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Prospecting for the incidence of genes involved in ochratoxin and fumonisin biosynthesis in Brazilian strains of *Aspergillus niger* and *Aspergillus welwitschiae*

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ABSTRACT

Aspergillus niger "aggregate" is an informal taxonomic rank that represents a group of species from the section *Nigri*. Among *A. niger* "aggregate" species *Aspergillus niger sensu stricto* and its cryptic species *Aspergillus welwitschiae* (=*Aspergillus awamori sensu* Perrone) are proven as ochratoxin A and fumonisin B_2 producing species. *A. niger* has been frequently found in tropical and subtropical foods. *A. welwitschiae* is a new species, which was recently dismembered from the *A. niger* taxon. These species are morphologically very similar and molecular data are indispensable for their identification. A total of 175 Brazilian isolates previously identified as *A. niger* collected from dried fruits, Brazil nuts, coffee beans, grapes, cocoa and onions were investigated in this study. Based on partial calmodulin gene sequences about one-half of our isolates were identified as *A. welwitschiae*. This new species was the predominant species in onions analyzed in Brazil. *A. niger* and *A. welwitschiae* differ in their ability to produce ochratoxin A and fumonisin B_2. Among *A. niger* isolates, approximately 32% were OTA producers, but in contrast only 1% of the *A. welwitschiae* isolates revealed the ability to produce ochratoxin A. Regarding fumonisin B_2 production, there was a higher frequency of FB_2 producing isolates in *A. niger* (74%) compared to *A. welwitschiae* (34%). Because not all *A. niger* and *A. welwitschiae* strains produce ochratoxin A and fumonisin B_2, in this study a multiplex PCR was developed for detecting the presence of essential genes involved in ochratoxin (polyketide synthase and *radH*flavin-dependent halogenase) and fumonisin (α-oxoamine synthase) biosynthesis in the genome of *A. niger* and *A. welwitschiae* isolates. The frequency of strains harboring the mycotoxin genes was markedly different between *A. niger* and *A. welwitschiae*. All OTA producing isolates of *A. niger* and *A. welwitschiae* showed in their genome the *pks* and *radH* genes, and 95.2% of the nonproducing isolates did not contain these genes. The α-oxoamine synthase gene was detected in 100% and 36% of the *A. niger* and *A. welwitschiae* isolates, respectively. The loss of ochratoxin A production in *A. niger* and *A. welwitschiae* is highly associated with gene deletions within the ochratoxin biosynthetic gene cluster. The loss of fumonisin production in *A. welwitschiae* is associated with gene deletions within the fumonisin biosynthetic gene cluster, but this is not the case with *A. niger*.

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1. Introduction

Aspergillus niger "aggregate" is an informal taxonomic rank that represents a group of morphologically very similar species of *Aspergillus* section *Nigri* demanding DNA sequence analysis of calmodulin (*CaM*) and β-tubulin (*benA*) genes for the identification of their members. The taxonomical status of *A. niger* "aggregate" has evolved continuously; for instance, the taxon *Aspergillus niger sensu stricto* was recently dismembered into *Aspergillus welwitschiae* and *A. niger* (Perrone et al., 2011). Despite the industrial importance of both these species, they are recognized as producers of mycotoxins, namely ochratoxin A (OTA) and fumonisin B_2 (FB_2) (Abarca et al., 1994; Frisvad et al., 2007, 2011; Perrone et al., 2011). Excluding *Aspergillus lacticoffeatus*, which is a color mutant of *A. niger* (see Varga et al., 2011), there are 10 *A. niger* "aggregate" species, only two of which were conclusively proven to be OTA and FB_2 producers.

Ochratoxin A is a nephrotoxic and potentially carcinogenic mycotoxin found in a variety of food commodities such as cereals, coffee beans, cocoa beans, grapes, dried fruits and spices (Malir et al., 2013; Ostry et al., 2013). Fumonisin B_2 is another mycotoxin frequently found in cereals that can cause a variety of toxic effects in different animal

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species and has been associated with esophageal cancers in humans (Rocha et al., 2014; Scott, 2012).

Interestingly, not all the strains of *A. niger* are able to produce OTA or FB_2. Before *A. niger* was dismembered into two taxa, Frisvad et al. (2011) analyzed a group of available industrial strains reporting that 33% and 83% of them were OTA and FB_2 producers respectively. Nowadays, there is a consensus that all *A. niger* isolates have to be carefully checked for mycotoxin production before they can be used for industrial purposes.

In general, genes encoding enzymes involved in mycotoxin biosynthesis are located physically adjacent constituting gene clusters that usually harbor genes for polyketide synthases (PKS), non-ribosomal peptide synthases (NRPS), hydrolases, oxidases, methylases, transporters, and regulatory proteins (Turner, 2010). The *in silico* analyses performed by Ferracin et al. (2012) have shown that the *pks-locus* tag An15g07920 located in the ochratoxin gene cluster of the ochratoxigenic *A. niger* CBS 513.88 strain is absent in the non-ochratoxigenic ATCC 1015 strain. Moreover, an *in vivo* analysis of several Brazilian strains has shown there is an association between the presence of this particular *pks* gene and the capability to synthesize OTA (Ferracin et al., 2012).

Regarding fumonisin, the presence of a putative fumonisin gene cluster in the *A. niger* genome, harboring at least 10 genes, was reported by Pel et al. (2007). The gene *fum8*, located in this gene cluster, encodes an α-oxoamine synthase (Fum8p), which is an essential enzyme for fumonisin biosynthesis, and the disruption of *fum8* was proven to result in the loss of FB_2 biosynthesis in *A. niger* (Shimizu et al., 2015).

Because *A. niger* and/or its cryptic species *A. welwitschiae* are very common in grapes, coffee beans, onions, Brazil nuts, and dried fruits, in this study the presence of genes encoding essential proteins for ochratoxin and fumonisin biosynthesis in a Brazilian isolate collection was examined, using a multiplex PCR developed by our group for this purpose.

2. Material and methods

2.1. Fungal isolates

Fungal isolates were provided by the following Brazilian institutions: Universidade Estadual de Londrina ($n = 85$) and Instituto de Tecnologia de Alimentos ($n = 90$). They were collected from dried fruits ($n = 19$), Brazil nuts ($n = 30$), coffee beans ($n = 27$), grapes ($n = 40$), cocoa ($n = 3$), and onions ($n = 56$). The Brazilian geographical regions where the samples were collected are shown in Supplementary Fig. 2 (see supplementary file Data in Brief, Massi et al., submitted for publication).

2.2. DNA extraction

Conidia of each isolate were inoculated into 7 mL of liquid complete medium (Pontecorvo et al., 1953) and incubated at 25 °C for 24 h. Mycelia were collected, frozen in liquid nitrogen and ground to a fine powder. Nucleic acids were purified using the BioPur Mini Spin Extraction Kit (Biometrix, Brazil), according to the manufacturer's instructions.

2.3. Partial calmodulin gene sequence analyses

Amplification of a *CaM* gene region was performed using the primers cmd5 (5′ CCG AGT ACA AGG AGG CCT TC 3′) and cmd6 (5′ CCG ATA GAG GTC ATA ACG TGG 3′) described in Hong et al. (2006), and the resulting fragments purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Sequencing reactions were then carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and their products processed in an ABI 3500XL Genetic Analyser (Applied Biosystems, USA). The sequences were subsequently aligned with those from *Aspergillus* section *Nigri* available in the GenBank database. A phylogeny reconstruction was obtained by using a distance-based Neighbor-Joining method (Saitou and Nei, 1987). The tree was drawn using MEGA 6.05 (Tamura et al., 2013) with 1000 bootstrap replicates for assessing node confidences.

2.4. Ochratoxin A production

The capacity of each isolate to produce ochratoxin A (OTA) was analyzed according to a qualitative method described by Filtenborg et al. (1983). Few modifications were made. The species were three point inoculated into Yeast Extract Sucrose Agar (YES Agar) and incubated at 25 °C for 7 days. An agar plug was removed from the center of the colony, the OTA extracted with methanol: chloroform (1:1) and the plug placed on a silica plate for thin layer chromatography. The mobile phase used was toluene: ethyl acetate: formic acid 90%: chloroform (7:5:2:5, *v*/*v*/*v*/*v*). The OTA was visualized under UV light at 254 and 365 nm. An OTA standard (Sigma, St. Louis, USA) was used to compare the fluorescence spectrum and the retention index of the strains' compounds relative to those of OTA.

2.5. Fumonisin production

The production of fumonisin B_2 by the isolates was tested using the methodology in Frisvad et al. (2007) with modifications. The isolates were inoculated onto agar Czapek Yeast Extract 20% Sucrose (CY20S Agar) and incubated at 25 °C for 7 days. Five small pieces of mycelium were removed (plugs) from the center of the colony and transferred into a vial. One milliliter of methanol was added and stirred by vortex for 3 min. Two filtrations were carried out with a Millex membrane; 0.45 μM and 0.22 μM, 55 μL of the extract was transferred to an HPLC vial, adding 55 μL of o-phthaldialdehyde reagent (OPA), according to the method described by Visconti et al. (2001). The vial was stirred in vortex for 30 s. A 20 μL aliquot of the extract was injected into the chromatograph. Detection and quantification of FB_2 was performed in a Shimadzu LC-10VP (Shimadzu, Japan) HPLC with a fluorescence detector (model RF-10AXL), set at 335 nm excitation and 440 nm emission. The chromatography column used was a YMC-Pack ODS-A (YMC Co., Japan) (5 mm, 4.6 × 150 mm) with a mobile phase of acetonitrile: water: acetic acid (51:47:02, *v*/*v*/*v*), a flow rate of 1 mL/min and an oven temperature of 40 °C. Samples as well as a fumonisin B_2 standard (Sigma, St. Louis, USA) were injected into the HPLC equipment. The detection limit for fumonisin B_2 was calculated as 0.07 μg/g.

2.6. Primer designing

PCR assays were developed for reliable fungal species identification and detection of genes involved in mycotoxin synthesis. The primer sets were designed to have similar melting temperatures and to avoid the formation of secondary structures, either for self or to another (Table 1). The primer-pair *benA*-An/Aw was designed to detect the species *A. niger* and *A. welwitschiae*. This involved retrieving the *benA* gene sequences of all black aspergilli from the GenBank databases. The sequences were then aligned using the BioEdit software (Hall, 1999), and visually checked for regions of complete similarity between *A. niger* and *A. welwitschiae*, but not other species belonging to section *Nigri* (Fig. 1). A second primer-pair was designed to detect the gene *radH* (locus tag An15g07880 of *A. niger* CBS 513.88), which encodes a flavin-dependent halogenase involved in OTA biosynthesis; similarly, a third primer-pair was designed to target another essential gene (*pks*), which encodes for a polyketide synthase (locus tag An15g07920 of *A. niger* CBS 513.88), developed by Ferracin et al.

Table 1
Primer sequences used in the multiplex PCR.

Primer name	Primer sequence (5′–3′)	Amplicon size (bp)	Reference
pks-F	TCC TAC GAC TTC ACC GAC AT	554	Ferracin et al., 2012
pks-R	CAT TTC GTT GAT CCC ATC G		
radH-F	AGG CAT CAT CAA ACC CAT CTT	328	This study
radH-R	GAA ATC AAA CAG GCG TCC GA		
fum8vnF1-F*	TCG TTT GAG TGG TGG CAG AAT	128	*Modified from Susca et al., 2010
fum8-R	GTT GGG CAC AGA TAC CAT TTG		
benA-An/Aw-F	TGG GCA AAG GGT TGG GTC TT	192	This study
benA-An/Aw-R	ACG AGG ACG GCA CGA GGA		

benA-An/Aw — designed for detection of *A. niger* and *A. welwitschiae*;
pks — designed for detection of the polyketide synthase gene involved in ochratoxin biosynthesis;
radH — designed for detection of the *radH* flavin-dependent halogenase gene involved in ochratoxin biosynthesis;
fum8 — designed for detection of the α-oxoamine synthase gene involved in fumonisin biosynthesis.

(2012). Finally, a primer-pair was designed to detect the gene *fum8* (locus tag An01g06870 of *A. niger* CBS 513.88), which encodes α-oxoamine synthase, an essential enzyme for FB_2 biosynthesis. The forward (fum8vn-F) consisted of the same sequence designed by Susca et al. (2010), denoted vnF1, but with five additional nucleotide residues. The reverse (fum8-R) was designed after the selection of a conserved region, 128 nucleotides away from the target sequence of the forward primer. All primers were analyzed *in silico* for specificity using "Primer-Blast" (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

The inclusiveness of *benA*-An/Aw was evaluated experimentally using the DNA from all strains of *A. niger* ($n = 89$) and *A. welwitschiae* ($n = 86$). The specificity of the *benA*-An/Aw primers was determined experimentally against the following non-target species: *Aspergillus uvarum*, *Aspergillus aculeatus*, *Aspergillus japonicus*, *Aspergillus neoniger*, *Aspergillus tubingensis*, *Aspergillus carbonarius*, *Aspergillus luchuensis*, *Aspergillus fijiensis* and *Aspergillus brasiliensis* (Fig. 2).

2.7. Multiplex PCR

Multiplex amplifications (mPCR) were carried out using the four primer-pairs in a single PCR mixture. All reactions were performed in a final volume of 10 μL containing 1× PCR buffer, 10 ng of DNA template, 0.4 pMoL of each primer, 0.2 mM of dNTP set (Invitrogen, Life Technologies, USA), 2.0 mM of $MgCl_2$ and 1.0 U of Platinum Taq DNA polymerase (Invitrogen, Life Technologies, USA). The DNA was amplified in a Veriti® Thermal Cycler (Applied Biosystems, USA) under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 40 s, annealing at 58 °C for 35 s and extension at 72 °C for 40 s followed by final extension of 72 °C for 30 min. The amplified products were analyzed using standard (1.4%) agarose gel and capillary electrophoresis. This involved resynthesizing all primers to incorporate a fluorescent dye (6-carboxy-fluorescine (FAM), on the 5′ end of pks-F, radH-F and fum8vnF1-F, and hexachloro-6-carboxy-fluorescine (HEX) on the 5′ end of *benA*-An/Aw-F). Each amplified sample was diluted 10× and 8.0 μL of (Hi-Di) formamide and 0.3 μL of GeneScan™ 600 LIZ® internal lane size standard (Applied Biosystems, USA) were added to 2 μL of this diluted sample. An ABI 3500XL Genetic Analyser (Applied Biosystems, USA) was used to separate and detect the fluorescently labeled PCR products which were analyzed using GeneMarker® Software (SoftGenetics®).

3. Results and discussion

3.1. Molecular species identification

Initially, a portion of the *CaM* gene was sequenced using all the isolates (175) collected from dried fruits, Brazil nuts, coffee beans, grapes, cocoa and onions, which were previously identified as *A. niger* (unpublished data). The analysis revealed the existence of 10 haplotypes and their sequences were deposited in the NCBI database

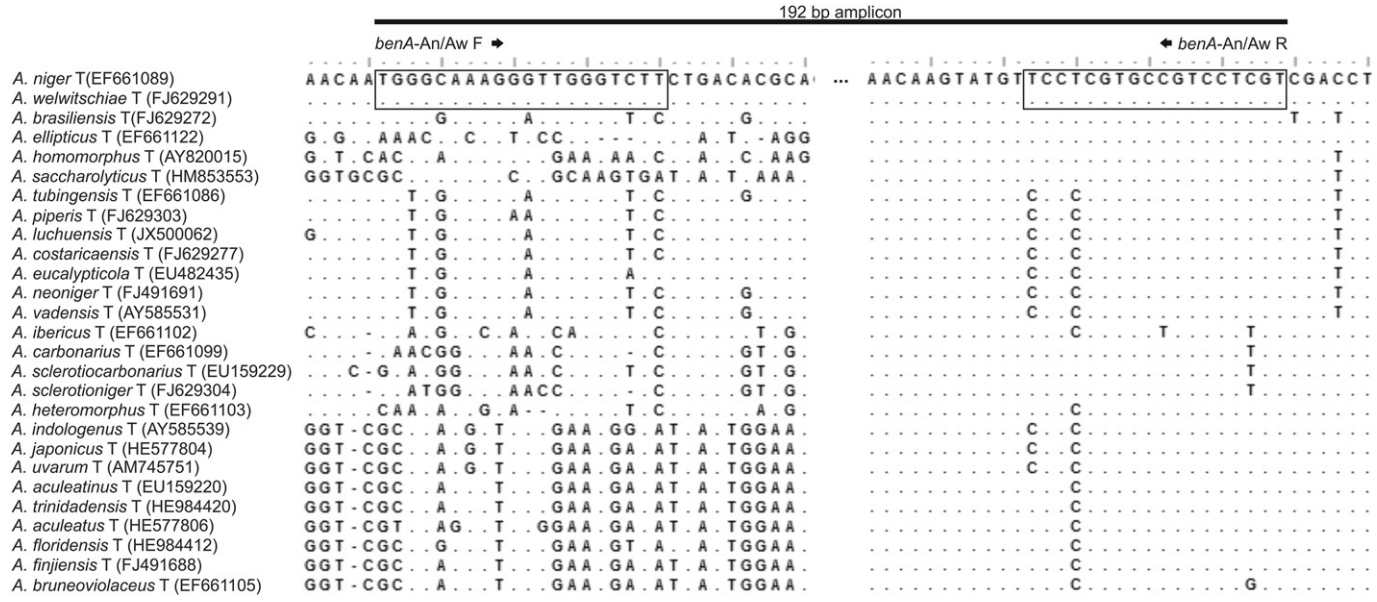


Fig. 1. Alignment of partial β-tubulin gene sequences of all black aspergilli species. The sequences were obtained from the GenBank databases. Priming sites of the primers *benA*-An/Aw-F and *benA*-An/Aw-R are boxed. ^{T} means type strain.

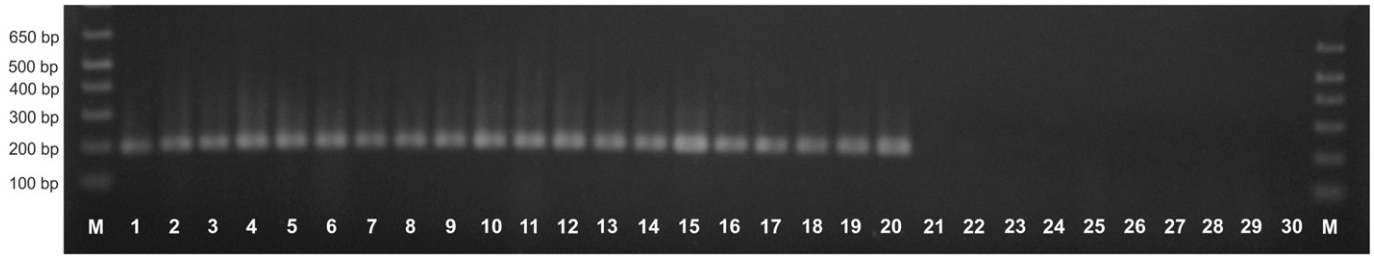


Fig. 2. Specificity of the *benA*-An/Aw primers for detecting of *Aspergillus niger* and *A. welwitschiae*. Note that a strong positive signal of 192 bp in length was detected when *A. niger* (lanes 1 to 10) and *A. welwitschiae* (lanes 11 to 20) DNAs were examined. No specific amplification occurred in any of the following non-target species: *Aspergillus uvarum*, *A. aculeatus*, *A. japonicus*, *A. neoniger*, *A. tubingensis*, *A. carbonarius*, *A. luchuensis*, *A. fijiensis* and *A. brasiliensis* (lanes 21 to 29, respectively). The molecular weight standard (lane M) is a 1 kb DNA ladder (Invitrogen Life Technologies, USA).

under GenBank accession numbers KT749947, KT749948, KT749949, KT749950, KT749951, KT749952, KT749953, KT749954, KT749955 and KT749956. Subsequently, the phylogenetic tree was reconstructed showing that the Brazilian isolates herein investigated split into two distinct clades: one grouping with *A. niger* CBS 554.65 type strain and the other with its sibling species, *A. welwitschiae* CBS 139.54 type strain

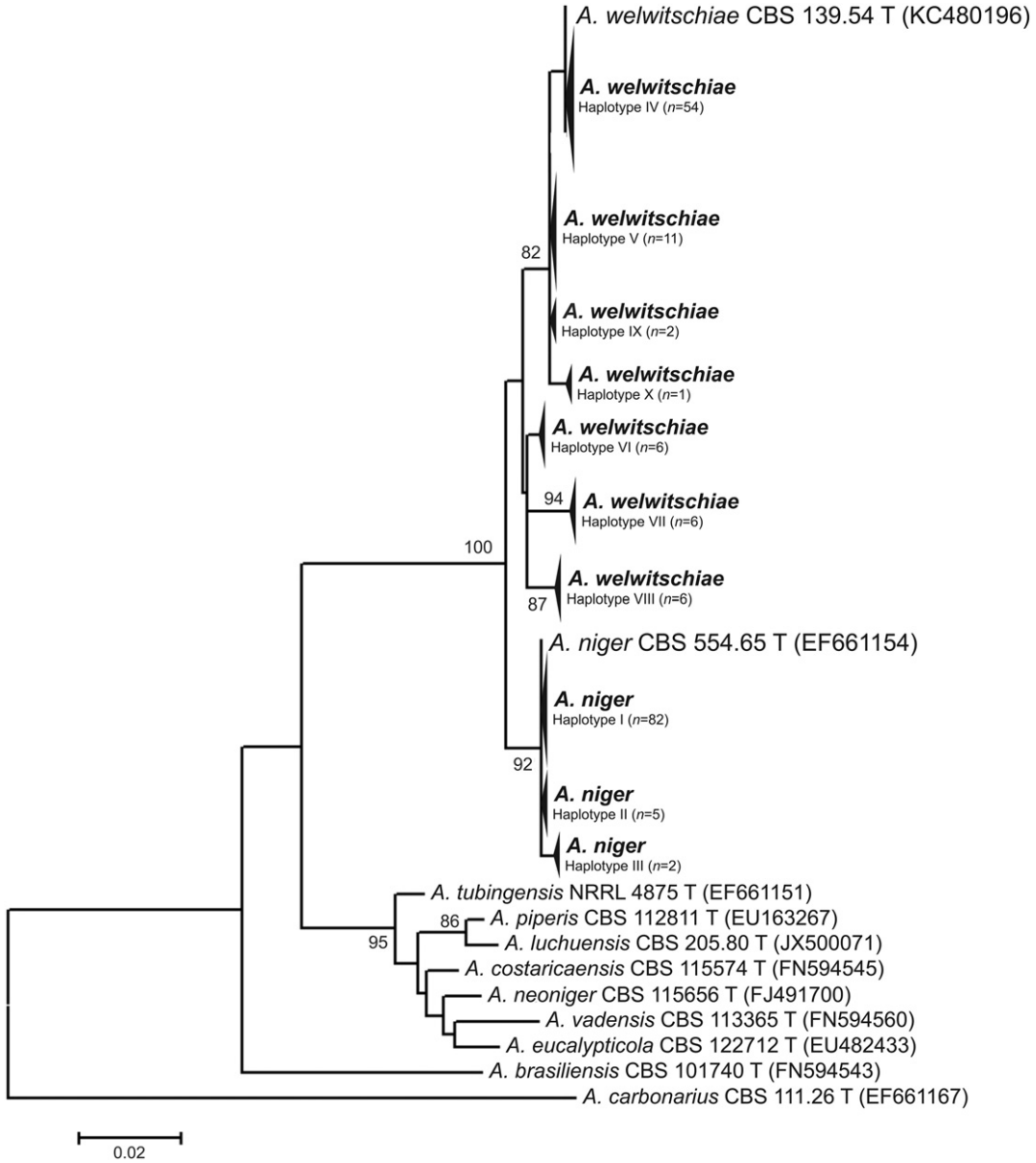


Fig. 3. Neighbor-joining tree reconstructed from the partial calmodulin gene sequences. The sequences of *A. niger*, haplotypes I (KT749947), II (KT749948), III (KT749949) and *A. welwitschiae* haplotypes IV (KT749950), V (KT749951), VI (KT749952), VII (KT749953), VIII (KT749954), IX (KT749955) and X (KT749956) were obtained in the present study. ^{T} means type strain sequences obtained from GenBank database. The accession numbers of nucleotide sequences are in parenthesis.

Table 2
Aspergillus niger and *A. welwitschiae* isolates found in dried fruits, Brazil nuts, coffee beans, grapes, cocoa and onions from Brazil, fumonisin and ochratoxin phenotypes, and mPCR profile detecting the presence or absence of genes involved in ochratoxin and fumonisin biosynthesis.

						Multiplex PCR profile			
Isolate	Substrate	Geographical origin	Fungal species[a]	Fumonisin phenotype[b]	Ochratoxin phenotype[b]	*A. niger*/*A. welwitschiae*[c]	*fum8*[d]	*radH*[e]	*pks*[f]
UEL 2.4	Grapes	Paraná	*A. niger*	+	+	+	+	+	+
UEL 2.5	Grapes	Paraná	*A. niger*	+	+	+	+	+	+
UEL 2.9	Grapes	Paraná	*A. niger*	+	+	+	+	+	+
UEL 1.1	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 1.2	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 6.59	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 10.108	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 10.109	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 10.116	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 10.118	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 10.120	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 10.134	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
ITAL 55.1166	Grapes	Pernambuco	*A. niger*	+	−	+	+	−	−
ITAL 56.1211	Grapes	Pernambuco	*A. niger*	+	−	+	+	−	−
UEL 10.126	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 10.132	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 10.137	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 10.140	Grapes	Paraná	*A. niger*	+	−	+	+	−	−
UEL 21.216	Grapes	Rio Grande do Sul	*A. niger*	+	−	+	+	−	−
UEL 2.3	Grapes	Paraná	*A. niger*	−	−	+	+	−	−
UEL 10.124	Grapes	Paraná	*A. niger*	−	−	+	+	−	−
UEL 4.17	Grapes	Paraná	*A. niger*	−	−	+	+	−	−
UEL 4.18	Grapes	Paraná	*A. niger*	−	−	+	+	−	−
UEL 4.19	Grapes	Paraná	*A. niger*	−	−	+	+	−	−
UEL 4.31	Grapes	Paraná	*A. niger*	−	−	+	+	−	−
ITAL 57.1278	Grapes	Pernambuco	*A. niger*	−	−	+	+	−	−
ITAL 53.1059	Grapes	Pernambuco	*A. niger*	−	−	+	+	−	−
ITAL 55.1161	Grapes	Pernambuco	*A. niger*	−	−	+	+	−	−
ITAL 4195	Brazil nuts	Amazonas	*A. niger*	+	+	+	+	+	+
ITAL 2636	Brazil nuts	Pará	*A. niger*	+	−	+	+	−	−
ITAL 6504	Brazil nuts	São Paulo	*A. niger*	+	−	+	+	−	−
ITAL 4138	Brazil nuts	Amazonas	*A. niger*	+	−	+	+	−	−
ITAL 7061	Brazil nuts	Amazonas	*A. niger*	+	−	+	+	−	−
ITAL 2334	Brazil nuts	Pará	*A. niger*	−	−	+	+	−	−
ITAL 7377	Brazil nuts	Amazonas	*A. niger*	−	−	+	+	−	−
ITAL 1357	Brazil nuts	Pará	*A. niger*	−	−	+	+	−	−
ITAL 2504	Brazil nuts	Pará	*A. niger*	−	−	+	+	−	−
ITAL 1551	Brazil nuts	Pará	*A. niger*	−	−	+	+	−	−
ITAL 902	Brazil nuts	Pará	*A. niger*	−	−	+	+	−	−
ITAL 1400	Brazil nuts	Pará	*A. niger*	−	−	+	+	−	−
ITAL 880	Brazil nuts	Pará	*A. niger*	−	−	+	+	−	−
ITAL 238	Brazil nuts	Amazonas	*A. niger*	−	−	+	+	−	−
UEL Ac 16.75	Onions	Paraná	*A. niger*	+	+	+	+	+	+
UEL Ac 23.115	Onions	Paraná	*A. niger*	+	+	+	+	+	+
ITAL 103	Coffee beans	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 213	Coffee beans	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 643	Coffee beans	Paraná	*A. niger*	+	+	+	+	+	+
ITAL 8219	Coffee beans	Espírito Santo	*A. niger*	+	+	+	+	+	+
ITAL 8216	Coffee beans	Espírito Santo	*A. niger*	+	+	+	+	+	+
ITAL 8191	Coffee beans	Espírito Santo	*A. niger*	+	+	+	+	+	+
ITAL 113	Coffee beans	São Paulo	*A. niger*	+	−	+	+	−	−
ITAL 121	Coffee beans	São Paulo	*A. niger*	+	−	+	+	−	−
ITAL 123	Coffee beans	São Paulo	*A. niger*	+	−	+	+	−	−
ITAL 186	Coffee beans	São Paulo	*A. niger*	+	−	+	+	−	−
ITAL 413	Coffee beans	Paraná	*A. niger*	+	−	+	+	−	−
ITAL 444	Coffee beans	Paraná	*A. niger*	+	−	+	+	−	−
UEL 15.03	Coffee beans	Minas Gerais	*A. niger*	+	−	+	+	−	−
ITAL 219	Coffee beans	São Paulo	*A. niger*	+	−	+	+	−	−
UEL 15.06	Coffee beans	Minas Gerais	*A. niger*	+	−	+	+	−	−
UEL 11.07	Coffee beans	Espírito Santo	*A. niger*	+	−	+	+	−	−
UEL 12.41	Coffee beans	Espírito Santo	*A. niger*	+	−	+	+	−	−
ITAL 104	Coffee beans	São Paulo	*A. niger*	+	−	+	+	−	−
ITAL 106	Coffee beans	São Paulo	*A. niger*	+	−	+	+	−	−
ITAL 109	Coffee beans	São Paulo	*A. niger*	+	−	+	+	−	−
ITAL 185	Coffee beans	São Paulo	*A. niger*	+	−	+	+	−	−
ITAL 446	Coffee beans	Paraná	*A. niger*	+	−	+	+	−	−
ITAL 418	Coffee beans	Paraná	*A. niger*	+	−	+	+	−	−
ITAL 642	Coffee beans	Paraná	*A. niger*	+	−	+	+	−	−
ITAL 8276	Coffee beans	Roraima	*A. niger*	−	−	+	+	+	+
ITAL 105	Coffee beans	São Paulo	*A. niger*	−	−	+	+	−	−
ITAL 430	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 331	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+

(continued on next page)

Table 2 (*continued*)

Isolate	Substrate	Geographical origin	Fungal species[a]	Fumonisin phenotype[b]	Ochratoxin phenotype[b]	Multiplex PCR profile			
						A. niger/*A. welwitschiae*[c]	*fum8*[d]	*radH*[e]	*pks*[f]
ITAL 499	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 428	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 429	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 180	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 152	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 318	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 150	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 501	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 494	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 249	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 437	Dried fruits	São Paulo	*A. niger*	+	+	+	+	+	+
ITAL 490	Dried fruits	São Paulo	*A. niger*	−	+	+	+	+	+
ITAL 493	Dried fruits	São Paulo	*A. niger*	−	+	+	+	+	+
ITAL 405	Dried fruits	São Paulo	*A. niger*	+	−	+	+	−	−
ITAL 500	Dried fruits	São Paulo	*A. niger*	−	−	+	+	−	−
ITAL 632	Cocoa	Bahia	*A. niger*	+	+	+	+	+	+
ITAL 1246	Cocoa	Bahia	*A. niger*	+	−	+	+	−	−
UEL 17.179	Grapes	Rio Grande do Sul	*A. welwitschiae*	+	−	+	+	−	−
UEL 2.6	Grapes	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL 2.7	Grapes	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL 2.8	Grapes	Paraná	*A. welwitschiae*	−	−	+	−	−	−
ITAL 47.514	Grapes	Pernambuco	*A. welwitschiae*	−	−	+	−	−	−
ITAL 58.1350	Grapes	Pernambuco	*A. welwitschiae*	−	−	+	−	−	−
ITAL 48.544	Grapes	Pernambuco	*A. welwitschiae*	−	−	+	−	−	−
ITAL 47.456	Grapes	Pernambuco	*A. welwitschiae*	−	−	+	−	−	−
ITAL 49.580	Grapes	Pernambuco	*A. welwitschiae*	−	−	+	−	−	−
ITAL 55.1175	Grapes	Pernambuco	*A. welwitschiae*	−	−	+	−	−	−
ITAL 58.1292	Grapes	Pernambuco	*A. welwitschiae*	−	−	+	−	−	−
UEL 33.351	Grapes	Rio Grande do Sul	*A. welwitschiae*	−	−	+	−	−	−
ITAL 1642	Brazil nuts	Pará	*A. welwitschiae*	+	−	+	+	−	−
ITAL 6951	Brazil nuts	Amazonas	*A. welwitschiae*	+	−	+	+	−	−
ITAL 6573	Brazil nuts	São Paulo	*A. welwitschiae*	+	−	+	+	−	−
ITAL 361	Brazil nuts	Pará	*A. welwitschiae*	+	−	+	+	−	−
ITAL 6591	Brazil nuts	São Paulo	*A. welwitschiae*	−	−	+	+	−	−
ITAL 716	Brazil nuts	Amazonas	*A. welwitschiae*	−	−	+	−	−	−
ITAL 6543	Brazil nuts	São Paulo	*A. welwitschiae*	−	−	+	−	−	−
ITAL 4410	Brazil nuts	Amazonas	*A. welwitschiae*	−	−	+	−	−	−
ITAL 670	Brazil nuts	Amazonas	*A. welwitschiae*	−	−	+	−	−	−
ITAL 714	Brazil nuts	Amazonas	*A. welwitschiae*	−	−	+	−	−	−
ITAL 684	Brazil nuts	Amazonas	*A. welwitschiae*	−	−	+	−	−	−
ITAL 748	Brazil nuts	Amazonas	*A. welwitschiae*	−	−	+	−	−	−
ITAL 797	Brazil nuts	Amazonas	*A. welwitschiae*	−	−	+	−	−	−
ITAL 987	Brazil nuts	Amazonas	*A. welwitschiae*	−	−	+	−	−	−
ITAL 3738	Brazil nuts	Pará	*A. welwitschiae*	−	−	+	−	−	−
ITAL 5614	Brazil nuts	Pará	*A. welwitschiae*	−	−	+	−	−	−
ITAL 15.931	Onions	São Paulo	*A. welwitschiae*	+	+	+	+	+	+
UEL Ac 4.10	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 8.32	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 13.79	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 15.64	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 17.88	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 19.99	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 22.110	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 28.147	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 28.152	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 30.164[a]	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 31.166	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 37.208	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 37.212	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 40.226	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 42.289	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 44.267	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 45.242	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 46.273	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 47.246	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 48.279	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 53.293	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 54.300	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 49.305	Onions	Paraná	*A. welwitschiae*	+	−	+	+	−	−
UEL Ac 23.117	Onions	Paraná	*A. welwitschiae*	−	−	+	+	−	−
ITAL 16.177	Onions	São Paulo	*A. welwitschiae*	−	−	+	−	+	+
ITAL 16.192	Onions	São Paulo	*A. welwitschiae*	−	−	+	−	+	+
ITAL 16.251	Onions	São Paulo	*A. welwitschiae*	−	−	+	−	+	+
UEL Ac 2.05	Onions	Paraná	*A. welwitschiae*	−	−	+	−	+	+
UEL Ac 13.83	Onions	Paraná	*A. welwitschiae*	−	−	+	−	+	+

Table 2 (*continued*)

Isolate	Substrate	Geographical origin	Fungal species[a]	Fumonisin phenotype[b]	Ochratoxin phenotype[b]	Multiplex PCR profile			
						A. niger/*A. welwitschiae*[c]	*fum8*[d]	*radH*[e]	*pks*[f]
UEL Ac 52.315	Onions	Paraná	*A. welwitschiae*	−	−	+	−	+	+
UEL Ac 1.02	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 4.14	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 9.78	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 17.84	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 21.103	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 24.127	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 25.132	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 27.140	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 27.143	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 29.154	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 36.203	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 38.215	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 40.231	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 42.288	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 45.236	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 47.248	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 49.304	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 51.314	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 6.21	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 14.53	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 48.282	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 53.295	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL Ac 55.316	Onions	Paraná	*A. welwitschiae*	−	−	+	−	−	−
UEL 13.09	Coffee beans	Paraná	*A. welwitschiae*	−	−	+	−	−	−
ITAL 424	Dried fruits	São Paulo	*A. welwitschiae*	−	−	+	−	−	−
ITAL 425	Dried fruits	São Paulo	*A. welwitschiae*	−	−	+	−	−	−
ITAL 1113	Cocoa	Bahia	*A. welwitschiae*	−	−	+	−	−	−

[a] Identification by means of *CaM* sequences.
[b] OTA or FB_2 production in Yeast Extract Sucrose Agar (YESA) and CYA with 20% Sucrose Agar (CY20S) respectively.
[c] Identified by the presence of the *ben*A amplicon (192 bp).
[d] Identified by the presence/absence of the *fum8* amplicon (128 bp).
[e] Identified by the presence/absence of the *radH* amplicon (328 bp).
[f] Identified by the presence/absence of the *pks* amplicon (554 bp).

(Fig. 3). The identification of all isolates based on *CaM* sequences is shown in Table 2.

A. niger is one of the most common species of the "aggregate" and has been frequently found in tropical and subtropical foods, including dried fruits, Brazil nuts, coffee beans, grapes, cocoa and onions (Bayman et al., 2002; Chiotta et al., 2013; Egbuta et al., 2015; Iamanaka et al., 2014; Palumbo et al., 2015; Passamani et al., 2012; Samson and Varga, 2010; Shehu and Muhammad, 2011; Susca et al., 2013; Tyson and Fullerton, 2004). *A. welwitschiae* (=*Aspergillus awamori sensu*, Perrone et al., 2011) is a new species, which was dismembered from the *A. niger* taxon (Hong et al., 2013). As mentioned above, these species are morphologically indistinguishable, but fixed nucleotide differences between them were suggested for their identification (Hong et al., 2013); three SNPs found in the partial *CaM* sequences have been useful for discriminating these sibling species (Samson et al., 2014).

Based on the *CaM* sequences, about one-half of our isolates were identified as *A. welwitschiae* (49%), predominant in onions (96%). *A. niger* was predominant in coffee beans (96%), dried fruits (90%) and grapes (70%). *A. niger* and *A. welwitschiae* were found to have a similar frequency in Brazil nuts, *e.g.* 47% and 53%, respectively.

According to Hong et al. (2013), *A. welwitschiae* has been found in dried fruits, grapes, coffee beans and cocoa. However, these authors did not mention its proportion with regard to *A. niger sensu stricto*. Examining onion samples from Saudi Arabia, Gherbawy et al. (2015) reported *A. welwitschiae* as the prevalent species, in line with the findings of our study.

3.2. Mycotoxin production

Fungal strains belonging to *A. niger* and *A. welwitschiae* have been reported to produce OTA and FB_2 in culture media as well as in natural substrates (Abarca et al., 1994; Frisvad et al., 2007; Logrieco et al., 2014). However, the impact of this property on human health is still unclear (Pitt et al., 2013), in part due to misidentification of the species and because of the limited knowledge we have concerning the frequency of isolates with potential for OTA and FB_2 production. According to Palumbo et al. (2013), the recent designation of *A. welwitschiae* as a distinct species should encourage the reexamination of FB_2 producing and nonproducing populations, especially since the ecological and toxicological characteristics of *A. niger* and *A. welwitschiae* overlap extensively.

In our study, we were able to recognize isolates that produce only OTA, only FB_2, both toxins, and also nonproducing isolates in YES Agar (OTA) or CY20S (FB_2) medium (Table 3). Regardless of the species, approximately 17% of our collection of isolates were OTA producers. This is in accordance with the low frequencies found by several authors investigating the ochratoxin producing abilities of *A. niger* isolates before it was split into two species (Bejaoui et al., 2006; Bellí et al., 2006; Chiotta et al., 2013; Frisvad et al., 2011; Iamanaka et al., 2005; Lasram et al., 2012; Serra et al., 2003; Soares et al., 2013). Similarly, with regard to the FB_2 production capability, we found that 54% of the isolates were producers, as reported by Logrieco et al. (2009); Palumbo et al. (2011) and Varga et al. (2010) examining Italian grapes, dried vine fruits from different geographical regions, and Californian grapes, respectively.

It is important to state that the ability to produce OTA and FB2 was found to be markedly different when considering the existence of two species. Among *A. niger* isolates, approximately 32% were detected as OTA producers, in contrast to only 1% of the *A. welwitschiae* isolates. Regarding FB2 production, a higher frequency of FB2-producing isolates in *A. niger* (74%) in comparison to *A. welwitschiae* (34%) was also observed. This is in line with the very few existing studies; for instance,

Table 3
Phenotypic characterization for production of ochratoxin A and fumonisin B_2 by Brazilian *A. niger* and *A. welwitschiae* isolates.

Species	Ochratoxin phenotype		Fumonisin phenotype		Both toxin phenotype	
	+	−	+	−	+/+	−/−
A. niger ($n = 89$)	28 (31.46%)	61 (68.53%)	66 (74.15%)	23 (25.84%)	26 (29.21%)	21 (23.59%)
A. welwitschiae ($n = 86$)	1 (1.16%)	85 (98.84%)	29 (33.72%)	57 (66.28%)	1 (1.16%)	57 (66.27%)
Total ($n = 175$)	29 (16.57%)	146 (83.42%)	95 (54.28%)	80 (45.71%)	27 (15.42%)	78 (44.57%)

Storari et al. (2012) did not find any OTA producers in A. welwitschiae collected from herbal teas, whereas 37% were FB2 producers. Gherbawy et al. (2015) examined 37 *A. welwitschiae* isolates collected from onions in Saudi Arabia and did not find any OTA producers, although half of them did produce FB2.

3.3. Incidence of genes involved in ochratoxin and fumonisin biosynthesis detected by multiplex PCR

Nowadays, the *A. niger* "aggregate" comprises a set of 10 species: *A. niger*, *A. welwitschiae*, *A tubingensis*, *Aspergillus piperis*, *A. luchuensis*, *A. neoniger*, *Aspergillus costaricaensis*, *Aspergillus vadensis*, *Aspergillus eucalypticola* and *A. brasiliensis.* Of all these morphologically similar species, only *A. niger* and *A. welwitschiae* are OTA and FB_2-producing species. This alone means that it is very important to develop a method of distinguishing these toxigenic species from the remainder of the "aggregate". As mentioned in the Material and methods section, a primer-pair (*benA*-An/Aw) for the simultaneous diagnosis of *A. niger* and *A. welwitschiae* was designed, and its specificity confirmed by an *in silico* analysis. Of the 20,343 *Aspergillus* blast hits analyzed, only the *A. niger* and *A. welwitschiae* (syn. *A. awamori*) strains had 100% sequence similarity. *In vitro*, all PCRs containing *A. niger* ($n = 89$) or *A. welwitschiae* ($n = 86$) template DNA produced a strong positive signal of 192 bp in length, although no strong fragment was detected when other *Aspergillus* species' DNA were examined (Fig. 2). The usefulness of PCR-based identification by means of the primer-pair herein designed was confirmed by sequence data.

Bearing in mind that not all *A. niger* and *A. welwitschiae* strains produce OTA and FB_2, a mPCR was idealized and used to look for the incidence of genes involved in these biosyntheses, and four PCR profiles were found (Table 4). Interestingly, the frequency of strains harboring the mycotoxin genes was markedly different between the two species. With regard to OTA, 32% of *A. niger* strains harbor *radH* and *pks* genes, while in *A. welwitschiae* only 8% of the strains harbor both these genes. There were no strains of either species that contained one or other gene, suggesting a single profile of deletion for all *A. niger* and *A. welwitschiae* strains.

These results are supported by the findings of Ferracin and coauthors indicating that the gene cluster involved in OTA biosynthesis (harboring the *pks*-locus tag An15g07920 and *radH*-locus tag An15g07880) is located at the end of the chromosome III, where deletions are thought to be more common than in other regions. These authors analyzed several *A. niger* strains (before it was split into two species) finding that the *pks* gene An15g07920 was present in 26% of them. Recently, Gherbawy et al. (2015) reported that this gene was missing in all *A. welwitschiae* strains obtained from Saudi Arabian onion samples.

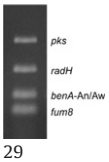
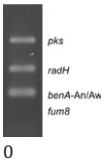
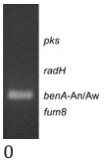
With regard to fumonisin, the mPCR assay revealed that 100% of *A. niger* isolates and 36% of *A. welwitschiae* isolates harbored the *fum8* gene in their genomes, in line with Susca et al.'s findings when studying *A. niger* and *A. welwitschiae* isolates recovered from grapes grown in the Mediterranean basin (Susca et al., 2014). These authors reported that *fum8* was present in all isolates of *A. niger*, but in only 40% of *A. welwitschiae*.

With the aim of at identifying the mPCR products more rapidly and with high precision, we used HEX- or FAM-labeled forward primers and automated high-resolution capillary electrophoresis (CE). This kind of procedure can separate, detect, and genotype up to 96 samples in one run and gives accurate genotyping answers in a few hours (Durney et al., 2015). The mPCR products derived from each isolate were identified according to size and color: *A. niger*/*A. welwitschiae* species (green, 192 bp); *radH* (blue, 328 bp); *pks* (blue, 554 bp); *fum8* (blue, 128 bp). The four profiles revealed by CE are shown in Supplementary Fig. 1 (see Data in Brief, Massi et al., submitted for publication). The mPCR products were very consistent, confirming the effectiveness of the approach herein adopted for addressing the presence/absence of the genes encoding essential proteins for OTA and fumonisin biosynthesis in *A. niger* and its cryptic species, *A. welwitschiae*.

3.4. Association between mPCR-based genotyping and mycotoxin production

All OTA producing isolates of *A. niger* (28) and *A. welwitschiae* (1) harbor in their genomes the *pks* and *radH* genes, and 95.2% of the nonproducing isolates (139/146) did not contain both genes (Tables 2 and 4). These results support the existence of a significant association

Table 4
Distinct genotypes of Brazilian *A. niger* and *A. welwitschiae* isolates revealed after mPCR and agarose gel electrophorese.

Species[a]	PCR genotype			
	Profile 1	Profile 2	Profile 3	Profile 4
				
A. niger ($n = 89$)	29	0	60	0
A. welwitschiae ($n = 86$)	1	6	30	49
Total ($n = 175$)	30	6	90	49

Size of 128 bp, *fum8* amplicon;
Size of 192 bp, *benA* amplicon specific for *A. niger*/*A. welwitschiae*;
Size of 328 bp, *radH* amplicon;
Size of 554 bp, the *pks* amplicon.
[a] Identification by means of *CaM* sequences.

between the presence of the *pks* gene and the production of OTA, pointed out by Ferracin et al. (2012).

All FB_2 producing strains of *A. niger* and *A. welwitschiae* (95) harbor in their genomes the *fum8* gene, and of the nonproducing isolates, only 68.7% (55/80) did not contain this gene.

Analyzing *A. welwitschiae* apart from *A. niger* isolates, it was possible to conclude that there is an association between the absence of *fum8* (genotype) and the FB_2 nonproduction phenotype in *A. welwitschiae*, but not in *A. niger*. The *fum8* gene was detected in all *A. niger* isolates, regardless of their ability to produce fumonisin. Conversely, *fum8* was not detected in 97% of the *A. welwitschiae* fumonisin non-producing isolates, meaning the loss of this phenotype is closely associated with gene deletions within the gene cluster. These results are supported by those obtained for *A. welwitschiae* recovered from samples of Mediterranean basin grapes (Susca et al., 2014) and Saudi Arabian onions (Gherbawy et al., 2015).

The extraordinary association found between the OTA negative phenotype and the absence of *pks* and *radH* genes, in both genome species, confirms that the PCR-based analysis developed herein may be useful to predict the OTA production ability in *A. niger* and *A. welwitschiae* strains. Nevertheless, because no association was found between the FUM negative phenotype and the absence of the *fum8* gene in *A. niger*, a method to efficiently predict FB_2 nonproduction in this species remains a challenging task.

In conclusion, though *A. niger* and *A. welwitschiae* overlap extensively in several ecological and morphological characteristics, they are differentiated in regard to the association between the presence of *fum8* gene and the FB_2 production phenotype.

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References

Abarca, M.L., Bragulat, M.R., Castellá, G., Cabañes, F.J., 1994. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. Appl. Environ. Microbiol. 60, 2650–2652.

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

Bejaoui, H., Mathieu, F., Taillandier, P., Lebrihi, A., 2006. Black aspergilli and ochratoxin A production in French vineyards. Int. J. Food Microbiol. 111, 46–52.

Bellí, N., Bau, M., Marín, S., Abarca, M.L., Ramos, A.J., Bragulat, M.R., 2006. Mycobiota and ochratoxin A producing fungi from Spanish wine grapes. Int. J. Food Microbiol. 111, 40–45.

Chiotta, M.L., Ponsone, M.L., Sosa, D.M., Combina, M., Chulze, S.N., 2013. Biodiversity of *Aspergillus* section *Nigri* populations in Argentinian vineyards and ochratoxin A contamination. Food Microbiol. 36, 182–190.

Durney, B.C., Crihfield, C.L., Holland, L.A., 2015. Capillary electrophoresis applied to DNA: determining and harnessing sequence and structure to advance bioanalyses (2009–2014). Anal. Bioanal. Chem. http://dx.doi.org/10.1007/s00216-015-8703-5.

Egbuta, M.A., Mwanza, M., Njobeh, P.B., Phoku, J.Z., Chilaka, C.A., Dutton, M.F., 2015. Isolation of filamentous fungi species contaminating some Nigerian food commodities. J. Food Res. 4, 38–50.

Ferracin, L.M., Fier, C.B., Vieira, M.L.C., Vitorello, C.B.M., Varani, A.M., Rossi, M.M., Santos, M.M., Taniwaki, M.H., Iamanaka, B.T., Fungaro, M.H.P., 2012. Strain-specific polyketide synthase genes of *Aspergillus niger*. Int. J. Food Microbiol. 155, 137–145.

Filtenborg, O., Frisvad, J.C., Svendsen, J.A., 1983. Simple screening method for molds producing intracellular mycotoxins in pure cultures. Appl. Environ. Microbiol. 45, 581–585.

Frisvad, J.C., Smedsgaard, J., Samson, R.A., Larsen, T.O., Thrane, U., 2007. Fumonisin B_2 production by *Aspergillus niger*. J. Agric. Food Chem. 55, 9727–9732.

Frisvad, J.C., Larsen, T.O., Thrane, U., Meijer, M., Varga, J., Samson, R.A., Nielsen, K., 2011. Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoS One 6, 1–6.

Gherbawy, Y., Elhariry, H., Kocsubé, S., Bahobial, A., Deeb, B.E., Altalhi, A., Varga, J., Vágvolgyi, C., 2015. Molecular characterization of black *Aspergillus* species from onion and their potential for ochratoxin A and fumonisin B_2 production. Foodborne Pathog. Dis. 12, 414–423.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hong, S.B., Cho, H.S., Shin, H.D., Frisvad, J.C., Samson, R.A., 2006. Novel *Neosartorya* species isolated from soil in Korea. Int. J. Syst. Evol. Microbiol. 56, 477–486.

Hong, S.B., Lee, M., Kim, D.H., Varga, J., Frisvad, J.C., Perrone, K.G., Yamada, O., Machida, M., Houbraken, J., Samson, R.A., 2013. *Aspergillus luchuensis*, an industrially important black *Aspergillus* in East Asia. PLoS One 8, 1–9.

Iamanaka, B.T., Taniwaki, M.H., Menezes, H.C., Vicente, E., Fungaro, M.H.P., 2005. Incidence of toxigenic fungi and ochratoxin A in dried fruits sold in Brazil. Food Addit. Contam. 22, 1258–1263.

Iamanaka, B.T., Teixeira, A.A., Teixeira, A.R.R., Copetti, M.V., Bragagnolo, N., Taniwaki, M.H., 2014. The mycobiota of coffee beans and its influence on the coffee beverage. Food Res. Int. 62, 353–358.

Lasram, S., Oueslati, S., Mliki, A., Ghorbel, A., Silar, P., Chebil, S., 2012. Ochratoxin A and ochratoxigenic black *Aspergillus* species in Tunisian grapes cultivated in different geographic areas. Food Control 25, 75–80.

Logrieco, A., Ferracane, R., Haidukowsky, M., Cozzi, G., Visconti, A., Ritieni, A., 2009. Fumonisin B_2 production by *Aspergillus niger* from grapes and natural occurrence in must. Food Addit. Contam. 26, 1495–1500.

Logrieco, A.F., Haidukowski, M., Susca, A., Mulè, G., Munkvold, G.P., Moretti, A., 2014. *Aspergillus* section *Nigri* as contributor of fumonisin B_2 contamination in maize. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 31, 149–155.

Malir, F., Ostry, V., Novotna, E., 2013. Toxicity of the mycotoxin ochratoxin A in the light of recent data. Toxin Rev. 32, 19–33.

Massi, F.P., Sartori, D., Ferranti, L.S., Iamanaka, B.T., Taniwaki, M.H., Vieira, M.L.C., Fungaro, M.H.P., 2016. Data on the presence or absence of genes encoding essential proteins for ochratoxin and fumonisin biosynthesis in *Aspergillus niger* and *Aspergillus welwitschiae*. Int. J. Food Microbiol. (Data in Brief, submitted for publication).

Ostry, V., Malir, F., Ruprich, J., 2013. Producers and important dietary sources of ochratoxin A and citrinin. Toxins 5, 1574–1586.

Palumbo, J.D., O'Keeffe, T.L., Vasquez, S.J., Mahoney, N.E., 2011. Isolation and identification of ochratoxin A-producing *Aspergillus* section *Nigri* strains from California raisins. Lett. Appl. Microbiol. 52, 330–336.

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, 277–284.

Palumbo, J.D., O'Keeffe, T.L., Ho, Y.S., Santillan, C.J., 2015. Occurrence of ochratoxin A contamination and detection of ochratoxigenic *Aspergillus* species in retail samples of dried fruits and nuts. J. Food Prot. 78, 836–842.

Passamani, F.R.F., Lopes, N.A., Pereira, G.E., Prado, G., Batista, L.R., 2012. *Aspergillus* section *Nigri* in grapes cultivated in the tropical winery region of Brazil. Food Public Health 2, 276–280.

Pel, H.J., de Winde, J.H., Archer, D.B., Dyer, P.S., Hofmann, G., Schaap, P.J., Turner, G., de Vries, R.P., Albang, R., Albermann, K., Andersen, M.R., Bendtsen, J.D., Benen, J.A.E., van den Berg, M., Breestraat, S., Caddick, M.X., Contreras, R., Cornell, M., Coutinho, P.M., Danchin, E.G.J., Debets, A.J.M., Dekker, P., van Dijck, P.W.M., van Dijk, A., Dijkhuizen, L., Driessen, A.J.M., d'Enfert, C., Geysens, S., Goosen, C., Groot, G.S.P., de Groot, P.W.J., Guillemette, T., Henrissat, B., Herweijer, M., van den Hombergh, J.P.T.W., van den Hondel, C.A.M.J.J., van der Heijden, R.T.J.M., van der Kaaij, R.M., Klis, F.M., Kools, H.J., Kubicek, C.P., van Kuyk, P.A., Lauber, J., Lu, X., van der Maarel, M.J.E.C., Meulenberg, R., Menke, H., Mortimer, M.A., Nielsen, J., Oliver, S.G., Olsthoorn, M., Pal, K., van Peij, N.N.M.E., Ram, A.F.J., Rinas, U., Roubos, J.A., Sagt, C.M.J., Schmoll, M., Sun, J., Ussery, D., Varga, J., Vervecken, W., van de Vondervoort, P.J.J., Wedler, H., Wösten, H.A.B., Zeng, A.P., van Ooyen, A.J.J., Visser, J., Stam, H., 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat. Biotechnol. 25, 221–231.

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. 115, 1138–1150.

Pitt, J.I., Taniwaki, M.H., Cole, M.B., 2013. Mycotoxin production in major crops as influenced by growing, harvesting, storage and processing, with emphasis on the achievement of food safety objectives. Food Control 32, 205–215.

Pontecorvo, G., Roper, J.A., Hemmons, L.M., MacDonald, K.D., Bufton, A.W.J., 1953. The genetics of *Aspergillus nidulans*. Adv. Genet. 5, 141–148.

Rocha, M.E.B., Freire, F.C.O., Maia, F.E.F., Guedes, M.I.F., Rondina, D., 2014. Mycotoxins and their effects on human and animal health. Food Control 36, 159–165.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Samson, R.A., Varga, J., 2010. Molecular systematics of *Aspergillus* and its teleomorphs. In: Machida, M., Gomi, K. (Eds.), *Aspergillus*: Molecular Biology and Genomics. Caister Academic Press, United Kingdom, pp. 19–40.

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., Kocsubé, S., Szigeti, G., Yaguchi, T., Frisvad, J.C., 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Stud. Mycol. 78, 141–173.

Scott, P.M., 2012. Recent research on fumonisins: a review. Food Addit. Contam. 29, 242–248.

Serra, R., Abrunhosa, L., Kozakiewicz, Z., Venâncio, A., 2003. Black *Aspergillus* species as ochratoxin A producers in Portuguese wine grapes. Int. J. Food Microbiol. 88, 63–68.

Shehu, K., Muhammad, S., 2011. Fungi associated with storage rots of onion bulbs in Sokoto. Nigeria. Int. J. Mod. Bot. 1, 1–3.

Shimizu, K., Nakagawa, H., Hashimoto, R., Hagiwara, D., Onji, Y., Asano, K., Kawamoto, S., Takahashi, H., Yokoyama, K., 2015. The α-oxoamine synthase gene fum8 is involved in fumonisin B_2 biosynthesis in *Aspergillus niger*. Mycoscience 56, 301–308.

Soares, C., Calado, T., Venâncio, A., 2013. Mycotoxin production by *Aspergillus niger* aggregate isolated from harvested maize in three Portuguese regions. Rev. Iberoam. Micol. 30, 9–13.

Storari, M., Dennert, F.G., Bigler, L., Gessler, C., Broggini, G.A.L., 2012. Isolation of mycotoxins producing black *aspergilli* in herbal teas available on the Swiss market. Food Control 26, 157–161.

Susca, A., Proctor, R.H., Mulè, G., Stea, G., Ritieni, A., Logrieco, A., Moretti, A., 2010. Correlation of mycotoxin fumonisin B_2 production and presence of the fumonisin biosynthetic gene *fum8* in *Aspergillus niger* from grape. J. Agric. Food Chem. 58, 9266–9272.

Susca, A., Perrone, G., Cozzi, G., Stea, G., Logrieco, A.F., Mulè, G., 2013. Multilocus sequence analysis of Aspergillus Sect. Nigri in dried vine fruits of worldwide origin. Int. J. Food Microbiol. 165, 163–168.

Susca, A., Proctor, R.H., Butchko, R.A.E., Haidukowski, M., Stea, G., Logrieco, A., Moretti, A., 2014. Variation in the fumonisin biosynthetic gene cluster in fumonisin-producing and nonproducing *black aspergilli*. Fungal Genet. Biol. 73, 39–52.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. Mega6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Turner, G., 2010. Genomics and secondary metabolism in *Aspergillus*. In: Machida, M., Gomi, K. (Eds.), *Aspergillus*: Molecular Biology and Genomics. Caister Academic Press, Norfolk, pp. 139–155.

Tyson, J.L., Fullerton, R.A., 2004. Effect of soil-borne inoculum on incidence of onion black mould (*Aspergillus niger*). Hortic. Arable Pathol. 141, 138–141.

Varga, J., Kocsubé, S., Suri, K., Szigeti, G., Szekeres, A., Varga, M., Tóth, B., Bartók, T., 2010. Fumonisin contamination and fumonisin producing black *aspergilli* in dried vine fruits of different origin. Int. J. Food Microbiol. 143, 143–149.

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.

Visconti, A., Solfrizzo, M., De Girolamo, A., 2001. Determination of fumonisins B_1 and B_2 in corn and corn flakes by liquid chromatography with immunoaffinity column cleanup: collaborative study. J. AOAC Int. 84, 1828–1838.