RESEARCH LETTER

Molecular strategy for identification in *Aspergillus* section *Flavi*

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Abstract

Aspergillus flavus is one of the most common contaminants that produces aflatoxins in foodstuffs. It is also a human allergen and a pathogen of animals and plants. *Aspergillus flavus* is included in the *Aspergillus* section *Flavi* that comprises 11 closely related species producing different profiles of secondary metabolites. A six-step strategy has been developed that allows identification of nine of the 11 species. First, three real-time PCR reactions allowed us to discriminate four groups within the section: (1) *A. flavus*/*Aspergillus oryzae*/*Aspergillus minisclerotigenes*/*Aspergillus parvisclerotigenus*; (2) *Aspergillus parasiticus*/*Aspergillus sojae*/*Aspergillus arachidicola*; (3) *Aspergillus tamarii*/*Aspergillus bombycis*/*Aspergillus pseudotamarii*; and (4) *Aspergillus nomius*. Secondly, random amplification of polymorphic DNA (RAPD) amplifications or SmaI digestion allowed us to differentiate (1) *A. flavus*, *A. oryzae* and *A. minisclerotigenes*; (2) *A. parasiticus*, *A. sojae* and *A. arachidicola*; (3) *A. tamarii*, *A. bombycis* and *A. pseudotamarii*. Among the 11 species, only *A. parvisclerotigenus* cannot be differentiated from *A. flavus*. Using the results of real-time PCR, RAPD and SmaI digestion, a decision-making tree was drawn up to identify nine of the 11 species of section *Flavi*. In contrast to conventional morphological methods, which are often time-consuming, the molecular strategy proposed here is based mainly on real-time PCR, which is rapid and requires minimal handling.

Introduction

Aspergillus section *Flavi* includes six economically important species that are very closely related morphologically and phylogenetically, and which are often separated into two groups on the basis of their impact on food or human health. The first group includes *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*, which can cause serious damage to stored food products such as wheat and rye grain, nuts, spices and peanuts (Kurtzman *et al*., 1987; Moody & Tyler, 1990a; Samson *et al*., 2000; Rigo *et al*., 2002; Hedayati *et al*., 2007). Furthermore, these species can produce carcinogenic secondary metabolites, the aflatoxins (Klich & Mullaney, 1987; Kurtzman *et al*., 1987; Yuan *et al*., 1995; Samson *et al*., 2000; Hedayati *et al*., 2007). After *Aspergillus fumigatus*, *A. flavus* is known as the second cause of human invasive aspergillosis (Denning, 1998; Latgé, 1999; Hedayati *et al*., 2007). Often, the name *A. flavus* is mistakenly used to describe the different species of *Aspergillus* section *Flavi*. Other recently described species are included in this group but these species are less important economically or rarely isolated. Indeed, *Aspergillus bombycis* was described by Peterson *et al*. (2001) from nine isolates collected in silk-worm-rearing houses. A variety of *A. flavus*, *A. flavus* var. *parvisclerotigenus*, has been raised to species level by Frisvad *et al*. (2005) as *Aspergillus parvisclerotigenus*. *Aspergillus arachidicola* and *Aspergillus minisclerotigenes* were described by Pildain *et al*. (2008). Seven strains of *A. arachidicola* were isolated in Argentina from *Arachis*. Some of the 15 strains of *A. minisclerotigenes* were been described for a long time as *A. flavus* group II by Geiser *et al*. (1998a, b, 2000), before being raised to the species rank by Pildain *et al*. (2008). Hence, many authors have shown evidence that *A. flavus sensu lato* may consist of several species (Geiser *et al*., 1998a, b, 2000; Pildain *et al*, 2008). The second group of the section *Flavi* comprises the nonproducing aflatoxin species *Aspergillus tamarii*, *Aspergillus oryzae* and *Aspergillus sojae*. The last two have lost the ability to produce aflatoxins (Samson *et al*., 2000) and are widely used as a koji mold for the production of fermented foods in some Asian countries.

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Goto *et al*. (1996) reported aflatoxin production by one isolate defined as *A. tamarii*; however, Ito *et al*. (2001) described this isolate as well as a second one as a new closely related species, *Aspergillus pseudotamarii*.

Because some species of the *Aspergillus* section *Flavi* have the ability to produce aflatoxins and cause several diseases in humans, an accurate identification of each species would provide fundamental information concerning their aflatoxigenic and pathogenic properties.

Classical identification methods of *Aspergillus* section *Flavi* strains are performed by examining several morphological traits observed on fungal cultures grown on different media (Samson *et al*., 2000). However, these procedures are time-consuming, require important mycological knowledge and are inaccurate because of intra- and interspecific morphological divergences (Klich & Pitt, 1988).

Several molecular genetic techniques have been tested to classify *Aspergillus* section *Flavi* strains: random amplification of polymorphic DNA (RAPD) (Yuan *et al*., 1995), amplified fragment length polymorphism (Montiel *et al*., 2003), DNA restriction fragment polymorphism (Klich & Mullaney, 1987; Moody & Tyler, 1990a, b), and sequence analyses of (1) the mitochondrial cytochrome *b* gene (Wang *et al*., 2001), (2) the internal transcribed spacer (ITS) region (Kumeda & Asao, 1996; Henry *et al*., 2000; Kumeda & Asao, 2001; Rigo *et al*., 2002) and (3) the aflatoxin gene cluster (Chang *et al*., 1995; Watson *et al*., 1999; Tominaga *et al*., 2006). Although these studies provided important information about the phylogenetic relationships between species, none of them used singly was able to solve problems of identification.

Based on these studies, it appears that two aflatoxin genes (*aflT* and *aflR*) and the ITS regions are good candidates for further taxonomic investigations. The *aflT* gene, which is present in the species of the section *Flavi*, encodes a major facilitator superfamily transporter (Chang *et al*., 2004). The *aflR* is a regulatory gene of several enzymatic steps involved in the aflatoxin biosynthetic pathway (Payne *et al*., 1992). Woloshuk *et al*. (1994) revealed similar sequences of *aflR* gene in four species of the section: *A. flavus*, *A. oryzae*, *A. parasiticus* and *A. sojae*. Kumeda & Asao (2001) showed that most sequence differences among *Aspergillus* section *Flavi* species were sparsely observed in the ITS1 and ITS2 genes.

In this paper, we have developed a six-step strategy using real-time PCR as the key tool, complemented if necessary by RAPD and DNA restriction enzyme fragment polymorphism technique, to set up a decision-making tree allowing an accurate identification process for nine of the 11 species described within the *Aspergillus* section *Flavi*. This method, focusing on the six most economical species, is proposed as a specific, sensitive and rapid diagnostic tool.

Materials and methods

Strains and media

Strains used in this study are listed in Table 1. Strains were routinely subcultured on potato dextrose agar (PDA, Sharlau, Barcelona, Spain) medium in Petri dishes. To produce biomass, fungal isolates were subcultured in a 2% malt extract broth medium (Duchefa, Haarlem, the Netherlands) and grown in the dark at 25 °C for 5 days on a rotary shaker (100 r.p.m.). Mycelium was harvested by centrifugation (2250 ***g***, 4 °C, 15 min), and the pellets were lyophilized.

Fungal genomic DNA preparation

Approximately 30 mg of lyophilized mycelium was disrupted in the Magna Lyser (Roche Diagnostics GmbH, Germany). Fungal DNA was extracted and purified using the EZNA fungal DNA miniprep kit (Omega Bio-tek, Doraville, GA), according to the manufacturer's recommendations. The purified DNAs were quantified using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany) and stored at − 80 °C.

Sequence alignment and primer design

Two primer sets were designed in the ITS1–5.8S rRNA gene–ITS2 and on the *aflT* gene sequences obtained in GenBank [National Center for Biotechnology Information (NCBI), National Institutes of Health], available for six and four species of the *Aspergillus* section *Flavi*, respectively. The sequence alignments were performed with the CLUSTALW program (NCBI), using the default parameters. Primers were designed with the LIGHTCYCLER® PROBE DESIGN software 2.0 (Roche Diagnostics GmbH) and selected in DNA regions with low homology between species. The primers were synthesized and purified by Sigma-Aldrich (St. Louis, MO).

Two previously designed primer sets were used for amplification and sequencing of aflatoxin genes. One primer set targeting the *aflT* gene (Aflt-F and Aflt-R) was designed by Tominaga *et al*. (2006) (Table 2). The targeted fragment is involved in the aflatoxin biosynthetic pathway and is present in both aflatoxin producer and nonproducer species of the section *Flavi*. The second primer set designed by Chang *et al*. (1995) (F1 and R1 renamed AflR-F and AflR-R) enables the amplification of an *aflR* gene fragment only in *A. flavus*, *A. oryzae*, *A. parasiticus* and *A. sojae*.

LIGHTCYCLER® PCR Sybr Green assay

The LIGHTCYCLER® 2.0 Instrument was used for the real-time PCR amplifications of the target DNA. PCR amplification and detection were performed in a single glass capillary (LIGHTCYCLER® capillaries; Roche Diagnostics GmbH). For PCR reaction, the LIGHTCYCLER®FastStart DNA Master^{plus}

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Table 1. Fungal strains used in this study and molecular results

Strains	Collection number	Aflt PCR	Afaflt PCR	AflR PCR	Anits PCR	Calmodulin	Real-time	RAPD	SmaI digestion	Strategy conclusion
	Results					Identification				
Aspergillus section *Flavi* economically important species										
A. flavus	CBS 569.65 (NT)	+	+	+	–	*A. flavus/A. oryzae*	*A. flavus/A. oryzae/A. parvsclerotigenus/A. minisclerotigenes*		*A. flavus/A. parvisclerotigenus*	*A. flavus/A. parvisclerotigenus*
A. nomius	CBS 260.88 (HT)	+	–	–	+	*A. nomius*	*A. nomius*			*A. nomius*
A. oryzae	CBS 466.91 (T)	+	+	+	–	*A. flavus/A. oryzae*	*A. flavus/A. oryzae/A. parvisclerotigenus/A. minisclerotigenes*		*A. oryzae*	*A. oryzae*
A. parasiticus	CBS 260.67 (T)	+	–	+	–	*A. parasiticus/A. sojae*	*A. parsiticus/A. sojae/A. arachidicola*	*A. parasiticus*		*A. parasiticus*
A. sojae	CBS 100928 (NT)	+	–	+	–	*A. parasiticus/A. sojae*	*A. parsiticus/A. sojae/A. arachidicola*	*A. sojae*		*A. sojae*
A. tamarii	CBS 104.13 (NT)	+	–	-	–	*A. tamarii*	*A. tamarii/A. pseudotamarii/ A. bombycis*	*A. tamarii*		*A. tamarii*
Other species of section *Flavi*										
A. arachidicola	CBS 117610 (T)	+	–	+	–	*A. arachidicola*	*A. parasiticus/A. sojae/A. arachidicola*	*A. arachidicola*		*A. arachidicola*
A. bombycis	CBS 117187 (T)	+	–	–	–	*A. bombycis*	*A. tamarii/A. pseudotamarii/ A. bombycis*	*A. bombycis*		*A. bombycis*
A. minisclerotigenes	CBS 117635	+	+	+	–		*A. flavus/A. oryzae/A. parvsclerotigenus/A. miniclerotigenes*		*A. minisclerotigenes*	*A. minisclerotigenes*
A. parvisclerotigenus	CBS 117633	+	+	+	–	*A. parvisclerotigenus*	*A. flavus/A. oryzae/A. parvsclerotigenus/A. miniclerotigenes*		*A. flavus/A. parvisclerotigenus*	*A. flavus/A. parvisclerotigenus*
A. pseudotamarii	CBS 766.67 (HT)	+	–	–	–	*A. pseudotamarii*	*A. tamarii/A. pseudotamarii/ A. bombycis*	*A. pseudotamarii*		*A. pseudotamarii*
Strains received as										
A. flavus	MUCL 13598	+	–	+	–	*A. parasiticus/A. sojae*	*A. parsiticus/A. sojae/A. arachidicola*	*A. parasiticus*		*A. parasiticus*
A. flavus	MUCL 13618	+	–	+	–	*A. parasiticus/A. sojae*	*A. parsiticus/A. sojae/A. arachidicola*	*A. parasiticus*		*A. parasiticus*
A. flavus	MUCL 15975	+	+	+	–	*A. flavus/A. oryzae*	*A. flavus/A. oryzae/A. parvisclerotigenus/A. minisclerotigenes*		*A. flavus/A. parvisclerotigenus*	*A. flavus/A. parvisclerotigenus*
A. flavus	MUCL 18554	+	+	+	–	*A. flavus/A. oryzae*	*A. flavus/A. oryzae/A. parvisclerotigenus/A. minisclerotigenes*		*A. flavus/A. parvisclerotigenus*	*A. flavus/A. parvisclerotigenus*
A. flavus	MUCL 18820	+	+	+	–	*A. flavus/A. oryzae*	*A. flavus/A. oryzae/A. parvisclerotigenus/A. minisclerotigenes*		*A. oryzae*	*A. oryzae*

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Table 1. Continued.

	Results					Identification				
Strains	Collection number	Aflt PCR	Afaflt PCR	AflR PCR	Anits PCR	Calmodulin	Real-time	RAPD	SmaI digestion	Strategy conclusion
A. flavus	MUCL 19006	+	+	+	–	*A. flavus/A. oryzae*	*A. flavus/A. oryzae/A. parvisclerotigenus/A. minisclerotigenes*		*A. oryzae*	*A. oryzae*
A. flavus	MUCL 35049	+	+	+	–	*A. flavus/A. oryzae*	*A. flavus/A. oryzae/A. parvisclerotigenus/A. minisclerotigenes*		*A. flavus/A. parvisclerotigenus*	*A. flavus/A. parvisclerotigenus*
A. flavus	MUCL 42670	+	+	+	–	*A. flavus/A. oryzae*	*A. flavus/A. oryzae/A. parvisclerotigenus/A. minisclerotigenes*		*A. flavus/A. parvisclerotigenus*	*A. flavus/A. parvisclerotigenus*
A. oryzae	MUCL 14492	+	+	+	–	*A. flavus/A. oryzae*	*A. flavus/A. oryzae/A. parvisclerotigenus/A. minisclerotigenes*		*A. oryzae*	*A. oryzae*
A. tamarii	MUCL 2127	+	–	–	–	*A. tamarii*	*A. tamarii/A. pseudotamarii/A. bombycis*			*A. tamarii*
A. sojae	MUCL 39332	+	–	+	–	*A. parasiticus/A. sojae*	*A. parasiticus/A. sojae/A. arachidicola*	*A. sojae*		*A. sojae*
Other *Aspergillus* species or genera										
Alternaria alternata	MUCL 30967	–	–	–	–					
A. niger	CBS 554.65 (NT)	–	–	–	–					
A. versicolor	CBS 583.65 (NT)	–	–	–	–					
Chaetomium globosum	MUCL 39882	–	–	–	–					
Cladosporium cladosporioides	MUCL 45643	–	–	–	–					
Eurotium herbariorum	MUCL 15641	–	–	–	–					
Paecilomyces variotii	MUCL 19015	–	–	–	–					
Penicillium chrysogenum	MUCL 19154	–	–	–	–					
Penicillium citrinum	MUCL 19781	–	–	–	–					
Penicillium commune	MUCL 34882	–	–	–	–					
Stachybotrys chartarum	MUCL 9480	–	–	–	–					

A., Aspergillus; T, type; HT, Holotype; NT, neotype; (+), amplification product detected; (–), no amplification product detected; CBS, Centraal Bureau voor Schimmelcultures; MUCL, Mycothèque de l'Université catholique de Louvain.

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Table 2. Primers used in this study

Primer name	Sequence	T_m (°C)	Target	Species specificity	References
Aflt-F	5′-GCACCAAATGGGTCTTTCTCGT-3′	68.2	Aflt gene	Section *Flavi*	Tominaga *et al*. (2006)
Aflt-R	5′-ATCCACGGTGAAGAGGGTAAGG-3′	67.1	Aflt gene	Section *Flavi*	Tominaga *et al*. (2006)
Afaflt-F	5′-CGCGCGAGATACTTCTTATACT-3′	61.3	Aflt gene	*A. flavus/A. oryzae*	Designed in this study
Afaflt-R	5′-GAGCCCACTTCGAAAATACC-3′	62.3	Aflt gene	*A. flavus/A. oryzae*	Designed in this study
AflR-F	5′-TCGGTACGTAAACAAGGAAC-3′	64.7	AflR gene	*A. parasiticus/A. sojae*	Chang *et al*. (1995)
AflR-R	5′-TCTGATGGTCGCCGAGTTGA-3′	60.6	AflR gene	*A. parasiticus/A. sojae*	Chang *et al*. (1995)
Anits-F	5′-ACACCACGAACTCTGAAC-3′	55.8	ITS region	*A. nomius*	Designed in this study
Anits-R	5′-CGAGGTCAACCTGGAAAGAATGGTTGTTT-3′	74	ITS region	*A. nomius*	Designed in this study

F, forward; R, reverse.

Sybr Green I kit (Roche Diagnostics GmbH) containing a ready-to-use reaction mix (Master Mix), was used as described by the manufacturers.

The amplification mix consisted of 4 μL of the Master Mix 5 × (containing dNTP mix, FastStart Taq DNA polymerase, $MgCl_2$, Sybr Green I dye), 0.5 μM of each primer and 5 μL of template DNA in a final volume of 20 μL. PCR was performed as follows: preincubation step at 95 °C for 10 min and 45 cycles of denaturation at 95 °C for 10 s, annealing at temperature T_m primer dependent for 2–10 s and with a temperature transition rate of 20 °C s^{-1}, and a final extension at 72 °C for a time (in seconds) depending on the amplicon length [amplicon (bp) 25 s^{-1}]. Following amplification, a melting curve analysis was performed by raising the sample temperature to 95 °C, and then immediately lowering it to 65 °C for 60 s, followed by slow heating at a transition rate of 0.1 °C s^{-1} to 95 °C. Fluorescence was monitored at regular intervals during the extension step and continuously during the melting. The experiment was completed in approximately 45 min. The target sequence is detected when the fluorescence curve turns abruptly upward above the threshold. Each DNA sample is characterized by this point of the curve, called the crossing point (C_p).

Determination of specificity of LightCycler® Sybr Green PCRs

The specificity of the primers tested on type strains was then validated using DNA extracted from a set of 11 *Aspergillus* section *Flavi* strains, two other *Aspergillus* species and six fungal genera commonly found in the environment (Table 1).

Within the section *Flavi*, PCR results were compared with the identification data obtained by means of the calmodulin gene sequencing as described previously (O'Donnell *et al*., 2000).

RAPD analysis

Three RAPD analyses were performed as described by Yuan *et al*. (1995) with the primers OPA-04, OPB-10, OPR-01, and sequences AATCGGGCTG, CTGCTGGGAC and TGCGGGTCCT, respectively. DNA amplification was carried out in a final volume of 25 μL containing 100 ng of template DNA, 5 pmol of primer (Sigma-Aldrich), 1 U of Taq DNA polymerase (Sigma-Aldrich), 1 × of Taq DNA buffer (Sigma-Aldrich), 100 μM of dNTPs and 1.5 mM $MgCl_2$. Amplification was performed in a thermocycler (Biometra, Tgradient, Göttingen, Germany) and the amplified products were separated by gel electrophoresis according to Yuan *et al*. (1995), except that the gel was stained with GelRed™ (Biotium Inc., Hayward, CA).

SmaI digestion

One microgram of DNA was digested with SmaI (Klich & Mullaney, 1987) under the following conditions: overnight incubation at 25 °C in a final volume of 25 μL containing 1 U of SmaI (Roche Diagnostics GmbH) and 1 × of buffer. Restriction was fractionated by electrophoresis on a 0.7% agarose gel stained with GelRed™ (Biotium Inc.).

Results

Primer design and specificity

Two primers, Afaflt-F (forward) and Afaflt-R (reverse), were designed on a region of the *aflT* gene presenting a low level of homology between *A. flavus*, *A. oryzae* and other four species of the section *Flavi* for which the gene sequences were available in GenBank (Fig. 1a). A second primer set, Anits-F (forward) and Anits-R (reverse), was designed on a region of the *A. nomius* ITS1–ITS2 region unique to this species (Fig. 1b).

Before PCR amplification, the theoretical specificity stringency of the primers designed for species of the *Aspergillus* section *Flavi* was evaluated using the basic local alignment search tool (BLAST, NCBI). For each set, no fungal species other than the target *Aspergillus* species were proposed, i.e. *A. oryzae* and *A. flavus* for Afaflt-F/Afaflt-R and *A. nomius* for Anits-F/Anits-R.

Different times and annealing temperatures were tested to define the optimal conditions required for each primer set specificity. The PCR program was the same for amplification with the AflT, AfAflt, AflR and Anits primer sets, except for

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the annealing temperature and time, which were 70 °C for 5 s, 70 °C for 2 s, 68 °C for 4 s, 65 °C for 3 s, respectively.

Strategy

The following six-step protocol discriminated nine of the 11 species *Aspergillus* species of the section *Flavi* – five of the economically important and widespread species and four recently described species.

First step

The primer set targeting the *aflT* gene designed by Tominaga *et al.* (2006) successfully amplified 11 type strains of *Aspergillus* section *Flavi*, but none of the other species and genera were tested.

Second step

Afaflt-F and Afaflt-R separated the 11 species into two groups. Species of the first group (*A. flavus*/*A. oryzae*/*A. minisclerotigenes*/*A. parvisclerotigenus*) presented the amplified target fragment, whereas no amplification was observed for species of the second group (*A. parasiticus*/*A. sojae*/*A. nomius*/*A. tamarii*/*A. arachidicola*/*A. bombycis*/*A. pseudotamarii*).

Third step

Within the second group, the AflR-F and AflR-R primers amplified the target products only for *A. parasiticus*, *A. sojae* and *A. arachidicola*, and not for *A. nomius*, *A. tamarii*, *A. bombycis* and *A. pseudotamarii*, confirming the data of Chang *et al.* (1995).

Fourth step

For the nonamplified species during the third step, the Anits-F and Anits-R primers amplified only *A. nomius*, as expected.

Fifth step

For the species group obtained in the second step (*A. flavus*/*A. oryzae*/*A. minisclerotigenes*/*A. parvisclerotigenus*), the

(a)

```
A.flavus   : CGATATTGGATGGTATGCGTCCGCCTATCTACTGACTACGTGTGGTAAGTCCG :  371
A.oryzae   : ..................................................... :  287
A.parasiti : ..................................................... :  287
A.nomius   : T.......................T........T................... :  371

                    Afaflt-F
A.flavus   : CGCGAGATACTTCTTATACTAAACCTCGAATTGCCATTCCACTGACGACCGGC :  424
A.oryzae   : ..................................................... :  340
A.parasiti : .......CTT........................................... :  340
A.nomius   : ..T....CGA.....T...CC..............C....G.....AC..C-- :  422

A.flavus   : GGGGGAGGACAGCCTTCCAATTACTATATGGCAAGTTATATGCCCTCTTCAGC :  477
A.oryzae   : ..................................................... :  393
A.parasiti : ..................................................... :  393
A.nomius   : -........T............G..............T............... :  474

                                                 Afaflt-R
A.flavus   : ACCAAATGGGTCTTTCTCGTCGCCCTGGGTATTTTCGAAGTGGGCTCTCTGAT :  530
A.oryzae   : ..................................................... :  446
A.parasiti : ...........................T......................... :  446
A.nomius   : .................T.........TT...A.....G.............. :  527
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Fig. 1. (a) Partial nucleotide sequence alignment for *aflT* gene of *Aspergillus flavus* (GenBank accession no. AY510451, complete sequence of the aflatoxin biosynthesis gene cluster), *Aspergillus oryzae* (GenBank accession no. AB076803 complete sequence of the aflatoxin biosynthesis gene), *Aspergillus parasiticus* (GenBank accession no. AY371490, complete sequence of the aflatoxin biosynthesis gene cluster), Afaflt-F and Afaflt-R represent the sequences of the primers. (b) Nucleotide sequence alignment for ITS1–ITS2 of rRNA of *A. flavus* (GenBank accession no. D84353), *A. oryzae* (GenBank accession no. AB000533), *A. parasiticus* (GenBank accession no. D84356), *Aspergillus tamarii* (GenBank accession no. D84358), *Aspergillus sojae* (GenBank accession no. D84357) and *Aspergillus nomius* (GenBank accession no. D84354). Anits-F and Anits-R represent the sequence of the primers.

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(b)

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A. flavus   : AAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTA-CCTTAGTTGCTTCGGCGGG :  80
A. oryzae   : .............................................................-................... :  80
A. parasiti : .............................................................-................... :  80
A. tamarii  : .............................................................A................... :  81
A. sojae    : .............................................................-................... :  80
A. nomius   : .............................................................-................... :  80

A. flavus   : CCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCC-GGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGT : 160
A. oryzae   : ...................................-............................................. : 160
A. parasiti : ......G.-................G-........-............................................. : 158
A. tamarii  : ......T.-T.A............GCA........C............................................. : 161
A. sojae    : ......G.-................G-........-............................................. : 158
A. nomius   : ......GC--.A............GCA..C....C..................................AAC........ : 159
                                                                                 Anits-F

A. flavus   : GAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAG : 241
A. oryzae   : ................................................................................. : 241
A. parasiti : ................................................................................. : 239
A. tamarii  : ................................................................................. : 242
A. sojae    : ................................................................................. : 239
A. nomius   : ................................................................................. : 240

A. flavus   : CGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCC : 322
A. oryzae   : ................................................................................. : 322
A. parasiti : ................................................................................. : 320
A. tamarii  : ................................................................................. : 323
A. sojae    : ................................................................................. : 320
A. nomius   : ................................................................................. : 321

A. flavus   : GGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCC--GGGGG : 401
A. oryzae   : ..........................................................................--..... : 401
A. parasiti : ..........................................................................--..... : 399
A. tamarii  : ........................................................................T.--..... : 402
A. sojae    : ..........................................................................--..... : 399
A. nomius   : .....................................................................C.CT.CG..... : 402

A. flavus   : GGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGC : 482
A. oryzae   : ................................................................................. : 482
A. parasiti : ................................................................................. : 480
A. tamarii  : ................................................................................. : 483
A. sojae    : ................................................................................. : 480
A. nomius   : ..........T...................................................................... : 483

A. flavus   : CGGCGCTTGCCGAACGCAAATCAATC--TTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA : 556
A. oryzae   : ..........................--....-............................--------------- : 540
A. parasiti : ....................A...C.AT................................................ : 556
A. tamarii  : ....................A...C.AT.C.............................................. : 559
A. sojae    : ....................A...C.AT................................................ : 556
A. nomius   : ....................A...C.AT.C.............................................. : 559
                              Anits-R
```

Fig. 1. Continued.

presence of a 3.8-kb band in the *A. flavus* SmaI restriction pattern only and of 2.7-kb and 1-kb bands in the *A. oryzae* restriction pattern differentiated *A. flavus* from *A. oryzae* (Fig. 2a), as previously demonstrated by Klich & Mullaney (1987). Furthermore, the SmaI pattern of *A. minisclerotigenes* did not present a 3.8-kb band (Fig. 2b). Unfortunately, *A. parvisclerotigenus* could not be differentiated from *A. flavus* after the SmaI digest (Fig. 2b).

Sixth step

This step consists in analyzing RAPD profiles of the unresolved groups *A. parasiticus*/*A. sojae*/*A. arachidicola* and *A. tamarii*/*A. bombycis*/*A. pseudotamarii*. The presence of a major 2.0-kb band in the *A. parasiticus* amplification pattern obtained with OPB-10 allowed us to distinguish *A. parasiticus* from *A. sojae* (Fig. 3a), as demonstrated previously by

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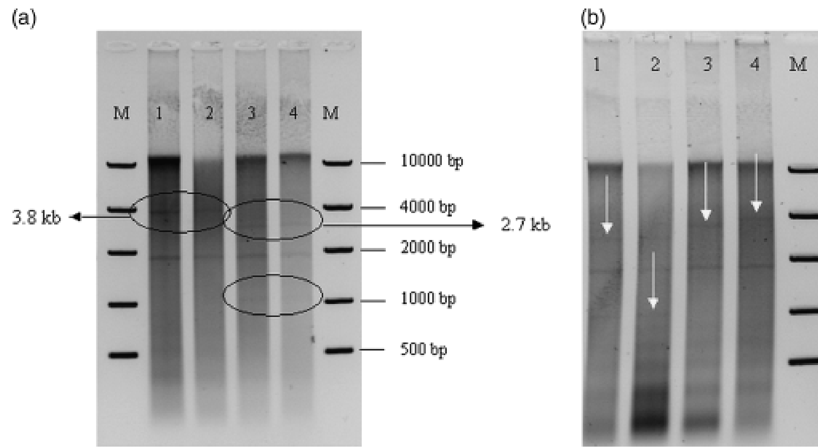

Fig. 2. (a) SmaI digest of *Aspergillus flavus* and *Aspergillus oryzae* genomic DNA electrophoresed on agarose gel. Lane 1: *A. flavus/A. oryzae* MUCL 16109. Lane 2: *A. flavus* CBS 569.65. Lane 3: *A. oryzae* CBS 125.59. Lane 4: *A. flavus/A. oryzae* MUCL 14492. The molecular weight ladder is FastRuler^{TM} High Range (Fermentas, Ontario, Canada). (b) SmaI digest of *A. oryzae*, *Aspergillus minisclerotigenes* and *Aspergillus parvisclerotigenus* genomic DNA electrophoresed on agarose gel. Lane 1: *A. oryzae* CBS 125.59. Lane 2: *A. oryzae* MUCL 14492. Lane 3: *A. minisclerotigenes* CBS 117635. Lane 4: *A. parvisclerotigenus* CBS 117633. M, the molecular weight ladder is FastRuler^{TM} High Range.

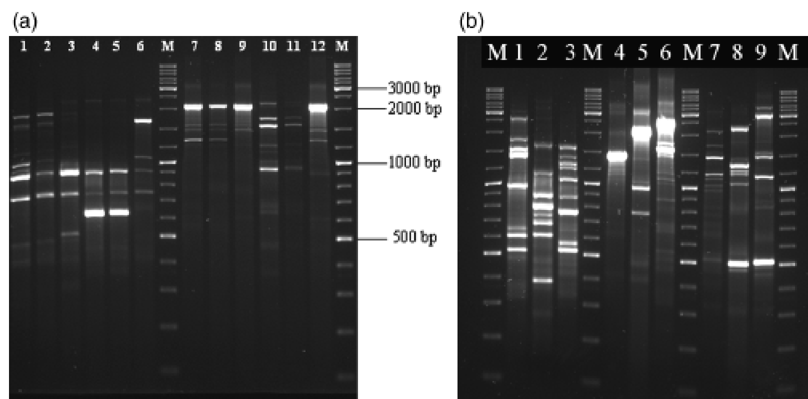

Fig. 3. (a) RAPD pattern of *Aspergillus parasiticus*, *Aspergillus sojae* and *Aspergillus arachidicola* with primers OPA-04 (lanes 1–6) and OPB-10 (lanes 7–12). Lanes 1, 7: *A. parasiticus/A. sojae* MUCL 13598. Lanes 2, 8: *A. parasiticus/A. sojae* MUCL 13618. Lanes 3, 9: *A. parasiticus* CBS 260.67. Lanes 4, 10: *A. sojae* CBS 100.928. Lanes 5, 11: *A. sojae* MUCL39332. Lanes 6, 12: *A. arachidicola* CBS 117610. M, molecular weight ladder (GeneRuler^{TM} DNA Ladder Mix, Fermentas). (b) RAPD pattern of *Aspergillus tamarii*, *Aspergillus pseudotamarii* and *Aspergillus bombycis* with primers OPA-04 (lanes 1–3), OPB-10 (lanes 4–6) and OPR-01 (lanes 7–9). Lanes 1, 4, 7: *A. tamarii* CBS 104.13. Lanes 2, 5, 8: *A. pseudotamarii* CBS 766.67. Lanes 3, 6, 9: *A. bombycis* CBS 117187. M, molecular weight ladder (GeneRuler^{TM} DNA Ladder Mix).

Yuan *et al.* (1995). Furthermore, using the OPA-04 primer, a major band of 1.7 kb was observed in the pattern of *A. arachidicola* and not in the two other patterns (Fig. 3a). The two RAPD amplifications thus allowed the discrimination of the three species. RAPD patterns of *A. bombycis* obtained with OPA-04, OPB-10 and OPR-01 were clearly different from those of *A. tamarii* and *A. pseudotamarii* (Fig. 3b). The *A. pseudotamarii* amplification pattern obtained with OPR-01 produces a 3000-bp and a 500-bp major band, allowing its discrimination from *A. tamarii*.

The PCR profiles (+ or −) obtained for the four primer sets are summarized in Table 1, as well as the RAPD and SmaI digestion results. Finally, a decision-making tree (Fig. 4) was set up and will serve as the molecular key tool for *Aspergillus* section *Flavi* strain identification. Therefore, the final protocol was simplified to avoid superfluous amplifications and subsequent costs as follows: within the section *Flavi*, the third (AflR) and the fourth (Anits) amplifications were performed only after (−) results for the second and the third amplifications, i.e. after an AfAflt − and an AflR − result, respectively.

Strategy validation

No amplification products were obtained for the other species and genera tested, which demonstrates the specificity of the primers for the targeted *Aspergillus* species. For the eight strains considered to belong to *A. flavus*, as well as for the strains of *A. oryzae*, *A. sojae* and *A. tamarii*, partial sequencing of their calmodulin gene confirmed their taxonomic identification, within the limit of the method's specificity (Table 1), i.e. the inability to distinguish *A. flavus* from *A. oryzae* and *A. parasiticus* from *A. sojae*. The expected real-time amplification profiles were obtained for each of

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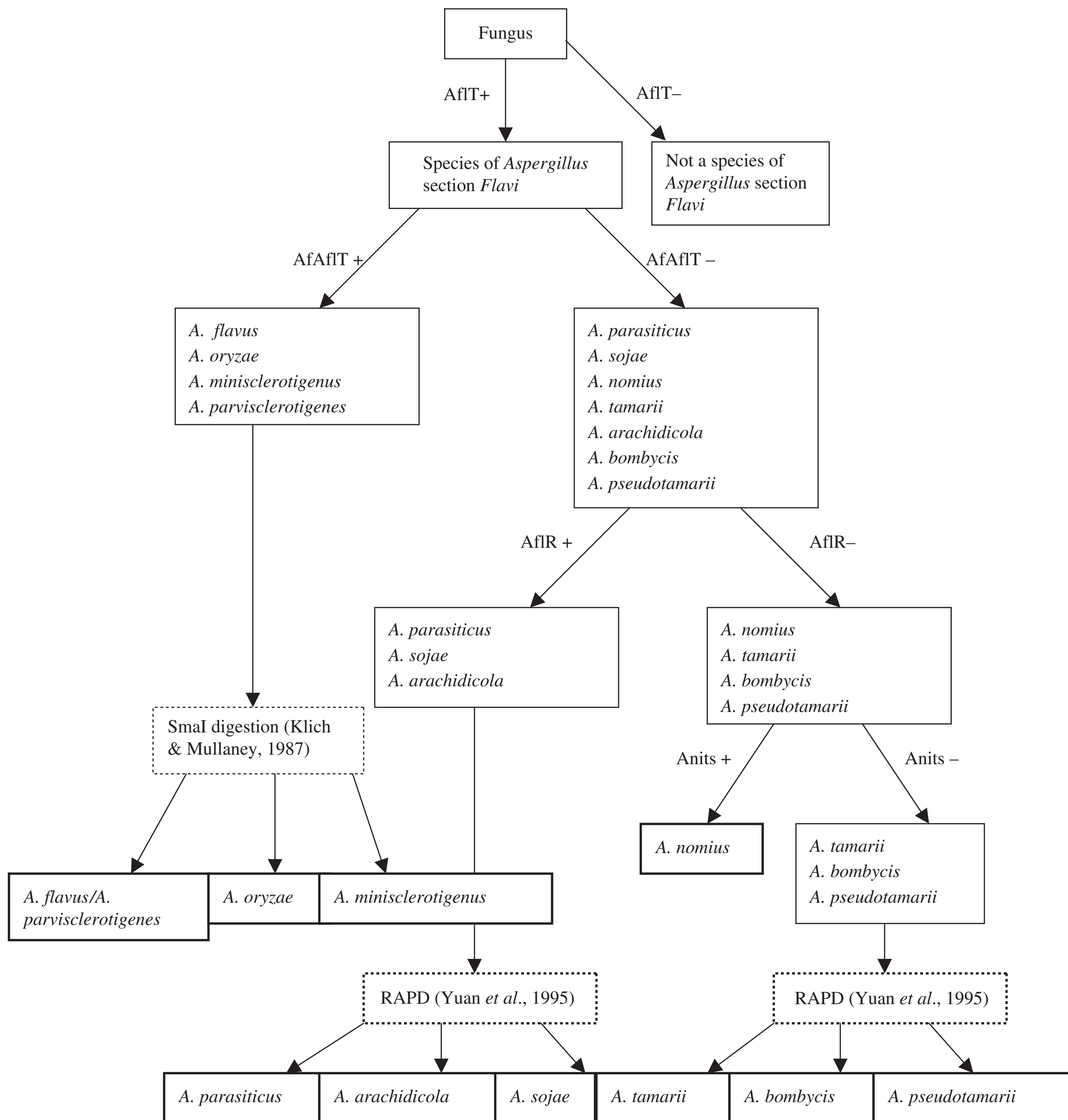

Fig. 4. Decision-making tree for a molecular identification of nine species within the *Aspergillus* section *Flavi*.

these strains compared with the type strains (Table 1). Finally, the follow-up of our strategy results in more precise identification than the calmodulin sequencing.

Discussion

The high frequency of fungal food contamination by *Aspergillus* section *Flavi* species, the potential mycotoxin production related to this process, and the subsequent danger for human and animal health highlight the importance of rapid detection of aflatoxin producers such as *A. flavus* and *A. parasiticus*, and an accurate taxonomical differentiation between the other species of the section. In this paper, we have developed a new easy-handling, rapid and specific molecular strategy for the identification of nine of the 11 species within the *Aspergillus* section *Flavi*.

This strategy, based on the first four steps of real-time PCR, allows preliminary distinction of four species groups and has several advantages. In contrast to conventional PCR followed by DNA sequencing, real-time amplification and

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detection are performed in the same reaction tube without agarose gel handling. In addition, the LightCycler® achieved 45 PCR cycles in 45 min because it uses air for heating and cooling and has an optimal surface-to-volume ratio to ensure a rapid equilibrium between the air and the reaction components.

The robustness of each real-time PCR assay was demonstrated for a wide range of template concentrations (10 ng–1 pg). The sensibility and efficacy are higher than for agarose gel detection after conventional PCR because real-time PCR collects fluorescence data during the linear phase of the exponential PCR, when the conditions of DNA amplification are optimal.

The LightCycler® probe design software analyzes the DNA sequence to find the more promising hybridization sites; however, these are not always the most discriminating sites observed in the alignment analysis. Moreover, to assure specificity, the discriminating nucleotide(s) must be located at the 3′ extremity of the primer. Nevertheless, it is very important that the set possesses a high score in the software to ensure acceptable PCR efficacy and avoid the risks of nonamplification, or of dimer or secondary structure formation. In summary, the primer sets are not always the best in terms of sequence differences or software score, but are often a compromise between the results of sequence alignment and software design. This could explain why *A. flavus*/*A. oryzae* and *A. parasiticus*/*A. sojae* cannot be differentiated with our real-time method. The validation on 11 species of this section demonstrated that identification results are more precise than those obtained by the single gene sequencing method.

From a taxonomic point of view, it is worth noting that the section *Flavi* is still a matter of debate. Indeed, although a lot of genetic approaches failed to identify interspecific differences between *A. flavus* and *A. oryzae*, or between *A. parasiticus* and *A. sojae* (Egel *et al*., 1994; Geiser *et al*., 1998a, b), other studies confirmed that *A. flavus* and *A. oryzae* are almost genetically identical, but show some slight differences at the level of the genes involved in the aflatoxin biosynthetic pathway (Watson *et al*., 1999; Geiser *et al*., 2000; Tominaga *et al*., 2006). Regrettably, these differences are minimal and do not allow researchers to design correct real-time primers assuring good PCR efficacy. Our tests on *aflT* and *aflR* genes to differentiate those two species were laborious and unsuccessful. Up to now, only genetic analyses based on the total DNA can differentiate these two pairs of species because they take genome differences into account. However, *A. oryzae* can be separated from *A. flavus* by SmaI digestion of total DNA (Klich & Mullaney, 1987), whereas *A. parasiticus* and *A. sojae* can be differentiated from each other only by RAPD analysis of the total DNA (Yuan *et al*., 1995).

Furthermore, *A. oryzae* and *A. sojae* are considered to be domesticated forms of *A. flavus* and *A. parasiticus*, respectively (Kurtzman *et al*., 1986; Klich & Pitt, 1988; Geiser *et al*., 1998a, b; Kumeda & Asao, 2001). According to several authors, the absence of interspecific variability provided no justification for maintaining the industrial species *A. oryzae* and *A. sojae* as individual species (Klich & Pitt, 1988; Kumeda & Asao, 2001). However, from a mycotoxigenic point of view, the proposition to meld taxonomically species used in the food-processing industry and aflatoxin-producing species was not received enthusiastically by food mycologists (Geiser *et al*., 2000).

From an ecological point of view, *A. flavus* and *A. parasiticus* are commonly found in the environment, whereas *A. oryzae* and *A. sojae*, used for industrial applications, would not live in the same niches as *A. flavus* and *A. parasiticus* (Yuan *et al*., 1995; Pitt & Hocking, 1999; Cruz & Buttner, 2008; Gonzalez-Salgado *et al*., 2008). Nevertheless, the necessary discrimination of *A. flavus* from *A. oryzae* and *A. parasiticus* from *A. sojae* at an industrial level is possible by analyzing the SmaI digestion patterns (Klich & Mullaney, 1987) and RAPD profiles (Yuan *et al*., 1995).

From a public health point of view, the most important aflatoxin producers are indubitably *A. flavus* and *A. parasiticus* (Pildain *et al*., 2008), which are widely distributed, as well as the aflatoxigenic *A. nomius* (Samson *et al*., 2000). Five new species of the section *Flavi* were tested with our strategy (*A. arachidicola*, *A. bombycis*, *A. minisclerotigenes*, *A. pseudotamarii* and *A. parvisclerotigenus*). Four of them were discriminated, but one species, *A. parvisclerotigenus*, could not be distinguished from *A. flavus*. However, *A. parvisclerotigenus* is also an aflatoxin-producing species and therefore represents a risk in terms of public health. Therefore, its detection simultaneously with *A. flavus*, also an aflatoxin producer, does not involve any economic or health issues for strategy users.

We do not question the descriptions of the five new species, but it must be noted that these species are much less important economically as well as in terms of public health, some are not found in foodstuffs in large numbers (*A. pseudotamarii*), or at all (*A. bombycis*), and some are rarely isolated (*A. arachidicola*), or are considered up to recently to be a variant of *A. flavus* (*A. parvisclerotigenus*) or included in *A. flavus* group II (*A. minisclerotigenes*).

In conclusion, the molecular strategy presented, based mainly on real-time PCR, is rapid and requires minimal handling, in contrast to conventional morphological methods or conventional PCR methods. Furthermore, RAPD and SmaI digestion allows an accurate identification of *Aspergillus* section *Flavi* species, in particular, to address toxigenic problems in the food fermentation industry.

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Statement

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