Food Microbiology 82 (2019) 240–248

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Significance of *Aspergillus niger* aggregate species as contaminants of food products in Spain regarding their occurrence and their ability to produce mycotoxins

Jéssica Gil-Serna*, Marta García-Díaz, Covadonga Vázquez, María Teresa González-Jaén, Belén Patiño

Department of Genetics, Physiology and Microbiology, Faculty of Biology, Complutense University of Madrid. Jose Antonio Nováis 12, 28040, Madrid, Spain

ARTICLE INFO

Keywords:
Ochratoxin A
Fumonisins
Food safety
Section *Nigri*

ABSTRACT

The *Aspergillus niger* aggregate contains 15 morphologically indistinguishable species which presence is related to ochratoxin A (OTA) and fumonisin B_2 (FB_2) contamination of foodstuffs. The taxonomy of this group was recently reevaluated and there is a need of new studies regarding the risk that these species might pose to food security. 258 isolates of *A. niger* aggregate obtained from a variety of products from Spain were classified by molecular methods being *A. tubingensis* the most frequently occurring (67.5%) followed by *A. welwitschiae* (19.4%) and *A. niger* (11.7%). Their potential ability to produce mycotoxins was evaluated by PCR protocols which allow a rapid detection of OTA and FB_2 biosynthetic genes in their genomes. OTA production is not widespread in *A. niger* aggregate since only 17% of *A. niger* and 6% of *A. welwitschiae* isolates presented the complete biosynthetic cluster whereas the lack of the cluster was confirmed in all *A. tubingensis* isolates. On the other hand, *A. niger* and *A. welwitschiae* seem to be important FB_2 producers with 97% and 29% of the isolates, respectively, presenting the complete cluster. The genes involved in OTA and FB_2 were overexpressed in producing isolates and their expression was related to mycotoxin synthesis.

1. Introduction

Aspergillus section *Nigri* (commonly known as Black Aspergilli) includes 27 species worldwide distributed which present dark colonies and uniseriate or biseriate conidial heads (Ismail, 2017). Many of these species are key microorganisms in biotechnological processes (Frisvad et al., 2011) although most of them are food spoilage fungi as well as mycotoxin producers in foodstuffs (Cabañes and Bragulat, 2018). *Aspergillus* section *Nigri* is one of the most difficult groups concerning species classification since morphological and chemical characteristics are quite similar among them and molecular approaches are essential to reach a correct identification (Perrone et al., 2011; Ismail, 2017). The taxonomy of this group is constantly changing and new species have been recently described. Included in *Aspergillus* section *Nigri*, there is a particular group of biseriate species known as *Aspergillus niger* aggregate. To date, this group contains 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).

The ability of *A. niger* aggregate species to produce mycotoxins is known for a long time but their contribution to mycotoxin content in foodstuffs as well as the differences in production ability among species are controversial topics. Some *A. niger* aggregate species have been traditionally considered important ochratoxin A (OTA) producers in foodstuffs, mainly in grapes and grape products (Gil-Serna et al., 2018b). 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., 2018a). 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., 2018a).

* Corresponding author.
E-mail address: jgilsern@ucm.es (J. Gil-Serna).

https://doi.org/10.1016/j.fm.2019.02.013
Received 3 September 2018; Received in revised form 10 January 2019; Accepted 24 February 2019
Available online 26 February 2019
0740-0020/

Frisvad et al. (2007) described for the first time the ability of *A. niger* to produce fumonisins. The authors found a putative fumonisin biosynthetic cluster in *A. niger* genome and detected fumonisins in fungal cultures. The fumonisin biosynthetic cluster in *A. niger* widely differs from that found in *Fusarium* species and some important genes are missing; therefore, black *Aspergilli* are not able to synthetized all types of fumonisins being fumonisin B_2 (FB_2) the most relevant one regarding food security (Frisvad et al., 2007). The fumonisin cluster in *A. niger* was acquired via horizontal gene transfer from an ancestor of *F. verticillioides* and then the cluster diverged by removing and reshuffling the genes (Khaldi and Wolfe, 2011). Up to now, both *A. niger* and *A. welwitschiae* strains have been reported to present the fumonisin cluster although, in some cases, a partial deletion occurs and the isolates are not able to produce FB_2 (Susca et al., 2014b, 2016).

As mentioned before, *A. niger* aggregate species are widespread and have been reported in a variety of commodities. However, their contribution to mycotoxin contamination is not clearly established yet since the description of the new species and their ability to produce FB_2 was firstly reported (Ismail, 2017; Cabañes and Bragulat, 2018). Grapes and derivatives have been extensively studied regarding their contamination by mycotoxigenic fungi (Gil-Serna et al., 2018b). The distribution of *A. niger* aggregate species in these products does not seem to be related to neither geographical nor climatological conditions. *A. tubingensis* has been reported as the most frequently occurring species in Mediterranean countries (Pantelides et al., 2017) and Argentina (Chiotta et al., 2011) whereas *A. niger* and *A. welwitschiae* are the predominant species on other South American countries such as Uruguay (Garmendia and Vero, 2016) and Brazil (Massi et al., 2016; De Souzza et al., 2018).

Aspergillus niger aggregate species occurrence and their ability to produce mycotoxins have been frequently reported in Spain but never after the description of *A. welwitschiae*. Therefore, the objectives of this work were: (I) to perform a comprehensive study regarding the occurrence of the main *A. niger* aggregate species (*A. niger*, *A. welwitschiae* and *A. tubingensis*) isolated from relevant products from Spain, (II) to unravel their relative importance to the contribution of mycotoxin content in foodstuffs by evaluating their potential ability to produce OTA and FB_2 using rapid PCR protocols which detect biosynthetic genes in fungal genomes, and (III) to study the expression of OTA and fumonisin biosynthetic genes along time by real time RT-PCR in the most relevant isolates obtained.

2. Materials and methods

2.1. Fungal isolates

The 258 isolates of the *Aspergillus niger* aggregate used in this study were collected from different commodities and locations in Spain in the course of different studies performed in our laboratory in the last 10 years. These include commodities considered to be the most relevant such as grapes and a variety of cereals, but also spices, onion or legumes, among others (Table 1). The *A. niger* aggregate isolates were initially identified taking into account their colony appearance and their morphological characteristics under microscopic visualization including the presence of biseriate conidiophores which produce small and non-ornamented conidia (Samson et al., 2007). Subsequently, all of them were analyzed using species specific PCR assays and DNA sequencing, when necessary, as described below. They were stored as spore suspensions in 15% glycerol at −80 °C and they were cultured on Potato Dextrose Agar (PDA) (Pronadisa, Madrid, Spain) when required.

2.2. Identification

2.2.1. DNA extraction

Genomic DNA was extracted from 4-day-old cultures of all the 258 *A. niger* aggregate isolates on PDA agar plates following the method

Table 1
Identification of the *A. niger* aggregate isolates analyzed in this work, their relative abundance (%), their origin (source/commodity/substrate) and the number of isolates showing the OTA and FB_2 clusters for each *Aspergillus* species.

Species	Source	Number of isolates	OTA cluster (%)	FUM complete cluster (%)	FUM truncated cluster (%)
Aspergillus niger (11.7%)	Anise	1	0	1 (100%)	0
	Air	1	0	1 (100%)	0
	Corn borer	11	0	10 (91%)	1 (9%)
	Grapes	3	1 (33%)	3 (100%)	0
	Hair	1	0	1 (100%)	0
	Maize	3	0	3 (100%)	0
	Oat	3	1 (33%)	3 (100%)	0
	Paprika	1	0	1 (100%)	0
	Pea	1	1 (100%)	1 (100%)	0
	Vetch	2	2 (100%)	2 (100%)	0
	Wheat	3	0	3 (100%)	0
	Total	**30**	**5 (17%)**	**29 (97%)**	**1 (3%)**
Aspergillus welwitschiae (19.4%)	Anise	2	0	0	2 (100%)
	Barley	2	0	2 (100%)	0
	Bean	1	1 (100%)	1 (100%)	0
	Chickpea	1	0	0	1 (100%)
	Corn borer	6	0	1 (17%)	5 (83%)
	Gold of pleasure	1	0	0	1 (100%)
	Grapes	17	1 (6%)	2 (12%)	14 (82%)
	Lupin	1	0	0	1 (100%)
	Maize	2	1 (50%)	1 (50%)	1 (50%)
	Oat	1	0	0	1 (100%)
	Onion	7	0	3 (43%)	4 (57%)
	Paprika	1	0	0	1 (100%)
	Plant roots	2	0	2 (100%)	0
	Rye	1	0	1 (100%)	0
	Soil	3	0	0	3 (100%)
	Sorghum	1	0	1 (100%)	0
	Wheat	1	0	0	1 (100%)
	Total	**50**	**3 (6%)**	**14 (28%)**	**35 (70%)**
Aspergillus tubingensis (67.8%)	Anise	9	0	0	0
	Barley	21	0	0	0
	Corn borer	7	0	0	0
	Gold of pleasure	5	0	0	0
	Grapes	42	0	0	0
	Maize	20	0	0	0
	Oat	11	0	0	0
	Onion	1	0	0	0
	Pea	2	0	0	0
	Peanut	1	0	0	0
	Rye	3	0	0	0
	Soil	6	0	0	0
	Sorghum	3	0	0	0
	Wheat	44	0	0	0
	Total	**175**	**0**	**0**	**0**
Aspergillus brasiliensis (1.1%)	Grapes	1	0	0	0
	Soil	2	0	0	0
	Total	**3**	**0**	**0**	**0**

described elsewhere (Querol et al., 1992). Mycelia were frozen with liquid nitrogen and grinded using a micropistille before DNA isolation. DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA).

2.2.2. Species-specific PCR assays

The integrity of the isolated genomic DNA and, therefore, its suitability for PCR amplification, was tested in all the samples using universal primers ITS1/ITS4 (White et al., 1990). Subsequently, species-specific PCR assays based on calmodulin gene were applied to identify the main ochratoxigenic *Aspergillus niger* aggregate species using the species specific primers and conditions reported by Palumbo and O'Keeffe (2014). The specific primer sets At F/R, An F/R and Aw F/R were used for the specific identification of *A. tubingensis, A. niger* and *A. welwitschiae*, respectively.

PCR assays were performed in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). All amplification reactions were carried out in volumes of 25 µL containing 100 ng of sample DNA, 1 µL of each primer (20 µM) (Metabion, Germany) and 12.5 µL NZYTaq II 2x Green Master Mix (nzytech, Lisboa, Portugal). PCR products were detected in 2% agarose ethidium bromide gels in TAE 1X buffer (Tris-acetate 40 mM and EDTA 1.0 mM). The NZYDNA Ladder V (nzytech, Lisboa, Portugal) was used as molecular size marker.

2.2.3. Sequencing

The partial sequence of calmodulin gene was obtained for those *A. niger* aggregate isolates negative for the three specific PCR assays described above using the primer set CF1L/CF4 (Peterson, 2008) and the following program: an initial denaturalization cycle of 5 min at 95 °C, 32 cycles of 60 s at 95 °C, 60 s at 68 °C and 60 s at 72 °C, and a final extension cycle of 5 min at 72 °C. The amplification products (about 600 bp long) were excised from the agarose gels and purified using the NZYGelpure kit (nzytech, Lisboa, Portugal). Sequencing was performed in an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Foster City, USA) according to manufacturer's instructions in Macrogen facilities (Madrid, Spain). All amplification products were sequenced in both directions. Sequences were assembled using the UGENE 1.29 package (Unipro, Novosibirsk, Russia). The sequences were compared with those deposited on NCBI nucleotide databases to reach the identification of the corresponding isolates at species level.

Similarly, the same partial sequence of calmodulin gene was obtained for all the *A. niger* aggregate isolates included in the phylogenetic analysis carried out in this work.

2.3. Presence of OTA and fumonisin biosynthetic genes

The presence of the five genes of the OTA biosynthetic cluster (*hal*, *bzip*, *p450*, *nrps* and *pks*) was tested in all the isolates using specific PCR protocols previously described in our group and reported in Gil-Serna et al. (2018a).

Additionally, the absence of the OTA biosynthetic cluster was assessed in the *A. niger*, *A. welwitschiae* and *A. tubingensis* isolates of this study.

The sequence of the two new set of primers were designed targeting the genes encoding an isopropanol dehydrogenase (IDH) and an oxide nitric synthase (ONS) located in the flanking regions of the OTA biosynthetic cluster (Fig. 1S). Primers ISODH-PKSTUBF (5′-CATGATCGC TCACCCACTCAC-3′) and ISODH-PKSTUBR (5′-GATGATGTTTGCAAGG TTATGCAT-3′) were specific for *A. tubingensis* whereas primers ISODH-PKSF/ISODH-PKSR (5′-GGTGACACAGTCGGGTCCAA-3’/5′-CACTGCGC CATAGCATTCTCA-3′) were specific for both *A. niger* and *A. welwitschiae*. In both cases, the PCR protocol was as follows: 1 cycle of 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 62 °C and 60 s at 72 °C, and a final extension of 7 min at 72 °C. Positive amplification (a band of approximately 1 kb long) indicates that the OTA biosynthetic cluster was absent.

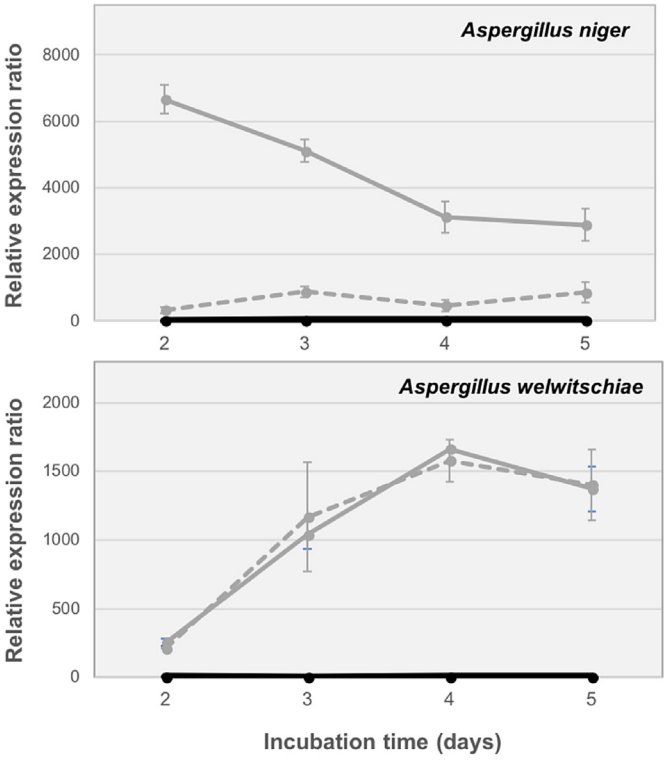

Fig. 1. Time course of the relative expression ratio of *fum1* gene quantified by real time RT-PCR for *Aspergillus niger* (above) and *A. welwitschiae* (below) isolates incubated in CYA plates for 2–5 days at permissive conditions for FB_2 production. Continuous and dotted grey lines corresponded to *A. niger* GRAPE.2 and VETCH.2, respectively, and *A. welwitschiae* RYE.4 and BEAN.1, respectively. Black lines correspond to the expression patterns of the FB2 non-producing isolates *A. niger* CORN-BORER.17 and *A. welwitschiae* GRAPE.55, showing the truncated version of the cluster. In both cases, the expression values were related to the corresponding non-producing strain, presenting the truncated FB_2 cluster at day 2 of incubation. Values indicate the average of the two replicates ± standard error.

The presence of the fumonisin biosynthetic cluster in all the isolates was assessed using the specific PCR protocols to detect fumonisin biosynthetic genes *fum1, fum8, fum14* (Palumbo et al., 2013) and *fum3, fum6, fum7, fum10, fum13, fum15, fum21* (Susca et al., 2016). The relative positions of the genes in the cluster either complete or truncated are shown in Fig. S2. The amplification program for *fum6, fum7* and *fum* 21 was as follows: 30 cycles of 30 s at 95 °C, 30 s at 58 °C and 50 s at 72 °C; for *fum3, fum10, fum13* and *fum15*, 30 cycles of 30 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C; and for *fum1, fum8, fum19* and *fum14,* 30 cycles of 45 s at 95 °C, 30 s at 66 °C and 30 s at 72 °C. All these programs started with 5 min of initial denaturation at 95 °C and finished with a cycle of 7 min at 72 °C for final elongation.

PCR reagents as well as the master mix composition were the same as described above. PCR products were observed and analyzed by electrophoresis in 2% agarose ethidium bromide gels in TAE 1X buffer prepared as mentioned above.

2.4. Genome sequencing of Aspergillus tubingensis

The cluster of genes involved in OTA biosynthesis was not detected by PCR in any of the 175 *A. tubingensis* isolates tested; therefore, to discard that the sequence of the cluster might be very different or present in other genomic location, the genome of one *A. tubingensis* isolate was studied. The sequence of the whole genome of *A. tubingensis PEANUT.1* was obtained by next generation sequencing in the lllumina HiSeq platform (Applied Biosystems, USA) (Stab-Vida, Portugal), using 100 bp paired-end sequencing reads. The analysis of the generated raw

sequence data was carried out using CLC Genomics Workbench 9.0 and the raw sequence data of the sample were *de novo* assembled using an algorithm based on Bruijn graphs. The genome size was estimated in 36.20 Mb.

Genomic DNA was isolated from 3-day-old cultures in potato-dextrose broth (Pronadisa, Spain) using the DNeasy Plant Mini Kit (QIAgen, Spain) following manufacturer's instructions. The DNA sample was used for library construction using the TruSeq DNA Whole genome library preparation kit (Illumina, USA).

2.5. Study on OTA and FB_2 production by selected Aspergillus niger aggregate isolates

2.5.1. Culture conditions

The ability to produce OTA and FB_2 and the expression of biosynthetic genes along time were studied *in vitro* in permissive conditions (Frisvad et al., 2007; Gil-Serna et al., 2018a) in a group of 9 selected isolates: *A. welwitschiae* BEAN.1, RYE.4, GRAPE.55 and GRAPE.58; *A. tubingensis* MAIZE.4 and PEANUT.1; and *A. niger* CORN-BORER.17, GRAPE.2 and VETCH.2. Spore suspensions (2 μl, 10^7 spores/ml) were placed in the centre of the plates containing CYA medium and the plates were incubated for 6 days at 28 °C. The experiment was carried out by triplicate. Colony diameter was measured daily to calculate growth rate and OTA and FB_2 concentrations were evaluated at the end of the incubation period.

2.5.2. OTA and FB_2 determination

Three agar plugs were excised from the centre, medium and outer edge of the colony and OTA and FB_2 were extracted by 1 mL of methanol and 1 mL of methanol:water 75:25, respectively (Bragulat et al., 2001; Frisvad et al., 2007). Mycotoxin measurements were performed in the Laboratorio Arbitral Agroalimentario (Madrid, Spain) following its standardized protocols.

OTA concentration was determined by High Performance Liquid Chromatography (HPLC). OTA was measured using a reverse phase C18 column (Tracer Extrasil ODS2; 5 μm, 4.6 mm × 250 mm; Teknokroma, Barcelona, Spain) at 45 °C in a Perkin Elmer Series 200 HPLC system coupled with a fluorescence detector (Perkin Elmer, Massachusetts, USA) at excitation and emission wavelengths of 330 and 470 nm respectively. The mobile phase comprised monopotassium phosphate 4 mM pH 2.5 and methanol (33:67) and the flow rate was 1 mL x min^{-1}. OTA was eluted and quantified by comparison with a calibration curve generated from OTA standards (OEKANAL®, Sigma-Aldrich, Steinheim, Germany). The limit of detection was 50 ng/g agar.

Fumonisin B_2 concentration was measured by LC MS/MS using a Varian 325 HPLC MS/MS (Varian, Palo Alto, USA) with EC-C18 Poroshell 120 column (Agilent, Waldbronn, Germany) at room temperature. The system was equipped with a binary solvent pump and a MS detector consisting of a API source configured as vESI (Vortex Electrospray Ionization). The flow rate of the mobile phase was 0.25 mL x min^{-1}. The system was programmed to elute with a gradient of 0.15% formic acid +0.5 mM ammonium formate in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B) for a run time of 30 min according to the following schedule: 3 min, 90% A; 12 min, 60% A; 30 s, 35% A; 7 min, 0% A; and 7 min 30 s, 90% A. Operating conditions were as follows: nebulizer and vortex pressures 50 and 30 psi, respectively; drying gas temperature 300 °C.

2.5.3. Expression of the OTA biosynthetic genes and the FB_2 biosynthetic *fum1* gene of *A. niger* and *A. welwitschiae* along time

The time course expression of the five OTA biosynthetic genes was studied in two OTA producing isolates (GRAPE.2 and VETCH.2) and one non-producing isolate (CORN-BORER.17) of *A. niger*. In the case of *A. welwitschiae*, the strains selected were the OTA-producing GRAPE.55 and BEAN.1 and the non-producing CORN-BORER.58. The expression of the FB_2 biosynthetic *fum1* gene was similarly studied in two FB_2 producing isolates of *A. niger* (GRAPE.2 and VETCH.2) and *A. welwitschiae* (BEAN.1 and RYE.4) and the non-producing CORN BORER.17 (*A. niger*) and GRAPE.55 (*A. welwitschiae*).

Sterile cellophane membranes (BioRad, Spain) were laid on CYA plates before fungal inoculation to facilitate mycelia removal after incubation. Spore suspensions (2 μl, 10^7 spores/ml) were located in the centre of the plates and the incubation was performed at 28 °C for 2, 3, 4 and 5 days. At the end of corresponding incubation period, cellophanes with the fungal mycelia were removed from the plates and frozen at −80 °C for RNA extraction. The experiment was carried out by duplicate.

Mycelia were frozen in liquid nitrogen and grinded using a mortar and a pestle. Cell lysis was performed using TRI reagent (Ambion, USA) and RNA was subsequently purified with chloroform and isolated by lithium chloride precipitation. Two treatments with DNAse I were carried out to ensure complete DNA removal following manufacturer's instructions (RNase-Free DNase set, QIAgen, Spain). RNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, USA). Retrotranscription was performed starting from 1 μg of isolated RNA using the PrimeScript RT reagent Kit (Takara, Japan).

The expression of each of the five OTA biosynthetic genes was quantified using real-time RT-PCR protocols previously developed in our group for *A. welwitschiae* and *A. niger* (Gil-Serna et al., 2018a). In the case of *fum1* gene, a new primer pair was designed on the basis of available *fum1* gene sequences retrieved from the NCBI database in order to quantify the expression of *fum1* gene in both species. This primer pair, qFUM1nigF (5′-CTCAGCATGTGGAGGTATTGGA-3′)/qFUM1nigR (5′-GATGCTGGCACGCGGAATGT-3′), produced a fragment of either 207 bp or 139 bp using genomic DNA or cDNA as template, respectively. The optimization of the protocol was tested by generating a standard curve with ten-fold serial dilution of cDNA from *A. welwitschiae* RYE.4 and BEAN.1. Amplification efficiencies, correlation coefficients and dissociation curves were analyzed to check the correct optimization of the method.

Normalized quantification was performed for all the real-time PCR analysis (OTA biosynthetic and fum1 genes) in relation to the constitutive expression of β-tubulin gene (*ßtub*) which was amplified using the primers previously described in our group (Gil-Serna et al., 2018a). The calibrator sample corresponded to the value of expression of the corresponding non-producing strain after 2 days of incubation. In all cases, gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) which can only be used if the difference in amplification efficiencies between the constitutive and target genes is less than 10% (Schmittgen and Livak, 2008).

The assays were carried out and monitored in a QuantStudio 12 k Flex (Applied Biosystems, Spain) in the Genomic Unit of the Complutense University of Madrid using 384-well plates. The final reaction volume (10 μl) consisted in: 5 μl SYBR Premix Ex Taq (Takara, Japan), 0.2 μl ROX, 0.4 μl forward primer 5 μM (Metabion, USA), 0.4 μl reverse primer 5 μM (Metabion, USA), 1 μl cDNA template prepared as described above and 3 μl molecular grade water. All the real time RT-PCR assays were performed using the standard program: one cycle of 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All reactions were carried out by triplicate.

2.6. Phylogenetical analysis

A phylogenetic analysis of 66 *Aspergillus niger* aggregate isolates identified in this study was carried out with the partial sequence of the calmodulin gene sequenced using CF1L and CF4 primers as mentioned above (Peterson, 2008). These isolates used for phylogenetic analysis were selected in order to maximize the diversity of the sample regarding their substrates and their potential ability to produce mycotoxins. The phylogenetic analysis was conducted using MEGA 5 (Tamura et al., 2011). Alignment of DNA sequences was performed

using CLUSTALW and the subsequent phylogeny was inferred by Maximum Parsimony algorithm. Phylogenetic analysis involved 67 calmodulin partial sequences including *A. tubingensis* (21), *A. welwitschiae* (27), *A. niger* (16) and *A. brasiliensis* (2) obtained in this work. Additionally, the calmodulin sequence of *A. carbonarius* NRRL4849 was retrieved from GenBank (accession number EF661168) and used as outgroup. Consistency index was calculated for parsimony-informative sites. The most parsimonious tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (100 replicates). Clade stability was assessed via 1000 bootstrap replications. All positions containing gaps and missing data were eliminated.

3. Results

3.1. Identification and characterization of A. niger aggregate isolates

A total of 258 isolates of *Aspergillus niger* aggregate were identified at species level and tested for the presence of OTA and FB_2 biosynthetic clusters. Table 1 displays these results indicating the *Aspergillus* species and the source of isolation. *A. tubingensis* was the most frequent species (175 isolates, 67.8%) followed by *A. welwitschiae* (50 isolates, 19.4%) and *A. niger* (30 isolates, 11.7%). Only three isolates needed to be identified using sequencing of the partial calmodulin gene which were all classified as *A. brasiliensis*.

Grapes were the most important source of *A. niger* aggregate isolates and *A. tubingensis* was the most frequently occurring in Spanish grapes (67%) followed by *A. welwitschiae* (27%) and *A. niger* (5%). Although these three species were isolated from almost all the matrices analyzed, the occurrence of *A. welwitschiae* was highest in grapes (34%) whereas *A. niger* appeared more frequently associated to maize and corn borer (46.6%) than to grapes (10%). On the other hand, *A. tubingensis* was found with similar values in grapes (24%) and in cereals, particularly wheat (25.1%). This species was the most frequently isolated in cereals (80, 92 and 91% of the isolates from maize, wheat and barley, respectively).

A. tubingensis was also the most important species in gold-of-pleasure (83%) and anise (82%) whereas *A. welwitschiae* was the predominant species in onion (88% of the isolates corresponded to this species).

The presence of the five genes of the OTA biosynthetic cluster was evaluated by conventional PCR. Positive amplification for all the genes was obtained in 17% of *A. niger* and 6% of *A. welwitschiae* isolates whereas all the *A. tubingensis* were negative for each of the five PCR tests. The application of a new PCR primers designed on the basis of the flanking regions of the OTA cluster allowed to confirm the deletion of the genes involved in OTA biosynthesis. All *A. tubingensis* isolates yielded the expected 1 kb long product for the PCR test which identifies the truncated version of the functional OTA biosynthetic cluster (Fig. 1S). Similarly, all *A. niger* and *A. welwitschiae* negative for the five OTA cluster genes were positive for the presence of the truncated version.

All the *A. niger* isolates obtained in this work had the complete cluster of FB_2 biosynthetic genes (97%), except for one isolate which contained a truncated version with only *fum1*, *fum19* and *fum15* genes present (Fig. 2S). This truncated version was present in most of the isolates of *A. welwitschiae* (70%), the complete FB_2 biosynthetic cluster was detected in 28% of the strains and only one isolate was negative for all the cluster genes. In the case of *A. tubingensis,* all the isolates were negative for any of the FB2 cluster genes tested.

In order to discard the presence of other possible structurally different clusters that could be putatively involved in OTA or FB_2 biosynthesis, the complete genome sequence of the *A. tubingensis* PEANUT.1 isolate was obtained in this work. This genomic sequence and that available for *A. tubingensis* CBS 134.48 at the JGI database (de Vries et al., 2017) were searched for the presence of key genes involved in the biosynthetic pathways of any of these two toxins. In particular, the search performed was focused on halogenase encoding genes, essential for the chlorination of the final product in OTA synthesis, or the FUM1 encoding gene, which is considered the key gene of FB_2 biosynthesis. However, no other regions containing similar genes were found in the genomes of the two isolates.

3.2. Study on mycotoxin production by selected Aspergillus niger aggregate isolates

Ochratoxin A and FB_2 production ability of the 9 selected isolates of the most important *A. niger aggregate* species (*A. tubingensis*, *A. niger* and *A. welwitschiae*) was evaluated in CYA plates at permissive conditions (Table 2). Table 2 also shows if the complete OTA and/or FB_2 clusters were present in the genomes of those isolates. In all cases, fungal growth rates were between 21 and 26 mm/day. In all the isolates, the presence of the complete OTA and FB_2 clusters was in agreement with production of OTA and FB_2 toxins, respectively (Table 2). Similarly, OTA and FB_2 values were below the detection limits in those plates inoculated with isolates lacking or having the truncated version of the corresponding biosynthetic clusters, showing that the presence of the complete version of the OTA and FB_2 biosynthetic can be an useful indicator of their ability to produce these mycotoxins.

3.3. Expression of the OTA biosynthetic genes and the FB_2 biosynthetic *fum1* gene of A. niger and A. welwitschiae along time

The time-course of the expression of OTA biosynthetic genes was also evaluated in *A. niger* and *A. welwitschiae* in relation to the expression of the isolate presenting the truncated version after 2 days of incubation (Table 3). The results of this analysis suggest that all OTA biosynthetic genes might be functional and that their expression patterns seem to be consistent at intraspecific level. The highest expression values were obtained by *A. niger* after 2 days of incubation and in 3-day-old cultures of *A. welwitschiae*. In both species, all five genes reached extremely high levels of expression. The halogenase and cytochrome p450 monooxygenase encoding genes showed the highest expression levels whereas the bZIP transcription factor showed the lowest values.

In the case of the FB_2 biosynthetic *fum1* gene, a new real-time PCR protocol was designed to evaluate its expression in *A. niger* and *A. welwitschiae*. The selected primer pair qFUM1nigF and qFUM1nigR amplified a region of 207 bp using genomic DNA as template and 139 bp using cDNA due to an intron within the amplification target region; this easily allows detection of genomic DNA present in cDNA samples. Amplification efficiencies of cDNA calculated from the standard curves were 96.1% and 98.7% in the case of *ßtub* (constitutive gene) and *fum1* respectively for RYE.4 strain and 100.5% and 104.7% for BEAN.1. Since the difference between the efficiencies of *ßtub* and *fum1* was less than 5% for both strains, the Livak method could be applied to perform relative quantification. Dissociation curves showed a single amplification peak indicating that non-specific products or primer dimers did not occur.

The results of the time course of *fum1* expression in *A. niger* and *A. welwitschiae* strains are shown in Fig. 1. The normalization was always performed in relation to the expression of the isolate presenting the truncated version of the cluster after 2 days of incubation. Both *A. niger* strains GRAPE.2 and VETCH.2, having the complete FB_2 biosynthetic cluster, showed induction of *fum1* gene expression, although the values were remarkably higher in GRAPE.2 in VETCH.2, in agreement with their levels of FB_2 production (Table 2). A similar pattern of induction of *fum1* was found in A. welwitschiae BEAN.1 and RYE.4, both reaching maximum expression levels after 4 days of incubation. No *fum1* expression was found for the strains *A. niger* CORN-BORER.17 and *A. welwitschiae* GRAPE.55, both having the truncated FB_2 cluster and C_T values were always similar to NTC.

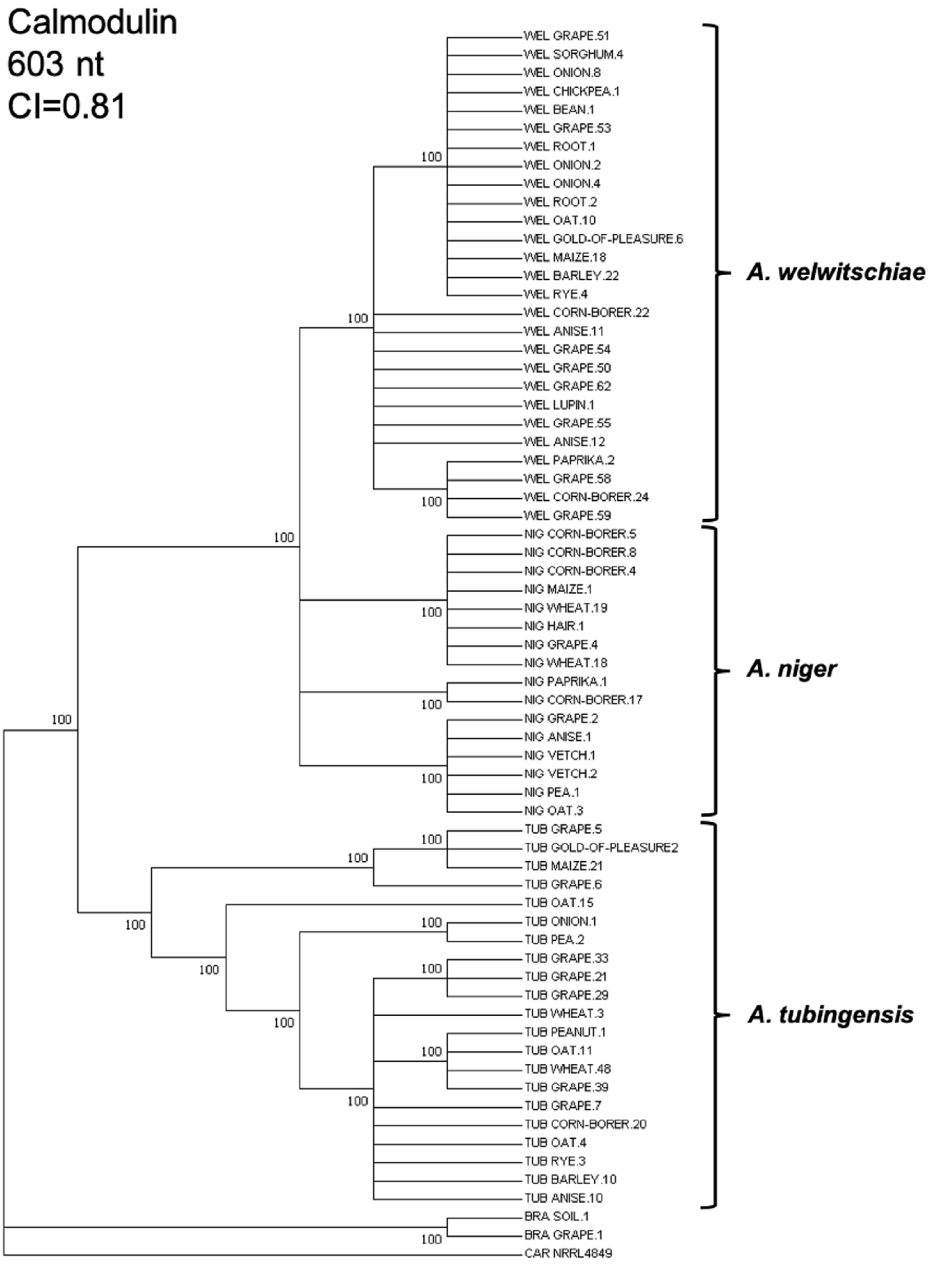

Fig. 2. Maximum parsimony tree generated from the partial calmodulin gene of *A. tubingensis*, *A. niger* and *A. welwitschiae* selected isolates. Numbers at the nodes are the bootstrap values.

Table 2
OTA and FB_2 production by isolates of *A. welwitschiae, A. tubingensis* and *A. niger* incubated in CYA medium for 6 days. The values represent the average of the concentration (μg/g). The presence of the OTA and FB_2 biosynthetic clusters in their genomes is also indicated. n.d. = Non detected (above the limits of detection).

Isolate	Species	OTA cluster	OTA (μg/g)	FB_2 cluster	FB_2 (μg/g)
BEAN.1	*A. welwitschiae*	+	0.08 ± 0.02	+	58.60 ± 5.45
RYE.4	*A. welwitschiae*	–	n.d.	+	35.46 ± 1.64
GRAPE.55	*A. welwitschiae*	+	0.15 ± 0.01	–	n.d.
GRAPE.58	*A. welwitschiae*	–	n.d.	–	n.d.
MAIZE.4	*A. tubingensis*	–	n.d.	–	n.d.
PEANUT.1	*A. tubingensis*	–	n.d.	–	n.d.
CORN-BORER.17	*A. niger*	–	n.d.	–	n.d.
GRAPE.2	*A. niger*	+	2.08 ± 0.01	+	29.85 ± 4.86
VETCH.2	*A. niger*	+	0.08 ± 0.02	+	0.39 ± 0.07

Table 3
Time course of the relative expression of the five OTA biosynthetic genes quantified by real timer RT-PCR obtained for isolates of *A. welwitschiae* (above) and *A. niger* (below) after 2–5 days of incubation in CYA plates at permissive conditions for OTA production. In all cases the expression values of OTA producing strains (OTA +) were related to the corresponding non-producing strain (OTA -), lacking the complete cluster, at day 2 of incubation. Values are the average of two replicates ± standard error.

	Isolate	Time (days)	HAL	BZIP	P450	NRPS	PKS
A. welwitschiae	**GRAPE.55**	2	83,450.78 ± 18,005.58	274.05 ± 65.03	112,723.20 ± 7470.34	21,798.30 ± 1501.01	209,886.15 ± 25,227.68
	OTA +	3	622,010.50 ± 6538.79	412.73 ± 7.34	795,672.93 ± 38,025.78	106,489.76 ± 25,234.92	248,594.09 ± 19,089.01
		4	230,298.14 ± 40,776.95	254.43 ± 70.39	297,879.66 ± 64,254.96	32,776.04 ± 177.96	176,033.13 ± 8838.78
		5	97,758.06 ± 10,942.48	115.04 ± 26.34	113,640.34 ± 4120.46	17,045.75 ± 342.60	181,112.35 ± 27,794.45
	BEAN.1 OTA	2	171,238.68 ± 60,638.12	198.32 ± 69.29	173,564.45 ± 63,841.96	25,502.70 ± 3988.53	184,764.33 ± 7136.21
	+	3	320,393.82 ± 182,072.25	340.65 ± 168.34	501,359.83 ± 336,200.39	82,122.64 ± 32,975.05	351,275.11 ± 8358.06
		4	155,565.78 ± 152,596.97	217.49 ± 205.22	207,907.30 ± 206,541.21	26,311.98 ± 25,446.21	173,763.06 ± 21,785.34
		5	51,813.46 ± 5728.77	113.35 ± 4.45	68,809.39 ± 13,732.23	7706.20 ± 649.24	174,716.73 ± 7483.64
	GRAPE.58	2	3.93 ± 2.93	0.94 ± 0.06	1.00 ± 0.01	0.67 ± 0.33	1.39 ± 0.39
	OTA -	3	3.61 ± 2.94	2.56 ± 2.29	2.10 ± 0.55	0.71 ± 0.01	3.08 ± 1.54
		4	9.70 ± 6.21	3.22 ± 2.41	16.88 ± 14.79	0.98 ± 0.22	3.13 ± 0.22
		5	17.85 ± 3.51	3.85 ± 0.46	12.93 ± 9.93	5.11 ± 3.86	6.99 ± 3.96
	Isolate	Time (days)	HAL	BZIP	P450	NRPS	PKS
A. niger	**GRAPE.2**	2	391,274.06 ± 146,260.99	308.25 ± 150.00	554,503.35 ± 238,517.44	117,740.19 ± 29,570.23	285,890.01 ± 61,211.89
	OTA +	3	215,836.66 ± 41,803.93	145.39 ± 1.97	234,989.54 ± 65,891.59	73,467.40 ± 9494.81	256,310.23 ± 12,426.62
		4	239,339.42 ± 14,167.66	149.10 ± 21.26	264,366.36 ± 33,265.02	65,073.50 ± 8106.73	232,127.79 ± 5415.98
		5	174,451.60 ± 36,321.61	131.08 ± 12.51	219,130.50 ± 46,519.22	37,849.45 ± 1512.09	228,736.99 ± 17,353.94
	VETCH.2	2	467,276.38 ± 113,249.28	595.31 ± 210.90	1,386,603.87 ± 527,185.97	86,251.16 ± 17,480.76	144,031.34 ± 16,278.19
	OTA +	3	311,594.91 ± 29,465.09	408.59 ± 2.08	905,018.06 ± 128,047.49	58,335.51 ± 10,054.56	133,746.16 ± 16,555.31
		4	63,046.27 ± 55,136.98	66.77 ± 52.81	124,083.87 ± 103,467.54	8410.95 ± 7394.85	54,501.48 ± 11,477.97
		5	110,283.05 ± 36,806.33	471.23 ± 345.56	281,044.62 ± 79,254.02	25,529.46 ± 13,759.41	111,045.67 ± 60,294.04
	CORN-	2	3.93 ± 2.93	0.94 ± 0.06	0.99 ± 0.01	0.67 ± 0.33	1.39 ± 0.39
	BORER.17	3	3.38 ± 3.17	2.56 ± 2.29	2.10 ± 0.55	0.71 ± 0.01	3.08 ± 1.54
	OTA -	4	9.70 ± 6.21	3.22 ± 2.41	16.88 ± 14.79	0.98 ± 0.22	3.13 ± 0.22
		5	17.85 ± 3.51	3.85 ± 0.46	12.93 ± 9.90	5.11 ± 3.86	6.99 ± 3.96

3.4. Phylogenetic analysis

The results of the phylogenetic analysis of the 66 isolates of *A. niger* (16), *A. tubingensis* (21), *A. welwitschiae* (27) and *A. brasiliensis* (2) was inferred using Maximum parsimony. The most parsimonious tree obtained is shown in Fig. 2. There were a total of 603 positions of the calmodulin partial sequence in the final dataset. In all cases, branches corresponding to partitions reproduced in less than 60% of bootstrap replicates were collapsed. Consistency index was 0.81. The phylogenetic tree clearly clustered the three species in different branches supported by high bootstrap values (100). No apparent relationship between isolation source and ability to produce OTA or FB_2 was found except for the case of OTA-producing strains of *A. niger* (GRAPE.2, VETCH.1, VETCH.2, PEA.1 and OAT.3) that appeared in the same branch.

4. Discussion

The study of *Aspergillus niger* aggregate group was performed in a wide and diverse sample of Spanish isolates (258), collected from a variety of agroclimatic regions and commodities which included cereals and grapes, important agronomic cultures in Spain. The identification of the isolates at species level was performed by species specific PCR protocols for the three main species of the complex (*A. niger*, *A. welwitschiae* and *A. tubingensis*). All the isolates, belonged to these species (98.8%) except for 3 isolates which were identified as *A. brasiliensis* identified by their partial sequence of the calmodulin gene. The results of the analysis revealed the highest incidence of *A. tubingensis* in Spain, in particular in cereal and grapes but also in many other substrates. Both *A. niger* and *A. welwitschiae* were also present in diverse substrates, including grapes and cereals, but at lower frequencies than *A. tubingensis*.

The description of *A. welwitschiae* (Perrone et al., 2011) is still recent to have substantial information available about its global occurrence. Massi et al. (2016) reevaluate the identification of 175 isolates from different sources in Brazil which had been previously identified as *A. niger* and found that one-half were indeed *A. welwitschiae*.

A recent review on the presence of *A. niger* aggregate species in grapes indicated that most of the studies had been published before *A. welwitschiae* description and, consequently, substantial reevaluation of the occurrence of *A. niger* aggregate species on grapes and grape products was strongly needed (Gil-Serna et al., 2018b). This evaluation is especially relevant regarding their potential ability to produce OTA or FB_2. A recent report from Cyprus indicated *A. tubingensis* as the main species contaminating grapes (Pantelides et al., 2017). This contrasts with studies from South America where *A. niger* and *A. welwitschiae* were the predominant species in this product (Garmendia and Vero, 2016; Massi et al., 2016; De Souzza et al., 2018). Although our work supported the highest relevance for *A. tubingensis* in grapes, both *A. niger* and *A. welwitschiae* might still be relevant enough to be considered.

Cereals are also an important source of *A. niger* aggregate isolates worldwide. In our work, *A. tubingensis* was also by far the most prevalent species followed by *A. welwitschiae* and *A. niger*. The first two species have been also reported as the most frequently species associated to maize in Italy whereas *A. niger* was the most prevalent in USA (Logrieco et al., 2014; Susca et al., 2014a). Moreover, both reports defended the capability of *A. niger* and *A. welwitschiae* to produce FB_2 and its important contribution to FB_2 accumulation in maize.

The presence of these species on other food less investigated might result in a potential risk of toxin contamination and, certainly, it would require more attention. This might be the case of onion, where Gherbawy et al. (2015) found that *A. welwitschiae* was the most prevalent species in onion samples from Saudi Arabia. Although none of the isolates showed ochratoxigenic potential, some of them were FB_2 producers. This situation is in agreement with the results we have obtained for our *A. welwitschiae* isolates from onion. Studies reporting many *A. niger* isolates having truncated versions of OTA and FB_2 biosynthetic clusters and, therefore, unable to synthetize the toxins (Susca et al., 2014b, 2016; Gil-Serna et al., 2018a) provide the possibility to

test for the presence of these clusters in *A. niger* aggregate species in order to predict their potential ability to produce OTA and FB_2. We have followed this approach to analyze the presence of both biosynthetic clusters in all the *A. tubingensis*, *A. welwitschiae* and *A. niger* isolates from Spain and using available DNA based tools as well as additional new protocols described in this work. *A. tubingensis* isolates showed no complete clusters for any of both toxins but the truncated versions of them. In contrast, *A. welwitschiae* and *A. niger* species could be considered potential producers for both OTA and FB2 toxins; however, their frequency of potential producing isolates for each toxin varies. OTA production is not general since only a few number of isolates of *A. niger* and *A. welwitschiae* present the complete version of the cluster. However, the expression of all the OTA biosynthetic genes showed a coordinate similar expression pattern, typical of clustered genes, with high level of induction in those strains analyzed having the complete cluster. Moreover, the isolates which were grown in *in vitro* permissive conditions did actually produce OTA, confirming that the genes were functional.

As mentioned above, the potential ability to produce FB_2 seems to be more frequent in *A. niger* than in *A. welwitschiae*. This fact agrees with reports by several authors who demonstrated that the complete version of FB_2 biosynthetic cluster was more frequently found in *A. niger* than *A. welwitschiae* (Susca et al., 2014a, 2016; Massi et al., 2016). The lack of FB_2 production in *A. welwitschiae* is reported to be associated with gene deletions although some cases of *A. niger* isolates having the complete cluster were unable to produce detectable levels of the toxin (Massi et al., 2016). In our work, we analyzed FB_2 by LC MS/MS and *fum1* expression by real-time PCR in two selected isolates of *A. niger* and *A. welwitschiae* having the complete cluster and one strain each having the truncated FB_2 biosynthetic cluster. In all cases, when the complete cluster was present, FB_2 was detected in the medium and *fum1* gene was expressed at high levels. In contrast, those isolates having the truncated FB_2 cluster did not express fum1 gene and FB_2 concentration was above detectable levels.

The ability of *A. tubingensis* to produce OTA has been often controversial and several authors have discussed if it is indeed an OTA producer (Accensi et al., 2001; Medina et al., 2005; Perrone et al., 2006). In our work, all the *A. tubingensis* isolates (175) had the truncated version of the OTA biosynthetic cluster detected by the analysis of each of the five cluster genes as well as by the positive amplification of the PCR product of the expected size using a primer set located in the flanking regions of the cluster. Furthermore, we have used next generation sequencing to obtain the genomic sequence of a strain of *A. tubingensis*. We have searched this genome and that available on JGI database for key genes required for OTA and FB_2 production without success. These findings were recently confirmed by Choque et al. (2018) which studied the genome of another *A. tubingensis* strain. In the case of other OTA-producing species such as *P. nordicum*, two different regions have been described that contained genes involved in OTA biosynthesis and the expression of one or the other seems to be related to environmental conditions (Gil-Serna et al., 2018a). However, *A. tubingensis* genome did not contain another putative region involved in OTA biosynthesis. Finally, OTA and FB_2 production analyses were carried out for two isolates grown on CYA plates at permissive conditions and none of the two toxins could be detected.

The fact that *A. tubingensis* was considered an important OTA producer for a long time might be due to several reasons. Most of the studies describing the ability of this species to produce OTA were published before the description of the new species included in *A. niger* aggregate by Perrone et al. (2011) and, therefore, these isolates might have been misclassified and they correspond indeed to *A. welwitschiae*. On the other hand, all works regarding *A. tubingensis* production described that these isolates usually reached very low levels of OTA even in highly permissive conditions and, up to our knowledge, when OTA was detected, OTA levels were measured by HPLC. Storari et al. (2012) reported other metabolites with retention times similar to OTA when *A. tubingensis* extracts were analyzed by HPLC coupled with a fluorescence detector. The detection of these compounds can lead to misinterpretation of the chromatograms and to the incorrect attribution of OTA production to *A. tubingensis*. Nielsen et al. (2009) also claimed that *A. tubingensis* is not able to produce OTA probably due to unspecific chemical analysis. Analytical methods have evolved quickly and to confirm if *A. tubingensis* isolates are able or not to produce OTA, an analysis by LC-MS might be necessary to unambiguously confirm the presence of the toxin in fungal cultures.

The phylogenetic analysis based on parsimony using the partial region of the calmodulin gene revealed a topology consistent with the general genetic relationships previously reported for these species (Samson et al., 2014). The intraspecific variability appeared to be higher in *A. tubingensis* than in *A. niger* and *A. welwitschiae*, showing no evident relationship of intraspecific clusters and the host where the strains had been isolated from. Similarly, no relationship became apparent regarding the presence of either OTA or FB_2 complete cluster in *A. welwitschiae*. Whether the inclusion of the 5 *A. niger* isolates having all the OTA biosynthetic genes in one cluster might have phylogenetic significance or not, it remains premature until more information is available. The similar truncated versions of OTA and FB_2 in the three species suggests that both could have been generated in their common ancestral population resulting in the loss of the ability of toxin biosynthesis along their speciation process at different rates depending on the species and the toxin considered.

We might conclude on the basis of the evidences obtained from the different analyses carried out in this work that *A. tubingensis*, in spite of its high incidence in Spanish commodities, might not contribute to OTA and FB_2 contamination of food products. In contrast, although *A. niger* and *A. welwitschiae* are less frequent in Spain than *A. tubingensis,* their potential ability to produce both toxins, particularly FB_2, need to be carefully studied in agrofood products.

Acknowledgements

This work was supported by the Spanish Ministry of Science and Innovation (AGL 2014-53928-C2-2-R). M. García-Díaz was supported by a FPI fellowship by the Spanish Ministry of Science and Innovation. (BES-2015-074533).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.02.013.

References

Accensi, F., Abarca, M.L., Cano, J., Figuera, L., Cabañes, F.J., 2001. Distribution of ochratoxin A producing strains in the *A. niger* aggregate. A. van Leeuw. J. Microb. 79, 365–370. https://doi.org/10.1023/A:1012003813985.

Bragulat, M.R., Abarca, M.L., Cabañes, F.J., 2001. An easy screening method for fungi producing ochratoxin A in pure culture. Int. J. Food Microbiol. 71, 139–144. https://doi.org/10.1016/S0168-1605(01)00581-5.

Cabañes, F.J., Bragulat, M.R., 2018. Black aspergilli and ochratoxin-A producing species. Curr. Opin. Food Sci. 23, 1–10. https://doi.org/10.1016/j.cofs.2018.01.006.

Chiotta, M.L., Susca, A., Stea, G., Mulè, G., Perrone, G., Logrieco, A., Chulze, S.N., 2011. Phylogenetic characterization and ochratoxin A – fumonisin profile of black Aspergillus isolated from grapes in Argentina. Int. J. Food Microbiol. 149, 171–176. https://doi.org/10.1016/j.ijfoodmicro.2011.06.002.

Choque, E., Klopp, C., Valiere, S., Raynal, J., Mathieu, F., 2018. Whole-genome sequencing of *Aspergillus tubingensis* G131 and overview of its secondary metabolism potential. BMC Genomics 19, 200. https://doi.org/10.1186/s12864-018-4574-4.

De Souzza, L., Fungaro, M.H.P., Massi, F.P., da Silva, J.J., Silva, R.E., Frisvad, J.C., Taniwaki, M.H., Iamanaka, B.T., 2018. Diversity of Aspergillus section Nigri on the surface of Vitis lambrusca and its hybrid grapes. Int. J. Food Microbiol. 268, 53–60. https://doi.org/10.1016/j.ijfoodmicro.2017.12.027.

de Vries, R.P., Riley, R., Wiebenga, A., Aguilar-Osorio, G., Amillis, S., Uchima, C.A., Anderluh, G., Asadollahi, M., Askin, M., Barry, K., Battaglia, E., Bayram, O., Benocci, T., Braus-Stromeyer, S.A., Caldana, C., Canovas, D., Cerqueira, G.C., Chen, F., Chen, W., Choi, C., Clum, A., Dos Santos, R.A., Damasio, A.R., Diallinas, G., Emri, T., Fekete, E., Flipphi, M., Freyberg, S., Gallo, A., Gournas, C., Habgood, R., Hainaut, M.,

Harispe, M.L., Henrissat, B., Hilden, K.S., Hope, R., Hossain, A., Karabika, E., Karaffa, L., Karanyi, Z., Krasevec, N., Kuo, A., Kusch, H., LaButti, K., Lagendijk, E.L., Lapidus, A., Levasseur, A., Lindquist, E., Lipzen, A., Logrieco, A.F., MacCabe, A., Makela, M.R., Malavazi, I., Melin, P., Meyer, V., Mielnichuk, N., Miskei, M., Molnar, A.P., Mule, G., Ngan, C.Y., Orejas, M., Orosz, E., Ouedraogo, J.P., Overkamp, K.M., Park, H.S., Perrone, G., Piumi, F., Punt, P.J., Ram, A.F., Ramon, A., Rauscher, S., Record, E., Riano-Pachon, D.M., Robert, V., Rohrig, J., Ruller, R., Salamov, A., Salih, N.S., Samson, R.A., Sandor, E., Sanguinetti, M., Schutze, T., Sepcic, K., Shelest, E., Sherlock, G., Sophianopoulou, V., Squina, F.M., Sun, H., Susca, A., Todd, R.B., Tsang, A., Unkles, S.E., van de Wiele, N., van Rossen-Uffink, D., Oliveira, J.V., Vesth, T.C., Visser, J., Yu, J.H., Zhou, M., Andersen, M.R., Archer, D.B., Baker, S.E., Benoit, I., Brakhage, A.A., Braus, G.H., Fischer, R., Frisvad, J.C., Goldman, G.H., Houbraken, J., Oakley, B., Pocsi, I., Scazzocchio, C., Seiboth, B., van Kuyk, P.A., Wortman, J., Dyer, P.S., Grigoriev, I.V., 2017. Comparative genomics reveals high biological diversity and specific adaptations in the industrially and medically important fungal genus Aspergillus. Genome Biol. 18, 28. https://doi.org/10.1186/s13059-017-1151-0.

Frisvad, J.C., Smedsgaard, J., Samson, R.A., Larsen, T.O., Thrane, U., 2007. Fumonisin B2 production by Aspergillus niger. J. Agric. Food Chem. 55, 9727–9732. https://doi.org/10.1021/jf0718906.

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, e23496. https://doi.org/10.1371/journal.pone.0023496.

Garmendia, G., Vero, S., 2016. Occurrence and biodiversity of Aspergillus section Nigri on Tannat grapes in Uruguay. Int. J. Food Microbiol. 216, 31–39. https://doi.org/10.1016/j.ijfoodmicro.2015.08.020.

Gherbawy, Y., Elhariry, H., Kocsube, S., Bahobial, A., El Deeb, B., Altalhi, A., Varga, J., Vágvölgyi, C., 2015. Molecular characterization of Black Aspergillus species from onion and their potential for ochratoxin and fumonisin B2 production. Foodb. Pathog. Dis. 12, 414–423. https://doi.org/10.1089/fpd.2014.1870.

Gil-Serna, J., García-Díaz, M., González-Jaén, M.T., Vázquez, C., Patiño, B., 2018a. Description of an orthologous cluster of ochratoxin A biosynthetic genes in Aspergillus and Penicillium species. A comparative analysis. Int. J. Food Microbiol. 268, 35–43. https://doi.org/10.1016/j.ijfoodmicro.2017.12.028.

Gil-Serna, J., Vázquez, C., González-Jaén, M.T., Patiño, B., 2018b. Wine contamination with ochratoxins: a review. Beverages 4, 6. https://doi.org/10.3390/beverages4010006.

Hong, S.B., Lee, M., Kim, D.H., Varga, J., Frisvad, J.C., Perrone, G., Gomi, K., Yamada, O., Machida, M., Houbraken, J., Samson, R.A., 2013. Aspergillus luchuensis, an industrially important black Aspergillus in East Asia. PLoS One 8, e63769. https://doi.org/10.1371/journal.pone.0063769.

Ismail, M.A., 2017. Incidence and significance of black aspergilli in agricultural commodities: a review, with a key to all species accepted to-date. Eur. J. Biol. Res. 7, 207–222. https://doi.org/10.5281/zenodo.834504.

Khaldi, N., Wolfe, K.H., 2011. Evolutionary origins of the fumonisin secondary metabolite gene cluster in Fusarium verticillioides and Aspergillus niger. Int. J. Evol. Biol., 423821. https://doi.org/10.4061/2011/423821.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Logrieco, A.F., Haidukowski, M., Susca, A., Mulè, G., Munkvold, G.P., Moretti, A., 2014. Aspergillus section Nigri as contributor of fumonisin B2 contamination in maize. Food Addit. Contam. Part A 31, 149–155. https://doi.org/10.1080/19440049.2013.862349.

Massi, F.P., Sartori, D., de Souzza, L., Iamanaka, B.T., Taniwaki, M.H., Carneiro, M.L., Fungaro, M.H.P., 2016. Prospecting for the incidence of genes involved in ochratoxin and fumonisin biosynthesis in Brazilian strains of *Aspergillus niger* and *Aspergillus welwitschiae*. Int. J. Food Microbiol. 221, 19–28. https://doi.org/10.1016/j.ijfoodmicro.2016.01.010.

Medina, A., Mateo, R., López-Ocaña, 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, 4696–4702. https://doi.org/10.1128/AEM.71.8.4696-4702.2005.

Nielsen, K.F., Mogensen, J.M., Johansen, M., Larsen, T.O., Frisvad, J.C., 2009. Review of secondary metabolites and mycotoxins from the Aspergillus niger group. Anal. Bioanal. Chem. 395, 1225–1242.

Palumbo, J.D., O'Keeffe, T.L., 2014. Detection and discrimination of four Aspergillus section Nigri species by PCR. Lett. Appl. Microbiol. 60, 188–195. https://doi.org/10.1111/lam.12358.

Palumbo, J.D., O'Keeffe, T.L., Gorski, L., 2013. Multiplex PCR analysis of fumonisin biosynthetic genes in fumonisin-non producing Aspergillus niger and A. awamori strains. Mycologia 105, 277–284. https://doi.org/10.3852/11-418.

Pantelides, I.S., Aristeidou, E., Lazari, M., Tsolakidou, M.D., Tsaltas, D., Christofidou, M., Kafouris, D., Christou, E., 2017. Biodiversity and ochratoxin A profile of Aspergillus section Nigri populations isolated from wine grapes in Cyprus vineyards. Food Microbiol. 67, 106–117. https://doi.org/10.1016/j.fm.2017.06.010.

Perrone, G., Mulè, G., Susca, A., Battilani, P., Pietri, A., Logrieco, A., 2006. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius, A. tubingensis* and *A. niger* strains isolated from grapes in Italy. Appl. Environ. Microbiol. 72, 680–685. https://doi.org/10.1128/AEM.72.1.680-685.2006.

Perrone, G., Stea, G., Epifani, F., Varga, J., Frisvad, J.C., Samson, R.A., 2011. *Aspergillus niger* contains the cryptic phylogenetic species *A. awamori*. Fungal Biol-UK 115, 1138–1150. https://doi.org/10.1016/j.funbio.2011.07.008.

Peterson, S.W., 2008. Phylogenetic analysis of Aspergillus species using DNA sequences from four loci. Mycologia 100, 205–226. https://doi.org/10.1080/15572536.2008.11832477.

Querol, A., Barrio, E., Huerta, T., Ramón, D., 1992. Molecular monitoring of wine fermentations conducted by active dry yeast strains. Appl. Environ. Microbiol. 58, 2948–2953.

Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C., Varga, J., 2007. Diagnostic tools to identify black aspergilli. Stud. Mycol. 59, 129–145. https://doi.org/10.3114/sim.2007.59.13.

Samson, R.A., Visagie, C.M., Houbraken, J., Hong, S.B., Hubka, V., Klaassen, C.H.W., Perrone, G., Seifert, K.A., Susca, A., Tanney, J.B., Varga, J., Kocsube, S., Szigeti, G., Yaguchi, T., Frisvad, J.C., 2014. Phylogeny, identification and nomenclature of the genus Aspergillus. Stud. Mycol. 78, 141–173. https://doi.org/10.1016/j.simyco.2014.07.004.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3, 1101–1108. https://doi.org/10.1038/nprot.2008.73.

Storari, M., Bigler, L., Gessler, C., Broggini, G.A.L., 2012. Assessment of the ochratoxin A production ability of Aspergillus tubingensis. Food Addit. Contam. A 29, 1450–1454. https://doi.org/10.1080/19440049.2012.698656.

Susca, A., Moretti, A., Stea, G., Villani, A., Haidukowski, M., Logrieco, A., Munkvold, G., 2014a. Comparison of species composition and fumonisin production in Aspergillus section Nigri populations in maize kernels from USA and Italy. Int. J. Food Microbiol. 188, 75–82. https://doi.org/10.1016/j.ijfoodmicro.2014.06.031.

Susca, A., Proctor, R.H., Butchko, R.A.E., Haidukowski, M., Stea, G., Logrieco, A., Moretti, A., 2014b. Variation in the fumonisin biosynthetic gene cluster in fumonisin-producing and nonproducing black aspergilli. Fungal Genet. Biol. 73, 39–52. https://doi.org/10.1016/j.fgb.2014.09.009.

Susca, A., Proctor, R.H., Morelli, M., Haidukowski, M., Gallo, A., Logrieco, A.F., Moretti, A., 2016. Variation in fumonisin and ochratoxin production with differences in biosynthetic gene content in Aspergillus niger and A. welwitschiae isolates from multiple crop and geographic origins. Front. Microbiol. 7, 1412. https://doi.org/10.3389/fmicb.2016.01412.

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, 2731–2739. https://doi.org/10.1093/molbev/msr121.

Varga, J., Frisvad, J.C., Kocsubé, S., Brankovics, B., Tóth, B., Szigeti, G., Samson, R.A., 2011. New and revisited species in Aspergillus section Nigri. Stud. Mycol. 69, 1–17. https://doi.org/10.3114/sim.2011.69.01.

White, T.J., Burns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelgard, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR protocols: a guide to methods and applications. Academic Press, New York, USA, pp. 315–322.