MOLECULAR IDENTIFICATION OF *Aspergillus* spp. ISOLATED FROM COFFEE BEANS

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ABSTRACT: Some species belonging to the genus *Aspergillus* are potential producers of ochratoxin A (OA), a mycotoxin with nephrotoxic, immunosuppressive, teratogenic and carcinogenic effects. The aim of the present study was to identify the species of *Aspergillus* that contaminate the inside of coffee beans collected in the stage of maturation and drying, from 16 producing areas located in the northern region of the State of Paraná, in the South of Brazil. A total of 108 isolates of *Aspergillus* spp. was identified at the species level, by sequencing the internal transcribed spacer (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA). The results revealed the presence of potentially ochratoxigenic species in 82% of the geographic regions studied, among which *Aspergillus niger* was the species most frequently detected, followed by *A. ochraceus* and *A. carbonarius*. The presence of *A. carbonarius* in immature coffee fruits harvested from trees is reported for the first time.
Key words: ITS, ribosomal DNA, ochratoxin, mycotoxin

IDENTIFICAÇÃO MOLECULAR DE *Aspergillus* spp. ISOLADOS DE GRÃOS DE CAFÉ

RESUMO: Algumas espécies pertencentes ao gênero *Aspergillus* possuem potencial para produção de Ocratoxina A (OA), uma micotoxina de efeitos nefrotóxicos, imunossupressivos, teratogênicos e carcinogênicos. Com o objetivo de identificar as espécies de *Aspergillus* que contaminam o interior de grãos de café, foram coletadas amostras em diferentes estádios de maturação do produto, em 16 propriedades produtoras do norte do estado do Paraná. Um total de 108 isolados de *Aspergillus* spp. foram identificados ao nível de espécie, pelo sequenciamento dos espaços internos transcritos (ITS1-5,8S-ITS2) do DNA ribossomal (rDNA). Os resultados revelaram a presença de espécies potencialmente ocratoxigênicas em 82% das regiões analisadas, sendo dentre estas, *Aspergillus niger* a espécie mais freqüentemente detectada, seguida por *A. ochraceus,* e *A. carbonarius*. É relatada pela primeira vez a presença de *A. carbonarius* em frutos de café coletados na árvore.
Palavras-chave: ITS, DNA ribossomal, ocratoxina, micotoxina

INTRODUCTION

The ochratoxin A (OA) is the mycotoxin of greatest significance found in coffee beans and beverage. The presence of OA in coffee was first reported by Levi et al. (1974) followed by several other studies (Abarca et al., 1994; Moss 1996; Bucheli et al., 1998; Batista et al., 2003; Taniwaki et al., 2003).

The presence of this mycotoxin in food has been considered a worldwide problem in public health. In countries with tropical climates, species of *Aspergillus* have been the main ones responsible for the production of OA in coffee (Taniwaki et al., 2003). Based on this, studies aimed at identifying the species of *Aspergillus* that contaminate coffee and that are potential OA producers have become of great importance in the last decade.

The identification of fungal species by classic taxonomy is based mainly on the use of morphological markers. However, the number of these markers available is generally low, which makes difficult the classification and/or identification of related species. The development of molecular biology techniques for the genetic differentiation of species has resulted in substantial advances in taxonomy due to their sensitivity and specificity. The amplification of internal transcribed spacer (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA) by the polymerase chain reaction (PCR) (Mullis & Faloona, 1987), combined with sequencing of the amplicon and analysis of

similarity between the sequences obtained and those already deposited in the gene bank, has been frequently employed for identification of fungal species. Despite this, no studies have been published using molecular approaches for the identification of *Aspergillus* spp. in coffee beans.

The aim of the present study was to isolate and identify, by sequencing of the ITS regions, *Aspergillus* spp. that contaminate the inside of coffee beans, produced in the northern region of the State of Parana, in the South of Brazil.

MATERIAL AND METHODS

Material - Samples of coffee (*Coffea arabica*) at different stages of maturation (immature fruits from trees, cherries from the drying yard and overripe cherries from soil) and from storage in barns were obtained from 16 coffee properties in northern region of state of Paraná, in the south region of Brazil. The sampling consisted of a random collection of 1 kg coffee beans. After external disinfecting of the beans with 1% solution of sodium hypochlorite, 200 beans were submitted to a Blotter test (Neergard, 1979) to expose the fungi. The differentiation of *Aspergillus* from other genera uncovered was conducted according to Pitt & Hocking (1997). A total of 108 isolates of *Aspergillus* spp. were purified for molecular identification at the species level. These isolates were coded as indicated in Table 1.

Extraction of genomic DNA - Conidia of each isolate was inoculated in liquid medium (Pontecorvo et al*.,* 1953) and incubated at 28ºC, for 24 h, at 180 rpm. Genomic DNA was extracted according to Azevedo et al. (2000) and quantified by a fluorimetric method (Dyna Quant, Phamarcia).

PCR of ITS region - Fragments of the ITS1-5.8S-ITS2 were amplified by the use of the primers ITS1 and ITS4 (White et al*.,* 1990). The final volume of each reaction was 25 μL, containing 2.5 μL of buffer (200 mM Tris-HCl, pH 8,4 - 500 mM KCl, 1x concentrated), 2.0 μL of dNTPs (2.5 mM), 1.5 μL of each of the primers ITS1 and ITS4 (Invitrogen – 10 pmol μL^{-1}), 1.0 μL $MgCl_2$ (50 mM), 0.2 μL Taq DNA polymerase (5 U μL^{-1}), 2 μL DNA (5 ng μL^{-1}) and 14.3 μL of distilled water. The reaction mixture was placed in a PTC-100 thermocycler (MJ Research, Inc.) programmed to conduct 35 cycles after an initial denaturation of 4 min at 92ºC. Each amplification

Table 1 - Isolates of *Aspergillus* spp. from coffee beans sampled from different origins.

Coffee sample	Maturation stage or storage	Coding of *Aspergillus* isolates
1	storage	A11, A12, A13
1	drying yard	A21
2	storage	A51, A52
2	drying yard	A61, A62, A63, A64, A65
2	Immature fruits from tree	A71
2	cherries from soil	A81
3	storage	A91, A92, A93
3	drying yard	A101, A102, A103, A104, A105
4T	storage	A131, A132
4T	drying yard	A141
4T	Immature fruits from tree	A151, A152
4T	cherries from soil	A161, A162
4	storage	4A, 4B, 4C, 4D, 4F
6	storage	6A
7	storage	7A
8	storage	8A1, 8A2, 8B
11	storage	11B, 11C, 11D, 11F, 11G
12	storage	12A, 12C, 12D, 12E, 12G, 12H, 12I, 12J, 12L
13	storage	13A, 13B, 13C,13D, 13E, 13F, 13G, 13H, 13I
14	storage	14B, 14C1, 14C2, 14D, 14E1, 14F, 14G, 14H, 14I, 14J, 14M, 14N, 14O
15	storage	15A, 15B1, 15B2, 15C, 15D, 15E, 15F1, 15F2, 15G, 15H, 15I2, 15J
16	storage	16A, 16B, 16C1, 16C2, 16D, 16E, 16F, 16G,16H, 16J, 16L, 16M
17	storage	17A, 17B, 17C, 17E, 17G, 17H, 17I, 17J
18	storage	18A1, 18A2

cycle consisted of three steps: denaturation (92ºC, 40 s), annealing (55ºC, 1 min and 30 s) and elongation (72ºC, 2 min). Final elongation at 72ºC for 5 min was used. The amplified fragments were analyzed by gel electrophoresis in 1x TEB buffer according to Sambrook & Russel (2001). The 1% agarose gels were treated with ethidium bromide and the bands visualized under UV light.

Sequencing of the ITS region - The fragments of ITS1-5.8S-ITS2 amplified by PCR were sequenced according Sanger et al. (1977). The sequencing reaction was 10 µL, containing 2.0 µL of ITS4 primer (Invitrogen-3.0 µ mL^{-1}), 4.0 µL of premix of DYEnamic ET terminator (Amersham Biosciences) and 4.0 µL of the rDNA amplification product (100 ng μL^{-1}). The reaction was submitted to a Mastercycler thermocycler gradient (Eppendorf), programmed to perform 35 cycles after initial denaturation (95ºC, 2 min). Each cycle consisted of three steps: denaturation (95ºC, 1 min), annealing (55ºC, 1 min) and elongation (60ºC, 1 min). At the end of the cycles, there was a final elongation step (60ºC, 5 min). Sequencing was performed on a MegaBACE™ 1000 sequencer (Amersham Biosciences). The conditions for injection and electrophoresis were 2 Kv/60 s and 6 Kv/230 min, respectively.

Sequence analysis - The quality of the sequences was examined by Phred/Phrap/Consed package. For the identification of the isolates, the nucleotide sequences obtained were compared with those already deposited in the data bank of the National Center for Biotecnology and Information (NCBI), using BLAST search tool (Altschul et al., 1990). The identification of the species was determined based on the best score.

***A. niger* and *A. tubingensis* discrimination -** The discrimination between the species *A. niger* and *A. tubingensis* was performed by RFLP analysis of rDNA *in silico,* by searching for the *Rsa*I restriction site (GT/AC at positions 295 and 303 bp) directly in the rDNA sequences of both species. The analysis was conducted with the aid of the programs Phred/Phrap/Consed and BioEdit (Hall, 1999).

RESULTS AND DISCUSSION

The amplification of the ITS1-5.8S-ITS2 region of rDNA for the 108 *Aspergillus* isolates, using the universal primers ITS1 and ITS4 (White et al*.,* 1990) originated a fragment of approximately 600 bp. This result is in accordance to the literature (Henry et al., 2000). These authors found ITS1-5.8S-ITS2 amplicons of sizes varying between 565 and 613 bp.

Analysis of the nucleotide sequences of the amplified fragments allowed the identification of the isolates at the species level (Table 2). The percentage of occurrence of each species among all *Aspergillus* identified is shown in Figure 1.

With the exception of *A. tubingensis* and *A. carbonarius,* the other species here identified have already been reported as contaminants of coffee samples from the state of Minas Gerais (Batista et al., 2003). According to these authors, among 155 isolates of *Aspergillus* identified by classical taxonomy, the species *A. ochraceus*, *A. sulphureus*, *A. tamarii*, *A. niger* and *A.*

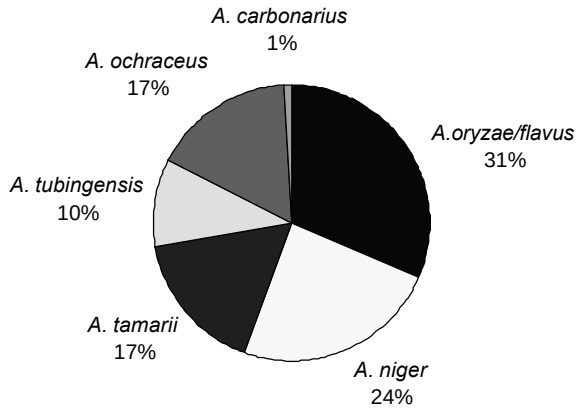

Figure 1 - Relative frequency of *Aspergillus* species in coffee bean samples.

Table 2 - Identification of *Aspergillus* species based on rDNA sequences.

Species	Isolate
A. flavus/oryzae (Section *Flavi*)	A64, A92, A93, A132, A161, 4A, 4B, 11C, 11F, 12D, 12E, 12G, 12I, 13A, 13B, 13C, 13D, 14E1, 14H, 14I, 15A, 15B1, 15B2, 15C, 15D, 16E, 16F, 16J, 16L, 16M, 17A, 17B, 17C, 18A1
A. niger (Section *Nigri*)	A11, A51, A61, A62, A81, A103, A104, A162, 6A, 8A1, 8A2,11G,12C, 12L, 13E, 13F,13G, 13H, 14C2, 14M, 14N,15G, 16G, 16H, 17E, 17G
A. ochraceus (*Section Circundati*)	A13, A21, A65, A91, A101, A105, A131, A141, A151, A152, 12J, 14B, 14C1, 14D, 14G, 14J, 14O, 16C2
A. tubingensis (Section *Nigri*)	A12, A52, A102, 4E, 7A, 8B, 14F, 15F1, 16C1, 16D, 17H
A. tamarii (Section *Flavi*)	A63, 4C, 4D, 11B, 11D, 12A, 12H, 13I, 15E, 15F2, 15H, 15I2, 15J, 16A, 16B, 17I, 17J, 18A2
A. carbonarius (Section *Nigri*)	A71

flavus represented 71% of the isolates. Despite *A. sulphureus* has been frequently detected in coffee produced in Minas Gerais, this species was not found in coffee samples from Paraná.

The isolates belonging to the section *Nigri* were detected in 93.75% of the geographical regions and represented 35% of the total *Aspergillus* spp. identified. Among the fungi of this section, 24% of the isolates were characterized as *A. niger*, 10% as *A. tubingensis* and 1% as *A. carbonarius*.

Currently, the presence of *A. tubingensis* in Brazilian coffee beans has not been described, probably because only classical taxonomy has been utilized, and it does not allow the discrimination of *A. tubingensis* from *A. niger.* These two species have a high morphological similarity and *A. tubingensis* was considered as a subspecies of *A. niger*. The utilization of molecular methods allowed a better distinction of the *A. niger* group until 1991 (Kusters-Van Someren et al*.,* 1991; Varga et al., 1993; 1994; Accensi et al*.,* 1999; 2001) and the classification of *A. niger* and *A. tubingensis* as distinct species.

In the present study, the distinction between *A. niger* and *A. tubingensis* was determined by RFLP analysis *in silico*. This analysis was based on the presence of the restriction site (at positions 295 and 303 bp) for the endonuclease *Rsa*I (GT/AC) in ITS1 sequence from *A. niger* and its absence in *A. tubingensis* (Accensi et al., 1999). The correct discrimination between these species is relevant because *A. niger* has been reported as OA producer in coffee, while *A. tubingensis* is a non-producer species of this mycotoxin.

Varga et al. (2000) described that some non-ochratoxigenic strains of *A. niger* has potential to decompose OA, through the secretion of a carboxypeptidase that converts OA to ochratoxin a and phenylalanine. Since *A. tubingensis* is unable to produce OA its potential to to control this mycotoxin in coffee should be addressed.

Of all the *Aspergillus* spp. identified, 48% included the section *Flavi* represented by the species *A. flavus/A. oryzae* (31.5%) and *A. tamarii* (16.5%), which were detected in 75% of the geographical regions analyzed. Reports in the literature on the taxonomy of this section have been conflicting. According to Varga et al. (2003), the section *Flavi* includes the clades "*A. flavus*" and "*A. tamarii,*" which includes respectively *A. flavus/ oryzae* and *A. tamarii*. The comparison between *A. flavus* and *A. oryzae*, based on molecular approaches, has demonstrated that they have high genetic similarity (Kurtzman et al*.,*1986; Chang et al., 1991; Nikkuni et al*.,* 1996). Nikkuni et al. (1998) studied the nucleotide sequence of rDNA from *Aspergillus* spp. and showed that this methodology did not allow the discrimination of the species *A. flavus* and *A. oryzae*. Identical findings were observed in the present study by analyzing the ITS1, 5.8S and ITS2 rDNA regions.

Although *A. oryzae* and *A. tamarii* belong to distinct clades of the section *Flavi*, Nikkuni et al. (1998) showed that these species differ in a single nucleotide and three gaps in the alignment of the ITS sequences. Although some isolates belonging to species of the section *Flavi* have potential to produce mycotoxins in cereal grains, none of them produce OA in coffee beans (Varga et al., 2003).

The species *A. ochraceus* (section *Circundati*) was detected in 44% of the geographical regions. Approximately 17% of the isolates here identified belong to this species (Figure 1). This frequency is similar to that found by Batista et al. (2003) in coffee samples from Minas Gerais. According to Taniwaki et al. (2003), 75% of the isolates of *A. ochraceus* have potential for producing OA. Therefore, the presence of this species in coffee beans should be a warning for good practices for farming, particularly during drying and storage.

Aspergillus species that are potential producers of OA were found in 82% of the geographical regions examined. Among these, the species identified most frequently was *A. niger,* followed by *A. ochraceus* and *A. carbonarius*. An extensive study conducted by Taniwaki et al. (2003), in coffee beans (408 samples) from the states of São Paulo and Minas Gerais also showed *A. niger* as the most common species. Although *A. niger* was found at higher frequency than the other two species, only 3% of the isolates were capable of producing AO.

The *A. carbonarius* species is considered a potent producer of OA, since approximately 77% of its isolates are ochratoxigenic. Taniwaki et al. (2003) detected *A. carbonarius* in samples originated from drying and storage areas at São Paulo state. In the present study we demonstrated, for the first time, the presence of *A. carbonarius* in samples of fruit taken from trees. Recently, Fungaro et al. (2004) developed a specific marker for the detection of *A. carbonarius* in coffee beans. The use of this marker to determine the presence of this species in coffee beans is strongly recommended.

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REFERENCES

ABARCA, M.L.; BRAGULAT, M.R.; CASTELLÁ, G.; CABAÑES, F.J. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. **Applied and Environmental Microbiology**, v.60, p.2650-2652, 1994.

ACCENSI, F.; CANO, J.; FIGUEIRA, L.; ABARCA, M.L.; CABAÑES, F.J. New PCR method to differentiate species in the *Aspergillus niger* aggregate. **FEMS Microbiology Letters**, v.180, p.191-196, 1999.

ACCENSI, F.; ABARCA, M.L.; CANO, J.; FIGUEIRA, L.; CABAÑES, F.J. Distribution of ochratoxin A producing strains in the *Aspergillus niger* aggregate. **Antonie van Leeuwenhoek**, v.79, p.365-370, 2001.

ALTSCHUL, S.F.; GISH, W.; MILLER, W.; MYERS, E.W.; LIPMAN, D.J. Basic local alignment search tool. **Journal of Molecular Biology**, v.215, p.403-410, 1990.

AZEVEDO, A.C.S.; FURLANETO, M.C.; SOSA-GOMEZ, D.R.; FUNGARO, M.H.P. Molecular characterization of *Paecilomyces fumosoroseus* (*Deuteromycotina: Hyphomycetes*) isolates. **Scientia Agricola**, v.57, p.729-732, 2000.

BATISTA, L.R.; CHALFOUN, S.M.; PRADO, G.; SCHWAN, R.F.; WHEALS, A.E. Toxigenic fungi associated with processed (green) coffee beans (*Coffea arabica L.*). **International Journal of Food Microbiology**, v.85, p.239-300, 2003.

BUCHELI, P.; MEYER, I.; PITTET, A.; VUATAZ, G.; VIANI, R. Industrial storage of green Robusta coffee under tropical conditions and its impact on raw material quality and ochratoxin A content. **Journal of Agricultural and Food Chemistry**, v.46, p.4507-4511, 1998.

CHANG, J.M.; OYAIZU, H.; SUGIYAMA, J. Phylogenetic-relationships among eleven select species of *Aspergillus* and associated teleomorphic genera estimated from 18S ribosomal-RNA partial sequences. **Journal of General and Applied Microbiology**, v.37, p.289-308, 1991.

FUNGARO, M.H.P.; VISSOTTO P.C.; SARTORI D.; VILAS-BOAS, L.A.; FURLANETO M.C.; TANIWAKI M.C. A molecular method for detection of *Aspergillus carbonarius* in coffee beans. **Current Microbiology**, v.49, p.121-125, 2004.

HALL, T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. **Nucleic Acids Symposium**, v.41, p.95-98, 1999.

HENRY, T.; IWEN, P.C.; HINRICHS, S.H. Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. **Journal of Clinical Microbiology**, v.38, p.1510-1515, 2000.

KURTZMAN, C.P.; SMILEY, M.J.; ROBNETT, C.J.; WICKLOW, D.T. DNA relatedness among wild and domesticated species in *Aspergillus flavus* group. **Mycologia**, v.78, p.955-959, 1986.

KUSTERS-VAN SOMEREN, M.A.; SAMSON, R.A.; VISSER, J. The use of RFLP analysis in classification of the black *Aspergilli*: reinterpretation of the *Aspergillus niger* aggregate. **Current Genetics**, v.19, p.21-26, 1991.

LEVI, C.P.; TRENK, H.L.; MOHR, H.K. Study of the occurrence of ochratoxin A in green coffee beans. **Journal of the AOAC International,** v.57, p.866-870, 1974.

MOSS, M.O. Mode of formation of ochratoxin A. **Food Additives and Contaminants**, v.13, p.5-9, 1996.

MULLIS, K.B.; FALOONA, F.A. Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction. **Methods in Enzymology**, v.155, p.335-350, 1987.

NEERGARD, P. **Seed pathology**. London: McMirlan, 1979. v.1, p.739-754.

NIKKUNI, S.; KOSAKA, N.; SUZUKI, C.; MORI, K. Comparative sequence analisys on the 18S rRNA gene of *Aspergillus oryzae, A. sojae, A. flavus, A. parasiticus*, *A. niger, A. awamori* and *A. tamarii*. **Journal of General and Applied Microbiology**, v.42, p.181-187, 1996.

NIKKUNI, S.; NAKAJIMA, H.; HOSHINA, S.; OHNO, M.; SUZUKI, C.; KASHIWAGI, Y.; MORI, K. Evolutionary relationships among *Aspergillus oryzae* and related species based on the sequences of 18S rRNA genes and internal transcribed spacers. **Journal of General and Applied Microbiology**, v.44, p.225-230, 1998.

PITT, J.I.; HOCKING, A.D. **Fungi and food spoilage**. 2.ed. Cambridge: Champman & Hall, 1997.

PONTECORVO, G.; ROPER, J.A.; HEMMONS, L.M.; MACDONALD, K.D.; BUFTON, A.W.J. The genetics of *Aspergillus nidulans*. **Advances in Genetics**, v.5, p.141-148, 1953.

SAMBROOK, J.; RUSSEL, D.W. **Molecular cloning.** A laboratory manual. 3.ed. New York: Cold Spring Harbor Laboratory Press, 2001.

SANGER, F.; NICKLEN, S.; COULSON, A.R. DNA sequencing with chain – terminating inhibitors. **Proceedings of the National Academy of Sciences of the USA**, v.74, p.5463-5467, 1977.

TANIWAKI, M.H.; PITT, J.I.; TEIXEIRA, A.A.; IAMANAKA, B.T. The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. **International Journal of Food Microbiology**, v.82, p.173-179, 2003.

VARGA, J.; KEVEI, F.; FEKETE, C.; COENEN, A.; KOZAKIEWICZ, Z.; CROFT, J.H.; Restriction fragment length polymorphisms in the mitochondrial DNAs of the *Aspergillus niger* aggregate. **Mycological Research**, v.97, p.1207-1212, 1993.

VARGA, J.; KEVEI, F.; VRIESEMA, A.; DEBETS, F.; KOZAKIEWICZ, Z.; CROFT, J.H. Mitochondrial DNA restriction fragment length polymorphisms in field isolates of the *Aspergillus niger* aggregate. **Canadian Journal of Microbiology**, v.40, p.612-621, 1994.

VARGA, J.; RIGÓ, K.; TÉREN, J. Degradation of ochratoxin A by *Aspergillus* species. **International Journal of Food Microbiology**, v.59, p.1-7, 2000.

VARGA, J.; RIGÓ, K.; TÓTH, B.; TÉREN, J.; KOZAKIEWICZ, Z. Evolutionary relationships among *Aspergillus* species producing economically important mycotoxins. **Food Technology and Biotechnology**, v.41, p.29-36, 2003.

WHITE, T.J.; BRUNS, T.; LEE, S.; TAYLOR, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: INNIS, M.A.; GELFAND, D.H.; SNINSKY, J.J.; WHITE, T.J. (Ed.) **PCR protocols:** A guide to methods and applications. San Diego: Academic Press, 1990. p.315-322.

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