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Aspergillus species from Brazilian dry beans and their toxigenic potential

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ABSTRACT – Aspergilli are common contaminants of food and feed and a major source of mycotoxins. In this study, 87 *Aspergillus* strains were isolated from beans from 14 different cities in Brazil and identified to the species level based on partial calmodulin and β-tubulin sequence data. All green spored isolates belonged to section *Flavi* and were identified as *A. flavus* (n=39) or *A. pseudocaelatus* (n=1). All black spored isolates belonged to section *Nigri* and were identified as *A. niger* (n=24) or *A. luchuensis* (n=10), while the yellow spored strains were identified as *A. westerdijkiae* (n=7), *A. ostianus* (n=3), *A. ochraceus* (n=1) or *A. wentii* (n=2). The toxigenic potential of these *Aspergillus* strains from beans was studied by the prospection of genes in three of the major mycotoxin clusters: aflatoxin (seven genes checked), ochratoxin A (four genes) and fumonisin (ten genes and two intergenic regions). Genes involved in the biosynthesis of aflatoxin were only detected in *A. flavus* isolates: 17/39 *A. flavus* isolates proved to contain all the aflatoxin genes tested, the others missed one or more genes. The full complement of fumonisin biosynthesis genes was identified in all *A. niger* isolates. Finally, no genes for ochratoxin A were detected in any of the isolates. Our work suggests that aflatoxin production by some *A. flavus* strains and fumonisin production by *A. niger* isolates form the largest mycotoxin risks in Brazilian beans.

Keywords: A. flavus; *A. niger*; *A. luchuensis*; *Aflatoxin*; Fumonisin; Ochratoxin A.

1. Introduction

Dry beans are a traditional staple food of great economic, social and nutrition importance in Brazil. Seven out of ten Brazilians consume beans daily, irrespective of their income level. Brazil is one of the major global producers of dry beans, with a total of 5.9 million tons harvested in 2016 growing seasons (CONAB – National Supply Company, 2017). A variety of beans from different groups and market classes are grown in Brazil: Groups being the botanical species *Phaseolus vulgaris* (I) and *Vigna unguiculata* (II), while the class identifies the beans according to their skin colours (black, white or mixed colors). The cream seeded variety

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Carioca belongs to group I and is most widely consumed, accounting for approximately 70% of total beans consumed, followed by black beans (several varieties grouped as *Preto*). *Carioca* and *Preto* beans correspond to around 85% of the Brazilian bean market (Ribeiro et al., 2014). Other types of beans are important regional foods, including the *Fradinho* bean (cowpea), which is popular in Northeastern Brazil, representing approximately 10% of the total Brazilian dry bean market (Vogt et al., 2011).

In the field, bean crops can be affected by a diverse range of organisms, including insects, nematodes, fungi, bacteria and viruses, which can reduce yields significantly (Graham and Ranalli, 1997). However, especially during the storage period, fungal species belonging to *Aspergillus* and *Penicillium* genera cause considerable loss due to their ability to grow under low humidity conditions. These fungi not only contaminate the seeds by fungal growth, but also affect the quality by the production of toxic secondary metabolites. The presence of *Aspergillus* on bean seeds has been reported before (Costa and Scussel, 2002; Domijan et al., 2005; Silva et al., 2008; Tseng et al., 1995), but in most reports, the molecular identification to species level or the capacity to produce mycotoxins was not performed. This lack of information may lead to a serious risk of food contamination, once these fungi produce toxins that are detrimental to humans and animals.

Aflatoxins are the most toxic and carcinogenic compounds among the known mycotoxins (Yu et al., 2004). In humans, they are capable of causing diseases such as hepatitis, liver cirrhosis, liver cancer, and gallbladder cancer (Koshiol et al., 2017; McKean et al., 2006). The four major types of aflatoxins are AFB_1, AFB_2, AFG_1 and AFG_2, which can be present on a wide range of commodities. *Aspergillus flavus* produces AFB_1 and AFB_2 and *A. parasiticus* produces AFB_1, AFB_2, AFG_1 and AFG_2, but other species like *A. nomius, A. pseudotamarii, A. ochraceoroseus, A. pseudocaelatus* and *A. ostianus* have also been reported to produce aflatoxin as reviewed by Bezerra da Rocha et al. (2014). Aflatoxin biosynthesis is regulated by an intricate group of genes clustered on a 70kb DNA segment. This cluster contains 25 genes involved in the complex reactions in the aflatoxin pathway (Yu, et al., 2004).

Ochratoxin A (OTA) is known as the most toxic member of the ochratoxin family of mycotoxins, displaying nephrotoxic, hepatotoxic, teratogenic, immunosuppressive and carcinogenic effects (JECFA, 2001). It is produced by certain *Aspergillus* and *Penicillium* species and it is commonly found as a contaminant in a wide variety of food commodities (Wang et al., 2016). *A. ochraceus* and *P. verrucosum* were considered for a long time the main OTA producers (Pitt, 2000). *A. ochraceus* strains have been shown to be capable of producing high amounts of OTA under certain circumstances, while eight other species, including *A. westerdijkiae* -were described as robust OTA producers (Frisvad et al., 2004). OTA-producing black aspergilli, such as *A. niger*, *A.*

welwitschiae and *A. carbonarius* are important contaminants of grape and wine (Einloft et al., 2017; Susca et al., 2016). The gene cluster involved in the production of ochratoxin A was identified for the first time in *Penicillium verrucosum* by Geisen et al. (2006). The OTA biosynthetic cluster in *Aspergillus* is not completely elucidated, but it has been demonstrated that at least a polyketide synthetase gene (PKS) and a non-ribosomal peptide synthase (NRPS) are involved in the pathway of OTA biosynthesis (Gallo et al., 2012a). In addition, three other genes are hypothesized to be part of the cluster (Ferracin et al., 2012; Susca et al., 2016).

Fumonisins were considered to be produced mainly by *Fusarium verticillioides* and *F. proliferatum* (Gelderblom et al., 1988), but recently, fumonisin B_2 production was detected in *A. niger* and *A. welwitschiae* (Frisvad et al., 2007; Hong et al., 2013; Perrone et al., 2011). The exposure to fumonisins can lead to carcinogenic, nephrotoxic and hepatotoxic effects in humans and animals (JECFA, 2001). The fumonisin biosynthetic gene cluster in *Aspergillus* consists of eleven homologues to *Fusarium* genes, and one additional gene (*sdr1*), a short-chain dehydrogenase gene not present in the *Fusarium* cluster (Pel et al., 2007; Susca et al., 2014).

The objective of the current study was to provide information about the occurrence of *Aspergillus* species in association with the most popular dry bean types in Brazil and to identify these isolates to species level using barcode sequences. Furthermore, we characterized their toxigenic potential by detecting the presence of genes involved in toxin biosynthesis. Hence, this paper gives an inventory of potential mycotoxins produced by *Aspergillus* species on dry beans.

2. Material and methods

2.1. Fungal isolates

Fungal strains were obtained from 35 seed lots originated from 14 different cities in Brazil (Figure 1; Table 2). The seed surface was disinfected by soaking seeds in sodium hypochlorite solution (NaClO 1%) during 1 minute and immediately rinsing them twice with sterile distilled water. After drying for 72h under aseptic conditions, 100 seeds were placed in four Petri dishes of 15cm diameter containing a sterile filter paper disc immersed in 0.5% water agar medium amended with 6% sodium chloride (NaCl) to reduce seed germination and favour *Aspergillus* growth in detriment of other fungi (Protocol from Brazilian Ministry of Agriculture, Livestock and Food Supply, 2009). After 7 days at 25°C, representative *Aspergillus* colonies based on spore colour (green, black and yellow) were selected for isolation, in order to avoid the selection of clones. Strains were subcultured on PDA and grown during 5-7 days at 25°C in a 12/12 hour photoperiod regime. From pure

cultures, spore suspensions were prepared in sterile water, and 50µL were spread on water agar medium. After 24-36h of incubation at 25°C, the plates were checked under a stereomicroscope. Single germinating spores were collected and transferred to a PDA plate to obtain single spore cultures.

Figure 1
Geographical origin of *Aspergillus* strains of this study. The letters indicate the name of Brazilian provinces: CE (Ceará), BA (Bahia), MG (Minas Gerais), SP (São Paulo), RS (Rio Grande do Sul).

2.2. DNA isolation, amplification and sequencing

Conidia from single spore cultures were inoculated on 2mL of Wickerham's medium and incubated at 25°C for DNA extraction. After 48 hours, mycelia were harvested and transferred to 1.5mL microtubes, washed with sterile distilled water and dried by centrifugation for 3min at 12,000rpm. DNA was isolated by using the Wizard® Genomic DNA Purification Kit according to the manufacturer's instructions. Amplification of part of the calmodulin gene (*caM*) and the β-tubulin gene (β-tub), was performed using the primers CMD5/CMD6 (Hong et al., 2005) and Bt2a/Bt2b (Glass and Donaldson, 1995) (Table 1). PCR reactions were performed in a 12.5µL-volume reaction, containing 0.5U Roche Taq DNA Polymerase, 1.25x Roche Taq DNA Polymerase buffer, 2mM $MgCl_2$, 200nM of each primer and 200µM dNTPs. The cycling protocol consisted of an initial denaturation at 95°C for 10min, 35 cycles of denaturation at 95°C for 50s, annealing for 30s at 55°C for *caM*, or at 58°C for β-tub and extension at 72°C for 40s, followed by a final extension at 72°C for 7min. Alternatively for those strains that did not amplify using CMD5/CMD6, primers CL1/CL2 (O'Donnell et al., 2000) were used to

obtain amplicons of the calmodulin gene using the same PCR conditions as described above. PCR products were sent to Macrogen Europe (Amsterdam, NL) for purification and sequencing.

2.3 Sequence analysis

DNA sequences were trimmed, assembled and aligned with CLC Genomic Workbench 9.5.1. Phylogenetic trees were obtained using MEGA 7.0.21 (Kumar et al., 2016), by the Maximum Likelihood construction method, using Tamura-Nei model with bootstrap support with 1,000 replicates.

2.4 Toxigenic potential

The presence of genes involved in aflatoxin (*afl*), ochratoxin (*ota*) or fumonisin (*fum*) biosynthesis was assessed by PCR using specific primers for genes and intergenic regions within the respective clusters (Figure 2; Table 1).

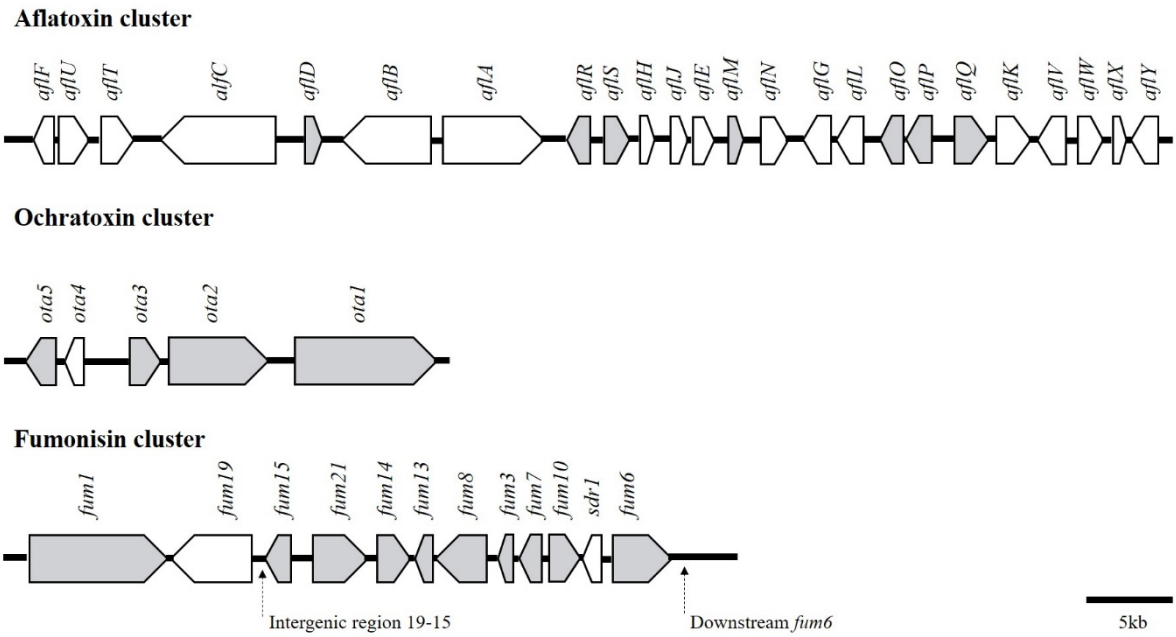

Figure 2
Graphical representation of the putative aflatoxin, ochratoxin and fumonisin biosynthetic gene clusters in *Aspergillus* (based on Yu et al., 2004, Susca et al., 2016 and Pel et al., 2007, respectively). Arrows represent genes and indicate direction of transcription. Genes targeted by PCR in this study are shaded in grey. In the fumonisin gene cluster also the intergenic region between *fum19* and *fum15* was targeted as well as the region downstream of *fum6* (dotted arrows).

The amplifications for all *afl, ota* and *fum* genes were performed in a final volume of 12.5µL, containing 0.5U Roche Taq DNA Polymerase, 1x Roche Taq DNA Polymerase buffer, 2.25mM $MgCl_2$, 300nM of both forward and reverse primer and 200µM dNTPs. The cycling conditions targeting the *afl* genes were described by Gallo et al. (2012b) consisting of an initial denaturation at 95°C for 10min, 30 cycles of denaturation at 95°C for 50s, annealing at 58°C for 50s and extension at 72°C for 2min, followed by a final

extension at 72°C for 5min. In case the *afl* amplifications did not give products or products of unexpected sizes, the reactions were repeated using Premix Ex Taq™ Hot Start Version (Takara) with 300nM of both forward and reverse primer using the same cycling conditions described above. The cycling conditions for *fum* and *ota* amplicons were the same as described by Susca et al. (2016), consisting of an initial denaturation at 95°C for 2min, followed by 35 cycles of denaturation at 94°C, annealing at 58 and 60°C, respectively, and extension at 72°C - each step performed for 50s for *fum* primers or 30s for *ota* primers and a final extension for 7min at 72°C.

Table 1

Primers used in this study.

Target genes/ cluster	Gene / Intergenic region	Primer sequence (5'-3')	Annealing temperature	Amplicon size	References
Barcodes	*caM* (CMD5/CMD6)	F: CCGAGTACAAGGARGCCTTC R: CCGATRGAGGTCATRACGTGG	55°C	600	Hong et al., 2005
	caM (CL1/CL2)	F: GARTWCAAGGAGGCCTTCTC R: TTTTGCATCATGAGTTGGAC	55°C	750	O'Donnell et al., 2000
	β-tub (Bt2a/Bt2b)	F: GGTAACCAAATCGGTGCTGCTTTC R: ACCCTCAGTGTAGTGACCCTTGGC	58°C	555	Glass and Donaldson, 1995
Aflatoxin	*aflD*	F: CACTTAGCCATCACGGTCA R: GAGTTGAGATCCATCCGTG	58°C	852	Gallo et al., 2012b
	aflR	F: AAGCTCCGGGATAGCTGTA R: AGGCCACTAAACCCGAGTA	58°C	1079	Gallo et al., 2012b
	aflS	F: TGAATCCGTACCCTTTGAGG R: GGAATGGGATGGAGATGAGA	58°C	684	Gallo et al., 2012b
	aflM	F: AAGTTAATGGCGGAGACG R: TCTACCTGCTCATCGGTGA	58°C	470	Gallo et al., 2012b
	aflO	F: TCCAGAACAGACGATGTGG R: CGTTGGCTAGAGTTTGAGG	58°C	790	Gallo et al., 2012b
	aflP	F: AGCCCCGAAGACCATAAAC R: CCGAATGTCATGCTCCATC	58°C	870	Gallo et al., 2012b
	aflQ	F: TCGTCCTTCCATCCTCTTG R: ATGTGAGTAGCATCGGCATTC	58°C	757	Gallo et al., 2012b
Ochratoxin	*ota5*	F: TCCCTCGGTAAGAGTATCCTCGT R: GCGAGTTCTTGGTTCATGACG	60°C	845	Susca et al., 2016
	ota3	F: TTAGACAAACTGCGCGAGGA R: GCGTCGCTATGCCCAGATA	60°C	613	Susca et al., 2016
	ota2	F: GGGAAYRCTGAYGTCGTGTTT R: TCCCACGAGCAWACAGCCTC	60°C	644	Susca et al., 2016
	ota1	F: CAATGCCGTCCAACCGTATG R: CCTTCGCCTCGCCCGTAG	60°C	776	Ferracin et al.,2012
Fumonisin	*fum1*	F: GGGTTCCAGGCAGAATCGTAC R: GAACTCACATCCTTTTGGGCC	58°C	701	Susca et al., 2014
	fum19-15 IGR	F: ACACCGCGAGAATTCCATG R: GCAGGCTGGTAGTAGCGACAT	58°C	868	Susca et al., 2014
	fum15	F: CGATTGGTAGCCCGAGGAA R: CTTGATATTGCGGAGTGGTCC	58°C	701	Susca et al., 2014
	fum21 region I	F: CATTTCATGGGACCTCAGCC R: AAGCACAGGTTCCGAATTTGA	58°C	703	Susca et al., 2014
	fum21 region II	F: GGGTCCCATTGCCTCAATT R: CAATGGAGTCGACGGTGTCAC	58°C	705	Susca et al., 2014
	fum14	F: TTGGGCTGATGTGCTCTGTC R: CCTCGTAGACGTAATTGAGTAGTCCT	58°C	730	Susca et al., 2014
	fum13	F: ATGCTCTTCACCTCCTCCGG R: CACTCAACGAGGAGCCTTCG	58°C	651	Susca et al., 2014
	fum8	F: TTCGTTTGAGTGGTGGCA R: CAACTCCATASTTCWWGRRAGCCT	58°C	862	Susca et al., 2010
	fum3	F: TACCATGGACCACTTTCCCG R: AAGTTCCTCAAGCGGCAGTC	58°C	651	Susca et al., 2014
	fum7	F: CAACAGCCCGAATCCCAGTA R: GCTCAGTCTTGCCCATCGTG	58°C	681	Susca et al., 2014
	fum10	F: GTCATTATTCCTCCGGCCCT R: TGGGATTCGAAAGCATACCG	58°C	651	Susca et al., 2014
	fum6	F: CTGTGAGGCCCTGGCACTT R: TCTGCCGGAGCTCAACGTA	58°C	849	Susca et al., 2014
	downstream fum6	F: CAAAAGACACCGCCCGTCT R: TTGACGCCCTGTACAAGGC	58°C	667	Susca et al., 2014

IGR: intergenic region between *fum19* and *fum15*

3. Results

The majority of the sampled bean lots proved to be infected with *Aspergillus*-like strains with green, black or yellow spores. From these we took a representative set of 87 strains (Table 2) for molecular identification, as it is difficult or even impossible to characterise *Aspergillus* strains to the species level using morphological tools. All strains were deposited in the Culture Collection of the Food Sciences Department (CCDCA) at Federal University of Lavras, Brazil. *Aspergillus* colonies were observed in the fast majority of the tested grains, with green strains being the most frequent, closely followed by black strains and finally by the yellow ones. Green strains were observed in 34 from 35 seed lots tested (97%), while black and yellow strains were found in 27 (77%) and 10 (29%) seed lots, respectively.

3.1 Species identification

Partial calmodulin and β-tubulin gene sequences were used to determine species identity of all *Aspergillus* strains collected from bean seeds (accession numbers MG746413 to MG746586). Sequences from both genes gave the same identification. Our work shows that 97.5% of the green strains are *A. flavus* (n=39) and 2.5% are *A. pseudocaelatus* (n=1). Within the black aspergilli, 70.6% of the strains are *A. niger* (n=24) and 29.4% were identified as *A. luchuensis* (n=10). Regarding the yellow group, 53.8% are *A. westerdijkiae* (n=7), 23.1% are *A. ostianus* (n=3), 15.4% are *A. wentii* (n=2), and 7.7% are *A. ochraceus* (n=1).

Table 2
Information on the analysed seed lots and molecular identification of the obtained strains.

Strain code	City	Province	Seed lot	Bean Group	Bean Class	Molecular identification (*caM* and β-tub)
CCDCA11411	Campo Belo	MG	20	I	Mixed colors	*A. niger*
CCDCA11412	Passos	MG	14	I	Mixed colors	*A. niger*
CCDCA11413	Madre de Deus de Minas	MG	35	I	Mixed colors	*A. flavus*
CCDCA11414	Sete Lagoas	MG	30	I	Mixed colors	*A. niger*
CCDCA11415	Ribeirão Vermelho	MG	23	I	Mixed colors	*A. flavus*
CCDCA11416	Ribeirão Vermelho	MG	23	I	Mixed colors	*A. niger*
CCDCA11417	Ribeirão Preto	SP	02	I	Mixed colors	*A. flavus*
CCDCA11418	Ribeirão Preto	SP	05	I	Mixed colors	*A. ostianus*
CCDCA11419	Ribeirão Preto	SP	06	I	Mixed colors	*A. flavus*
CCDCA11420	Ribeirão Preto	SP	32	I	Mixed colors	*A. niger*
CCDCA11421	Ribeirão Preto	SP	16	I	Mixed colors	*A. westerdijkiae*
CCDCA11422	Santo Anastácio	SP	15	I	Mixed colors	*A. niger*
CCDCA11423	Cruz das Almas	BA	27	II	White	*A. flavus*
CCDCA11424	Sete Lagoas	MG	10	I	Mixed colors	*A. westerdijkiae*
CCDCA11425	Cruz das Almas	BA	29	I	Black	*A. pseudocaelatus*
CCDCA11426	Ribeirão Preto	SP	08	I	Mixed colors	*A. niger*
CCDCA11427	Ribeirão Preto	SP	04	I	Mixed colors	*A. niger*

CCDCA11428	Ribeirão Preto	SP	03	I	Mixed colors	*A. flavus*
CCDCA11429	Sete Lagoas	MG	31	I	Mixed colors	*A. flavus*
CCDCA11430	Sete Lagoas	MG	10	I	Mixed colors	*A. niger*
CCDCA11431	Sete Lagoas	MG	01	I	Mixed colors	*A. flavus*
CCDCA11432	Ribeirão Preto	SP	12	I	Mixed colors	*A. niger*
CCDCA11433	Cruz das Almas	BA	28	I	Mixed colors	*A. luchuensis*
CCDCA11434	Ribeirão Preto	SP	07	I	Black	*A. niger*
CCDCA11435	Ribeirão Preto	SP	08	I	Mixed colors	*A. flavus*
CCDCA11436	Santo Anastácio	SP	15	I	Mixed colors	*A. flavus*
CCDCA11437	Ribeirão Preto	SP	33	I	Mixed colors	*A. flavus*
CCDCA11438	Sete Lagoas	MG	09	I	Mixed colors	*A. luchuensis*
CCDCA11439	Ribeirão Preto	SP	13	I	Mixed colors	*A. flavus*
CCDCA11440	Sete Lagoas	MG	31	I	Mixed colors	*A. luchuensis*
CCDCA11441	Patos de Minas	MG	25	I	Mixed colors	*A. niger*
CCDCA11442	Ribeirão Preto	SP	04	I	Mixed colors	*A. flavus*
CCDCA11443	Campo Belo	MG	20	I	Mixed colors	*A. luchuensis*
CCDCA11444	Ribeirão Preto	SP	16	I	Mixed colors	*A. flavus*
CCDCA11445	Ribeirão Preto	SP	07	I	Black	*A. flavus*
CCDCA11446	Cruz das Almas	BA	29	I	Black	*A. flavus*
CCDCA11447	Itutinga	MG	22	I	Black	*A. westerdijkiae*
CCDCA11448	Ribeirão Preto	SP	12	I	Mixed colors	*A. flavus*
CCDCA11449	Sete Lagoas	MG	10	I	Mixed colors	*A. flavus*
CCDCA11450	Ribeirão Preto	SP	11	I	Black	*A. flavus*
CCDCA11451	-	CE	18	II	White	*A. flavus*
CCDCA11452	Ribeirão Vermelho	MG	23	I	Mixed colors	*A. niger*
CCDCA11453	Cana Verde	MG	24	I	Mixed colors	*A. westerdijkiae*
CCDCA11454	Cruz das Almas	BA	27	II	White	*A. ostianus*
CCDCA11455	Camaquã	RS	17	II	White	*A. flavus*
CCDCA11456	Cruz das Almas	BA	26	I	Mixed colors	*A. niger*
CCDCA11457	Cana Verde	MG	24	I	Mixed colors	*A. flavus*
CCDCA11458	Ribeirão Preto	SP	11	I	Black	*A. niger*
CCDCA11459	Ribeirão Preto	SP	05	I	Mixed colors	*A. flavus*
CCDCA11460	Patos de Minas	MG	25	I	Mixed colors	*A. flavus*
CCDCA11461	Sete Lagoas	MG	09	I	Mixed colors	*A. flavus*
CCDCA11462	Ribeirão Vermelho	MG	19	I	Mixed colors	*A. flavus*
CCDCA11463	Sete Lagoas	MG	09	I	Mixed colors	*A. westerdijkiae*
CCDCA11464	Sete Lagoas	MG	09	I	Mixed colors	*A. ochraceus*
CCDCA11465	Ribeirão Preto	SP	03	I	Mixed colors	*A. niger*
CCDCA11466	Campo Belo	MG	20	I	Mixed colors	*A. luchuensis*
CCDCA11467	Perdões	MG	21	I	Mixed colors	*A. niger*
CCDCA11468	Ribeirão Preto	SP	06	I	Mixed colors	*A. niger*
CCDCA11469	Passos	MG	14	I	Mixed colors	*A. westerdijkiae*
CCDCA11470	Ribeirão Preto	SP	34	I	Mixed colors	*A. luchuensis*
CCDCA11471	Sete Lagoas	MG	01	I	Mixed colors	*A. niger*
CCDCA11472	Cruz das Almas	BA	28	I	Mixed colors	*A. flavus*
CCDCA11473	Ribeirão Preto	SP	34	I	Mixed colors	*A. flavus*

CCDCA11474	Sete Lagoas	MG	30	I	Mixed colors	*A. flavus*
CCDCA11475	Camaquã	RS	17	II	White	*A. niger*
CCDCA11476	Passos	MG	14	I	Mixed colors	*A. flavus*
CCDCA11477	Ribeirão Vermelho	MG	23	I	Mixed colors	*A. flavus*
CCDCA11478	Ribeirão Preto	SP	16	I	Mixed colors	*A. niger*
CCDCA11479	Passos	MG	14	I	Mixed colors	*A. flavus*
CCDCA11480	Itutinga	MG	22	I	Black	*A. niger*
CCDCA11481	Cana Verde	MG	24	I	Mixed colors	*A. niger*
CCDCA11482	Itutinga	MG	22	I	Black	*A. flavus*
CCDCA11483	Perdões	MG	21	I	Mixed colors	*A. flavus*
CCDCA11484	Ribeirão Vermelho	MG	23	I	Mixed colors	*A. flavus*
CCDCA11485	Cruz das Almas	BA	26	I	Mixed colors	*A. flavus*
CCDCA11486	Campo Belo	MG	20	I	Mixed colors	*A. flavus*
CCDCA11487	Itutinga	MG	22	I	Black	*A. westerdijkiae*
CCDCA11488	Campo Belo	MG	20	I	Mixed colors	*A. flavus*
CCDCA11489	Patos de Minas	MG	25	I	Mixed colors	*A. luchuensis*
CCDCA11490	Cana Verde	MG	24	I	Mixed colors	*A. luchuensis*
CCDCA11491	Itutinga	MG	22	I	Black	*A. niger*
CCDCA11492	Patos de Minas	MG	25	I	Mixed colors	*A. flavus*
CCDCA11493	Patos de Minas	MG	25	I	Mixed colors	*A. ostianus*
CCDCA11494	Itutinga	MG	22	I	Black	*A. luchuensis*
CCDCA11495	Ribeirão Preto	SP	33	I	Mixed colors	*A. luchuensis*
CCDCA11496	Ribeirão Preto	SP	33	I	Mixed colors	*A. wentii*
CCDCA11497	Ribeirão Preto	SP	33	I	Mixed colors	*A. wentii*

CCDCA: Culture Collection of the Food Sciences Department at Federal University of Lavras, Brazil
-: not specified
Province: CE (Ceará), BA (Bahia), MG (Minas Gerais), RS (Rio Grande do Sul), SP (São Paulo)
Bean group: I (*Phaseolus vulgaris*), II (*Vigna unguiculata*)

The sequences obtained from the 87 strains were compared to the reference sequences available at GenBank and shown to be 98-100% similarity to the type strain of each species (Figure 3). The *β-tub* sequences presented a single haplotype identical to the reference strain for each species, except for the two *A. wentii* isolates that both showed 1% variation with the type strain CBS 104.07 (EF652106). In contrast, the calmodulin (*caM*) sequences revealed variations up to 2% compared to the reference sequence, and up to 1% comparing among the isolates from this study (Table 3). The strains identified as *A. luchuensis* were the most divergent from the reference CBS 205.80 (2%), even though they all form a single haplotype, with no internal variation. The *A. flavus* isolates showed the highest internal variation, representing six haplotypes, all divergent from the reference CBS 569.65. The majority of SNPs are located in introns, but two SNPs reside in exons, leading to a non-synonymous mutation in the amino acid 81 of the haplotype IV (Ile>Leu) and a synonymous mutation at the amino acid 50 of the haplotype III (Figure 4).

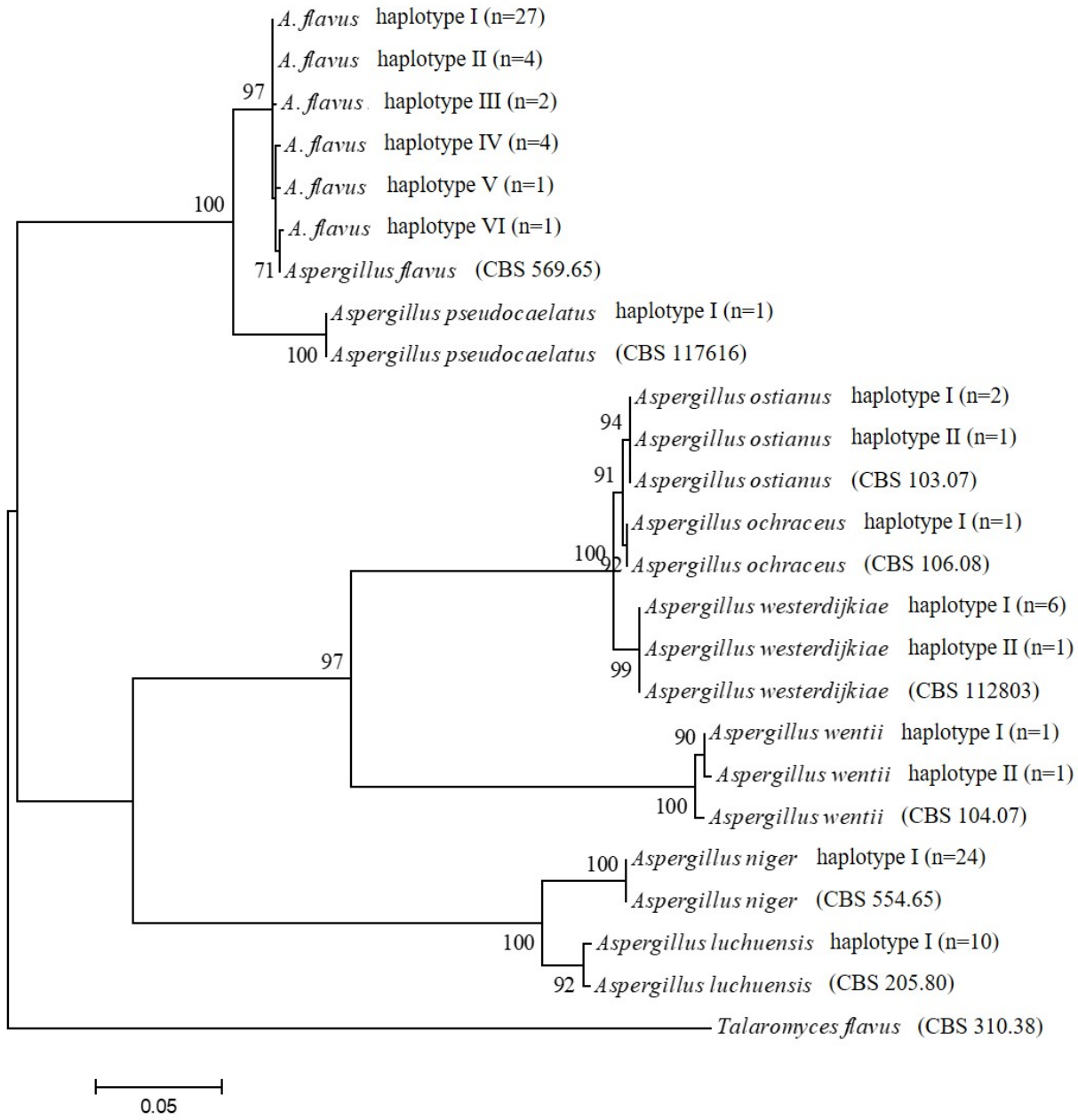

Figure 3

Maximum likelihood tree obtained from partial calmodulin and β-tub concatenated sequences. Bootstrap values over 70 are shown.

Table 3
Sequence variation among Aspergilli isolated from Brazilian dry beans, number of haplotypes found and similarity to the reference strains.

Species	n	β-tub				*caM*			
		Similarity to reference strain (%)	Reference accession[a]	Internal Variation[b] (%)	Haplotypes[c]	Similarity to reference strain (%)	Reference accession[a]	Internal Variation[b] (%)	Haplotypes[c]
A. flavus	39	100	EF661485	0	1	99	EF661508	1	6
A. niger	24	100	EF661089	0	1	100	EF661154	0	1
A. luchuensis	10	100	JX500062	0	1	98	JX500071	0	1
A. westerdijkiae	7	100	EF661329	0	1	99	EF661360	1	2
A. ostianus	3	100	EF661324	0	1	99-100	EF661385	1	2
A. wentii	2	99	EF652106	0	1	99	EF652131	1	2
A. ochraceus	1	100	EF661322	-	1	99	EF661381	-	1
A. pseudocaelatus	1	100	EF203128	-	1	99	EF202037	-	1

[a]Accession numbers of *caM* and β-tub sequences from reference strains (Samson et al., 2014)
[b]Variation observed between the strains analysed in this study
[c]Number of haplotypes found among strains from this study
-: not applicable since only one strain of the species was obtained in this study.

Figure 4
Alignment of partial calmodulin sequences presenting the SNPs observed in seven *A. flavus* haplotypes. Numbers indicate the position on calmodulin sequence of the reference strain *A. flavus* CBS 569.65 (EF661508) starting at the first nucleotide of calmodulin gene. Matching residues are show as dots. SNPs on the coding region in the haplotypes III and IV are boxed.

3.2 Toxigenic characterization

The presence of genes involved in mycotoxin biosynthesis was assessed by PCR, targeting seven, four and ten genes in the aflatoxin, ochratoxin and fumonisin biosynthetic clusters, respectively. In the fumonisin biosynthetic cluster, the presence of a second region of *fum 21*, one intergenic region (*fum 19-15*) and a region downstream the gene *fum 6* was also determined (Figure 2).

204 Within the studied species, *A. flavus*, *A. ostianus* and *A. pseudocaelatus* are known as potential
205 aflatoxin producers. None of the *A. ostianus* and *A. pseudocaelatus* strains gave the expected amplification
206 products, except *A. wentii* isolate CCDCA 11479, where an amplified fragment of approximately 650bp with
207 primers *aflO* was obtained. This amplicon was sequenced and compared within NCBI databases using BLAST.
208 The fragment had no similarity with the *aflO* gene, but its translation and comparison to protein sequences
209 (blastx) gave 100% identity to a hypothetical protein in *A. wentii* (OJJ31152). The 39 *A. flavus* strains in this
210 study represented 13 different amplification patterns (Table 4), varying from the presence of all tested genes
211 (n=17) to the absence of all of them (n=2), suggesting that a large part of the *A. flavus* population on beans in
212 Brazil (43%) is capable of producing aflatoxin.
213

Table 4
Amplification patterns of aflatoxin genes observed within the studied strains. Positive results are shaded in grey.

Species	*aflD*	*aflR*	*aflS*	*aflM*	*aflO*	*aflP*	*aflQ*
A. flavus (n=17)	+	+	+	+	+	+	+
A. flavus (n=5)	-	+	+	+	+	+	+
A. flavus (n=1)	+	-	+	+	+	+	+
A. flavus (n=3)	-	-	+	+	+	+	+
A. flavus (n=2)	+	+	+	-	+	+	+
A. flavus (n=1)	-	+	+	+	+	-	-
A. flavus (n=2)	-	-	+	+	-	-	+
A. flavus (n=1)	-	-	+	-	+	-	+
A. flavus (n=1)	-	-	-	+	+	+	-
A. flavus (n=1)	-	-	-	+	+	-	+
A. flavus (n=1)	-	-	-	+	-	+	-
A. flavus (n=2)	-	-	-	+	-	-	-
A. flavus (n=2)	-	-	-	-	-	-	-

+: amplicon with the expected size
-: no amplicon detected

Regarding fumonisin genes, amplicons were only observed in *A. niger* strains. In all 24 *A. niger* isolates from this work the expected amplicons were obtained for all 13 primer sets, indicating that all *A. niger* strains harbour the 10 genes checked in this pathway, as well as the two intergenic regions (Table 5). On the other hand, none of the ochratoxin genes was detected in any of the 87 studied strains (data not shown).

Table 5
Amplification patterns of fumonisin genes and intergenic regions observed within the studied strains.

Species	*fum1*	*fum19-15*	*fum15*	*fum21 I*	*fum21 II*	*fum14*	*fum13*	*fum8*	*fum3*	*fum7*	*fum10*	*fum6*	*downstream fum6*
A. flavus (n=39)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. niger (n=24)	+	+	+	+	+	+	+	+	+	+	+	+	+
A. luchuensis (n=10)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. westerdijkiae (n=7)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. ostianus (n=3)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. wentii (n=2)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. ochraceus (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	-
A. pseudocaelatus (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	-

+: amplicon with the expected size
-: no amplicon detected

4. Discussion

The presence of *Aspergillus* strains on beans is the first indication of a potential risk of mycotoxin contamination. Especially in large parts of Brazil, this commodity constitutes the basic diet of the population, increasing the chances of mycotoxin intake leading to public health issues. This is the first study to perform molecular identification of Brazilian strains of *Aspergillus* associated with beans as well as a prospection of

genes involved on aflatoxin, fumonisin and ochratoxin biosynthesis. The presence of *A. flavus* in 34 out of the 35 seed lots tested and the presence of all the scanned aflatoxin genes in 43% of them reinforces the necessity of legislation on acceptable mycotoxin limits and trading conditions. In addition the occurrence of a single *A. niger* lineage harbouring the whole fumonisin cluster (n=24) in all provinces surveyed, alerts to the possibility of fumonisin contamination in many regions of Brazil. Furthermore, our findings indicate the necessity of further studies on *Aspergillus* populations on Brazilian commodities, mainly concerning their toxigenic potentials and the environmental conditions triggering toxin production.

Species identification was based on two barcoding genes, calmodulin and β-tubulin: *A. flavus* and *A. niger* strains which are the most common reported food-borne *Aspergillus* species (Dijksterhuis et al., 2013) were found in 97% and 71% respectively of the seed lots tested. In Brazil, *A. flavus* was also shown to be the prevalent species in peanuts and Brazil nuts (Martins et al., 2017; Reis et al., 2014).

The subsequently most frequently identified species was *A. luchuensis*. This species was recently reported to be atoxigenic and hence considered as safe for food and beverage fermentation purposes (Hong et al. 2013). In Korea, *A. luchuensis* is commonly isolated from meju, a product based on dried fermented soybeans (Hong et al., 2013). Second to *A. niger A. welwitschiae* was most frequently isolated species in crops such as grapes, onions, Brazil nuts and coffee (Ferranti et al., 2017; Massi et al., 2016). However, in our survey, we did not encounter *A. welwitschiae* among 35 dry bean samples.

Additional aspergilli identified in this work were *A. westerdijkiae* (n=7), *A. ochraceus* (n=1), *A. ostianus* (n=3) and *A. wentii* (n=2). In Brazil, *A. westerdijkiae* and *A. ochraceus* have been frequently reported on coffee and grapevine crops as the main agents of OTA contamination in associated beverages (Morello et al., 2007; Taniwaki et al., 2003). In our samples, *A. ostianus* and *A. wentii* were found in lower frequencies, similarly to frequencies reported in other Brazilian food products (Abe et al., 2015; Batista et al., 2003).

Sequence variation observed in *A. flavus* calmodulin sequences revealed six haplotypes, including two SNPs in the coding part of the gene. At position 452 of the calmodulin gene, there is synonymous SNP in the haplotype IV, while there is a nonsynonymous SNP at position 543 of haplotype III. However, the substitution of an isoleucine by a leucine residue is unlikely to cause significant changes in the structure of the protein.

Among the *A. flavus* strains of this study, 17/39 harbour all aflatoxin genes examined, suggesting that these strains possibly contain the entire aflatoxin gene cluster. These presumably toxigenic strains were found in 15 out of 35 seed lots (43%), which may indicate a potential aflatoxin contamination level higher than that observed by Lutfullah and Hussain (2012), that found 20% of aflatoxin contamination in beans from Pakistan.

Our data reveal a substantial risk of aflatoxin contamination on beans in Brazil that can be exacerbated by the low level of technology employed by small farmers and the uncontrolled humidity and temperature conditions during storage.

Although the high incidence of aflatoxin have been reported on beans (Silva et al., 2002; Tseng et al., 1995), Telles et al. (2017) suggested that phenolic compounds found in this grain constitute a defence mechanism against fungal attack and aflatoxin production. Nevertheless, *A. flavus* is the most frequent species in Brazilian beans tested in this work, what reinforces the need to monitor *A. flavus* populations on beans to support either the defence mechanism proposed by Telles et al. (2017) or to inforce actions to reduce aflatoxin intake by the population.

In addition to isolates that appear to contain all the genes of the *afl*-cluster (n=17), the most frequent amplification patterns comprise strains lacking *aflD* (n=5) or both *aflD* and *aflR* (n=3). These patterns are in accordance with larger deletions (>1kb) at the left end of the cluster observed in several genotypes of non-aflatoxigenic strains used as biocontrol agents (Adhikari et al., 2016). The complete absence of amplicons for all the tested genes as observed in two strains also indicates large deletions, possibly comprising the entire cluster may occur. It has been suggested that the most frequent deletions in the aflatoxin cluster occur at end of the gene cluster closest to the telomeric end of the chromosome (Adhikari et al., 2016). Nonetheless, we observed nine amplification patterns that lack genes in the central part of the cluster, resembling the results found by Fakruddin et al. (2015) using the same sets of primers. These unexpected patterns can be explained by the occurrence of small (<1kb) deletions, which were also reported by Adhikari et al. (2016). The occurrence of strains lacking tested *afl*-genes strongly suggests the presence of local non-aflatoxigenic strains on beans, which must be further studied in order to confirm them as candidate biocontrol agents in Brazil. Reduction of aflatoxins using non-toxigenic *A. flavus* strains requires the selection of local strains that occur endemically on target crops in target regions (Mehl et al., 2012).

Calmodulin and β-tubulin sequences revealed a single *A. niger* haplotype 100% identical to the reference strain, and a single *A. luchuensis* haplotype 98% identical to the reference strain. The lack of variation was also observed in the fumonisin cluster: our results showed that all 24 *A. niger* strains tested, harbour the whole biosynthetic cluster. Our observation of complete absence of the fumonisin cluster in *A. luchuensis* (n=10) is in contrast to the results obtained by Susca et al. (2014) who reported the presence of the genes *fum1* and *fum15*. The results described here suggest the occurrence of specific lineages of *A. niger* and *A. luchuensis* affecting beans in all surveyed regions, which may not be different in other parts of the country.

Although the presence of the complete fumonisin cluster was also reported in non-producing *A. niger* strains (Susca et al., 2014), it is necessary to highlight the risk of fumonisin contamination on beans. This toxin is produced by *Fusarium verticillioides* and other Fusaria. Therefore, the current legislation in Brazil regulates the tolerable limits of fumonisins only on corn as the main ecological niche for *F. verticillioides* and corn-based products (Anvisa - The Brazilian Health Regulatory Agency, 2011). Considering the present and previous studies, it is clear that the laws concerning fumonisin obligatory assessments must be extended to products highly affected by *A. niger*, including beans.

Among the strains analyzed in this study, three species were reported to be able to produce ochratoxin: *A. niger, A. westerdijkiae* and *A. ochraceus*, however none of the four genes examined was found among the strains tested in the present study. This result confirms previous studies reporting that only a minority of *A. niger* strains can simultaneously produce fumonisin and ochratoxin (Massi et al., 2016). Susca et al. (2014) reported that 100% of the Brazilian strains analysed were OTA non-producers with two possible *ota* amplicon patterns: either presence or absence of all four involved genes. These authors also suggested that the deletion of the *ota* cluster occurred in an *A. niger* ancestor and resulted in the formation of two alleles: an intact and a deleted ota-cluster allele., On the other hand, the reasons why no amplification was observed in *A. westerdijkiae* and *A. ochraceus* strains can be the result of specificity problems of the primers employed, since they were designed based on the *A. niger* ochratoxin gene cluster and may be unable to generate amplicons in *A. westerdijkiae* and *A. ochraceus*.

A recent study performed by Gil-Serna et al. (2018) compared the ochratoxin A biosynthetic genes in different *Aspergillus* species, corroborating the hypothesis that the primers used in the present work are specific for *A. niger* and incapable of amplifying *A. westerdijkiae* and *A. ochraceus* ota genes. Furthermore, these results endorse our findings that the *A. niger* strains tested in this study do not contain the OTA cluster. We also performed PCRs using general primers (F1OT and R1OT Luque et al., 2013), which were supposed to work on both *Aspergillus* and *Penicillium* strains. All these PCRs were not successful, although sometimes shorter amplicon than the expected (459bp) were obtained (data not included). Subsequently, we decided to use the primers designed from Susca et al. (2016). We assume the three used sets of Aspergillus primers for the OTA cluster will detect at least some of the OTA regions when enough similarity exists between potential clusters in different species and sections.

In conclusion, the current study revealed that *Aspergillus* species containing toxigenic gene clusters are frequently found on beans in Brazil what suggests a potentially high risk of daily intake of mycotoxins by the

population. Therefore, we emphasize the need for further studies to elucidate the *Aspergillus* diversity in Brazil and contribute to strategies for preventing toxin contamination in food in the world. In addition, we demonstrated the requirement of reliable tests and a strict regulation on the tolerable mycotoxin levels especially in staple foods.

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