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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.