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New PCR method to differentiate species in the *Aspergillus niger* aggregate

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Abstract

The DNA that encodes the 5.8S gene of the ribosomal RNA and the two intergenic spacers ITS1 and ITS2 of the two proposed type strains of the *Aspergillus niger* aggregate (*A. niger* and *Aspergillus tubingensis*) have been sequenced. By comparison of sequences we have found that both species could be differentiated by *Rsa*I digestion of the PCR products of the mentioned regions. This method could be a useful tool in the identification of strains of the *A. niger* aggregate, especially in studies that involve a large number of isolates.

Keywords: Taxonomy; 5.8S rDNA; PCR; RFLP analysis; *Aspergillus niger*; *Aspergillus tubingensis*

1. Introduction

Black aspergilli are cosmopolitan fungi. Because of their ability to produce extracellular organic acids some of them are commonly used in food industry. Moreover *A. niger* Tiegh. is one of the fungi that has been labelled with the GRAS (generally recognized as safe) status from the US Food and Drug Administration [1], despite the fact that ochratoxin A is produced by members of the Section *Nigri* [2–4]. The taxonomy of the black aspergilli has been carefully studied based on morphological criteria [5–7]. One such study created the *A. niger* aggregate formed by six varieties and two formae [6]. Recently, it was proposed to divide the *A. niger* aggregate into two species, *A. niger* and *A. tubingensis* Mosseray, by means of RFLP analysis of total DNA and Southern blot analysis [8]. Those findings were confirmed by RFLP analysis of total DNA with different endonucleases, combined with rDNA hybridization probes [9], RFLP analysis of mtDNA [10,11] and also with RAPD analysis [9]. On the other hand, there are studies that have found strains from the *A. niger* aggregate with RFLP patterns different from *A. niger* or *A. tubingensis* both in field isolates [11] and collection strains [12]. The aim of our study was to find a rapid and simple molecular technique to differentiate *A. niger* from *A. tubingensis*. The 5.8S rDNA, ITS1 and ITS2 regions from strains of the two proposed species [8] have been sequenced and compared

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Table 1
Strains from the *A. niger* aggregate examined in our study

Species	Origin
A. niger (NT)	CBS 554.65
A. hennebergi (T)	CBS 118.35
A. phoenicis	CBS 126.49
A. usami (T)	CBS 139.52
A. niger	ATCC 22343
A. awamori (T)	IMI 211394
A. niger (*A. foetidus*)*	NRRL 3122
A. foetidus	CBS 618.78
A. tubingensis (T)	CBS 134.48
A. pulverulentus	CBS 558.65
A. niger	CBS 117.32
A. tubingensis	IMI 172296
A. awamori	IMI 63764
A. niger	ATCC 26036
'*A. brasiliensis*'	607 (J. Varga)

Abbreviations: ATCC, American Type Culture Collection, Rockville, MD, USA; CBS, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands; IMI, International Mycological Institute, Egham, UK; NRRL, Agricultural Research Service Collection, Peoria, IL, USA
(T) Type strain.
(NT) Neotype strain.
*Strain reclassified as *A. foetidus* by Varga et al. [10].

in order to find a method that allows their differentiation by means of RFLP analysis.

2. Materials and methods

2.1. Strains

The strains used in our study belong to the *A. niger* aggregate. These strains have been classified into *A. niger* or *A. tubingensis* in previous reports [8,10]. We have included also one strain isolated from Brazilian soil that showed an RFLP pattern different from those of *A. niger* or *A. tubingensis* which is called '*Aspergillus brasiliensis*' by the authors [11]. The strains are listed in Table 1.

2.2. DNA extraction

Fungal DNA was extracted as described by Estruch et al. [13] with some modifications. The strains were inoculated in 1.5 ml Eppendorf tubes containing 500 μl of Sabouraud broth (2% glucose, w/v; 1% peptone w/v) supplemented with chloramphenicol (1 mg l^{-1}), and incubated overnight in an orbital shaker at 300 rpm and 30°C. Mycelium was recovered after centrifugation and washed with NaCl 0.9% (w/v), frozen in liquid nitrogen and ground to a fine powder with a pipette tip. The powder was incubated for 1 h at 65°C in 500 μl extraction buffer (Tris-HCl 50 mM, EDTA 50 mM, SDS 3% and 2-mercaptoethanol 1%). The lysate was extracted with phenol: chloroform (1:1, v/v), 3 M NaOAc and 1 M NaCl. DNA was recovered by isopropanol precipitation. The pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8). DNA was cleaned with 'Geneclean kit II' (BIO 101, La Jolla, CA, USA), according to the manufacturer's instructions.

2.3. PCR amplification

ITS rDNA and 5.8S rDNA were amplified as described by Gené et al. [14], using a Perkin Elmer 2400 thermal cycler (Perkin Elmer Cetus Corporation, Emeryville, CA, USA). The primer pairs ITS5 (3′ GGAAGTAAAAGTCGTAACAAGG 5′) and ITS4 (3′ TCCTCCGCTTATTGATATGC 5′) used were described by White et al. [15]. The amplification process consisted of a pre-denaturation step at 94°C, for 5 min, followed by 35 cycles of denaturation at 95°C/30 s, annealing at 50°C/1 min and extension at 72°C/1 min, plus a final extension of 7 min at 72°C. The molecular masses of the amplified DNA were estimated by comparison with the 100-bp DNA ladder (Gibco BRL) standard lane.

2.4. Sequencing

The protocol '*Taq* Dye Deoxy Terminator Cycle Sequencing kit' (Applied Biosystems, Gouda, The Netherlands) was used for sequencing. The primers ITS5 and ITS4 described by White et al. [15] were used in the sequencing reaction. An Applied Biosystems mod. 310 sequencer was used to obtain the DNA sequences. The sequences were aligned by using the software program Clustal W (1.5) [16].

2.5. RFLP analysis

The PCR products were digested overnight with

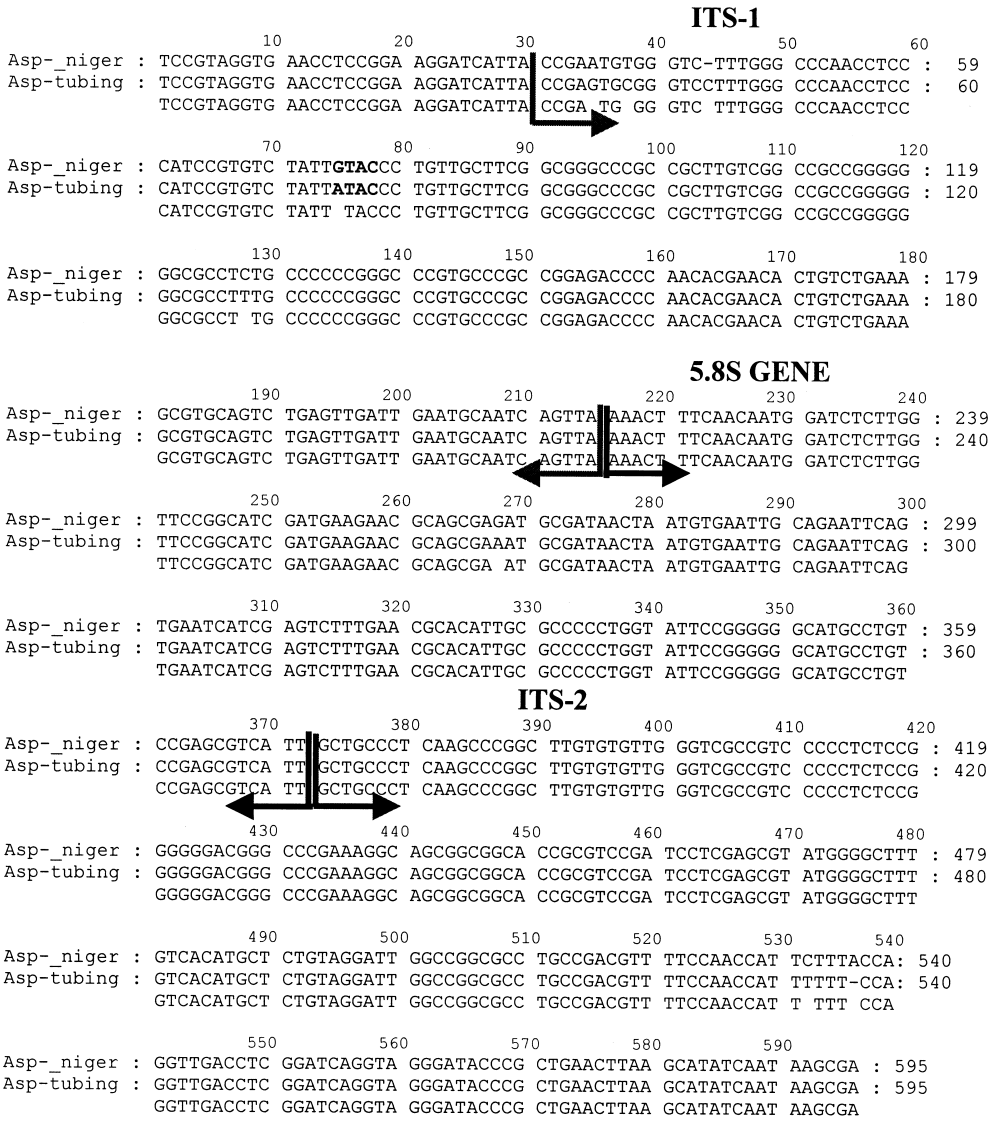

Fig. 1. DNA sequences of the ITS1, 5.8S rRNA gene and ITS2 of *A. niger* CBS 554.65 (first line) and *A. tubingensis* CBS 134.48 (second line). GenBank accession numbers are AJ223852 and AJ223853, respectively. The third line is the consensus sequence, which has a gap every time that there is a difference between the sequences of *A. niger* and *A. tubingensis*. The numbers on the right margin show the real number of bases in each line. The section of DNA sequenced from *A. niger* included 595 base pairs, with 112A, 176C, 172G and 135T and the section of DNA sequenced from *A. tubingensis* included 595 base pairs, with 112A, 176C, 171G and 136T.

the restriction endonuclease *Rsa*I (GT/AC), following the recommendations of the manufacturer (Boehringer Mannheim). RFLP analysis was performed by loading the product of the digestion reaction onto a 2% Agarose MP (Boehringer Mannheim) gel and stained for 30 min in ethidium bromide as described by Sambrook et al. [17].

2.6. Nucleotide sequence accession number

The *A. niger* and *A. tubingensis* sequences have been deposited in the GenBank database under accession number AJ223852 and AJ223853, respectively.

3. Results

The two aligned sequences of the 5.8S rDNA with its two intergenic spacers ITS1 and ITS2 of *A. niger* CBS 554.65 and *A. tubingensis* CBS 134.48 are shown in the Fig. 1. The section of DNA sequenced from *A. niger* included 595 base pairs. The ITS1 region occupied nucleotides 31 to 214, the 5.8S rDNA gene from nucleotides 215 to 371 and ITS2 from nucleotides 372 to 595. The section of DNA sequenced from *A. tubingensis* included 595 bp. The ITS1 region occupied nucleotides 31 to 215, the 5.8S rDNA gene from nucleotides 216 to 372 and ITS2 from nucleotides 373 to 595. Few differences were found between the two sequences, but a target for the endonuclease *Rsa*I was detected (located in position 75) in the ITS1 of the rDNA of *A. niger* that does not exist in the sequence of *A. tubingensis* (bold in Fig. 1). A pair of differentiable RFLP patterns could therefore be obtained after a digestion of the PCR products. The PCR-amplified 5.8S rDNA of *A. niger* was digested into two fragments of 519 and 76 base pairs, while the PCR amplicons of *A. tubingensis* remained as undigested fragments of 595 base pairs. These patterns were denoted N and T, corresponding to *A. niger* and *A. tubingensis*, respectively. Representatives of these two patterns are shown in Fig. 2. The 76-bp fragment was too small to remain on the gel. The results of the *Rsa*I digestion of the strains' PCR products are shown in Table 2.

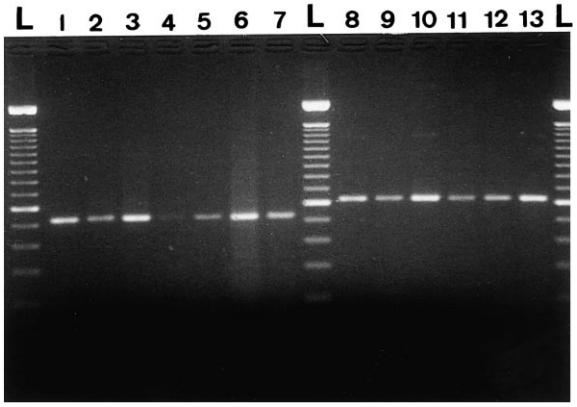

Fig. 2. PCR products cleaved by *Rsa*I and separated on a 2% agarose gel. Lanes L are the 100-bp DNA ladder (Gibco BRL) used as size marker. Lanes 1–7: pattern N. Lanes 8–13: pattern T. Lane 1, CBS 554.65; Lane 2, CBS 126.49; Lane 3, NRRL 3122; Lane 4, ATCC 22343; Lane 5, CBS 618.78; Lane 6, IMI 211394; Lane 7, CBS 118.35; Lane 8, CBS 134.48; Lane 9, CBS 117.32; Lane 10, IMI 172296; Lane 11, IMI 63764; Lane 12, CBS 558.65; Lane 13, ATCC 26036. The 76-bp fragment of pattern N was too small to remain in the gel.

Table 2
5.8S rDNA, rDNA and mtDNA types of the strains examined

Strain	rDNA type*	mtDNA type+	5.8S rDNA, ITS1 and ITS2type
CBS 554.65	I	1a	N[a]
CBS 118.35	I	1b	N
CBS 126.49	I′	1a	N
CBS 139.52	I′	1a	N
ATCC 22343	I′	1a	N
IMI 211394	I	1c	N
NRRL 3122	I	1a	N
CBS 618.78	I′	1a	N
CBS 134.48	II	–	T[b]
CBS 558.65	II	2a	T
CBS 117.32	II	2a	T
IMI 172296	II	2a	T
IMI 63764	II	2a	T
ATCC 26036	II	2a	T
607 (J. Varga)	III	3	N

[a]Corresponding to *A. niger*.
[b]Corresponding to *A. tubingensis*.
*The rDNA type results have been taken from previous reports [8,10,11].
+The mtDNA type results have been taken from previous reports [10,11].

4. Discussion

Our method, based on the PCR amplification of the 5.8S rDNA and its two intergenic spacers ITS1 and ITS2 followed by the subsequent digestion of the amplicon with the restriction endonuclease *Rsa*I, proved to be a useful tool for classifying the isolates of the *A. niger* aggregate into *A. niger* or *A. tubingensis*. All the collection strains that previously [8,10] exhibited an rDNA type I or I' or a mtDNA type 1a, 1b or 1c (corresponding to *A. niger*) have been classified by us as type N and all the collection strains with a rDNA type II or mtDNA type 2a (corresponding to *A. tubingensis*) have been classified by us as type T. So, the strains grouped in pattern N are isolates of *A. niger* and the strains grouped in pattern T are *A. tubingensis* isolates. The '*A. brasiliensis*'

strain, previously shown to exhibit an rDNA type III and mtDNA type 3, was described as more closely related to *A. niger* than to *A. tubingensis* [11]. Our results are in agreement with this study since this strain showed a type N pattern.

The ribosomal repeat unit is formed by conserved regions encoding the 18S, 5.8S and 28S rRNA genes and non-coding regions (ITS, ETS, NTS) whose sequences are not conserved [18]. The sequences of the non-coding regions are commonly used in taxonomic studies in order to differentiate between taxa at different levels [19]. The similarity of the sequences that we have obtained confirmed the proximity of the two species. In spite of that fact, differences were found which allowed them to be separated into *A. niger* and *A. tubingensis*. The differences within the RFLP patterns in the *A. niger* rDNA were shown previously to be due to differences in restriction sites in the non-coding regions of the ribosomal repeat [9,20]. This is in agreement with the present study since differences in ITS1 were observed which enabled us to differentiate between *A. niger* and *A. tubingensis*.

This PCR based technique is suitable for studies that involve a large number of isolates on account of its rapid DNA extraction. Because of the initial small quantity of mycelium used, this procedure saves reagents and time by allowing several extractions to be made simultaneously. The extraction provides us with only a small amount of DNA from each isolate but that is just what is needed to amplificate the 5.8S rDNA. Another of the advantages of this PCR procedure is the possibility of working with specific fragments of the ribosomal RNA genes without working with Southern blot DNA hybridization techniques. RAPD analysis requires small amounts of DNA too but in contrast it needs a large set of primers that are quite expensive. Moreover, the patterns N and T are, in fact, easier to recognize than the RFLP patterns resulting from single or double digestions of chromosomal DNA as employed by other authors [8–12] or the patterns that result after a RAPD analysis [9].

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