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FOOD MICROBIOLOGY - RESEARCH PAPER

Molecular analysis of *Aspergillus* section *Nigri* isolated from onion samples reveals the prevalence of *A. welwitschiae*

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Abstract

The aim of this study was to isolate *Aspergillus* section *Nigri* from onion samples bought in supermarkets and to analyze the fungal isolates by means of molecular data in order to differentiate *A. niger* and *A. welwitschiae* species from the other non-toxigenic species of black aspergilli, and detect genes involved in the biosynthesis of ochratoxin A and fumonisin B_2. *Aspergillus* section *Nigri* were found in 98% (94/96) of the onion samples. Based on the results of multiplex PCR (performed on 500 randomly selected strains), 97.4% of the *Aspergillus* section *Nigri* strains were recognized as *A. niger/A. welwitschiae*. Around half of them were subjected to partial sequencing of the *CaM* gene to distinguish one from the other. A total of 97.9% of the isolates were identified as *A. welwitschiae* and only 2.1% as *A. niger*. The *fum8* gene, involved in fumonisin B_2 biosynthesis, was found in 36% of *A. welwitschiae* isolates, but *radH* and *pks* genes, involved in ochratoxin A biosynthesis, were found in only 2.8%. The presence/absence of *fum8* gene in the *A. welwitschiae* genome is closely associated with ability/inability of the isolates to produce fumonisin in vitro. Based on these results, we suggest that in-depth studies are conducted to investigate the presence of fumonisins in onion bulbs.

Keywords *Aspergillus* section *Nigri* · *A. welwitschiae* · Ochratoxin A · Fumonisin B_2 · Onion bulbs

Introduction

It is not easy to recognize the macro- and micro-morphological differences between some species of *Aspergillus* section *Nigri* (black aspergilli). The use of molecular methods has proved essential for distinguishing these species. Nowadays, 27 species are included in *A.* section *Nigri* which, based on calmodulin gene sequences, is split into seven clades: *A. tubingensis*, *A. niger*, *A. brasiliensis*, *A. carbonarius*, *A. heteromorphus*, *A. homomorphus*, and *A. aculeatus* [1, 2].

Highlights
- *Aspergillus welwitschiae* is very frequently found in onions marketed in Brazil.
- An essential gene involved in fumonisin biosynthesis (*fum8*) is present in 36% of *Aspergillus welwitschiae* strains.

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Ten morphologically very similar taxa, *A. costaricaensis*, *A. luchuensis*, *A. neoniger*, *A. piperis*, *A. tubingensis*, *A. eucalypticola* and *A. vadensis* (*A. tubingensis* clade), *A. niger* and *A. welwitschiae* (*A. niger* clade), and *A. brasiliensis* (*A. brasiliensis* clade), are generally referred as *Aspergillus niger* aggregate [2]. The significance of this group of fungi as contaminants of food products regarding their occurrence and their ability to produce mycotoxins has been investigated worldwide. Two mycotoxins commonly associated with *Aspergillus niger* aggregate are fumonisin B_2 (FB_2) and ochratoxin A (OTA). However, only fungal strains belonging to *A. niger* and *A. welwitschiae* have been reported to be able to produce these mycotoxins in culture media as well as on natural substrates [3–5]. *A. welwitschiae* is a species which, not too long ago, was dismembered from the *A. niger* taxon [3, 6]. No other species belonging to *A. niger* aggregate have been described as OTA and/or FB_2 producers. OTA is a nephrotoxic and carcinogenic mycotoxin and FB_2 is neurotoxic and hepatotoxic to animal species, and has also been associated with esophageal cancers in humans [7, 8].

Considering the inherent risk of the occurrence of *A. niger* and *A. welwitschiae* in foodstuffs, several molecular methods have been developed for distinguishing these toxigenic species

from non-toxigenic *A. niger* aggregate species, including PCR using the primer-pair *benA*-An/Aw designed by Massi et al. [9]. Using this primer-pair, only those PCRs containing *A. niger* or *A. welwitschiae* template DNA produce an amplicon of 192 bp in length. Because not all strains of *A. niger* and *A. welwitschiae* are able to produce OTA or FB_2, some authors have investigated the presence of some essential genes involved in OTA or FB_2 synthesis in *A. niger* and *A. welwitschiae* strains isolated from foodstuffs, with the aim of predicting the contamination risk [9, 10]. It is consensual that the *fum8* (α-oxoamine synthase) and the *pks* An15g07920 (polyketide synthase) genes are required respectively for the production of FB_2 and OTA by *Aspergillus* [9–11]. Based on this confirmation, Massi et al. [9] developed a multiplex PCR technique to detect the frequency of *A. niger*/*A. welwitschiae* strains harboring these genes essential for the production of OTA and FB_2.

A. niger aggregate species are frequently responsible for post-harvest losses of some vegetables. Black mold is one of the predominant post-harvest onion diseases, and was initially thought to be caused by *A. niger* [12]. However, there are very few molecular biology–based studies to distinguish *A. niger* and *A. welwitschiae* from other *A. niger* aggregate species isolated from onion samples [9, 13, 14]. Gherbawy et al. [14] detected black aspergilli in 92.5% of onion samples produced in the Taif region of Saudi Arabia. Using molecular data, the authors showed that the most common fungus isolated from the onion bulb was *A. welwitschiae*. Of 37 isolates of *A. welwitschiae* analyzed by Gherbawy et al. [14], 18 isolates were fumonisin producers, while none of them was able to produce ochratoxins.

Given that onions are one of the most widely consumed fresh vegetables in Brazil, the aim of this study was to isolate *Aspergillus* section *Nigri* from onion bulb samples, bought in supermarkets over a period of 24 consecutive months, and to analyze the fungal isolates by means of molecular data in order to differentiate *A. niger* and *A. welwitschiae* species from the other non-toxigenic species of black aspergilli, and detect genes involved in the biosynthesis of OTA and FB_2.

Materials and methods

Isolation of fungi from onion samples

The yellow variety of onions (*Allium cepa* L.) sold in supermarkets in Paraná State, southern Brazil, was analyzed for the presence of fungi. A total of ninety-six samples purchased over 24 consecutive months in Paraná State, Brazil, were collected from four different supermarkets. Approximately 0.3 kg of onion samples, apparently with no ongoing disease, was selected randomly from the supermarket gondolas, placed in plastic bags and taken to the laboratory.

The dry outer and fleshy inner scales of the onions were cut into pieces of 1 × 1 cm and disinfected superficially by immersion in 0.4% sodium hypochlorite solution (NaOCl) for 2 min [15]. The excess moisture was removed by blotting on sterile filter paper. One hundred pieces (dry and fleshy) from the bulb were plated on Dichloran 18% Glycerol agar (DG18 agar) [16]. A portion containing adventitious roots was also disinfected and plated. The plates were incubated at 25 °C for 5–7 days. The incidence of fungal genera was determined morphologically [2, 15, 17]. Colonies belonging to *Aspergillus* section *Nigri* were isolated, purified on Czapek Yeast Autolysate agar (CYA agar - [Pitt 1979]) and incubated at 25 °C for 7 days.

DNA extraction from *Aspergillus* section *Nigri*

Five hundred *Aspergillus* section *Nigri* isolates were grown in 7 mL of liquid complete medium [18] at 25 °C for 24 h. The mycelia were collected, frozen in liquid nitrogen, and ground to a fine powder. Genomic DNA was extracted using the BioPur Mini Spin Extraction Kit® (Biometrix, Brazil), according to the manufacturer's instructions.

Multiplex PCR

Five hundred *Aspergillus* section *Nigri* isolates were analyzed using the multiplex PCR method described by Massi et al. [9] in order to differentiate *A. niger*/*A. welwitschiae* species from the remainder of *A.* section *Nigri* species and also to characterize the strains in terms of the presence or absence of the two genes required for ochratoxin biosynthesis (*radH* gene, locus tag An15g07880 of *A. niger* CBS 513.88, and the *pks* gene, locus tag An15g07920 of *A. niger* CBS 513.88), respectively, encoding a flavin-dependent halogenase and a polyketide synthase, and the gene required for FB_2 synthesis (*fum8* gene, locus tag An01g06870 of *A. niger* CBS 513.88) encoding an α-oxoamine synthase.

Partial calmodulin gene sequence analysis

Two hundred fifty *Aspergillus* section *Nigri* isolates were subjected to partial sequencing of the calmodulin (*CaM*) gene. The *CaM* amplicon obtained using the primer-pair cmd5/cmd6, according to the protocol described by Hong et al. [19], was purified using ExoProStar™ 1-Step (GE Healthcare Life Sciences, UK). The purified amplicon was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in an ABI3500XL genetic analyzer (Applied Biosystems, USA). The sequences obtained were aligned with sequences from all *Aspergillus* section *Nigri* type strains in the NCBI database (http://www.ncbi.nlm.nih.gov/) using ClustalW and BioEdit software [20]. Phylogenetic analysis was run using MEGA7

software [21] and the maximum likelihood (ML) method with the best model. To determine the support for each clade, bootstrap analysis was run on 1000 replicates.

FB_2 production by *A. welwitschiae* strains

A total of 50 *A. welwitschiae* strains (24 PCR-positive for *fum*8 and 26 PCR-negative for *fum*8) were analyzed for FB_2 production. Briefly, the strains were inoculated onto Czapek Yeast Extract 20% Sucrose agar (CY20S agar). The plates were incubated for 7 days at 25 °C. The toxin was extracted from five small plugs of mycelium with methanol. The extract was filtered and FB_2 derivatized using ortho-phthaldialdehyde reagent (OPA) and then injected into a Shimadzu LC-10VP (Shimadzu, Japan) HPLC system, with a fluorescence detector set to 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). The respective flow rate, oven temperature, and injection volume were 1 mL/1 min, 40 °C, and 20 μL. The methodology is described in detail in Ferranti et al. [22].

Results

All outer dry scales, inner fleshy scales, and all adventitious root samples analyzed herein showed fungal infection. Based on macroscopic and microscopic examinations, we identified *Penicillium* (51.1%) and *Aspergillus* (43.4%) as the predominant fungal genera in all onion samples. Other fungal genera, such as *Fusarium* and *Cladosporium*, were observed at low frequency (5.4%). Among the *Aspergillus* counted in the onion samples, those belonging to the *Aspergillus* section *Nigri* were the most frequent (99.8%) and found in 98% (94/96) of the onion samples. The highest incidence of *Aspergillus* section *Nigri* was found in the inner scale (50.2%), where water activity values corroborate with the highest number of fungal contaminations, followed by outer dry scales (41.5%) and adventitious roots (8.3%). The frequency of occurrence and average of infection by *Aspergillus* section *Nigri* species are given in Table 1.

Based on the results of multiplex PCR (performed on 500 strains randomly selected), 97.4% of the *Aspergillus* section *Nigri* strains ($n = 487$) were recognized as *A. niger/A. welwitschiae*. Only 13 strains (2.6%) did not reveal the predicted amplicon when using the specific primers developed for detecting *A. niger/A. welwitschiae*.

The *Aspergillus* section *Nigri* strains ($n = 13$) that did not reveal any multiplex PCR product were subjected to partial sequencing of the calmodulin (*CaM*) gene for identification purposes. Four strains were phylogenetically identified as *A. brasiliensis*, three as *A. japonicus*, two as *A. tubingensis*, two as *A. luchuensis*, one as *A. neoniger*, and one as *A. heteromorphus* (Fig. 1).

Table 1 Frequency of occurrence and average of infection with *Aspergillus* section *Nigri* species on inner scales, outer scales, and adventitious roots of onion samples ($n = 96$)

	Inner scales	Outer scales	Adventitious roots
FO (%)	90.6	80.2	90.6
AI (%)	49.9	41.6	47.9

FO, frequency of occurrence (number of samples infected with *Aspergillus* section *Nigri*/number of samples); *AI*, average of infection (sum of percentage infection of samples/number of samples)

Of the 487 strains recognized as *A. niger/A. welwitschiae*, approximately half ($n = 242$) were also subjected to partial sequencing of the *CaM* gene to distinguish one from the other. Based on the percentage identity found using BLASTn and a phylogenetic inference (data not shown), 237 strains (97.9%) were identified as *A. welwitschiae* and only five (2.1%) as *A. niger*.

The gene *fum8*, essential for FB_2 synthesis, was found in 36% of *A. welwitschiae* isolates, whereas the *radH* and *pks* genes, involved in OTA biosynthesis, were found in only 2.8% of the isolates. Because the incidence of *fum*8 was high, we examined FB_2 production in a sample of 50 *A. welwitschiae* strains (24 PCR-positive for *fum*8 and 26 PCR-negative for *fum*8). Approximately 96% of the PCR-positive strains produced FB_2 in Czapek Yeast Extract 20% Sucrose (CY20S) agar. On the other hand, 100% of PCR-negative strains were FB_2 non-producing, meaning that the loss of this capability is closely associated with gene deletions within the *A. welwitschiae* genome. In the present study, the number of positive strains for OTA genes was low ($n = 7$) and the association between strains that are positive for the *radH* and *pks* genes and their actual production of OTA was not investigated.

Discussion

There are very few studies on the presence of toxigenic fungi in onion bulbs and the potential risk associated with mycotoxin production. In our study, we investigated fungal incidence in onion samples purchased over 24 consecutive months in Paraná State, Brazil. In line with the findings for onion bulbs marketed in Korea [23], Hungary [13], Saudi Arabia [14], Bangladesh [24], and Nigeria [25], *Penicillium* and *Aspergillus* were the genera most frequently found.

Aspergillus section *Nigri* was isolated from 98% of the onion samples. The multiplex PCR allowed *A. niger/A. welwitschiae* strains to be differentiated from the remainder of *Aspergillus* section *Nigri* species. Of the 500 randomly sampled fungal isolates, *A. niger/A. welwitschiae* were much more frequent than other *A. niger* aggregate species throughout the period herein investigated. These results are in line with studies that have shown *A. niger* [25,

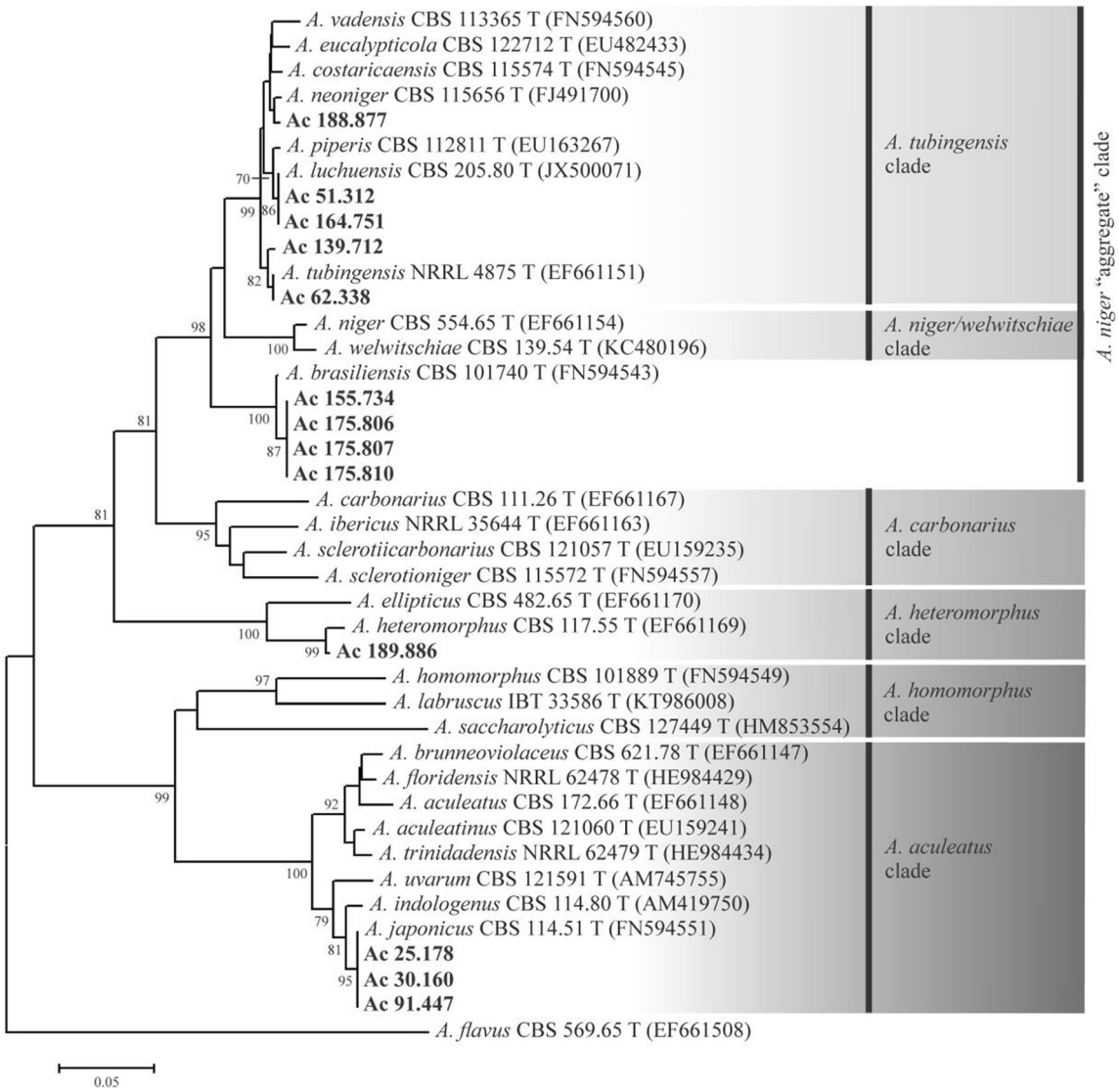

Fig. 1 Maximum likelihood (ML) phylogenetic tree reconstructed from the partial calmodulin gene sequence aligned with the corresponding sequence of type strains (T) from *Aspergillus* section *Nigri* deposited in the NCBI database. Bootstrap values ≥ 70% are shown. *Aspergillus flavus* was used as the outgroup

26] or *A. welwitschiae* [9, 13, 14] to be the prevalent species in onion bulbs.

Perrone and coauthors have shown that the *CaM* sequence can be used to differentiate *A. niger* and *A. welwitschiae* [3] and based on this type of gene sequence, some authors have reported that the frequency of occurrence of *A. niger* and *A. welwitschiae* can vary in different hosts [27–29]. In our study, every month for 24 months, we selected *A. niger*/*A. welwitschiae* isolates for partial sequencing of *CaM* with the aim of differentiating one from the other. Based on the percentage identity provided by BLASTn and a phylogenetic tree (data not shown), 98% of the strains were identified as *A. welwitschiae*. The number of isolates herein analyzed (n = 250) was substantially greater than that in the previous studies, which allows us to consistently reveal that *A. welwitschiae* is the most frequent black aspergilli found throughout the year.

The 13 *Aspergillus* section *Nigri* isolates that did not result in any PCR product when using *A. niger/A. welwitschiae*–specific primers were identified as *A. brasiliensis* (n = 4), *A. japonicus* (n = 3), *A. tubingensis* (n = 2), *A. luchuensis* (n = 2), *A. neoniger* (n = 1), and *A. heteromorphus* (n = 1). None of these species is OTA and/or FB_2 producers.

In fungal genomes, genes associated with mycotoxin biosynthesis are often clustered, co-regulated, and co-expressed. These gene clusters usually harbor genes encoding one or more types of "core" enzyme responsible for biosynthesis of the metabolite backbone structure [30]. They may contain polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS), which are adjacent to genes encoding regulatory proteins, hydrolases, oxidases, methylases, etc., that are involved in chemical transformations of the backbone structure to produce the final metabolite [31]. Ferracin et al. [32] investigated a gene cluster harboring one

pks gene, annotated as An15g07920 in the genome of *A. niger* CBS 513.88, predicted by Pel et al. [33] to be involved in OTA biosynthesis. This gene cluster is located near to the end of chromosome III. In many eukaryotic genomes, chromosome ends are sites of higher rates of rearrangement, such as deletion, compared to the rest of the genome. Since not all the strains of *A. niger* are able to produce OTA [9, 14, 34], Ferracin et al. [32] investigated 119 *A. niger* strains for the presence/absence of this *pks* gene and also for the ability to produce OTA. The *pks* gene was detected in all strains that produced OTA ($n = 31$), but not detected in any of the non-producing strains ($n = 88$), revealing a positive association between the ability to produce OTA and the presence of the *pks* locus in the *A. niger*/*A. welwitschiae* genome. Castellá and Cabanes [35] also showed the importance of the An15g07920 *pks* gene for specific detection of OTA-producing strains of *A. niger* aggregate. It is important to mention that the authors' findings were reported before *A. niger* sensu stricto was dismembered into two taxa, *A. niger* and *A. welwitschiae* [3, 6]. Gherbawy et al. [14] analyzed *A. welwitschiae* strains ($n = 37$) isolated from onion samples from Saudi Arabia. The authors found that all strains were non-OTA producing and the *pks* gene involved in OTA biosynthesis was missing in all of them. In our study, we analyzed a large number of *A. welwitschiae* strains ($n = 237$) isolated from onions and showed that the great majority (97.2%) of the *A. welwitschiae* isolates do not have the two essential genes involved in ochratoxin biosynthesis.

The fumonisin biosynthetic gene cluster in *A. niger* contains at least 14 *fum* genes [36]. Surveys of some of these genes in the fumonisin biosynthetic gene (*fum*) cluster have indicated that fumonisin-non-producing isolates of *Aspergillus welwitschiae* lack some of them, but non-producing isolates of *Aspergillus niger* do not [9, 27, 29]. In our study, the use of the multiplex PCR procedure revealed that 36% of the *A. welwitschiae* strains obtained from onions were positive for the *fum*8 gene, which encodes an alpha-oxoamine synthase [10]. The presence/absence of this gene in the *A. welwitschiae* genome is closely associated with ability/inability of the isolates to produce fumonisin in vitro. In line with our findings, Gherbawy et al. [14] reported that 18 of the 37 *A. welwitschiae* isolates collected from onion samples purchased at markets in Saudi Arabia were potential producers of FB_2. These authors also examined the FB_2 content in onion samples and detected this mycotoxin in 37.5%. Mycotoxin contamination of Brazilian onion bulbs has not been investigated, but currently we are working to check for the presence of FB_2 in several samples of Brazilian onions.

Conclusion

We conclude that *A. welwitschiae* is very frequently found in onion bulbs purchased at markets. Of the *Aspergillus* species, *A. welwitschiae* is the most prevalent throughout the year. Essential genes involved in OTA biosynthesis are missing in almost all *A. welwitschiae* strains, but the *fum8* gene required for fumonisin biosynthesis is present in approximately 36% of the strains. The presence/absence of the *fum*8 gene in *A. welwitschiae* strains is closely associated with ability/inability to produce fumonisin in vitro. The presence of fumonisins in Brazilian onion bulbs needs to be investigated in order to clarify the significance of these observations.

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Compliance with ethical standards

Competing interests The authors declare that they have no conflict of interest.

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