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1 **PCR identification of *Aspergillus niger* and *Aspergillus tubingensis***
2 **based on calmodulin gene.**

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ABSTRACT

Aspergillus niger and *A. tubingensis*, species belonging to section *Nigri*, are commonly found in plant products and processed food, such as grapes, cereals, coffee and derived products. These two species are very difficult to differentiate by classical morphological criteria and some isolates are known to produce ochratoxin A. The exact identification of these two species is very important to avoid overestimation of toxicological contamination and related risks. A PCR-based identification and detection assay was developed as a tool to identify *A. niger* and *A. tubingensis*, using molecular differences obtained by sequencing the calmodulin gene. Two pairs of species-specific primers were designed and empirically evaluated for PCR identification of *A. niger* and *A. tubingensis*. Species-specific PCR products generated by each primer set were 505 bp (*A. tubingensis*) and 245 bp (*A. niger*) in length, which could be potentially useful for a multiplex PCR assay. The sensitivity of this assay was about 10 pg DNA in 25 µl PCR reaction volume, using pure total DNA of the two species. The method described in this study represents a rapid and reliable procedure to assess the presence in food products of two ochratoxigenic species of section *Nigri*.

KEYWORDS: *Aspergillus niger*, *Aspergillus tubingensis*, ochratoxin A, species-specific primers, PCR, calmodulin gene

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Introduction

Aspergillus species belonging to section *Nigri* are distributed worldwide. Many species of them cause food spoilage, and several are used in the fermentation industry (Bennett and Klich, 1992), or candidate in the biotechnology industries, such as *A. niger* which has been granted the GRAS (Generally Regarded As Safe) status by the Food and Drug Administration of the US Government. Recently, the significance of these species, commonly known as “black aspergilli” has completely changed since they were identified as the main fungi responsible for the ochratoxin A (OTA) accumulation in grapes and wine (Cabanès et al. 2002; Da Rocha Rosa et al., 2002; Battilani and Pietri, 2002; Magnoli et al., 2003). OTA is a potent nephrotoxin diffusely distributed in food and feed products such as grains, legumes, coffee, dried fruits, beer, wine, and meats. Since *A. tubingensis* and *A. niger*, together with *A. carbonarius*, are known to produce OTA, their exact identification within the *A. niger* aggregate group of species is very important to avoid overestimation of contamination and toxicological risk (Medina *et al.*, 2005; Perrone *et al.*, 2006a). In spite of this, the identification of species within the *A. niger* “aggregate”, a group of species within section *Nigri*, is still controversial. Molecular studies reduced the number of synonym names of described species within the black aspergilli and supported the division of this “aggregate” in two to four morphologically indistinguishable species: *A. niger*, *A. tubingensis*, *A. foetidus* and *A. brasiliensis* (Kuster van Someren et al., 1991; Accensi et al., 1999; Parenicova et al., 2000; Abarca et al., 2004). These four species are very difficult to differentiate by classical morphological criteria, such as conidial shape, colour and size (Samson *et al.*, 2004). However, effectiveness in the estimation of OTA contamination is dependent on

the association between species and OTA production, hence the use of primers identified on genes involved in the metabolic pathway would be more helpful. Although two primers were recently developed to detect OTA producers by Polymerase Chain Reaction (PCR) (Dao et al., 2005), genes involved in steps of OTA biosynthesis have not yet been identified. A PCR-based identification and detection of species could be an useful tool for identifying potential ochratoxigenic *Aspergillus* spp. The calmodulin gene has proved to be highly useful in discrimination of species belonging to section *Nigri*, since it contains some species specific diagnostic traits, suitable for diagnostic purposes. Molecular differences were exploited to set up a qualitative PCR assay for *A. carbonarius* and *A. japonicus/A. aculeatus* detection (Perrone et al., 2004). Moreover a quantitative real-time PCR assay using TaqMan chemistry was recently set up for *A. carbonarius* DNA quantification on grapes (Mulè et al., 2006).

The objective of this work was to set up a species-specific PCR assay based on differences in sequences found in the calmodulin gene for an accurate identification of *A. niger* and *A. tubingensis* in pure cultures.

Material and methods

Fungal strains

One hundred and fifty strains were analyzed in this study: 10 type-strains belonging to *Aspergillus* sect. *Nigri*, *A. aculeatus* ITEM 7046 (GenBank accession no. for calmodulin gene sequences, AJ964877), *A. brasiliensis* ITEM 7048 (AM295175), *A. carbonarius* ITEM 4503 (AJ964873), *A. ellipticus* ITEM 4505 (AM117809), *A. foetidus* ITEM 4507 (AM419749), *A. heteromorphus* ITEM 7045 (AM421461), *A.*

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1 *ibericus* ITEM 6600 (AJ971806), *A. japonicus* ITEM 4497 (AJ582717), *A. niger*
2 ITEM 4502 (AJ964872), *A. tubingensis* ITEM 7040 (AJ964876); 136 strains of “black
3 aspergilli” isolated from grapes grown in different geographical areas: 1 *A. aculeatus*, 4
4 *A. ibericus*, 4 *A. brasiliensis*, 31 *A. carbonarius*, 23 *A. japonicus*, 44 *A. niger*, 29 *A.*
5 *tubingensis* (Perrone et al., 2006b); and 4 strains as outgroup: 2 *A. ochraceus*, 1 *Botrytis*
6 *cinerea* (ITEM 3715) and 1 *Saccharomyces cerevisiae* (ITEM 6904). All strains were
7 obtained from the culture collection of the Institute of Sciences of Food Production,
8 with accession number ITEM. Further information about the strains (year of isolation,
9 depositor, toxin production, etc.) are available in the ITEM web site:
10 <http://www.ispa.cnr.it/Collection>.

11
12 **DNA extraction**

13 Fungal strains were grown in shaken cultures (150 rpm) in Wikerham’s medium (40 g
14 of glucose, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and water up to 1 L).
15 About 40 mg of filtered, frozen and lyophilized mycelium from each strain were used
16 for total DNA extraction using the EZNA Fungal DNA Miniprep Kit (Omega Bio-tek,
17 Doraville, USA). DNA was recovered and dissolved in sterile water. Concentrations of
18 DNA were determined by gel electrophoresis, and by measuring the ultraviolet-induced
19 fluorescence emitted by ethidium bromide molecules intercalated into DNA, and
20 comparing the fluorescent yield of the samples with a standard (100 bp DNA ladder,
21 New England Biolabs, Beverly, MA, USA).

22
23 **Fungal DNA amplification and sequencing**

1 Total DNA extracted from all strains (excluding *B. cinerea* and *S. cerevisiae*) was used
2 for PCR amplification of approximately 700 bp fragments of the calmodulin gene with
3 CL1 and CL2A primers (O'Donnell et al., 2000). Amplifications of the partial
4 calmodulin gene were set up in 100 µl reaction mixtures containing 2.5 U of Taq Gold
5 DNA polymerase (Applied Biosystems, Foster City, CA, USA), 30 pmol of each
6 primer, 20 nmol of each deoxynucleoside triphosphate (Applied Biosystems), and
7 approximately 10 ng of fungal template DNA. PCR reactions were performed using the
8 following conditions: denaturation at 94 °C for 10 min; 35 cycles of denaturation at 94
9 °C for 50 s, annealing at 55 °C for 50 s, extension at 72 °C for 1 min; final extension at
10 72 °C for 7 min, followed by cooling at 4 °C until recovery of the samples. After
11 amplification, PCR products were purified by Microcon-PCR Filter Units (Millipore,
12 Bedford, MA, USA).

13
14 Sequence analysis was performed with the “Big-Dye Terminator Cycle Sequencing
15 Ready Reaction Kit” for both strands, and the sequences were aligned with the “MT
16 Navigator” software (Applied Biosystems). All the sequencing reactions were purified
17 by gel filtration through columns containing Sephadex G-50 (Pharmacia, Uppsala,
18 Sweden) equilibrated in double-distilled water and were analyzed on the automatic
19 sequencer “310-Genetic Analyzer” (Applied Biosystems). The calmodulin sequence
20 data of all 150 isolates were aligned by the Clustal-W method.

21 22 **Primer design and testing**

23 Conservative and polymorphic calmodulin gene sequences were sought in a
24 multialignment of 11 *Aspergillus* species belonging to Section *Nigri* using Clustal-W

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method. Based on these data, two species-specific primer pairs were designed from the highly conserved region in *A. tubingensis* and *A. niger* species, exploiting polymorphic regions of the calmodulin gene in *Aspergillus* spp. using Primer Express software (Applied Biosystems, Foster City, CA, USA). The sequence identity between the designed primers and the calmodulin regions was analyzed with a BLAST search (<http://www.ncbi.nlm.nih.gov>).

PCR products were separated in 1.5% Tris–acetate–EDTA–agarose gels and were visualized with ethidium bromide and ultraviolet illumination (Sambrook et al., 1989). As negative controls, strains belonging to 8 different species of Section *Nigri* (*A. aculeatus*, *A. ibericus*, *A. brasiliensis*, *A. carbonarius*, *A. ellipticus*, *A. etheromorphus*, *A. japonicus*, *A. foetidus*) were used. Strains of *A. ochraceus*, *Botrytis cinerea*, and *Saccharomyces cerevisiae*, obtained from ITEM Culture Collection were used as outgroup. Positive controls of PCR reaction were prepared replacing the species-specific primer designed with rDNA primers ITS1 and ITS4 (White et al., 1990). Images were captured and stored using a Kodak Easy-Share DX3215 Zoom Digital Camera. The PCRs containing species-specific primers were set up in 25 µl reaction mixtures containing 1.25 U of Taq Gold DNA polymerase (Applied Biosystems), 7.5 pmol of each primer, 5 nmol of each deoxynucleoside triphosphate (Applied Biosystems, Foster City, CA, USA), and approximately 3 ng of fungal template DNA. Reactions were performed using the following PCR conditions: denaturation at 95 °C for 5 min; 25 cycles of denaturation at 94 °C for 50 s; annealing at 60 °C for 50 s; extension at 72 °C for 1 min; final extension at 72 °C for 7 min, followed by cooling at

4 °C until recovery of the samples. Amplification products were checked on 1.5% agarose gel stained with ethidium bromide.

Results

We evaluated the usefulness of two PCR assays, that target a region of calmodulin gene, for identifying *Aspergillus niger* and *Aspergillus tubingensis* within *Aspergillus niger* “aggregate” of species.

The partial calmodulin gene sequence has both conserved and polymorphic regions corresponding respectively mainly to exons and introns of the gene in the examined species, useful for designing species-specific primers for *A. niger* and *A. tubingensis*.

Calmodulin sequences showed 92 % identity (figure 1) between *A. niger* and *A. tubingensis*. In this analysis were included the type strains of *A. tubingensis* ITEM 7040 (AJ964876) and *A. niger* ITEM 4501 (AJ964872) respectively; their sequences were previously deposited in the EMBL nucleotide sequence database (Perrone et al., 2006a).

The visual inspection of the aligned calmodulin partial gene sequences readily identified unique regions within the fragment. Primers were chosen to target a region displaying high nucleotide divergence at the 3'-end among the species considered in this study.

Two pairs of species-specific primers for *Aspergillus* species belonging to Section *Nigri* were designed after consideration of product size and melting temperature (table 1). All primers were designed in order to: a) operate at same high annealing temperatures (60°C), b) work simultaneously under the same PCR conditions and c) prevent the co-amplification of non-specific target of DNA. Specific primer pair NIG1/ NIG2 amplified a 245 bp fragment in *A. niger*, whereas TUB1/ TUB2 amplified a 505 bp fragment in *A. tubingensis* and in the type strain of *A. phoenicis* (CBS 136.52), recently

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1 included in *A. tubingensis* species according to the revision made by Samson et al.
2 (2004) using extrolites and β -tubulin sequencing (figure 2).

3
4 PCR conditions using the two primer pairs were optimized and then tested on 44
5 *Aspergillus* strains (table 2). The sensitivity of this assay was about 10 pg DNA in 25 μ l
6 PCR reaction volume, using pure total DNA of the two species (data not shown).

7
8 Primers NIG1/NIG2 amplify only DNA belonging to *A. niger* strains and primers
9 TUB1/TUB2 amplify only DNA belonging to *A. tubingensis*, confirming their
10 specificity (figure 3). PCR amplicons were confirmed as originating from *Aspergillus*
11 *tubingensis* and *Aspergillus niger* by sequencing in both directions.

12
13 Moreover no amplification was obtained with other fungal/yeast species occurring
14 frequently on grapes.

15
16 **Discussion**

17 PCR assays have been introduced successfully for the identification and detection of
18 fungal species based on sequence analysis. PCR technology to detect fungi uses primers
19 which can target two kinds of sequences:

- 20 (i) fungus-specific regions of conserved proteins such as calmodulin, beta-
21 tubulin, elongation factor 1 alfa, and
22 (ii) repeated sequences such as those of rDNA and mitochondrial DNA. Setting
23 up PCR assay requires definition of oligonucleotide primers to amplify a

DNA region. Oligonucleotides binding species-specific region could assume diagnostic meaning.

Specific PCR assays have been developed in this study for detection of *A. niger* and *A. tubingensis*, two OTA producer species within the *A. niger* aggregate group.

Specific PCR assays are available (Perrone et al., 2004) for the other two species of section *Nigri* widely occurring in plant products: *A. carbonarius* and *A. japonicus/laculeatus*, OTA producer and OTA non-producer, respectively. Further specific primers for PCR detection have been also described for *A. carbonarius* and *A. ochraceus* (Mulè et al., 2006; Patino et al., 2005; Pelegrinelli-Fungaro et al., 2004; Schmidt et al., 2004) and *A. niger/A. tubingensis* (Gonzalez-Salgado et al., 2005; Kanbe et al., 2002; Sugita et al., 2004). This study offers one step assay which is a faster alternative to molecular assay previously set up by Accensi et al. (1999), consisting of a PCR amplification of ITS rDNA and 5.8S rDNA followed by *RsaI* digestion.

Specific primers described in this work have been designed on the basis of calmodulin gene sequence comparisons of several strains of *Aspergillus* species and taking into consideration the phylogenetic and taxonomic analysis made in previous publications (Abarca et al., 2004; Samson et al., 2004). In accordance with previous studies (Perrone et al., 2004; Mulè et al., 2006) we demonstrate that the calmodulin-based PCR method has high degree of specificity for the identification of *Aspergillus* at species level within the black aspergilli. In contrast, ITS regions showed low degree of specificity consisting in variation of only three different nucleotides between these species (Parenicova et al., 2000).

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1 The PCR assay can be used to identify these species without the need for morphological
2 analysis and consequent risks of misidentification. In addition, this method is suitable
3 for investigations that involve a large number of fungal isolates due to rapid DNA
4 extraction and simple PCR amplification.

6 The importance of a molecular tool for species identification is due to the difficulty of
7 morphological recognition of species within this section, and specifically within the *A.*
8 *niger* “aggregate” group. These primers could be a useful tool for investigations of a
9 high number of unidentified strains isolated from grapes. The availability of reliable
10 molecular markers for *Aspergillus* section *Nigri* occurring on grapes is of great interest
11 also since an early diagnosis of *A. carbonarius*, *A. niger* and *A. tubingensis* occurrence
12 in the field would provide important information on possible OTA contamination in
13 grapes. Because of *A. tubingensis* is recently described as a new OTA producer species
14 within the morphologically undistiguishable *A. niger* “aggregate” group (Perrone et al.,
15 2006a; Medina et al., 2005), having a molecular tool for species identification is very
16 important to predict OTA risk in foodstuffs.

18 Species-specific primers NIG1/2 and TUB1/2 allowed discrimination of this two
19 ochratoxigenic species within the other atoxigenic species of the *A. niger* aggregate
20 group. In this respect, these primers together with CARBO1/2 (Perrone et al., 2004)
21 could be helpful in the prediction of the related risks of OTA contamination on grapes
22 and by-products and in further development of PCR assays from vegetative material.
23 Moreover, the sets of developed primers amplify PCR products with different molecular
24 weight (245 and 505 bp), potentially useful for a multiplex PCR assay.

In conclusion, the novel primers designed could be used to detect and identify *A. tubingensis* and *A. niger* strains simultaneously under the same PCR conditions. Furthermore, this protocol could facilitate the early and accurate identification of *Aspergillus* contamination in natural grape samples to prevent OTA risk. However further experiments will be necessary to determine the conditions needed to detect *Aspergillus* directly from environmental samples. The specific assays reported in this study together with other assays developed for the two main important OTA-producing fungi, *A. carbonarius* and *A. ochraceus*, represent a quick and reliable means for a rapid, sensitive and accurate detection of ochratoxigenic fungi present in food product.

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3	<i>A. tubingensis</i>	TGCTCCCTGAATGAACCCCGACCACTCAAATTGATGTCCTATCTTAACCGGCTCATAATGCTAATGTATTTTCAAACCTCAATAGGACAAGGATGGCGAT	100
4	<i>A. niger</i>	TGCTCCCTGAATGACCTCCGATCATCTGATCGATGAGCTATCTTAACCGGAGCATAATGCTAATGTGTTTTCGGACTTAATAGGACAAGGATGGCGAT	100
5	Consensus	tgctccctgaatga cc ccga ca c at gatg ctatctt accgg cataatgctaagt ttttc act aataggacaaggatggcgat	
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7	<i>A. tubingensis</i>	GGTGGGTGGAATTCTGTCCCTTCACGTTTTACCTGTAGCGCCGATCCGACCGCGGGATTTTCGACAGCTATTTCCCTTCGATCTGAATCATAATACT	200
8	<i>A. niger</i>	GGTGGGTGGAATCTATCCCTTCACATTTTACCTGTAGCGCTCGATCCGACCGCGGGATTTTCGACAGC.ATTTCTCAGAATTATTGGATCATAATACT	199
9	Consensus	ggtgggtggaat ct tccccttcac ttttacctgttagcgc cgatccgacgcgggatttcgacagc atttc c at tg atcataatact	
10			
11	<i>A. tubingensis</i>	GATGTAATCTG.GAAATAGGCCAGATCACCACCAAGGAGCTCGGCACTGTGATGCGCTCCCTCGGCCAGAACCCCTCCGAGTCTGAGCTTCAGGACATGA	299
12	<i>A. niger</i>	AATTTAATCGGTGAATCAGGCCAGATCACCACCAAGGAGCTCGGCACTGTGATGCGCTCCCTTGGCCAGAACCCCTCCGAGTCTGAGCTTCAGGACATGA	299
13	Consensus	at taatc g gaa aggccagatcaccaccaaggagctcggcactgtgatgcgctccct ggccagaacccctccgagctctgagcttcaggacatga	
14			
15	<i>A. tubingensis</i>	TCAACGAGGTTGACGCTGACAACAACGGAACGATCGACTTCCCCGGTATGTGATAGATCTACGCCTGTAAGGCGGGAATGCCGTATGGATTGTGATTGAC	399
16	<i>A. niger</i>	TCAACGAGGTTGACGCTGACAACAACGGAACGATCGACTTCCCCGGTATGTGTTAGATTACGCCTGTAAGGCGGGAATGCCGGCTGGATTGTGATTGAC	399
17	Consensus	tcaacgaggttgacgctgacaacaacggaacgatcgacttccccggatgtg tagat tacgcctgtaaggcgg aatgc g tggattgtgattgac	
18			
19	<i>A. tubingensis</i>	TTTTGCCGCCAGAATTCCTTACCATGATGGCTCGTAAGATGAAGGACACCGACTCCGAGGAGGAAATCCGCGAGGCTTTCAAGGTCTTCGACCGCGACAA	499
20	<i>A. niger</i>	TTTTGCCGCCAGAATTCCTTACCATGATGGCTCGTAAGATGAAGGACACCGACTCCGAGGAGGAAATCCGCGAGGCTTTCAAGGTCTTCGACCGCGACAA	499
21	Consensus	ttttgccgccagaatttccttaccatgatggctcgtaagatgaaggacaccgactccgaggaggaaatccgcgaggctttcaaggtctt gaccgcgacaa	
22			
23	<i>A. tubingensis</i>	CAATGGTTTCAATCTCCGCCGCGGAGTTGCGCCACGTCATGACCTCCATTGGCGAGAAGCTCAGTACGACGAAGTCGATGAGATGATCCGTGAGGCTGAC	599
24	<i>A. niger</i>	CAATGGTTTATCTCCGCCGCGGAGCTGCGCCACGTCATGACCTCCATTGGCGAGAAGCTCACCGACGACGAAGTCGATGAGATGATCCGTGAGGCGGAC	599
25	Consensus	caatggttt atctccgcgcggag tgcgccacgtcatgacctccattggcgagaagctcac gacgacgaagtcgatgagatgatccgtgaggc gac	
26			
27	<i>A. tubingensis</i>	CAGGACGGTGATGGCCGCATCGACTGTATGTTTCCATTCCCTGA.TATGCCCGTGATATGACATGCTAAC	668
28	<i>A. niger</i>	CAGGACGGTGATGGCCGCATCGACTGTATGTTTACCATGCCGATATATACTCATATCATAACATACTGAC	669
29	Consensus	caggacggtgatggccgcacatcgactgtatgttt ccat cc ga tat c c t at acat ct ac	

Fig.1

Sequence alignment between calmodulin gene representative of *A. tubingensis* and *A. niger* spp. Dark regions correspond to high conserved regions and light regions correspond to species-specific regions, considered in primer's designing.

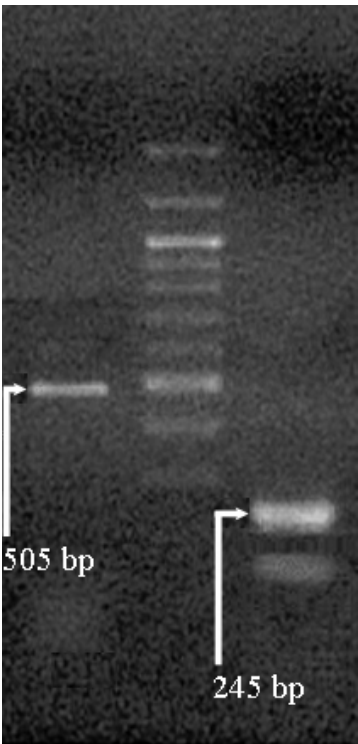


Fig. 2
Species-specific PCR product obtained amplifying *A. tubingensis* DNA (505 bp) in lane 1 and *A. niger* DNA (245 bp) in lane 2.

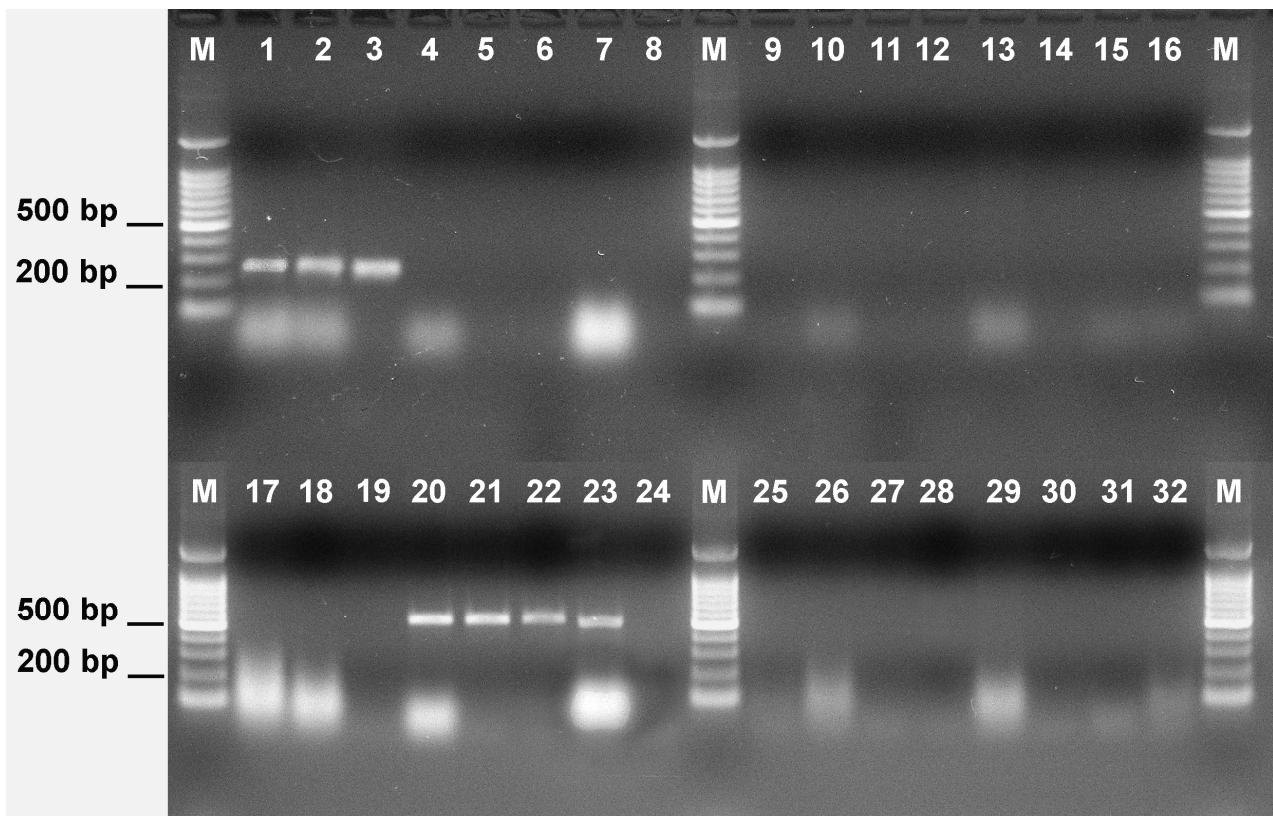


Fig. 3 PCR products obtained amplifying *Aspergillus* spp. genomic DNA with NIG F/R (lanes 1-16) and TUB F/R (lanes 17-32): *A. niger* (lanes 1-3 and 17-19), *A. tubingensis* (lanes 4-6 and 20-22), *A. phoenicis/A. tubingensis* (lanes 7 and 23), *A. awamori/niger* (lanes 8 and 24), *A. ellipticus* (lanes 9 and 25), *A. brasiliensis* (lanes 10 and 26), *A. heteromorphus* (lanes 11 and 27), *A. japonicus* (lanes 12 and 28), *A. ochraceus* (lanes 13 and 29), *A. foetidus* (lanes 14 and 30), *A. carbonarius* (lanes 15 and 31), *A. aculeatus* (lanes 16 and 32).

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Table 1 Details of the two species-specific PCR primer pairs for *A. niger* and *A. tubingensis*

Name	Sequence	Species-specificity
NIG1	5'-GATTTTCGACAGCATTT(CT/TC)CAGAA-3'	<i>A. niger</i>
NIG2	5'-AAAGTCAATCACAATCCAGCCC-3'	
TUB1	5'-TCGACAGCTATTTCCCCCTT-3'	<i>A. tubingensis</i>
TUB2	5'-TAGCATGTCATATCACGGGCAT-3'	

Table 2 Fungal strains tested by PCR reaction with the species-specific primers for *A. niger* (NIG1/NIG2) and *A. tubingensis* (TUB1/TUB2).

Species	ITEM Strains
<i>A. aculeatus</i>	7046*, 7047
<i>A. brasiliensis</i>	7048*
<i>A. carbonarius</i>	4503*, 4504, 4544
<i>A. ellipticus</i>	4505*
<i>A. foetidus</i>	4506*
<i>A. heteromorphus</i>	7045*
<i>A. ibericus</i>	6600
<i>A. japonicus</i>	7044*, 7047
<i>A. niger</i>	4501*, 4502*, 4774, 4775*, 4717, 4730*, 4541, 4547, 4551*, 4552*, 5219, 5276, 4509, 7042
<i>A. ochraceus</i>	7043*
<i>A. phoenicis</i>	4498*
<i>A. tubingensis</i>	4208*, 4500*, 4542, 4543, 4546*, 4720, 4721*, 4725, 4728, 4815*, 4816, 4817, 4818, 7040*, 7041, 4510

*= representative strains utilized for PCR reactions showed in figure 3