Differentiation between *Aspergillus flavus* and *Aspergillus parasiticus* from Pure Culture and Aflatoxin-Contaminated Grapes Using PCR-RFLP Analysis of *aflR-aflJ* Intergenic Spacer

André El Khoury, Ali Atoui, Toufic Rizk, Roger Lteif, Mireille Kallassy, and Ahmed Lebrihi

Abstract: Aflatoxins (AFs) represent the most important single mycotoxin-related food safety problem in developed and developing countries as they have adverse effects on human and animal health. They are produced mainly by *Aspergillus flavus* and *A. parasiticus*. Both species have different aflatoxinogenic profile. In order to distinguish between *A. flavus* and *A. parasiticus*, gene-specific primers were designed to target the intergenic spacer (IGS) for the AF biosynthesis genes, *aflJ* and *aflR*. Polymerase chain reaction (PCR) products were subjected to restriction endonuclease analysis using *BglII* to look for restriction fragment length polymorphisms (RFLPs). Our result showed that both species displayed different PCR-based RFLP (PCR-RFLP) profile. PCR products from *A. flavus* cleaved into 3 fragments of 362, 210, and 102 bp. However, there is only one restriction site for this enzyme in the sequence of *A. parasiticus* that produced only 2 fragments of 363 and 311 bp. The method was successfully applied to contaminated grapes samples. This approach of differentiating these 2 species would be simpler, less costly, and quicker than conventional sequencing of PCR products and/or morphological identification.

Keywords: *Aspergillus flavus*, *Aspergillus parasiticus*, *aflJ-aflR*, IGS, PCR, RFLP

Introduction

Aflatoxins (AFs) are derived secondary metabolites of a polyketide family produced by several species of the *Aspergillus* spp. They are considered as potent hepatotoxins and carcinogens causing mortality and/or reducing the productivity of farm animals. Contaminated foodstuffs by these mycotoxins have also been associated with a high incidence of liver cancer in human (Stark 1980; Berry 1988; Ventura and others 2004; Magan and Olsen 2004; European Commission 2006; Giorni and others 2007).

The major AFs of concern are designated as B1, B2, G1, and G2 (Ventura and others 2004; Barros and others 2006). However, AFB1 is usually the most predominant and the most toxic metabolite within this family. AFB1 is also known as being one of the most potent genotoxic agent and hepatocarcinogen identified (Busby and Wogan 1984; Sharma and Salunkhe 1991; Miller and Trenholm 1994; Wang and others 1998). In fact, the International Agency for Research on Cancer (IARC) classified AFB1 as a human carcinogen (group 2A) (IARC, 1993).

The worldwide occurrence of AFs contamination of food and feed has been well documented. The most pronounced contamination has been encountered in corn, peanuts, cottonseed, and other grain crops being most frequently contaminated (Jelinek and others 1989; Gourama and Bullerman 1995; Chen and others 2002; Somashekar and others 2004; Ventura and others 2004). Recently the occurrence of AFB1 in wine grapes and musts has been reported (El Khoury and others 2006; 2008).

The principal filamentous fungi involved in the AF production are *A. flavus* and *A. parasiticus* (Deiner and others 1987; Giorni and others 2007). Taxonomically, these 2 species belong to the section *flavi* of the *Aspergillus* genus (Gams and others 1985) that are phylogenetically related (Kutrzman and others 1987; Egel and others 1994). Thus, morphologically differentiation between these species is very difficult and microscopic identification requires experts in filamentous fungi taxonomy. However, other species belonging to the section *flavi* have also been reported as AF producers namely *A. nominus* (Kutrzman and others 1987; Cotty and others 1994; Scholl and Groopman, 1995), *A. tamarii* (Goto and others 1997), and *A. pseudotamarii* (Ito and others 2001).

The identification of fungal species by conventional methods of standard taxonomic systems is based mainly on the use of morphological markers such as the shape of conidiophores and conidia dimension of each species.

Rodrigues and others (2007) reported that contemporary diagnosis of *A. flavus* and *A. parasiticus* species is based on the descriptions and keys of Raper and Fennell (1965). The primary separation being the presence of metulae and phialides (biseriate conidial head) for *A. flavus* and phialides only (uniseriate conidial head) for *A. parasiticus*. Herein lies the problem. In the key for *A. parasiticus*, the words "strictly uniseriate" replace the former terms of "usually" or "mostly uniseriate" as used in previous

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keys (Thom and Church 1926). Examination of a large number of *A. parasiticus* isolates (Kozakiewicz 1995) has shown that up to 10% of conidial heads in an *A. parasiticus* colony can have metulae and phialides (biseriate). Furthermore, not all *A. flavus* isolates consistently produce metulae (Klich and Pitt 1988; Kozakiewicz 1995). Consequently, the number of these available markers is generally low, which makes difficult the classification and/or the identification of related species beside that these methods are time consuming and require very expert taxonomists.

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 (ITS) of ribosomal DNA (rDNA) by the Polymerase chain reaction (PCR) (Criseo and others 2001; Chen and others 2002), combined with sequencing of the amplicons and analysis of similarity between the sequences obtained and those deposited in the Gene bank, has been frequently employed for identification of fungal species (Chen and others 2002).

The variation in DNA sequence can be detected by restriction fragment length polymorphism (RFLP) analysis, which can detect minor nucleotide variations that may not be expressed at protein level and can detect changes in noncoding regions of DNA (Feinberg and Vogelstein 1984). RFLP can be used as fingerprints to distinguish between closely related organisms and to infer phylogenetic relationships.

In recent years, PCR-based RFLP (PCR-RFLP) has been widely used in the detection and differentiation between mycotoxigenic species. Somashekar and others (2004) were able to differentiate *A. parasiticus* from *A. flavus* based on the RFLP resulting from digesting an *aflR* gene fragment with the restriction enzyme *Pvu*II. Martinez-Culebras and Ramon (2007) developed a method to identify black *Aspergillus* isolates responsible for ochratoxin A contamination in grapes and wine using an ITS-RFLP. Recently, an RFLP analysis of the 5.8S-ITS genes was performed by using a *TaqI* restriction enzyme in order to characterize the toxigenic molds of paprika of the different genera and species of *Fusarium*, *Aspergillus*, *Penicillium*, *Cladosporium*, *Mucor,* and *Phlebia* (Ruiz-Moyano and others 2009).

The aim of this study was to determine the existence of genotypic differences between *A. flavus* and *A. parasiticus*, the most predominant aflatoxigenic species of the section *flavi* contaminating foodstuffs. This differentiation was accomplished by PCR-RFLP targeting the *aflR-aflJ* intergenic spacer (IGS) of the AF biosynthetic cluster.

Materials and Methods

Fungal isolates

Aflatoxigenic and nonaflatoxigenic fungal strains used in this study are described in Table 1. Strains were stored as spore suspensions in 20% glycerol at –20 °C.

Culture medium

The culture medium used in this study was Czapek yeast extract agar (CYA), which contained per liter of distilled water: 30 g sucrose (Fisher Labosi, Elancourt cedex, France); 1 mL trace metal (Cu + Zn) solution (Fisher Labosi); 1 g K_2HPO_4 (Acros, Geel, Belgium); 10 mL Czapek concentrate; 5 g yeast extract (Difco, Fisher Labosi); and 15 g agar (Difco, Fisher Labosi) (Pitt and Hocking 1997).

In order to obtain a young mycelium for DNA extraction, all fungal isolates were grown in petri dishes containing CYA medium for 2 d at 25 °C.

Table 1–Fungal cultures used for PCR-RFLP of *aflR-aflJ* intergenic spacer fragment.

Name	Produced mycotoxin	Source
A. flavus NRRL 35691	Aflatoxins B1, B2	Atoui and others 2007
A. parasiticus CBS 100926	Aflatoxins B1, B2, G1, G2	Prof. J.C. Frisvad
A. flavus I5	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus I21	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus F17	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus F54	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus F66	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus F87	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus F89	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus M8	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus M14	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus M18	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus M21	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus M30	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus M34	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus S5	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus S7	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus S8	Aflatoxins B1, B2	EL Khoury and others 2008
A. flavus S10	Aflatoxins B1, B2	EL Khoury and others 2008
A. parasiticus WT12	Aflatoxins B1, B2, G1, G2	Olivier Puel
A. parasiticus WT25	Aflatoxins B1, B2, G1, G2	Olivier Puel
A. westerdijkiae NRRL 3174	Ochratoxin A	Olivier Puel
A. fumigatus NRRL 35693	Gliotoxin, fumagillin	Atoui and others 2007
A. niger CBS 120166	Ochratoxin A	Atoui and others 2007
A. carbonarius CBS 120168	Ochratoxin A	Atoui and others 2007
A. sulfureus NRRL 4077	Ochratoxin A	Atoui and others 2007
Penicillium nordicum	Ochratoxin A	Olivier Puel
P. expansum NRRL 35694	Patulin	Olivier Puel
P. citrinum NRRL 1843	Citrinin	Atoui and others 2007
P. verrucosum NRRL 3711	Ochratoxin A	Atoui and others 2007
Fusarium graminareum NRRL 5883	Zearalenone, Deoxinivalenol	Olivier Puel

DNA extraction from pure fungal cultures

A rapid DNA extraction from fungal strains was performed according to Lui and others (2000). To a 1.5-mL Eppendorf tube containing 500 mL of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM ethylene diaminetetra acetic acid [EDTA] [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate), a small lump of mycelia from young culture is added by using a sterile toothpick, with which the lump of mycelia is disrupted. The tube is then left at room temperature for 10 min. After adding 150 mL of potassium acetate (pH 4.8; which is made of 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, and 28.5 mL of distilled water), the tube is vortexed briefly and spun at 10000 × *g* for 1 min. The supernatant is transferred to another 1.5-mL Eppendorf tube and centrifuged again as described above. After transferring the supernatant to a new 1.5-mL Eppendorf tube, an equal volume of isopropyl alcohol is added. The tube is mixed by inversion briefly. The tube is spun at 10000 × *g* for 2 min, and the supernatant is discarded. The resultant DNA pellet is washed in 300 mL of 70% ethanol. After the pellet is spun at 10000 rpm for 1 min, the supernatant is discarded. The DNA pellet is air dried and dissolved in 50 mL of deionized H_2O, and 1 mL of the purified DNA is used in 25 to 50 mL of PCR mixture. The extractions were done in duplicate assays for each sample.

Fungal DNA extraction from grape samples

In this study, 5 AF-contaminated grape samples were collected from the Lebanese vineyard during the study of El Khoury and others (2008). DNA extractions were performed in duplicate according to the method described by Atoui and others (2007). In this method, a portion of fresh and frozen grape berries (300 mg) was weighed and incubated with 1.5-mL extraction buffer (1 M Tris-HCl [pH 8], 1.4 M NaCl, 20 mM EDTA, 3% cetyttrimethyl ammonium bromide [CTAB]) and 15 μL β-mercaptoethanol for 90 min at 65 °C under constant shaking on a orbital shaker. After incubation, samples were centrifuged at 6500 rpm for 5 min at 4 °C and the supernatant was collected in a 2-mL Eppendorf tube. One volume of chloroform/isoamyl alcohol (24:1) was added and samples were mixed and centrifuged at 6500 rpm for 20 min at 4 °C. The upper aqueous phase was transferred into another tube, adding 0.1 volume of 10% CTAB. Again, one volume of chloroform/isoamyl alcohol (24:1) was added and samples were mixed and centrifuged at 6500 rpm for 20 min at 4 °C. The upper aqueous phase was transferred into another tube, adding 0.1 volume of cold 2-propanol. Samples were then incubated for 60 min at −80 °C and centrifuged for 20 min at 13000 rpm. The pellet was dissolved in 300 μL of sterile H_2O and processed according to the EZNA Fungal DNA Miniprep Kit protocol, starting from step 8 of protocol B that implies DNA cleanup through Hi-bond® (Biofidal, Vaulx en Velin, France) spin column. In the final step, DNA was eluted in 100 μL of deionized H_2O.

Polymerase chain reaction

The PCR was performed, in duplicate, with the *Taq* recombinant polymerase (Invitrogen, Cergy Pontoise, France). Amplification was carried out in 50 μL reaction mixture containing: 5 μ of *Taq* polymerase buffer 10 ×, 1.5 μL of 50 mM $MgCl_2$, 1 μL of dNTP 10 mM of each (Promega, Charbonniéres, France), 1 μM of each primer, 1.5 units of *Taq*, about 50 ng of genomic DNA, H_2O up to 50 μL. Reaction conditions were: 94 °C for 4 min, (94 °C for 40 s, 58 °C for 40 s, and 72 °C for 1 min) × 35 cycles followed by an incubation at 72 °C for 10 min. The amplified products were examined by 0.8% w/v agarose (Promega) gel electrophoresis.

Primer design

The IGS for the AF biosynthesis genes *aflJ* and *aflR* was used as a target in order to discriminate *A. flavus* and *A. parasiticus*. Sequences from several isolates were obtained from Gene bank and then aligned using Clustal X package version 1.83 (Figure 1). A primer pair IGS-F/IGS-R was designed to amplify the available published regions from those isolates (Ehrlich and others 2003, 2007) that correspond to a PCR product of 674 bp. The sequences of the primers used are as follows: IGS-F, 5′-AAGGAATTCAGGAATTCTCAATTG-3′; IGS-R, 5′-GTCCACCGGCAAATCGCCGTGCG-3′. The β-tubulin gene was used as positive control and primer sequences were: TubF: CTCGAGCGTATGAACGTCTAC; TubR: AAACCCTGGAGGCAGTCGC, which amplified a 340 bp fragment on genomic DNA. Primer synthesis was performed in Distribio, France.

Restriction site analysis of PCR products

The PCR products were subjected to endonuclease restriction enzyme digestion using *BglII* (FERMENTAS GMBH, Opelstrasse, Germany). The reactions were performed in a total volume of 40 μL containing 15 units of enzyme, 4 μL of buffer, 15 μL of PCR product, and Ultrapure water up to 40 μL. The reaction mixture was incubated at 37 °C for 3 h. Then the resulting fragments were separated by electrophoresis on a 2% w/v agarose (Promega) gel for 1 h 45 min at 100 V.

Results and Discussion

Available sequences of *aflR-aflJ* intergenic region of *A. flavus* and *A. parasiticus* isolates were obtained from GenBank database and then aligned using Clustal X. Figure 1 showed the result of alignment as well as the location of the primer pair IGS-F/IGS-R selected on the basis of sequence alignment to amplify the whole aligned fragment of 674 bp.

In order to check the PCR's specificity, all fungal strains listed in Table 1 were amplified using the primer pair IGS-F/IGS-R. As shown in Figure 2A, these primers were highly specific for *aflR-aflJ* IGS fragment. Only *A. flavus* and *A. parasiticus* DNA was amplified yielding amplicons of the expected size of 674 bp and no additional or nonspecific bands were observed. In addition, none of the other species gave a positive result with this PCR primer set used (Figure 2A, Lane 21 to Lane 30). The β-tubulin gene was used as a positive control with a fragment of 340 bp obtained in the same PCR conditions for all fungal DNA (Figure 2B for nonaflatoxigenic species).

Variation in DNA sequence can be detected by PCR-RFLP, which can detect minor nucleotide variations (Feinberg and Vogelstein 1984). A detailed comparison of the restriction maps of the PCR product of *aflR-aflJ* intergenic region fragment allowed the identification of a restriction endonuclease, *BglII*, which could be used to differentiate *A. flavus* and *A. parasiticus* (Figure 1). According to the sequence analysis, there are 2 restriction sites for *BglII* in the sequence of *A. flavus* that should cleave the PCR products into 3 fragments of 362, 210, and 102 bp. However, there is only one restriction site for this enzyme in the sequence of *A. parasiticus* that should produce 2 fragments of 363 and 311 bp. Therefore in order to verify the above restriction sequences analysis, PCR-RFLP was carried out. As expected, PCR-RFLP patterns of aflatoxigenic

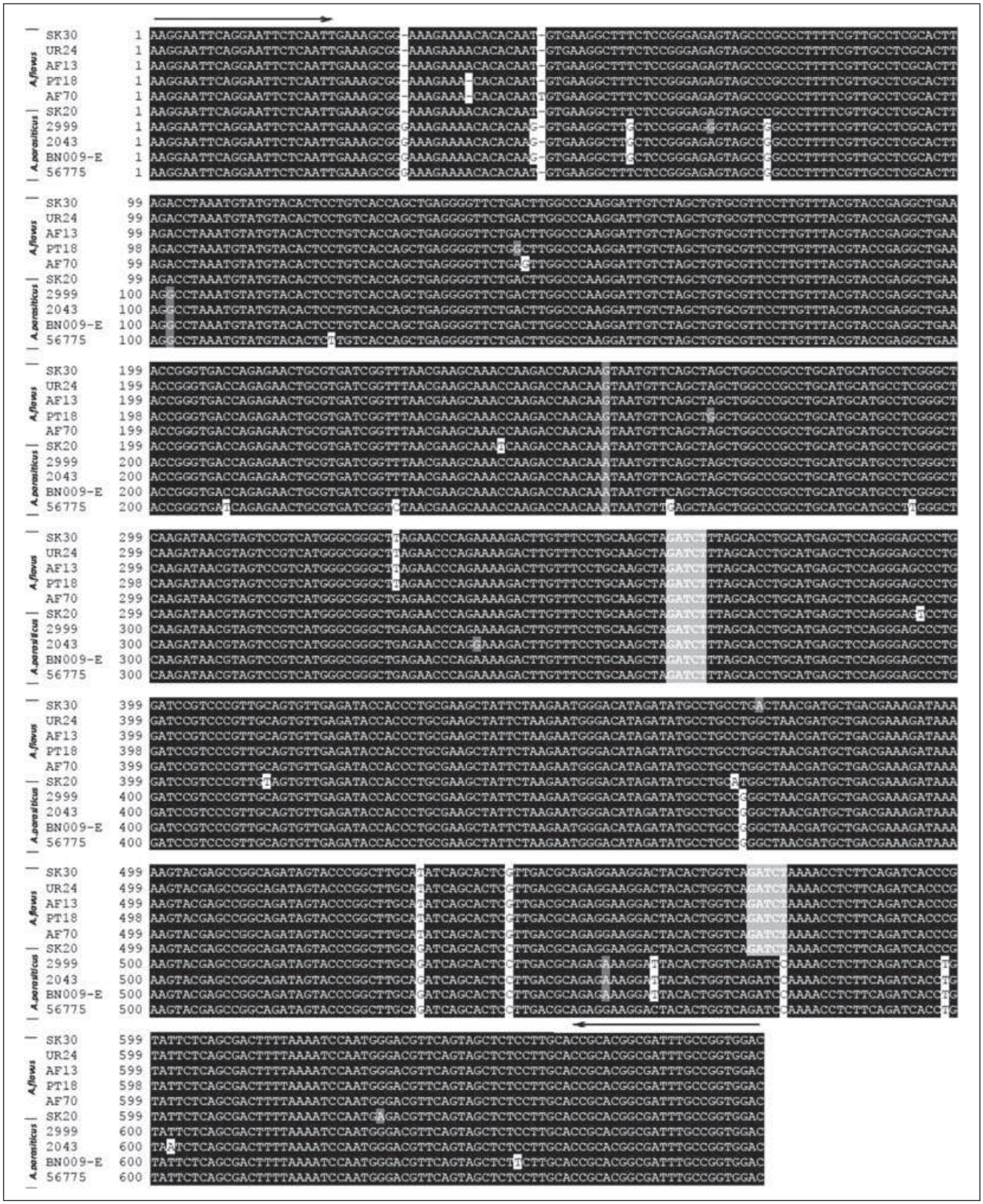

Figure 1–Alignment of *aflR-aflJ* intergenic spacer region sequences in 10 strains of *A. flavus* isolates (SK30, GenBank accession number: DQ467939.1; UR24, GenBank accession number: DQ467936.1; AF13, GenBank accession number: DQ467938.1; PT18, GenBank accession number: DQ467941.1; AF70, GenBank accession number: DQ467940.1; SK20, GenBank accession number: DQ467948.1) and *A. parasiticus* isolates (2999, GenBank accession number: DQ467949; 2043, GenBank accession number: AF441438.1; BN009-E, GenBank accession number: AF441436.1; 56775, GenBank accession number: AF452809.1). The location of primers IGS-F/IGS-R is represented by bold arrows. The regions shadowed in pale gray represent the restriction site for *BglII* endonuclease enzyme.

isolates (Table 1), obtained with *BglII*, showed enough differences to distinguish *A. flavus* and *A. parasiticus* (Figure 3).

Molecular methods have been widely applied in the identification of a large number of *Aspergillus* species. Several approaches have been developed for fungal systematic studies, including random amplified polymorphic DNA (RAPD) analysis, specific diagnostic PCR primers (Nicholson and others 1998), and DNA sequencing (O'Donnell and others 1998; Paterson 2006). However, the methods more currently used are often based on the analysis of rRNA gene (or rDNA) sequences that are universal and contain both conserved and variable regions, allowing discrimination at different taxonomic levels (Ferrer and others 2001; Paterson 2006). Restriction analysis of PCR-amplified rDNA sequences has been shown to be a suitable method for taxonomic studies in many *Fusarium* and *Aspergillus* species (Mirete and others 2003; Gonzalez-Salgado and others 2005; Paterson 2006; Martinez-Culebras and Ramon 2007).

Genes involved in mycotoxin biosynthesis are considered to be more variable within closely related species (Geiser and others 2000). Genes involved in AF biosynthesis have been identified, cloned, and studied. They include regulatory genes *aflJ*, *aflR,* and several structural genes (Chang and others 1993; Payne and others 1993; Bennett and others 1994; Yu and others 2004; Paterson 2006). However, *A. flavus* group species are difficult to differentiate even genetically. *Aspergillus flavus*, *A. parasiticus*, have shown to possess high degrees of DNA relatedness and similar genome size. Chang and others (1995) found the *aflR* gene to be virtually identical in *A. flavus* and *A. parasiticus*, but Somashekar and others (2004), using a limited number of strains, were able to differentiate *A. parasiticus* from *A. flavus* based on the RFLP resulting from digesting an *aflR* gene fragment with the restriction enzyme *Pvu*II.

When dealing with both food safety and plant pathology, correct as well as fast identification of the fungal species are essential. In food industry, it is important to know if toxigenic fungi are present

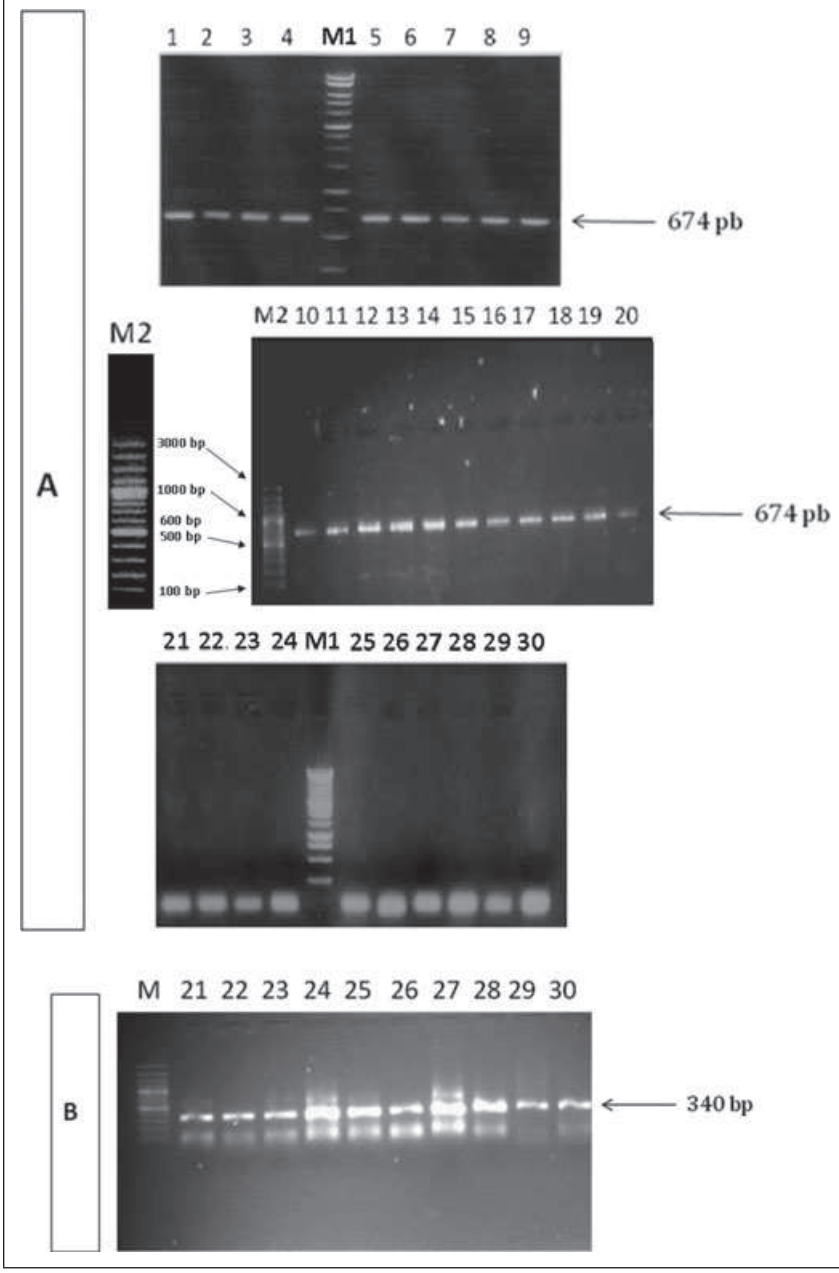

Figure 2–(A) A total of 0.8% of agarose gel electrophoresis of PCR products with IGSF/IGSR primers. Lane 1— *A. flavus* I5; Lane 2— *A. flavus* I21; Lane 3— *A. flavus* F17; Lane 4— *A. flavus* F54; Lane M1—1 kb DNA marker (Promega); Lane 5— *A. flavus* F66; Lane 6— *A. flavus* F87; Lane 7— *A. flavus* F89; Lane 8—*A. flavus* NRRL 35691; Lane 9— *A. parasiticus* CBS100926; Lane M2—100 bp DNA marker (GeneRuler, Fermentas); Lane 10— *A. flavus* M8; Lane 11— *A. flavus* M14; Lane 12— *A. flavus* M18; Lane 13— *A. flavus* M21; Lane 14— *A. flavus* M30; Lane 15— *A. flavus* M34; Lane 16— *A. flavus* S5; Lane 17— *A. flavus* S7; Lane 17— *A. flavus* S8; Lane 18— *A. flavus* S10; Lane 19— *A. parasiticus* S7; Lane 20— *A. parasiticus* S45; Lane 21— *A. westerdijkiae* NRRL 3174; Lane 22— *A. fumigatus* NRRL 35693; Lane 23— *A. niger* CBS 120166; Lane 24— *A. carbonarius* CBS 120168; Lane M1—1kp DNA marker (Promega); Lane 25— *A. sulfureus* NRRL 4077; Lane 26— *Penicillium nordicum*; Lane 27— *P. expansum* NRRL 35694; Lane 28— *P. citrinum* NRRL 1843; Lane 29— *P. verrucosum* NRRL 3711; Lane 30— *F. graminareum* NRRL5883. (B) 0.8% of agarose gel electrophoresis of PCR products of DNA from nonaflatoxigenic species with β-tubulin primer pair (TubF/TubR). Lane M—100 bp DNA marker (GeneRuler, Fermentas); Lane 21— *A. westerdijkiae* NRRL 3174; Lane 22— *A. fumigatus* NRRL 35693; Lane 23— *A. niger* CBS 120166; Lane 24— *A. carbonarius* CBS 120168; Lane 25— *A. sulfureus* NRRL 4077; Lane 26— *Penicillium nordicum*; Lane 27— *P. expansum* NRRL 35694; Lane 28— *P. citrinum* NRRL 1843; Lane 29— *P. verrucosum* NRRL 3711; Lane 30— *F. graminareum* NRRL5883.

in the raw material prior to production, and in agriculture it is equally important to know if plant pathogenic fungi are present in order to rapidly employ the correct spraying regime (Andersen and others 2006). In the case of *Aspergillus* section, *Flavi* differentiation of *A. flavus* from *A. parasiticus* is important because of the difference in their metabolite production. *Aspergillus parasiticus* produces both "B" and "G" type toxins, whereas *A. flavus* produces only "B" type toxins. Taxonomically, *A. flavus* has finely roughened conidia mostly produced from heads bearing both metulae and phialides, while conidia of *A. parasiticus* are usually conspicuously roughened and most heads bear phialides alone (Pitt and Hocking 1985).

The *aflR-aflJ* IGS-RFLP assay, developed in this study, is proposed as a rapid and easy method to differentiate between *A. flavus* and *A. parasiticus* species isolated from foodstuffs. Thus, this method will help us to understand the epidemiology and distribution of *A. flavus* and *A. parasiticus* in foodstuff, where vast numbers of isolates have to be screened in a short time. Accurate identification of the both species in the section *Flavi* is also of great importance in determining toxicological risks because the toxic profile of each species could be different.

The applicability of the developed PCR assay in grapes was also analyzed using naturally contaminated samples. To accomplish this, fungal DNA extraction from grapes samples was performed followed by amplification using IGS-F/IGS-R. As shown in Figure 4, the PCR-RFLP patterns, obtained with *BglII*, successfully allowed the detection and identification of *A. flavus* contaminating these samples. Since we could not get *A. parasiticus* strains from grapes samples screened for aflatoxigenic fungi (El Khoury and others 2008), the study was restricted to the comparison of a single reference strain of *A. parasiticus* with several *A. flavus* strains.

In conclusion, the method described in this study represents a much quicker and more reliable detection and differentiation between *A. flavus* and *A. parasiticus* from pure culture as well as in AF-contaminated grape samples. It rendered results in less than 24 h, saving reasonable time and effort in comparison to conventional methods that required fungal isolation from contaminated

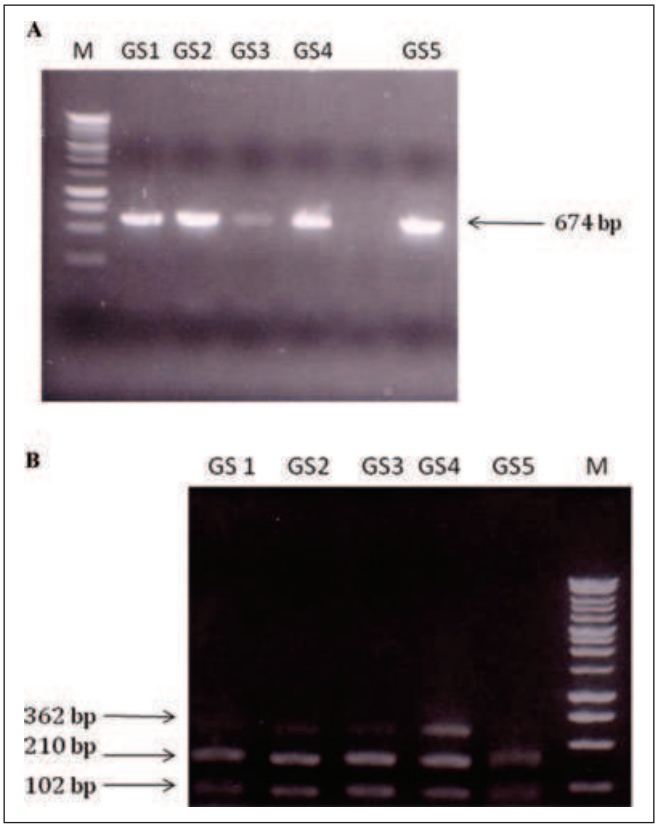

Figure 4–(A) PCR-based detection of *A. flavus* in 5 grapes samples amplified with IGSF/IGSR primers. Lane M—1 kb DNA marker (Promega); Lane GS1—Grape sample 1; Lane GS2— Grape sample 2; Lane GS3—Grape sample 3; Lane GS4—Grape sample 4; Lane GS5—Grape sample 5. (B) Electrophoretic analysis showing the restriction profiles of the *aflR-aflJ* intergenic spacer PCR product from 5 grape samples after digestion with *BglII*. Lane GS1—Grape sample 1; Lane GS2—Grape sample 2; Lane GS3—Grape sample 3; Lane GS4—Grape sample 4; Lane GS5—Grape sample 5, Lane M—100 bp DNA marker (GeneRuler, Fermentas).

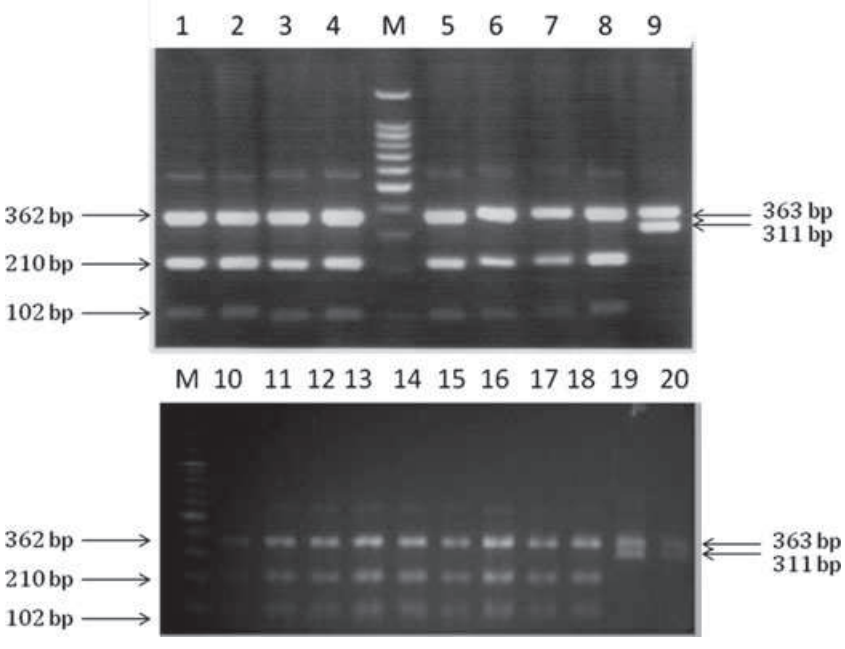

Figure 3–Electrophoretic analysis showing the restriction profiles of the *aflR-aflJ* intergenic spacer PCR product digested with *BglII*. Lane 1— *A. flavus* I5; Lane 2— *A. flavus* I21; Lane 3— *A. flavus* F17; Lane 4— *A. flavus* F54; Lane M—100 bp DNA marker (GeneRuler, Fermentas); Lane 5— *A. flavus* F66; Lane 6— *A. flavus* F87; Lane 7— *A. flavus* F89; Lane 8—*A. flavus* NRRL 35691; Lane 9— *A. parasiticus* CBS100926; Lane M—100-bp DNA marker (GeneRuler, Fermentas); Lane 10— *A. flavus* M8; Lane 11— *A. flavus* M14; Lane 12— *A. flavus* M18; Lane 13— *A. flavus* M21; Lane 14— *A. flavus* M30; Lane 15— *A. flavus* M34; Lane 16— *A. flavus* S5; Lane 17— *A. flavus* S7; Lane 17— *A. flavus* S8; Lane 18— *A. flavus* S10; Lane 19— *A. parasiticus* S7; Lane 20— *A. parasiticus* S45.

food samples and difficult taxonomical identification. In addition to that the differentiation between *A. flavus* and *A. parasiticus* by metabolite analysis requires many steps starting from culture and mycotoxins production (10 d) followed by extraction and purification of these molecules ending by chromatographical analysis.

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