular genetic studies help to rearrange the species number in Section *Nigri,* which has now been reduced to 10 (Houbraken et al., 2020), adding 4 more species later, for a total of 14 (Silva et al., 2020; Khuna et al., 2021). Bian et al. (2022) proposed a new reduced number of accepted species in *Aspergillus* Sect. *Nigri*, currently at 6, morphologically indistinguishable, but distinguishable with DNA sequencing. The importance of the correct identification relies on the ability of some species included in the section to produce OTA, a potent nephrotoxic and carcinogenic mycotoxin. The ability of *A. tubingensis* to produce OTA is still not completely elucidated. Previous studies reported this species as OTA producing species (Medina et al., 2005; Perrone et al., 2006; Storari et al., 2012; Lahouar et al., 2017; Gil-Serna et al., 2019; Singh et al., 2024), under water activity of 0.950–0.965 and 20 °C (Chiotta et al., 2014), and some other studies as OTA non-producing (Abarca et al., 2004; Samson et al., 2004; Frisvad et al., 2011; Tavakol Noorabadi et al., 2020) consistently with the lack of some genes in the OTA cluster (Susca et al., 2016; Gil-Serna et al., 2020). However, 30 out of 30 black *Aspergilli* detected on Turkish pistachio nuts were identified as *A. tubingensis* by phylogenetic analysis, based on *Aspergillus* Sect. *Nigri* taxonomy proposed by Houbraken et al. (2020). None of them was found to be ochratoxigenic when cultured on YES medium and using a specific IMA column for OTA, confirming the inability of *A. tubingensis* to produce OTA as suggested by many previous studies, but still not excluding a mixture of chemotypes in the species, similarly to what observed in other taxa, e.g. *A. niger* (Palumbo et al., 2013; Susca et al., 2016) or in *Fusarium equiseti* species complex (Xia et al., 2019; Villani et al., 2019).

The *A. clavatus* is the economically most important species of its section and is possibly a cosmopolitan fungus, because it has been isolated from different sources e.g. soil and dung, but also from stored products (mainly cereals), inadequately stored rice, corn, and millet (Varga et al., 2007; Zutz et al., 2013; Houbraken et al., 2020). *A. clavatus* is also able to strongly resist to alkaline conditions, allowing to act as decomposer in situations where other fungi usually do not act. Even though this species produces potentially toxic metabolites, such as patulin (Li et al., 2015; Botha et al., 2018), its detection as a unique isolate, in the present semi-quantitative study, leads to consider the species as occasionally associated with pistachio nuts.

5. Conclusions

The adverse health effects of these mycotoxins both in humans and animals highlight the necessity of a better management of pistachio nuts production along food chain.

This study confirmed that Turkish pistachio nuts are susceptible to the growth of *A. tubingensis* and *A. flavus* species, and could be potentially contaminated by aflatoxin, assessed to be produced *in vitro*. However, the availability of naturally AFs non-producing strains could be deeper investigated for double purposes, elucidating genetic mechanism related to AFs production and verifying possibility to be exploited as biocontrol agents in programs for AFs reduction contamination.Nucleotide biodiversity in AFs biosynthetic gene cluster of *A. flavus,* investigated by multiplex PCR, confirmed nucleotide variability in AFs gene cluster and identified, for the first time, a new genotype in for *A. flavus* non-producing strains.

The absence of OTA production *in vitro,* for all *A. tubingensis* tested strain isolated from Turkish pistachio nuts, is congruent with a large related literature (Medina et al., 2005; Perrone et al., 2006; Storari et al., 2012; Lahouar et al., 2017; Gil-Serna et al., 2019; Singh et al., 2024), leading to suppose that the species could be a mixture of OTA producing and non-producing strains, a condition which should be deeper investigated by genome sequencing approach in strains of both chemotypes.

In consideration of the difficulty of mycotoxin detoxification methods, the prevention is still the most effective control measure for mycotoxin contamination, especially if it offers the possibility for early and not expensive chemotype identification.

Competing interests

The author declares that he has no competing interests.

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Declarations of interest

None.

CRediT authorship contribution statement

Pamela Anelli: Investigation, Visualization, Writing – original draft. **Miriam Haidukowski:** Formal analysis, Investigation, Writing – original draft. **Massimo Ferrara:** Investigation, Writing – review & editing. **Asli Kisikkaya:** Resources, Writing – review & editing. **Ceyda Pembeci:** Resources, Writing – review & editing. **Hayrettin Ozer:** Resources, Writing – review & editing. **Giuseppina Mulè:** Writing – review & editing. **Martina Loi:** Writing – review & editing. **Antonio Moretti:** Funding acquisition, Project administration. **Antonia Susca:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2024.07.009.

References

Abarca, M.L., Accensi, F., Cano, J., Cabañes, F.J., 2004. Taxonomy and significance of black aspergilli. Antonie Leeuwenhoek 86, 33–49.

Ak, B.E., Karadag, S., Sakar, E., 2016. Pistachio production and industry in Turkey: current status and future perspective. In: Kodad, O., López-Francos, A., Rovira, M., Socias i Company, R. (Eds.), XVI GREMPA Meeting on Almonds and Pistachios Options Méditerranéennes: Série A Séminaires Méditerranéens, pp. 323–329 n 119 CIHEAM, Zaragoza.

Akinola, S.A., Ateba, C.N., Mwanza, M., 2019. Polyphasic assessment of aflatoxin production potential in selected Aspergilli. Toxins 11 (12), 692.

Al-Musallam, A., 1980. Revision of the Black *Aspergillus* Species. University of Utrecht, Utrecht, The Netherlands. Ph.D. thesis.

Aydogdu, M.H., Şahin, Z., Sevinç, M.R., Cançelik, M., Doğan, H.P., Küçük, N., 2020. Analysis of recent trends in pistachio (pistacia vera l.) production in Turkey. International Journal of Humanities and Social Science Invention 9 (3), 40–46.

Bars, T., 2022. Pistachio, January-2022 agricultural products market report, Republic of Turkey Ministry of agriculture and Forestry. Agricultural Economics and Policy Development Institute/AEPDI [Online]. https://arastirma.tarimorman.gov.tr. (Accessed 14 September 2023). tepge/Belgeler/PDF%20Tar%C4%B1m%20%C3%9Cr%C3%BCnleri%20Piyasalar%C4%B1/2022-Temmuz%20Tar%C4%B1m%20%C3%9Cr%C3%BCnleri%20Raporu/1-ANTEPFISTI%C4%9EI%20T%C3%9CP%20TEMMUZ%202022.pdf.

Bayman, P., Baker, J.L., Mahoney, N.E., 2002. *Aspergillus* on tree nuts: incidence and associations. Mycopathologia 155, 161–169.

Bian, C., Kusuya, Y., Sklenář, F., D'hooge, E., Yaguchi, T., Ban, S., Visagie, C.M., Houbraken, J., Takahashi, H., Hubka, V., 2022. Reducing the number of accepted species in *Aspergillus* series Nigri. Stud. Mycol. 102 (1), 95–132.

Botha, C.J., Truter, M., Sulyok, M., 2018. Multimycotoxin analysis of South African *Aspergillus clavatus* isolates. Mycotoxin Res. 34, 91–97.

Cabañes, F.J., Bragulat, M.R., 2018. Black aspergilli and ochratoxin A-producing species in foods. Curr. Opin. Food Sci. 23, 1–10.

Callicott, K.A., Cotty, P.J., 2015. Method for monitoring deletions in the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* with multiplex PCR. Lett. Appl. Microbiol. 60 (1), 60–65.

Camiletti, B.X., Moral, J., Asensio, C.M., Torrico, A.K., Lucini, E.I., Giménez-Pecci, M.D.L. P., Michailides, T.J., 2018. Characterization of Argentinian endemic *Aspergillus flavus* isolates and their potential use as biocontrol agents for mycotoxins in maize. Phytopathology 108 (7), 818–828.

Chang, P.K., Horn, B.W., Dorner, J.W., 2005. Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. Fungal Genet. Biol. 42 (11), 914–923.

Chang, P.K., Scharfenstein, L.L., Ehrlich, K.C., Wei, Q., Bhatnagar, D., Ingber, B.F., 2012. Effects of laeA deletion on *Aspergillus flavus* conidial development and hydrophobicity may contribute to loss of aflatoxin production. Fungal Biol. 116 (2), 298–307.

Chethana, K.T., Manawasinghe, I.S., Hurdeal, V.G., Bhunjun, C.S., Appadoo, M.A., Gentekaki, E., Raspé, O., Promputtha, I., Hyde, K.D., 2021. What are fungal species and how to delineate them? Fungal Divers. 109 (1), 1–25.

Dadzie, M.A., Oppong, A., Ofori, K., Eleblu, J.S.Y., Ifie, B.E., Blay, E.T., Obeng-Bio, E., Appiah-Kubi, Z., Warburton, M.L., 2019. Distribution and genetic diversity among *Aspergillus flavus* isolates across three agro-ecologies essential for maize cultivation in Ghana. Plant Pathol. 68 (8), 1565–1576.

Denizel, T., Jarvis, B., Rolfe, E.J., 1976. A field survey of pistachio (*Pistacia vera*) nut production and storage in Turkey with particular reference to aflatoxin contamination. J. Sci. Food Agric. 27 (11), 1021–1026.

DIN EN 14132, 2009. In: Foodstuffs: Determination of Ochratoxin A in Barley and Roasted Coffee HPLC Method with Immunoaffinity Column Clean-Up, vol. 18.

FAO, 2022. FAOSTAT database. Available at: https://wwwfaoorg/faostat/en/#data/QCL.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39 (4), 783–791.

Fernane, F., Sanchis, V., Marin, S., Ramos, A.J., 2010. First report on mould and mycotoxin contamination of pistachios sampled in Algeria. Mycopathologia 170, 423–429.

Frisvad, J.C., Samson, R.A., 2004. Polyphasic taxonomy of *Penicillium* subgenus Penicillium A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Stud. Mycol. 49 (1), 1–174.

Frisvad, J.C., Larsen, T.O., Thrane, U., Meijer, M., Varga, J., Samson, R.A., Nielsen, K.F., 2011. Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoS One 6 (8), e23496.

Frisvad, J.C., Hubka, V., Ezekiel, C.N., Hong, S.B., Nováková, A.A., Chen, A.J., Arzanlou, M., Larsen, T.O., Sklenář, F., Mahakarnchanakul, W., Samson, R.A., Houbraken, J., 2019. Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. Stud. Mycol. 93 (1), 1–63.

Gallo, A., Stea, G., Battilani, P., Logrieco, A., Perrone, G., 2012. Molecular characterization of an *Aspergillus flavus* population isolated from maize during the first outbreak of aflatoxin contamination in Italy. Phytopathol. Mediterr. 51 (1), 198–206.

García-Cela, E., Crespo-Sempere, A., Ramos, A.J., Sanchis, V., Marin, S., 2014. Ecophysiological characterization of *Aspergillus carbonarius*, *Aspergillus tubingensis* and *Aspergillus niger* isolated from grapes in Spanish vineyards. Int. J. Food Microbiol. 173, 89–98.

Geisen, R., 2007. Molecular detection and monitoring. In: Dijksterhuis, J., Samson, R.A. (Eds.), Food Mycology - a Multifaceted Approach to Fungi and Food. CRC Press, Boca Raton, pp. 255–278.

Georgianna, D.R., Payne, G.A., 2009. Genetic regulation of aflatoxin biosynthesis: from gene to genome. Fungal Genet. Biol. 46 (2), 113–125.

Gil-Serna, J., García-Díaz, M., Vázquez, C., González-Jaén, M.T., Patiño, B., 2019. Significance of *Aspergillus niger* aggregate species as contaminants of food products in Spain regarding their occurrence and their ability to produce mycotoxins. Food Microbiol. 82, 240–248.

Gil-Serna, J., Vázquez, C., Patiño, B., 2020. The genomic regions that contain ochratoxin A biosynthetic genes widely differ in Aspergillus section circumdati species. Toxins 12 (12), 754.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61 (4), 1323–1330.

Hedayati, M.T., Pasqualotto, A.C., Warn, P.A., Bowyer, P., Denning, D.W., 2007. *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. Microbiology 153 (6), 1677–1692.

Hepsag, F., Golge, O., Kabak, B., 2014. Quantitation of aflatoxins in pistachios and groundnuts using HPLC-FLD method. Food Control 38, 75–81.

Houbraken, J., de Vries, R.P., Samson, R.A., 2014. Modern taxonomy of biotechnologically important *Aspergillus* and *Penicillium* species. Adv. Appl. Microbiol. 86, 199–249.

Houbraken, J., Kocsubé, S., Visagie, C.M., Yilmaz, N., Wang, X.C., Meijer, M., Kraak, B., Hubka, V., Bensch, K., Samson, R.A., Frisvad, J.C., 2020. Classification of *Aspergillus, Penicillium, Talaromyces* and related genera (Eurotiales): an overview of families, genera, subgenera, sections, series and species. Stud. Mycol. 95, 5–169.

Khuna, S., Suwannarach, N., Kumla, J., Frisvad, J.C., Matsui, K., Nuangmek, W., Lumyong, S., 2021. Growth enhancement of Arabidopsis (*Arabidopsis thaliana*) and onion (*Allium cepa*) with inoculation of three newly identified mineral-solubilizing fungi in the genus *Aspergillus* section *Nigri*. Front. Microbiol. 12, 705896.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Klich, M.A., 2007. *Aspergillus flavus*: the major producer of aflatoxin. Mol. Plant Pathol. 8 (6), 713–722.

Kozakiewicz, Z., 1989. *Aspergillus* Species on Stored Products. Mycological paper no. 161. CAB International Mycological Institute, Kew, UK, p. 1, 1989.

Kulahi, A., Kabak, B., 2020. A preliminary assessment of dietary exposure of ochratoxin A in Central Anatolia Region, Turkey. Mycotoxin Res. 36 (3), 327–337.

Lahouar, A., Marin, S., Crespo-Sempere, A., Saïd, S., Sanchis, V., 2017. Influence of temperature, water activity and incubation time on fungal growth and production of ochratoxin A and zearalenone by toxigenic Aspergillus tubingensis and Fusarium incarnatum isolates in sorghum seeds. Int. J. Food Microbiol. 242, 53–60.

Li, B., Zong, Y., Du, Z., Chen, Y., Zhang, Z., Qin, G., Tian, S., 2015. Genomic characterization reveals insights into patulin biosynthesis and pathogenicity in *Penicillium* species. Mol. Plant Microbe Interact. 28 (6), 635–647.

Liu, Y.J., Whelen, S., Hall, B.D., 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. Mol. Biol. Evol. 16 (12), 1799–1808.

López-Mendoza, M.C., Crespo-Sempere, A., Martínez-Culebras, P.V., 2009. Identification of *Aspergillus tubingensis* strains responsible for OTA contamination in grapes and wine based on the acyl transferase domain of a polyketide synthase gene. Int. J. Food Sci. Technol. 44 (11), 2147–2152.

Maxwell, L.A., Callicott, K.A., Bandyopadhyay, R., Mehl, H.L., Orbach, M.J., Cotty, P.J., 2021. Degradation of aflatoxins B1 by atoxigenic Aspergillus flavus biocontrol agents. Plant Dis. 105 (9), 2343–2350.

Medina, A., Mateo, R., López-Ocana, L., Valle-Algarra, F.M., Jiménez, M., 2005. Study of Spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. Appl. Environ. Microbiol. 71 (8), 4696–4702.

Moral, J., Garcia-Lopez, M.T., Camiletti, B.X., Jaime, R., Michailides, T.J., Bandyopadhyay, R., Ortega-Beltran, A., 2020. Present status and perspective on the future use of aflatoxin biocontrol products. Agronomy 10 (4), 491.

Mosseray, R., 1934. Les *Aspergillus* de la section "*Niger*" Thom et Church. La Cellule 43, 203–285.

Niessen, L., Bechtner, J., Fodil, S., Taniwaki, M.H., Vogel, R.F., 2018. LAMP-based group specific detection of aflatoxin producers within *Aspergillus* section *Flavi* in food raw materials, spices, and dried fruit using neutral red for visible-light signal detection. Int. J. Food Microbiol. 266, 241–250.

Nugraha, A., Khotimah, K., Rietjens, I.M., 2018. Risk assessment of aflatoxin B1 exposure from maize and peanut consumption in Indonesia using the margin of exposure and liver cancer risk estimation approaches. Food Chem. Toxicol. 113, 134–144.

Oliveri, C., Torta, L., Catara, V., 2008. A polyphasic approach to the identification of ochratoxin A-producing black Aspergillus isolates from vineyards in Sicily. Int. J. Food Microbiol. 127 (1–2), 147–154.

O'Donnell, K., Nirenberg, H.I., Aoki, T., Cigelnik, E., 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. Mycoscience 41 (1), 61–78.

Palumbo, J.D., O'Keeffe, T.L., Gorski, L., 2013. Multiplex PCR analysis of fumonisin biosynthetic genes in fumonisin-nonproducing *Aspergillus niger* and *A. awamori* strains. Mycologia 105 (2), 277–284.

Payne, G.A., Nierman, W.C., Wortman, J.R., Pritchard, B.L., Brown, D., Dean, R.A., Bhatnagar, D., Cleveland, T.E., Machida, M., Yu, J., 2006. Whole genome comparison of *Aspergillus flavus* and *A. oryzae*. Med. Mycol. 44 (1), 9–11.

Perrone, G., Mule, G., Susca, A., Battilani, P., Pietri, A., Logrieco, A., 2006. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius, Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. Appl. Environ. Microbiol. 72 (1), 680–685.

Raper, K.B., Fennell, D.I., 1965. The Genus *Aspergillus*. Williams & Wilkins, Baltimore, Md.

Rokas, A., Payne, G., Fedorova, N.D., Baker, S.E., Machida, M., Yu, J., Georgianna, D.R., Dean, R.A., Bhatnagar, D., Cleveland, T.E., Wortman, J.R., Maiti, R., Joardar, V., Amedeo, P., Denning, D.W., Nierman, W.C., 2007. What can comparative genomics tell us about species concepts in the genus *Aspergillus*? Stud. Mycol. 59 (1), 11–17.

Samson, R.A., Houbraken, J.A.M.P., Kuijpers, A.F., Frank, J.M., Frisvad, J.C., 2004. New ochratoxin A or sclerotium producing species in Aspergillus section Nigri. Stud. Mycol. 50 (1), 45–56.

Sedaghati, E., Nikkhah, M., Zare, R., Fotuhifar, K., Kocsubé, S., Vágvölgyi, C., Varga, J., 2011. Molecular identification of potentialy mycotoxigenic black Aspergili contaminating pistachio nuts in Iran. Acta Aliment. 40 (1), 65–70.

Sedefoğlu, C., 2013. Antep Fıstıklarında Okratoksin A Ve Aflatoksin Varlığının Incelenmesi/Determination of Ochratoxin A and Aflatoxins in Pistachio Nuts. İstanbul Technical University, Institute of Science and Technology. M.Sc. Thesis.

Silva, J.J.D., Iamanaka, B.T., Ferranti, L.S., Massi, F.P., Taniwaki, M.H., Puel, O., Lorber, S., Frisvad, J.C., Fungaro, M.H.P., 2020. Diversity within *Aspergillus niger* clade and description of a new species: *Aspergillus vinaceus sp. nov*. Journal of Fungi 6 (4), 371.

Singh, P., Mehl, H.L., Orbach, M.J., Callicott, K.A., Cotty, P.J., 2022. Genetic diversity of *Aspergillus flavus* associated with chili in Nigeria and identification of haplotypes with potential in aflatoxin mitigation. Plant Dis. 106 (7), 1818–1825.

Singh, P., Jaime, R., Puckett, R.D., Lake, J., Papagelis, A., Gabri, V.M., Michailides, T.J., 2024. Ochratoxin A contamination of California pistachios and identification of causal agents. Plant Dis. 108 (6), 1591–1601.

Soares Mateus, A.R., Barros, S., Pena, A., Sanches Silva, A., 2021. Mycotoxins in pistachios (*Pistacia vera L.*): methods for determination, occurrence, decontamination. Toxins 13 (10), 682.

Storari, M., Bigler, L., Gessler, C., Broggini, G.A., 2012. Assessment of the ochratoxin A production ability of *Aspergillus tubingensis*. Food Addit. Contam. 29 (9), 1450–1454.

Susca, A., Proctor, R.H., Morelli, M., Haidukowski, M., Gallo, A., Logrieco, A.F., Moretti, A., 2016. Variation in fumonisin and ochratoxin production associated with differences in biosynthetic gene content in *Aspergillus niger* and *A. welwitschiae* isolates from multiple crop and geographic origins. Front. Microbiol. 7, 1412.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28 (10), 2731–2739.

Tavakol Noorabadi, M., Babaeizad, V., Zare, R., Asgari, B., Haidukowski, M., Epifani, F., Stea, G., Moretti, A., Logrieco, A.F., Susca, A., 2020. Isolation, Molecular identification, and mycotoxin production of *Aspergillus* species isolated from the rhizosphere of sugarcane in the South of Iran. Toxins 12 (2), 122.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., Fisher, M.C., 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet. Biol. 31 (1), 21–32.

Varga, J., Due, M., Frisvad, J.C., Samson, R., 2007. Taxonomic revision of *Aspergillus* section *Clavati* based on molecular, morphological and physiological data. Stud. Mycol. 59 (1), 89–106.

Varga, J., Houbraken, J., Samson, R.A., Frisvad, J.C., 2008. Chapter 9 - molecular diversity of *Aspergillus* and *Penicillium* species on fruits and vegetables. In: Barkai-Golan, R., Paster, N. (Eds.), Mycotoxins in Fruits and Vegetables. Academic Press, pp. 205–223.

Villani, A., Proctor, R.H., Kim, H.S., Brown, D.W., Logrieco, A.F., Amatulli, M.T., Moretti, A., Susca, A., 2019. Variation in secondary metabolite production potential in the Fusarium incarnatum-equiseti species complex revealed by comparative analysis of 13 genomes. BMC Genom. 20 (1), 314.

Visagie, C.M., Houbraken, J., Frisvad, J.C., Hong, S.B., Klaassen, C.H., Perrone, G., Seifert, K.A., Varga, J., Yaguchi, T., Samson, R.A., 2014. Identification and nomenclature of the genus *Penicillium*. Stud. Mycol. 78 (1), 343–371.

Wen, Y., Hatabayashi, H., Arai, H., Kitamoto, H.K., Yabe, K., 2005. Function of the cypX and moxY genes in aflatoxin biosynthesis in *Aspergillus parasiticus*. Appl. Environ. Microbiol. 71 (6), 3192–3198.

Xia, J.W., Sandoval-Denis, M., Crous, P.W., Zhang, X.G., Lombard, L., 2019. Numbers to names - restyling the *Fusarium incarnatum-equiseti* species complex. Persoonia 43, 186–221.

Yu, J., Chang, P.K., Ehrlich, K.C., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Payne, G.A., Linz, J.E., Woloshuk, C.P., Bennett, J.W., 2004. Clustered pathway genes in aflatoxin biosynthesis. Appl. Environ. Microbiol. 70 (3), 1253–1262.

Zutz, C., Gacek, A., Sulyok, M., Wagner, M., Strauss, J., Rychli, K., 2013. Small chemical chromatin effectors alter secondary metabolite production in *Aspergillus clavatus*. Toxins 5 (10), 1723–1741.