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Real-time PCR-based method for rapid detection of *Aspergillus niger* and *Aspergillus welwitschiae* isolated from coffee

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ABSTRACT

Some species from *Aspergillus* section *Nigri* are morphologically very similar and altogether have been called *A. niger* aggregate. Although the species included in this group are morphologically very similar, they differ in their ability to produce mycotoxins and other metabolites and their taxonomical status has evolved continuously. Among them, *A. niger* and *A. welwitschiae* are ochratoxin A and fumonisin B_2 producers and their detection and/or identification is of crucial importance for food safety. The aim of this study was the development of a real-time PCR-based method for simultaneous discrimination of *A. niger* and *A. welwitschiae* from other species of the *A. niger* aggregate isolated from coffee beans. One primer pair and a hybridization probe specific for detection of *A. niger* and *A. welwitschiae* strains were designed based on the *BenA* gene sequences, and used in a Real-time PCR assay for the rapid discrimination between both these species from all others of the *A. niger* aggregate. The Real-time PCR assay was shown to be 100% efficient in discriminating the 73 isolates of *A. niger/A. welwitschiae* from the other *A. niger* aggregate species analyzed as a negative control. This result testifies to the use of this technique as a good tool in the rapid detection of these important toxigenic species.

1. Introduction

Species in *Aspergillus* section *Nigri* are widely distributed in the environment. They are important food contaminants and may be present at different stages of processing such as pre-harvest, drying, storage and transport (Samson et al., 2010; Taniwaki and Pitt, 2013). The taxonomy of *A.* section *Nigri* still raises controversy, probably because for many years the classification was based only on morphological and physiological criteria and, in most cases, it is not possible to notice differences between species only through these criteria (Varga et al., 2011). Some species from *A.* section *Nigri* are morphologically very similar and altogether have been called *Aspergillus niger* aggregate. The taxonomical status of *A. niger* aggregate has evolved continuously and nowadays there are 10 species (Massi et al., 2016; Varga et al., 2011). Although the species included in the *A. niger* aggregate are morphologically very similar, they differ in their ability to produce mycotoxins and other metabolites. Among them, only *Aspergillus welwitschiae* (Bresadola) Hennings apud Wehmer (=*Aspergillus awamori sensu* Perrone) and *Aspergillus niger* Thiegh are ochratoxin A and/or fumonisin B_2 producers (Abarca et al., 1994; Ferranti et al., 2017; Frisvad et al., 2007; Frisvad et al., 2011; Perrone et al., 2011). Therefore, the discrimination between these both species from all from the others of the *A. niger* aggregate is relevant in food safety.

Ochratoxin A (OTA) is known as a nephrotoxic compound and is classified by the International Agency of Research on Cancer (IARC (International Agency for Research on Cancer), 2002) as a Group B2, *i.e.* the metabolite is a probable human carcinogen. OTA production in food is influenced by various factors and can increase depending on climatic conditions and geographic region; varieties and crop systems; damage caused by insects; fungal infection or excessive irrigation/rainfall (Ostry et al., 2013). Fumonisins are carcinogenic mycotoxins and are among the most important toxins regarding food and feed safety (Frisvad et al., 2007). Fumonisin B_1 is the most studied fumonisin, but when compared to toxicity, fumonisin B_2 is more cytotoxic than B_1 (Gutleb et al., 2002). Ochratoxin A and fumonisin B_2 have been found in a variety of food commodities, including coffee and, in part,

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the source of these toxins in foods has been attributed to *A. niger* and *A. welwitschiae* strains (Mogensen et al., 2010; Noonim et al., 2009). The presence of toxigenic fungi and toxins in coffee has been extensively revised elsewhere (Bucheli and Taniwaki, 2002; Paterson et al., 2014).

During the last 15 years several PCR-based assays have been developed to overcome the difficulties of the traditional schemes for identification of toxigenic fungi from food samples. Although conventional PCR has been recognized as a very valuable tool for detecting toxigenic fungi (Fungaro and Sartori, 2009; Gherbawy et al., 2015; Massi et al., 2016; Palumbo and O'Keeffe, 2015; Schmidt et al., 2004), Real-time PCR does not require gel electrophoresis, and consequently reduces time and manual labor, making it appropriate for large-scale analyses. In this study, we developed a Real-time PCR for simultaneous discrimination of *A. niger* and *A. welwitschiae* from all other species of the *A. niger* aggregate. Considering the risk inherent with the presence of *A. niger/A. welwitschiae* species in foods and consequently the need for rapid detection and/or identification, we recognize the importance of this technical development.

2. Material and methods

2.1. Fungal isolation from coffee bean samples

A total of 23 samples of *Coffea canephora*, each of approximately 1 kg, was collected in the state of Espírito Santo, Brazil. Four samples came from fields and 19 from storage places.

For fungal isolation, approximately 100 g of each sample was taken randomly, and surface disinfected with sodium hypochlorite solution (0.4%) for 1 min. Fifty beans were then distributed evenly in five Petri dishes containing Dichloran 18% Glycerol Agar (DG18) and incubated at 25 °C for 5 d, according to Pitt and Hocking (2009).

2.2. Morphological identification

Isolates that had the appearance of belonging to *Aspergillus niger* aggregate were transferred to Czapek Yeast Extract Agar (CYA; Pitt and Hocking, 2009) and incubated at 25 °C for 7 d. Isolates were then examined on standard identification media for *Aspergillus* species CYA, at 25 °C and 37 °C and Malt Extract Agar (MEA; Samson et al., 2010) at 25 °C (Pitt and Hocking, 2009) and incubated for 7 d.

2.3. DNA extraction

A total of 79 isolates representing *A. niger* aggregate isolated from each coffee sample as shown in Table 1, were submitted to DNA extraction. For this, the isolates were grown in 40 mL malt extract broth for 48 h at 25 °C, with no shaking. The superficial mycelia and conidia were recovered and macerated with liquid nitrogen and then the extraction was carried out according to the manufacturer's protocol of PowerLyzerTM Power Soil® kit (Mo Bio Laboratories, Carlsbad, CA, USA). Quantification of DNA was performed on Nanodrop 2000 (Nanodrop Technologies, Walthan, USA).

2.4. DNA sequencing

After extracting the genomic DNA, all the isolates were submitted to sequencing of a portion of the calmodulin gene (*CaM*), since this gene has been pointed out as the most appropriate for identification of *Aspergillus* species (Samson et al., 2014). The PCR amplification of the *CaM* gene region was performed using the primer pair cmd5/cmd6 (Hong et al., 2006). Amplifications were conducted on a Veriti™ thermal cycler (Applied Biosystems, CA, USA) using a 25 µL reaction volume containing 10 ng of DNA template, 1× PCR buffer, 1.0 mM of $MgCl_2$, 0.25 mM of dNTP, 0.4 µM of each PCR primer, and 1 U of Taq DNA polymerase. The mixture was subjected to the following amplification program: initial denaturation at 95 °C for 1 min, followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, and a final elongation for 10 min at 72 °C. The resulting fragments were purified using the Wizard® SV Gel and PCR clean-up system (Promega, WI, USA), according to the manufacturer's recommended protocol. Amplicons were sequenced with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) under the following conditions: denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 15 s, extension at 60 °C for 1 min and a final extension at 60 °C for 1.5 min. The sequencing products were resuspended in Hi-Di formamide (10 µL) and processed in an ABI 3500XL Genetic Analyzer (Applied Biosystems, CA, USA).

The nucleotide sequences here obtained were aligned using the ClustalW algorithm (Thompson et al., 1994) with the ex-type or neotype strain sequences retrieved from the GenBank database. The Kimura-2-Parameter model (Kimura, 1980) was used for the construction of Maximum Likelihood tree (ML) in MEGA 7.0 software package (Kumar et al., 2015). To determine the support for each clade, a non-parametric bootstrap analysis was performed with 1000 resamplings.

2.5. Primers and probe design

We explored the nucleotide sequence variability in a region of the β-tubulin gene to design a primer and a TaqMan® MGB probe to amplify target DNA from *A. niger* and *A. welwitschiae*. This development involved the analysis of a *BenA* gene segment (approximately 500 bp in length), retrieved from the GenBank database, of all species belonging to *A. niger* aggregate. The analysis involved the alignment of the sequences, using ClustalW (Thompson et al., 1994), and the visual search for regions of complete similarity to *A. niger* and *A. welwitschiae,* but not to other species belonging to the *A. niger* aggregate (Fig. 1). The primer-pair *benA*-An/Aw (5′ GGG CAA AGG GTT GGG TCT TC 3′ and 5′ GAC GAG GAC GGC ACG AGG A 3′) and the TaqMan® MGB probe *benA*-An/Aw (5′ NED-CGT CTA CTT CAA CGA GGT GAG ATC CAT-MGB 3′) sequences were checked on BLASTN (https://goo.gl/Hu4o5Z) for correct specificity. Hairpin and dimer formations were checked using OligoAnalyzer 3.1 (https://goo.gl/ZRpoQy). The TaqMan® MGB probe *benA*-An/Aw, labeled at the 5′ end with the reporter dye NED and at the 3′end with the chemical compound minor groove binder (MGB), was purchased from Applied Biosystems (CA, USA).

2.6. Real-time PCR

The PCR conditions with the primer pair/probe here designed were optimized and then tested on DNA of the 67 isolates identified as *A. niger* and on the six identified as *A. welwitschiae*. The PCR amplification procedure was performed with a total volume of 10 µL containing 10 ng of DNA, 1× PCR buffer, 0.5 mM dNTPs, 3 mM $MgCl_2$, 1.25 U of platinum Taq DNA polymerase, 0.6 µM of each primer and 0.2 µM of the probe. The reaction was conducted at 95 °C for 1 min, followed by 40 cycles of 92 °C for 15 s and 58 °C for 1 min. TaqMan Real-time PCR was performed in an ABI 7500 real-time PCR system (Applied Biosystems, CA, USA). The analyses were carried out in triplicate. As negative control, *Aspergillus luchuensis*, *Aspergillus tubingensis* and *Aspergillus neoniger* strains were used. The strain UEL 2.9, meticulously identified as *A. niger* in a previous study (Massi et al., 2016), was used as positive control.

3. Results and discussion

3.1. Fungal isolates from coffee

The infection of *A. niger* aggregate in coffee samples ranged from 2% to 82% in field samples and 2% to 88% in storage samples, as shown in Table 1. Some samples were highly infected with *A. niger* aggregate. The presence of black aspergilli in *Coffea canephora* has been observed

Table 1
Aspergillus niger aggregate isolated from coffee, origin, percentage of infection (%), real-time PCR profile and phylogenetic identification.

Coffee sample	Local of collection	Range of infection	Isolate ID	Identification by real-time PCR		Phylogenetic identification
#		(%)		Amplification profile	Cycle threshold (CT)	More similar táxon
CC1	Storage	74	8191 N	+	17	*A. niger*
			8193 N	+	15	*A. niger*
			8205 N	+	19	*A. niger*
			8207 N	+	17	*A. welwitschiae*
			8208 N	+	14	*A. niger*
			8214 N	+	18	*A. niger*
			8216 N	+	18	*A. niger*
			8221 N	−	−	*A. neoniger*
			8223 N	+	19	*A. welwitschiae*
CC2	Field	2	8250 N	+	18	*A. niger*
			8257 N	−	−	*A. luchuensis*
			8271 N	+	21	*A. niger*
			8279 N	+	25	*A. welwitschiae*
CC3	Storage	72	8280 N	+	19	*A. niger*
			8284 N	+	22	*A. niger*
			8287 N	+	18	*A. niger*
			8292 N	+	19	*A. welwitschiae*
			8300 N	+	20	*A. niger*
			8305 N	+	20	*A. niger*
			8306 N	+	21	*A. niger*
CC4	Storage	60	8309 N	+	20	*A. niger*
			8317 N	+	18	*A. welwitschiae*
			8321 N	+	18	*A. niger*
			8325 N	+	19	*A. niger*
CC5	Storage	4	8330 N	+	18	*A. niger*
			9164 N	+	19	*A. niger*
			9165 N	+	20	*A. niger*
CC6	Field	40	9168 N	+	22	*A. niger*
			9177 N	+	19	*A. niger*
			9180 N	+	19	*A. niger*
			9181 N	+	23	*A. niger*
			9205 N	+	19	*A. niger*
CC7	Storage	40	9214 N	+	18	*A. niger*
			9218 N	+	17	*A. niger*
CC8	Storage	4	9220 N	+	22	*A. niger*
			9221 N	+	20	*A. welwitschiae*
CC9	Storage	2	9223 N	+	19	*A. niger*
			9233 N	+	19	*A. niger*
CC10	Storage	20	9236 N	+	17	*A. niger*
			9239 N	+	15	*A. niger*
			9240 N	+	19	*A. niger*
CC11	Storage	4	9256 N	−	−	*A. luchuensis*
			9257 N	+	20	*A. niger*
			9274 N	+	19	*A. niger*
			9276 N	+	21	*A. niger*
			9287 N	+	17	*A. niger*
			9290 N	+	19	*A. niger*
			9292 N	−	−	*A. luchuensis*
CC12	Storage	88	9294 N	+	19	*A. niger*
			9296 N	+	19	*A. niger*
			9303 N	+	18	*A. niger*
			9309 N	+	19	*A. niger*
			9310 N	+	18	*A. niger*
			9311 N	+	17	*A. niger*
			9314 N	+	18	*A. niger*
CC13	Storage	44	9321 N	+	21	*A. niger*
			9326 N	−	−	*A. tubingensis*
CC14	Storage	2	9346 N	+	19	*A. niger*
CC15	Storage	24	9357 N	+	18	*A. niger*
CC16	Storage	10	9363 N	+	15	*A. niger*
			9370 N	+	19	*A. niger*
			9380 N	+	23	*A. niger*
CC17	Field	82	9389 N	+	21	*A. niger*
			9394 N	+	20	*A. niger*
CC18	Storage	6	9427 N	+	19	*A. niger*
CC19	Field	2	9432 N	+	19	*A. niger*
CC20	Storage	20	9445 N	+	20	*A. niger*
			9452 N	+	19	*A. niger*
			9460 N	−	−	*A. tubingenis*
			9461 N	+	19	*A. niger*
			9466 N	+	16	*A. niger*

(*continued on next page*)

Table 1 (*continued*)

Coffee sample	Local of collection	Range of infection	Isolate ID	Identification by real-time PCR		Phylogenetic identification
#		(%)		Amplification profile	Cycle threshold (CT)	More similar táxon
CC21	Storage	78	9473 N	+	20	*A. niger*
			9488 N	+	14	*A. niger*
			9495 N	+	21	*A. niger*
			9497 N	+	18	*A. niger*
CC22	Storage	72	9503 N	+	20	*A. niger*
			9525 N	+	20	*A. niger*
CC23	Storage	70	9558 N	+	19	*A. niger*
			9560 N	+	20	*A. niger*

in several studies (Joosten et al., 2001; Noonim et al., 2008). According to Noonim et al. (2008) although some isolates of *A. niger* produce ochratoxin A, the amount produced is usually lower compared to *Aspergillus carbonarius* and the percentage of *A. niger* producing OTA varies from 3 to 13% (Noonim et al., 2008; Taniwaki et al., 2003) indicating that *A. niger* is not the main OTA source in coffee. However, studies have shown the ability of fumonisin B_2 production from 73 to 81% and 18 to 33% in *A. niger* and *A. welwitschiae*, respectively, making these two species a concern for public health (Ferranti et al., 2018; Frisvad et al., 2011; Massi et al., 2016; Palumbo and O'Keeffe, 2015; Perrone et al., 2011).

A total of 341 strains of *A. niger* aggregate was distinguished by micro and macro morphology and physiological characteristics, such as growth at 25 °C and 37 °C with black colonies reaching 60 mm diameter, reverse pale or occasionally deep brown. Vesicles spherical, bisseriate with rough conidia.

3.2. Molecular identification

Among these 341 strains recognized as belonging to *A. niger* aggregate, 79 isolates representing each coffee samples were chosen for molecular identification as shown in Table 1. Maximum Likelihood (ML) tree was reconstructed from the partial calmodulin gene (*CaM*) sequences (Fig. 2). Five species were found: *A. niger* ($n = 67$), *A. welwitschiae* ($n = 6$), *A. luchuensis* ($n = 3$), *A. neoniger* ($n = 1$) and *A. tubingensis* ($n = 2$). As expected *A. niger* isolates were the most common, because this species is one of the most common of the *A. niger* aggregate and has been frequently found in tropical and subtropical foods, including coffee beans (Massi et al., 2016; Noonim et al., 2008; Taniwaki et al., 2003).

3.3. Real-time PCR for A. niger/A. welwitschiae

The genetic variation found between species of the *A.* section *Nigri* in a region of the β-tubulin gene allowed us to design a primer-pair and a TaqMan® MGB probe specific to amplify the target DNA from *A. niger* and *A. welwitschiae* (Fig. 1). It is important to state that the primer-pair (*benA*-An/Aw2) is *slightly* modified compared to those previously reported by Massi et al. (2016) for use in a conventional PCR. The primer-pair specificity was confirmed by an *in silico* analysis. Of the 959 *Aspergillus* blast hits analyzed, only the *A. niger* and *A. welwitschiae* strains had 100% sequence identity.

Using the real-time PCR conditions described in Material and Methods section, the primer-pair *benA*-An/Aw and the TaqMan® MGB probe *benA*-An/Aw produced a 195 bp-amplicon from all *A. niger* ($n = 67$) and *A. welwitschiae* ($n = 6$) isolates tested and as expected no PCR product was obtained from DNA used as negative control (*A. luchuensis, A. neoniger* and *A. tubingiensis*) (Fig. 3).

Table 1 shows the 79 isolates of *A. niger* aggregate that were analyzed, and the results obtained in the Real-time PCR. Cycle threshold (CT) values refer to the numbers of PCR cycles that were detectable for amplification measurements. For these reasons, the analyses of results were carried out taking account of CT values and amplification curves. The results obtained in the Real-time PCR corroborate with the phylogenetic analyses. The isolates not amplified by Real-time PCR (Table 1) were identified as *A. tubingensis*, *A. luchuensis* or *A. neoniger*, confirming the specificities of the primer-pair/probe. Fig. 3 shows the amplification curves of representatives of all species found in this study.

No mutations found in the amplification region of the primers show the necessity of sequencing methodology to be used for further separation of the species of *A. niger* and *A. welwitschiae*. As reported by Varga et al. (2011)*A. niger* and *A. welwitschiae* are cryptic species in the process of separation and thus differentiation between these two species is very difficult and requires more than one molecular marker such as β-tubulin and calmodulin sequences. Methodologies to distinguish *A. niger* from *A. welwitschiae* by means of PCR are already available; however, although useful, these methodologies are based on conventional PCR (Gherbawy et al., 2015; Palumbo and O'Keeffe, 2015) and two PCR reactions are required to detect *A. niger* and *A. welwitschiae.* Our goal was to develop a Real-time PCR for discriminating *A. niger/A. welwitschiae* from others belonging to the *A. niger* species aggregate, because these species are the only ones of the *A. niger* aggregate

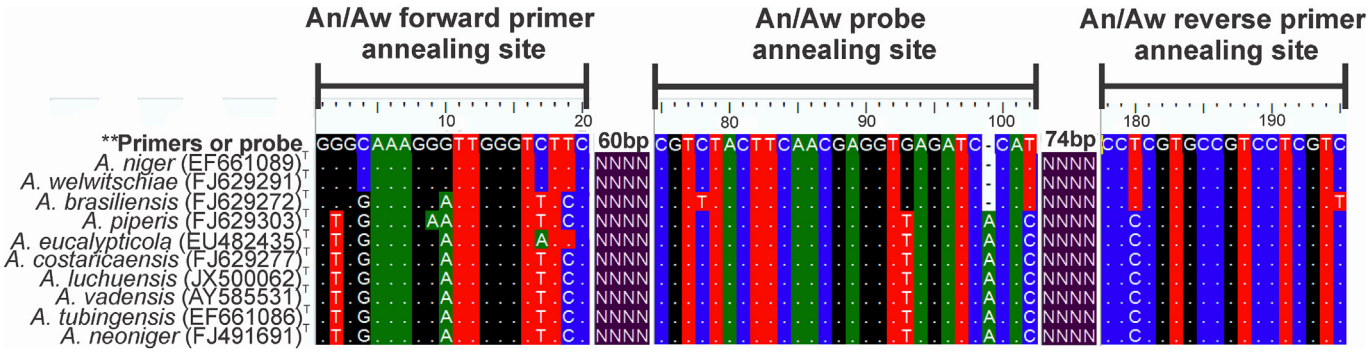

Fig. 1. Alignment of partial *BenA* gene sequences of *A. niger* aggregate (T superscript means type strain). The boxes marked by arrows indicate the annealing site of the An/Aw primer pair and the An/Aw probe. ** An/Aw-F or An/Aw-R or An/Aw probe, sequences.

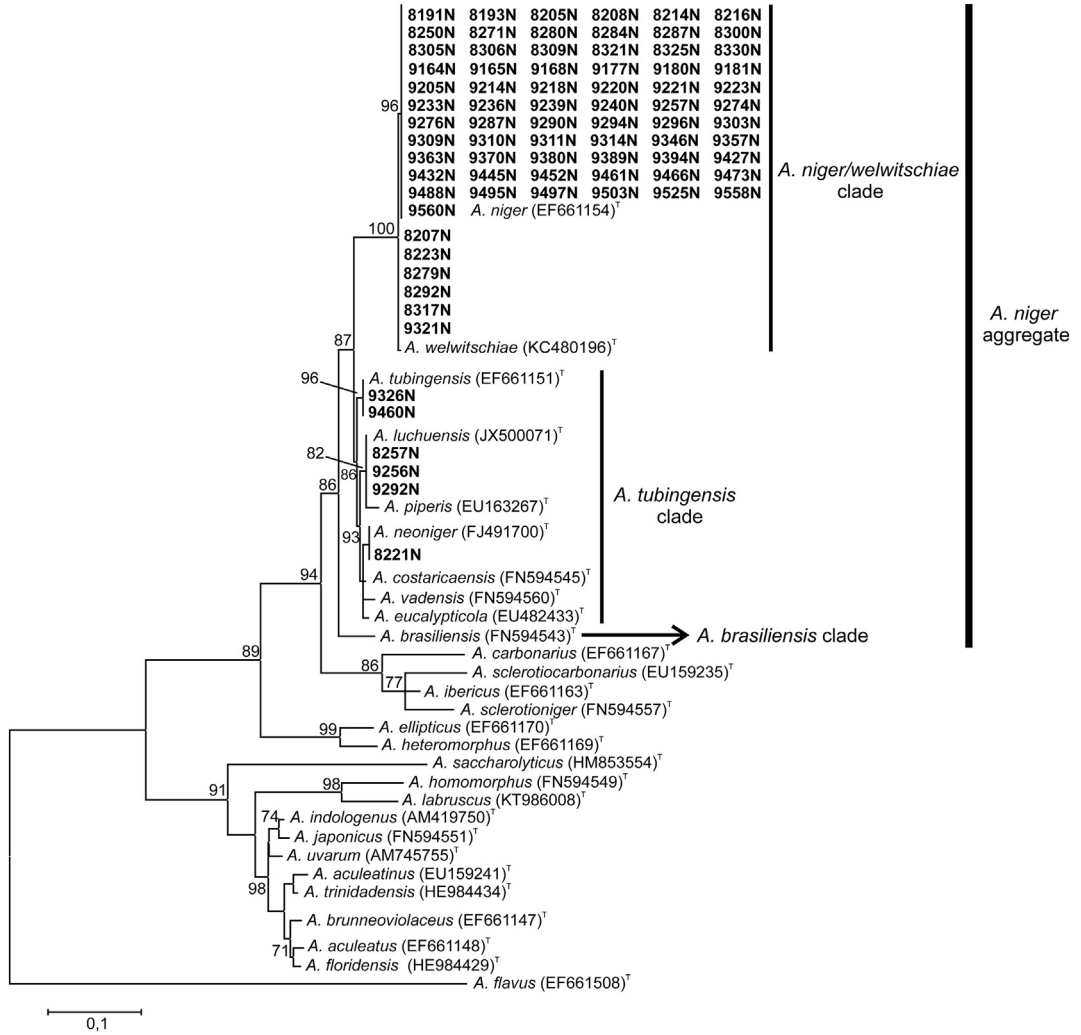

Fig. 2. Maximum Likelihood (ML) tree reconstructed from the partial calmodulin gene sequences aligned with corresponding sequences of type strains from *Aspergillus* section *Nigri*. The bootstrap values (≥70%) are indicated in each node. *A. flavus* is the outgroup.

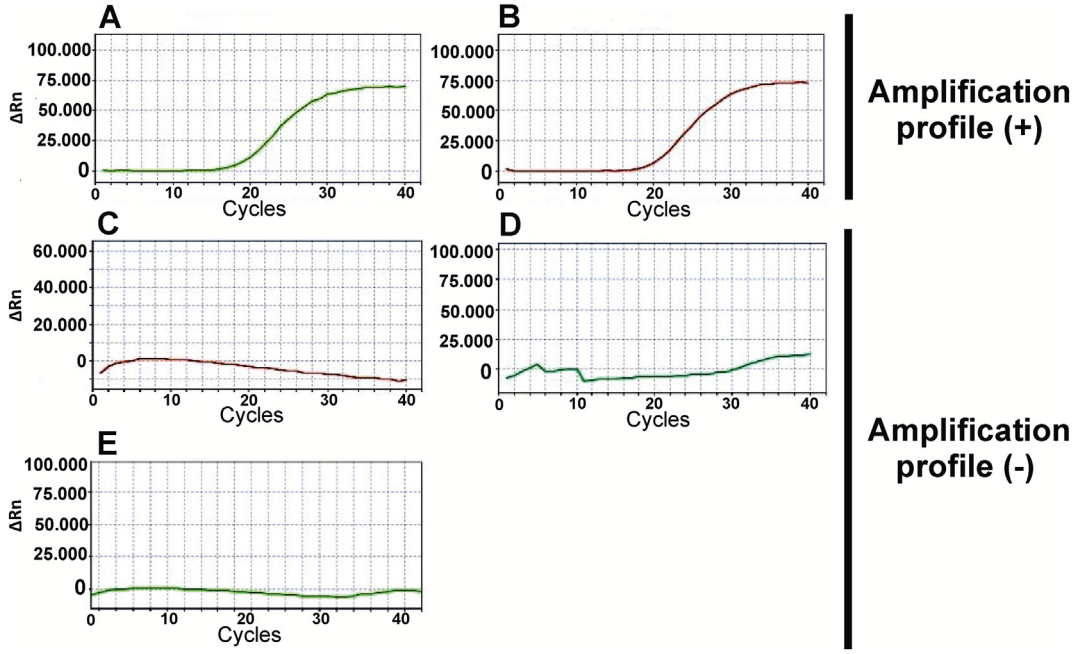

Fig. 3. Amplification plot of representatives of all species found in this study. A) *Aspergillus niger*; B) *A. welwitschiae*; C) *A. luchuensis*; D) *A. neoniger*; E) *A. tubingensis*.

described as producers of OTA and FB_2.

The primer-pair *benA*-An/Aw and the TaqMan® MGB probe *benA*-An/Aw described in this study were very accurate to separate strains of *A. niger/A. welwitschiae* from other species in the *A. niger* aggregate, 100% of the isolates showed coincidence on Real-time PCR and DNA sequencing. This methodology showed to be very promising and practical as a screening methodology for *A. niger* aggregate isolated from food. The main advantages of TaqMan real-time PCR assays are their economy of time and labor, high efficiency, sensitivity and high specificity, and no post-PCR steps, which reduce the risks of contamination (Lim et al., 2008). The TaqMan real-time PCR assay as here proposed is very useful for detection of *A. niger/A. welwitschiae*, which may harbor strains potentially producing OTA and FB_2.

4. Conclusions

In conclusion, the primer-pair/probe selected for identification of the *A. niger/welwitschiae* species proved to be very efficient in the Real-time PCR technique. This methodology may be a simple and useful alternative for discrimination of *A. niger/A. welwitschiae* from all other species of the *A. niger* aggregate.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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.

Bucheli, P., Taniwaki, M.H., 2002. Research on the origin, and the impact of postharvest handling and manufacturing on the presence of ochratoxin A in coffee. Food Addit. Contam. 19, 655–665.

Ferranti, L.S., Correa, B., Fungaro, M.H.P., Iamanaka, B.T., Massi, F.P., Phippen, C.B.W., Frisvad, J.C., Taniwaki, M.H., 2017. Occurrence and fumonisin B_2 producing potential of *Aspergillus* section *Nigri* in Brazil nuts. Mycotoxin Res. 33, 49–58.

Ferranti, L.S., Fungaro, M.H.P., Massi, F.P., Silva, J.J., Penha, R.E.S., Frisvad, J.C., Taniwaki, M.H., Iamanaka, B.T., 2018. Diversity of *Aspergillus* section *Nigri* on the surface of *Vitis labrusca* and its hybrid grapes. Int. J. Food Microbiol. 268, 53–60.

Frisvad, J.C., Smedsgaard, J., Samson, R.A., Larsen, T.O., Thrane, U., 2007. Fumonisin B2 production by *Aspergillus niger*. J. Agric. Food Chem. 55, 9727–9732.

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.

Fungaro, M.H.P., Sartori, D., 2009. An overview on molecular markers for detection of ochratoxigenic fungi in coffee beans. Braz. Arch. Biol. Technol. 52, 1–9.

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 B2 production. Foodborne Pathog. Dis. 12, 414–423.

Gutleb, A.C., Morrison, E., Murk, A.J., 2002. Cytotoxicity assays for mycotoxins produced by *Fusarium* strains: a review. Environ. Toxicol. Pharmacol. 11, 309–320.

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.

IARC (International Agency for Research on Cancer), 2002. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. In: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. vol. 82 International Agency for Research on Cancer, Lyon, France.

Joosten, H.M.L.J., Goetz, J., Pittet, A., Schellenberg, M., Bucheli, P., 2001. Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. Int. J. Food Microbiol. 65, 39–44.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Kumar, S., Stecher, G., Tamura, K., 2015. Mega7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Lim, S.Y., Kim, B.J., Lee, M.K., Kim, K., 2008. Development of a real-time PCR-based method for rapid differential identification of *Mycobacterium* species. Lett. Appl. Microbiol. 46, 101–106.

Massi, F.P., Sartori, D., Ferranti, L.S., Iamanaka, B.T., Taniwaki, M.H., Vieira, M.L.C., Fungaro, M.H.P., 2016. Prospecting for the incidence of genes involved in ochratoxin and fumonisin biosynthesis in Brazilian strains of *Aspergillus niger* and *Aspergillus welwitschiae*. Int. J. Food Microbiol. 221, 19–28.

Mogensen, J.M., Frisvad, J.C., Thrane, U., Nielsen, K.F., 2010. Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. J. Agric. Food Chem. 58, 954–958.

Noonim, P., Mahakarnchanakul, W., Nielsen, K.F., Frisvad, J.C., Samson, R.A., 2008. Isolation, identification and toxigenic potential of ochratoxin A-producing *Aspergillus* species from coffee beans grown in two regions of Thailand. Int. J. Food Microbiol. 128, 197–202.

Noonim, P., Mahakarnchanakul, W., Nielsen, K.F., Frisvad, J.C., Samson, R.A., 2009. Fumonisin B_2 production by *Aspergillus niger* in Thai coffee beans. Food Addit. Contam. A 26, 94–100.

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

Palumbo, J.D., O'Keeffe, T.L., 2015. Detection and discrimination of four *Aspergillus* section *Nigri* species by PCR. Lett. Appl. Microbiol. 60, 188–195.

Paterson, R.R.M., Lima, N., Taniwaki, M.H., 2014. Coffee, mycotoxins and climate change. Food Res. Int. 61, 1–15.

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., Hocking, A.D., 2009. Fungi and Food Spoilage, 3rd ed. Springer, New York.

Samson, R.A., Houbraken, J., Thrane, U., Frisvad, J.C., Andersen, B., 2010. Food and Indoor Fungi. CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.

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, A.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.

Schmidt, H., Bannier, M., Vogel, R.F., Niessen, L., 2004. Detection and quantification of *Aspergillus ochraceus* in green coffee by PCR. Lett. Appl. Microbiol. 38, 464–469.

Taniwaki, M.H., Pitt, J.I., 2013. Mycotoxins. In: Buchanan, R.L. (Ed.), Food Microbiology: Fundamentals and Frontiers Doyle MP, 4th edn. ASM Press, Washington, pp. 597–618.

Taniwaki, M.H., Pitt, J.I., Teixeira, A.A., Iamanaka, B.T., 2003. The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. Int. J. Food Microbiol. 82, 173–179.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Varga, J., Frisvad, J.C., Kocsubé, S., Brankovics, B., Tóth, B., Szigeti, G., Samson, A., 2011. New and revisited species in *Aspergillus* section *Nigri*. Stud. Mycol. 69, 1–17.