ORIGINAL PAPER
Polish Journal of Microbiology
2022, Vol. 71, No 4, 589–599
https://doi.org/10.33073/pjm-2022-048

Molecular Characterization of *Aspergillus flavus* Strains Isolated from Animal Feeds

HADJER SABER[1], YAHIA CHEBLOUNE[2]* and ABDALLAH MOUSSAOUI[1]

[1] Laboratory of Plant Resources Valorization and Food Safety in Semi-Arid Areas of Southwestern Algeria, Department of Biology, University of Bechar, Bechar, Algeria
[2] USC 1450 INRAE/UGA Lentiviral Pathogenesis and Vaccination Laboratory, Department of Biology, University of Grenoble Alpes, Saint-Martin-d'Hères, France

Submitted 9 June 2022, accepted 21 September 2022, published online 19 December 2022

Abstract

Aflatoxin (AF)-producing fungi such as *Aspergillus flavus* commonly contaminate animal feeds, causing high economic losses. *A. flavus* is the most prevalent and produces AFB1, a potent mutagen, and carcinogen threatening human and animal health. *Aspergillaceae* is a large group of closely related fungi sharing number of morphological and genetic similarities that complicate the diagnosis of highly pathogenic strains. We used here morphological and molecular assays to characterize fungal isolates from animal feeds in Southwestern Algeria. These tools helped to identify 20 out of 30 *Aspergillus* strains, and 15 of them belonged to the *Aspergillus* section *Flavi*. Further analyses detected four out of 15 as belonging to *Aspergillus flavus-parasiticus* group. PCR targeting the AF genes' *aflR-aflS(J)* intergenic region amplified a single 674 bp amplicon in all four isolates. The amplicons were digested with a *Bgl*II endonuclease, and three specific fragments were observed for *A. flavus* but *A. parasitucus* lacked two typical fragments. Sequencing data of four amplicons confirmed the presence of the two *Bgl*II restriction sites yielding the three fragments, confirming that all four strains were *A. flavus*. In addition, this analysis illustrated the genetic variability within the *A. flavus* strains.

K e y w o r d s: *Aspergillus flavus*, *aflR-aflS(J)*, IGS, PCR-RFLP, diagnosis tools

Introduction

Aspergillus producing AF from contaminated animal feed, a secondary metabolite, is a global problem with high economic impacts on animal and human health. Contaminations occur before harvest, and during the storage and distribution of seeds. In sub-Saharan Africa, the global agricultural loss associated with AF contamination was estimated at 40% of the global production, corresponding to $450 million (Gbashi et al. 2018). Aflatoxins refer to a family of over 18 related compounds, principally B1, B2, G1, and G2 (Ibrahim 2019), produced primarily by the closely related fungi, *Aspergillus flavus* and *Aspergillus parasiticus* (Rao et al. 2020). Aflatoxins are polyketide-derived secondary metabolites. *AflR* encodes a protein containing a zinc-finger binding domain that interacts with target sequence DNA (Lee et al. 2006). *AflS* encodes a costimulatory regulatory protein that enhances the transcription activity (Liao et al. 2020).

A. flavus and *A. parasiticus* are dominant species found in crops (Kim et al. 2017) that share morphological similarities and closely cluster in phylogeny (Godet and Munaut 2010). Thus, the distinction between these species is difficult because microscopic identification requires experts in filamentous fungi taxonomy (El Khoury et al. 2011). In addition, the morphological methods of identification are inaccurate because of close inter-species similarities. In contrast, molecular techniques save labor time and their sensitivity and specificity, provide distinct species closeness and distance estimations without sophisticated culture and further confirmation steps (Ahmad et al. 2010).

Recently, several molecular approaches have been used to differentiate among many *Aspergillus* species, including random amplified polymorphic DNA (RAPD)

* Corresponding author: Y. Cheboune, Lentiviral Pathogenesis and Vaccination Laboratory, PAVAL Lab., Department of Biology, University of Grenoble Alpes, Saint-Martin-d'Hères, France; **e-mail: yahia.cheboune@inrae.fr**

(Yin et al. 2009; Daghriri et al. 2018), amplified fragment length polymorphism (AFLP), with specific diagnostic PCR primers, polymorphic microsatellite marker analysis, microsatellite length polymorphism (Healy et al. 2004), and DNA sequencing (Nicholson et al. 1998).

The commonly used molecular approaches often target the analysis of the coding sequences for rRNA that contain both conserved and variable regions (Paterson 2006). Fungal rDNA operons have been shown to contain variability regions within genera (Levy et al. 2001). However, PCR-RFLP has been used to identify variations in DNA sequences, and it was shown to recognize closely related organisms using inter-phylogenetic relations (Zarrin and Erfaninejad 2016). This technique has been proven to be fast, sensitive, and reliable in determining the genetic variability among *A.* section *Flavi* species (Bagyalakshmi et al. 2007; Mohankumar et al. 2010; Lavkor 2020). Currently, there is a limited understanding of molecular variability among populations of *A. flavus* isolated from feed sources. Characterization of *A. flavus* requires an expert polyphasic approach to fully confirm its taxonomic status and develop an effective control strategy (Singh et al. 2018; Frisvad et al. 2019; Martinez-Miranda et al. 2019). Using molecular biology methods combined with morphology has revealed the diversity and genetic variability within the *A. flavus* species (Okoth et al. 2018).

In the present study, we used the PCR-RFLP targeting the *aflR-aflS(J)* intergenic spacer (IGS) of the AF biosynthesis cluster to complement the findings of the morphological characterization. The main objectives were to establish and assess the genetic relationships and variability patterns of *A. flavus* strains isolated from feeds in the southwestern Saharan region of Algeria using PCR and sequence analysis of the IGS region, followed by phylogenetic analysis.

Experimental

Materials and Methods

Fungal isolates and morphological identification. Thirty fungal isolates were obtained from various animal feeds. Out of these, twenty were identified as *Aspergillus*, and 15 out of 20 were identified as *A.* section *Flavi*. Samples were collected in the livestock feed manufacturing unit's storage facilities in Southwestern Algeria. As described earlier, the strains were isolated using Potato Dextrose Agar (PDA) (Pitt and Hocking 2009). Strains identification included macroscopic (colony color and morphology) and microscopic (head seriation, conidia morphology, and size) characteristics. The identification was performed according to the criteria defined for the taxonomic keys and guidelines for the Aspergillus genus (Pitt and Hocking 2009). All isolates were cultured on *Aspergillus flavus*/*parasiticus* agar (AFPA) (bacteriological peptone 10 g; yeast extract 20 g; ferric ammonium citrate 0.5 g; bacteriological agar 15 g; dichloran 2 mg (0.2% in ethanol, 1.0 ml); distilled water 1 l). One hundred microliters of spore suspension were inoculated in the center of 60 mm petri dishes and then incubated at 25°C for 48 h in the absence of light (Abriba et al. 2013; Hossain et al. 2018) as a particular growth condition for rapid identification of *Aspergillus* belonging to *A.* section *Flavi* (Pitt et al. 1983). At 48 h of culture on AFPA, the colonies of *A. flavus* and *A. parasiticus* strains appear with an orange color in the bottom of the culture, whereas the other species of *Aspergillus* section *Flavi* do not (Bothast and Fennell 1974; Hamsa and Ayres 1977; Pitt et al. 1983).

Molecular identification of fungal strains. Since the isolation and characterization by culture fail to differentiate between *A. parasiticus* and *A. flavus*, we used the molecular tools to perform this analysis.

Culture preparation. Fungal mycelia were produced by inoculating the fungal conidia with a glass Pasteur pipette in 250 ml Erlenmeyer flasks containing 150 ml Potato Dextrose Broth (PDB) and incubated for 48 hours at 25°C under orbital agitation at 150 rpm. Mycelia were harvested following filtration on 0.45 µm pore size filters and then snapped frozen into liquid nitrogen. Frozen mycelia were grounded into a fine powder using a mortar and pestle and then stored at –80°C until use.

DNA extraction. One hundred mg of powdered, frozen mycelia were transferred into a 1.5 ml microcentrifuge tube, thawed, and resuspended into 800 µl of DNA extraction buffer (100 mM TRIS-HCl (pH 7.4), 20 mM EDTA (pH 8.0), 1.4 mM NaCl, 2% sodium dodecyl sulfate) as described by (Lee et al. 2006). Subsequently, 10 µl of Proteinase K (20 mg/ml) was added, and the solution was incubated for 20 min at 60°C. The cell lysate was supplemented with 800 µl of phenol-chloroform-isoamyl alcohol (25/24/1, v/v/v) and then mixed by inversion until an emulsion was obtained. Following centrifugation for 5 min at 13,000 rpm at 20°C, the upper (aqueous) phase was transferred into a new microfuge tube. Four hundred microliters of chloroform/isoamyl alcohol (24:1) were added and mixed by inversion, and then the mixture was centrifuged for 10 min at 13,000 rpm at 4°C. The upper aqueous phase was transferred into a new microfuge tube and supplemented with 0.7 ml of cold 2-propanol for instant DNA precipitation. The precipitate was centrifuged for 10 min at 4°C, 13,000 rpm, and then the supernatant was discarded. The pellet was rinsed with 500 µl of 70% ethanol to remove residual salts. Following the last centrifugation at 13,000 rpm for 5 min at 4°C, ethanol was discarded, and the DNA pellet air dried. DNA pellet was then resuspended into 50 µl of sterile

ultrapure water. To remove the contaminant RNA, 5 µl of RNase (20 mg/ml) was added, incubated for 15 min at 37°C, and then heat-inactivated for 20 min at 70°C. The quality of DNA was checked following DNA separation by electrophoresis in a 0.8% agarose gel in TAE (40 mM Tris, 20 mM sodium acetate, and 1 mM EDTA, pH = 8.6) buffer and a high molecular weight marker. DNA concentration was measured at the optical density (OD) of 260 nm and the purity at 240 and 280 nm wavelengths using a UV spectrophotometer.

Primers. The non-coding intergenic sequences (IGS) that separate the *aflR* and *aflS(J)* genes of fungal ribosomal DNA operon were used as a target for PCR amplification to detect *A. flavus* and *A. parasiticus*. Sequences of the primers used were as follows: IGS-F, 5'-AAGGAATTCAGGAATTCTCAATTG-3' and IGS-R, 5'-GTCCACCGGCAAATCGCCG-TGCG-3' (Ehrlich et al. 2003; 2007; El Khoury et al. 2011).

PCR amplification. PCR was performed using 200 ng of a fungal DNA template (5 µl), 1 µl of each of the IGS-R and IGS-F (20 pM) primers (Eurofins Genomics, France), 25 µl of Econo Taq Plus Green 2× Master Mix (Euromedex, France), and 18 µl of sterile H_2O. The Master Mix contained: 0.1 U/µl of Econo Taq DNA polymerase – reaction buffer (pH 9.0), 400 µM each (dATP, dGTP, dCTP, and dTTP); 3 mM $MgCl_2$ and blue and yellow tracking dyes. Microtubes containing the PCR mix were transferred into a thermocycler and submitted to 34 cycles of amplification following an initial denaturation of DNA at 94°C for 5 min. Each cycle consisted of a denaturation step at 95°C for 1 min, an annealing step at 46°C for 1 min, and an extension step at 56°C for 1.5 min, with a final extension at 60°C for 10 min added to the last cycle. After amplification, the PCR products were stored at 4°C until used. PCR products were analyzed by electrophoresis separation on a 1.5 % agarose gel, and PCR-specific bands visualized by UV trans-illumination. The size of the amplicons was estimated using a 1 kb DNA ladder. The PCR products were purified using the clean-up Nucleospin kit (Machery Nagel, France), using the manufacturer's instructions.

Restriction enzyme digestion of PCR-IGS amplicons: PCR-IGS-RFLP. PCR-RFLP using *Bgl*II restriction endonuclease to digest the IGS product was shown to distinguish between *A. parasiticus* and *A. flavus* (El Khoury et al. 2011). Here we used this technique to examine whether the strains identified as *A.* section *Flavi* could be identified as *A. parasiticus* and/or *A. flavus.* The PCR-IGS amplicons were digested with the restriction endonuclease *Bgl*II (Promega, France). The reactions were performed using a total of 0.5 µg of purified PCR product in 10 µl, 2 µl of 10× *Bgl*II incubation buffer, 1 µl of *Bgl*II (10 U/µl), and 7 µl ultrapure H_2O in a total volume of 20 µl. The mixture was incubated at 37°C for 2 h, and then separated by electrophoresis in a 2% agarose gel. Restriction bands were revealed using a UV trans-illumination and a Gel-doc Image Lab system (Bio-Rad, France).

Sequencing, sequence alignment, and phylogenetic analysis. A total of 1 µg of purified PCR product of each amplicon was shipped to Eurofins Genomics (Germany) for sequencing using *IGS-F* primer. Sequence data were sent as electronic files to the lab for examination and use for bioinformatic analyses.

DNA BLAST analysis of obtained sequences was done online using the NCBI/NIH website to identify the closest sequences that match our new sequences. Multiple sequence alignment was performed using Clustal Omega software (The EMBL-EBI search and sequence analysis tools APIs in 2019). Sequences were submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov) under the reported accession numbers (Table I). Available sequences of *aflR-aflS(J)* intergenic region of reference strains were obtained from the GenBank database. The phylogenetic trees of isolates and their close relatives based on *aflR-aflS(J)* intergenic region were derived from sequence alignment using Clustal Omega.

Table I
Aspergillus flavus isolates identity based on the BLAST NCBI data.

Strain	Identity (%)
FZM1	*Aspergillus flavus* strain A9 chromosome 3 (97.48%)
FAK45	*Aspergillus flavus* strain A9 chromosome 3 (98.12%)
FSZ47	*Aspergillus flavus* strain SU-16 chromosome 3 (99.37%)
FDY50	*Aspergillus flavus* strain SU-16 chromosome 3 (99.67%)

Results and Discussion

The *flavus* group of *Aspergillus* was reported to be a significant cause of yearly economic losses reaching over 25% (Klingelhöfer et al. 2018), because of contamination of agriculture and feed components. This type of aflatoxin-producing fungi are opportunistic pathogens that contaminate corn, wheat, peanuts, and other food crops in many places of the world and causes severe diseases when ingested. Out of 30 fungal isolates, 20 were identified as *Aspergillus*, and 15 out of the 20 were characterized as *A.* section *Flavi*. Macroscopic and microscopic examinations of these 15 strains showed yellow-green colonies after growth on PDA, with spherical and rough spores/smooth to finely rough and globose conidia.

AFPA was used to identify both *A. parasiticus* and *A. flavus* (Hossain et al. 2018). Four colonies with yellow to green color with a white border surrounding the yellow to the greenish surface and an intense yellow-orange reverse color were selected as *A. flavus* and

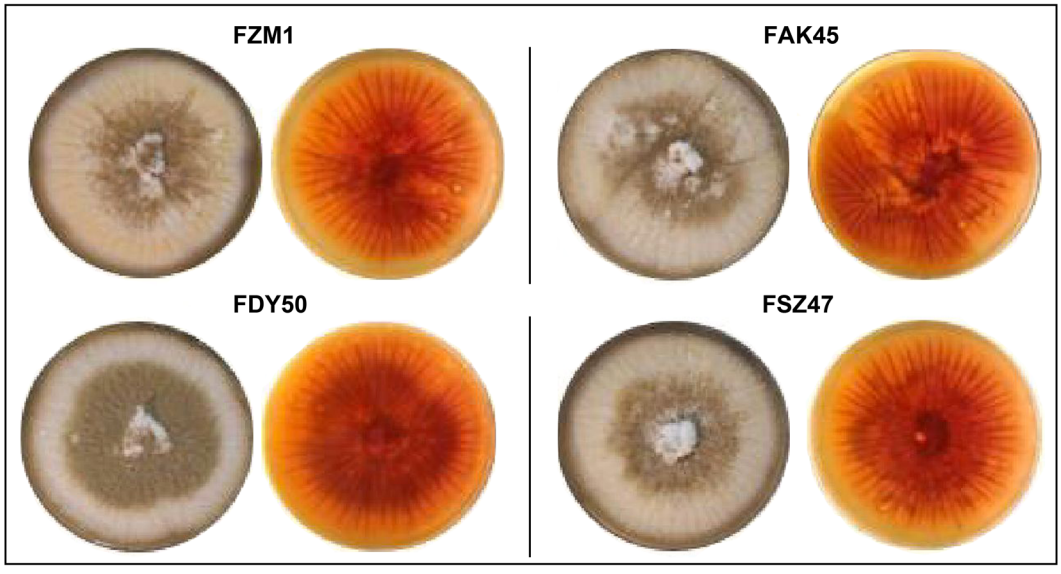

Fig. 1. Phenotypic characterization of *A. flavus* isolates grown on *A. flavus*/*parasiticus* agar (AFPA) medium. Colony color: white-green colony diameter 18 mm, colony reverse color: yellowish orange after seven days of incubation at 25°C.

A. parasiticus (Fig. 1). This orange color is due to the reaction of ferric citrate with aspergillic acid, forming a colored complex. The eleven remaining colonies produced creamy reverse color on AFPA, characteristic of *Aspergillus oryzae* species (Pitt et al. 1983; Rodrigues et al. 2009; Frisvad et al. 2019).

In previous studies, AFPA alone was used to identify *A. flavus* and *A. parasiticus* (Abriba et al. 2013; Fakruddin et al. 2015; Hossain et al. 2018; Krulj et al. 2020). However, based only on the phenotypic characteristics, it was impossible to distinguish between the two closely related species *A. flavus* and *A. parasiticus* because of overlapping morphological features (Rodrigues et al. 2009). Thus, molecular tools are necessary to differentiate these two species further. In the present study, we performed a molecular genetic characterization of the isolates selected to examine whether the four isolates could be *A. parasiticus* or *A. flavus.*

DNA samples from the four isolates were used as templates for PCR amplification using IGS-specific primers. These primers were highly specific for *aflR-aflS(J)* IGS fragments. DNA samples from all four fungal strains produced the expected 674 bp IGS amplicon with no additional or non-specific bands (Fig. 2). However, this 674 bp product was identical for both *A. flavus* and *A. parasiticus*. Therefore, we needed to examine the PCR product by RFLP further.

Previous studies indicated that PCR-IGS-RFLP using restriction enzymes that cleave the PCR products into sub-fragments are valuable tools for detecting and differentiating between *A. flavus* and *A. parasiticus* (El Khoury et al. 2011; Nikolic et al. 2018; Lavkor 2020). Therefore, we used the restriction endonuclease *Bgl*II to digest the four PCR products. Results shown in Fig. 3 clearly show that digestion of all the PCR products with *Bgl*II resulted in an identical profile yielding three fragments of 362, 210, and 102 bp. This typical profile demonstrated that all species were *A. flavus*. In contrast, *Bgl*II digestion should produce a profile with only two fragments of 311 bp and 362 bp with an IGS amplicon of *A. parasiticus* (El Khoury et al. 2011). Our analysis failed to detect this last profile among the four analyzed.

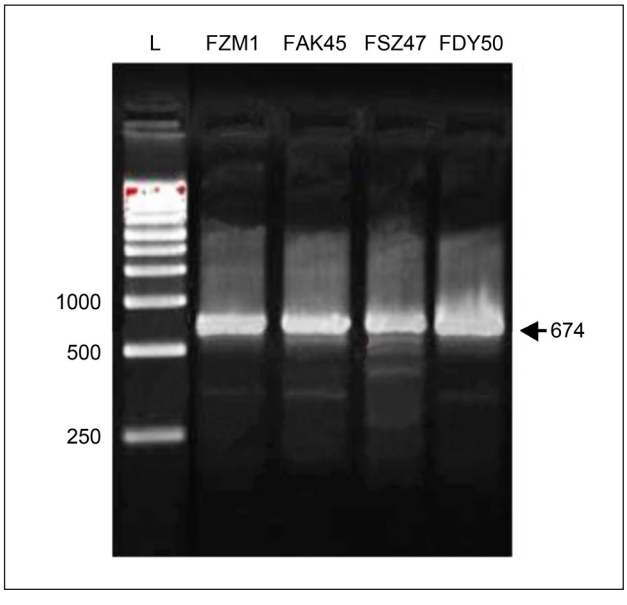

Fig. 2. PCR amplification of intergenic sequences of the four isolates. IGS-F and IGS-R primers were used for PCR amplification of the intergenic sequences in total DNA isolated from the four fungal isolates as described in Material and Methods. A total of 5 µl of each PCR product was separated in a 1.5% agarose gel by electrophoresis. Lane 1 kb ladder (L) DNA marker, *Aspergillus flavus* isolates are indicated on the top of each lane. The sizes of selected DNA fragments are indicated on the sides of the panels.

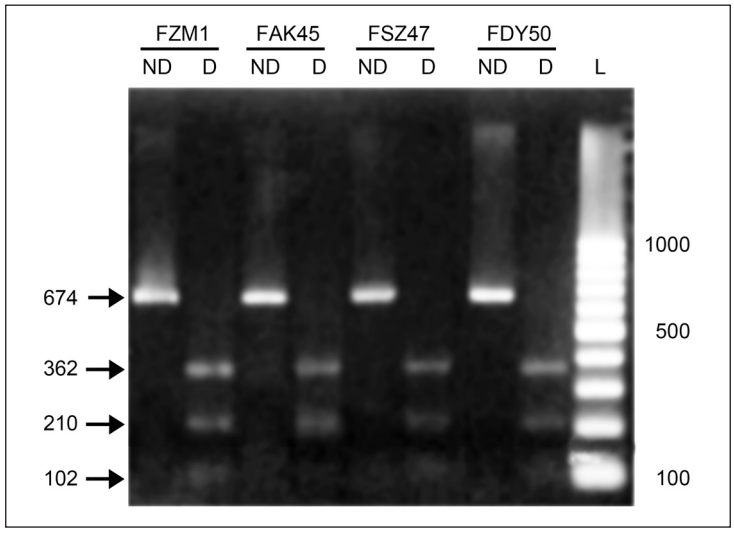

Fig. 3. PCR and RFLP analyses of fungal amplicons. A total of 5 µl of PCR products of each of the isolates (FZM1, FAK45, FSZ47 and FDY50) was digested with *Bgl*II restriction endonuclease as described in Materials and Methods. Nondigested (ND) and digested (D) products were loaded and separated by electrophoresis in a 2% agarose gel. A 100 bp DNA ladder (L) was used as molecular weight marker, the positions of 100, 500 and 1,000 bp fragments are indicated. Positions of the 362, 210 and 102 bp fragments generated by *Bgl*II digestion are indicated.

Our findings were similar to those reported by (Kana et al. 2013), which indicate that all *A. flavi* isolates recovered from all the commodities (feedstuffs and animal feed) were *A. flavus*. In contrast, other studies (Stanković et al. 2015; Nikolic et al. 2018) found the presence of *A. parasiticus* on wheat kernels and corn. This difference may result from the geographic isolation and the local atmosphere conditions (temperature, humidity), which might cause shifts in mycobiota distribution and composition of cereals observed among different regions.

Accurate identification and differentiation between this aflatoxigenic species are of great importance in determining toxicological risks due to the difference in the toxic profile of each species in the section Flavi. (Martínez-Culebras and Ramón 2007). It is well known that *A. parasiticus* produces only "B" and "G" type toxins, whereas *A. flavus* produces "B" type toxins but also cyclopiazonic acid, versicolorin, and sterigmatocystin (Wilson et al. 2002; Bailly et al. 2018). Metabolite analysis is a multistep process that requires long term culture and mycotoxin production (14 d) followed by extraction and purification for chromatographical analysis. In addition, this method requires multiple and expensive instruments to perform all these processes. In contrast, PCR-RFLP is a rapid and sensitive method requiring minimal investment and was shown to be a reliable method for taxonomic studies of *Aspergillus* species (Martínez-Culebras and Ramón 2007).

The *Aspergillus* genome is organized into eight chromosomes where the genes encoding aflatoxins are located in the 54th cluster, 80 kb from the telomere of chromosome 3 (Georgianna and Payne 2009). They include regulatory genes *aflR*, *aflS(J),* and several structural genes (Yu et al. 2004; Paterson 2006; Price et al. 2006). The *aflR* and *aflS(J)* genes are separated by a 737-bp intergenic region (Yu 2012). Sequence variability within this intergenic fragment has been used to establish the phylogenetic organization of *A. flavus* genotypes (Al-Wadai et al. 2013).

To confirm the PCR-RFLP data, suggesting that all our four isolates were *A. flavus*, we sequenced their amplicons using the IGS-F primer. The sequences were deposited in NCBI/Genbank under the following accession numbers (Table I). Sequence data were used for blast analysis against the Genbank DNA sequence database and to produce multiple sequence alignments (Fig. 4). The highest homology of the sequences of the isolates with those of sequences of the GenBank reference strains was used to define the isolates. All four isolates were identified as *A. flavus* with a similarity percentage ranging from 97.48% to 99.67% to *A. flavus* RefSeq from NCBI (Table II and III). In addition, sequence analysis confirmed the presence of the two restriction sites for *Bgl*II in the amplicon that yielded the three fragments (362, 210, and 102 bp). Thus, the *aflR-aflS(J)* IGS-RFLP assay with *Bgl*II is sufficient to detect *A. flavus* without sequencing. Therefore, this assay can be used for epidemiological studies to detect the prevalence of *A. flavus*.

Furthermore, this sequence analysis showed variation among the *A. flavus* isolates. This variation included single and multiple substitutions, insertions, inversions, and deletions. Sequence comparison of FSZ47 and FDY50 isolates with that of the reference strain SU 16 showed the highest sequence similarity (99,37%,

```
          1         10         20         30         40         50         60
SU-16     CGCTGAGAAT ACGGGTGATC TGAAGAGGTT TTAGATCTGA CCAGTGTAGT CCTTCCTCTG
FZM1      --C------- ---------- ---------- ---------- ---------T ----------
FDY50     --T------- ---------- ---------- ---------- ---------T ----------
FSZ47     --C------- ---------- ---------- ---------- ---------T ----------
FAK45     --C------- ---------- ---------- ---------- ---------C ----------
A9        --C------- ---------- ---------- ---------- ---------T ----------
K54A      --C------- ---------- ---------- ---------- ---------T ----------
Tox4      --C------- ---------- ---------- ---------- ---------T ----------

                   70         80         90        100        110        120
SU-16     CGTCAAGGAG TGCTGATCTG CAAGCCGGGT ACCATCTGCC GGCTCGTACT TTTTATCTTT
FZM1      ------C--- -------A-- ---------- --C------- --T------- ----------
FDY50     ------G--- -------C-- ---------- --C------- --C------- ----------
FSZ47     ------G--- -------C-- ---------- --C------- --C------- ----------
FAK45     ------C--- -------A-- ---------- --T------- --T------- ----------
A9        ------C--- -------A-- ---------- --T------- --C------- ----------
K54A      ------C--- -------A-- ---------- --T------- --C------- ----------
Tox4      ------C--- -------A-- ---------- --T------- --C------- ----------

                  130        140        150        160        170        180
SU-16     CGTCAGCATC GTTAGCCAGG CAGGCATATC TATGTCCCAT .TCTTAGAAT AGCTTCGCAG
FZM1      ---------- ---------- ---------- ---------- G--------- ----------
FDY50     ---------- ---------- ---------- ---------- .--------- ----------
FSZ47     ---------- ---------- ---------- ---------- .--------- ----------
FAK45     ---------- ---------- ---------- ---------- .--------- ----------
A9        ---------- ---------- ---------- ---------- .--------- ----------
K54A      ---------- ---------- ---------- ---------- .--------- ----------
Tox4      ---------- ---------- ---------- ---------- .--------- ----------

                  190        200        110        120        230        240
SU-16     GGTGGTATCT CAACACTGCA ACGGGACGGA TCCAGGGCTC CCTGGAGCTC ATGCAGGTGC
FZM1      ---------- ---------- ---------- ---------- ---------- ----------
FDY50     ---------- ---------- ---------- ---------- ---------- ----------
FSZ47     ---------- ---------- ---------- ---------- ---------- ----------
FAK45     ---------- ---------- ---------- ---------- ---------- ----------
A9        ---------- ---------- ---------- ---------- ---------- ----------
K54A      ---------- ---------- ---------- ---------- ---------- ----------
Tox4      ---------- ---------- ---------- ---------- ---------- ----------

                  250        260        270        280        290        300
SU-16     TAAAGATCTA GCTTGCAGGA AACAAGTCTT TTCTGGGTTC TCAGCCCGCC CATGACGGAC
FZM1      ---------- ---------- ---------- ---------- -C-------- ----------
FDY50     ---------- ---------- ---------- ---------- -C-------- ----------
FSZ47     ---------- ---------- ---------- ---------- -C-------- ----------
FAK45     ---------- ---------- ---------- ---------- -C-------- ----------

A9        ---------- ---------- ---------- ---------- -A-------- ----------
K54A      ---------- ---------- ---------- ---------- -A-------- ----------
Tox4      ---------- ---------- ---------- ---------- -A-------- ----------
```

```
                310        320        330        340        350        360
SU-16     TACGTTATCT TGAGCCCGAG GCATGCATGC AGGCGGGCCA GCAAGCTGAA CATTATTTGT
FZM1      ---------- ---------- ---------- ---------- --T------- -----T----
FDY50     ---------- ---------- ---------- ---------- --A------- -----T----
FSZ47     ---------- ---------- ---------- ---------- --A------- -----T----
FAK45     ---------- ---------- ---------- ---------- --T------- -----C----
A9        ---------- ---------- ---------- ---------- --T------- -----C----
K54A      ---------- ---------- ---------- ---------- --T------- -----C----
Tox4      ---------- ---------- ---------- ---------- --T------- -----C----

                370        380        390        400        410        420
SU-16     TGCTCTTGGT TCGCTTCGTT AAACCGATAA CGCAGTTCTC TGGTCACCCG GTTTCAGCCT
FZM1      --G------- -T-------C ----T---C- ---------- ---------- ---T------
FDY50     --C------- -C-------T ----C---A- ---------- ---------- ---T------
FSZ47     --C------- -C-------T ----C---A- ---------- ---------- ---T------
FAK45     --G------- -T-------C ----C---C- ---------- ---------- ---C------
A9        --G------- -T-------T ----C---C- ---------- ---------- ---T------
K54A      --G------- -T-------T ----C---C- ---------- ---------- ---T------
Tox4      --G------- -T-------T ----C---C- ---------- ---------- ---T------

                430        440        450        460        470        480
SU-16     CGGTACGTAA ACAAGGAACG CACAGCTAGA CAATCCTTGG GCCAAGTCAG AACCCCTC..
FZM1      ---------- ---------- ---------- ---------- ---------- ----G-CTAC
FDY50     ---------- ---------- ---------- ---------- ---------- ----C-TC..
FSZ47     ---------- ---------- ---------- ---------- ---------- ----C-TC..
FAK45     ---------- ---------- ---------- ---------- ---------- ----C-TC..
A9        ---------- ---------- ---------- ---------- ---------- ----C-TC..
K54A      ---------- ---------- ---------- ---------- ---------- ----C-TC..
Tox4      ---------- ---------- ---------- ---------- ---------- ----C-TC..

                490        500        510        520        530        540
SU-16     .AGCTGGTGA CAGGAGTGTA CATACATTTA GGTCTAAGTG CGAGGCAACG AAAAGGGTGG
FZM1      AGT------- ---------- -------C-- -------T-- ---------- -------T--
FDY50     .AG------- ---------- -------T-- -------G-- ---------- -------T--
FSZ47     .AG------- ---------- -------T-- -------G-- ---------- -------T--
FAK45     .AG------- ---------- -------T-- -------G-- ---------- -------C--
A9        .AG------- ---------- -------T-- -------G-- ---------- -------C--
K54A      .AG------- ---------- -------T-- -------G-- ---------- -------C--
Tox4      .AG------- ---------- -------T-- -------G-- ---------- -------C-

                550
SU-16     GCTACTCTCC CGGAGA
FZM1      ---T------ ------
FDY50     ---A------ ------
FSZ47     ---A------ ------
FAK45     ---A------ ------
A9        ---A------ ------
K54A      ---A------ ------
Tox4      ---A------ ------
```

Fig. 4. Nucleotide sequence alignment of the four isolates with four reference strains. The names of the four isolates are in green. Nucleotide sequence alignment was performed using Clustal software (–) represent similarity, (.) represent deletion. Nucleotide changes are shown in bold font.

Table II
Genbank accession numbers of the *Aspergillus flavus* isolates.

Isolate	Accession number	Strain
FZM1	OL944584.1	*A. flavus* strain FZM1 aflR-aflJ intergenic region, partial sequence
FAK45	OL944586.1	*A. flavus* strain FAK45 aflR-aflJ intergenic region, partial sequence
FSZ47	OL944587.1	*A. flavus* strain FSZ47 aflR-aflJ intergenic region, partial sequence
FDY50	OL944588.1	*A. flavus* strain FDY50 aflR-aflJ intergenic region, partial sequence

Table III
Genbank accession numbers
of the *Aspergillus flavus* reference strains.

Accession number	Strain
CP051037.1	*A. flavus* strain A9 chromosome 3
CP051085.1	*A. flavus* strain K54A chromosome 3
CP051045.1	*A. flavus* strain Tox4 chromosome 3
CP047251.1	*A. flavus* strain SU-16 chromosome 3

and 99.67%, respectively). Since the reference strains A9, K54A, and Tox4 are 100% similar in the studied segment, their similarity with FZM1 and FAK45 isolates was found to be 97.48% and 98.12% respectively. Therefore, the highest divergence compared to the existing reference strains was seen for the sequences of the FZM1 isolate. A total of 13 positions of divergence involving single nucleotide polymorphisms (SNPs) as well as double and multiple nucleotide polymorphisms (MNPs) were observed (Fig. 4). In contrast, only minor variations were seen in FDY50 isolate corresponding to SNPs at the positions 3, 5 and 28 of the sequence. On the opposite side, the isolate FAK45 showed the highest genetic similarity with *A. flavus* reference strains A9, K54A, and Tox4 with only minor variations at the positions 50 and 103. Most of the observed polymorphisms were nucleotide substitutions, with only 10% being insertions and inversions, as shown in Table IV. Nucleotide deletions and SNPs in AF coding genes are often associated with the inability of *A. flavus* to produce aflatoxins. Deletion strains of *A. flavus* were reported in many studies (Chang et al. 2005; Donner et al. 2010; Adhikari et al. 2016; Hua et al. 2018).

However, not all AF non-producing strains have deletions in the AF biosynthesis gene cluster. The strain NRRL 30797 known as a biocontrol agent has a few substitutions in the aflatoxin biosynthesis gene cluster (Chang et al. 2012). This genetic variability may complicate the diagnosis and, therefore, the control of *A. flavus* infection and aflatoxin contamination of food and feed. Detecting particular strains of *A. flavus*, such as NRRL 30797, requires sequencing or a special PCR-RFLP capable of detecting the single substitutions. In the present study, the IGS region has been used as a target for PCR and sequencing for phylogenetic analysis. The selection of this region was motivated by the sequence variability between different species but only minor variations within isolates of the same species. The phylogenetic tree of isolates and their close relatives based on the *aflR-aflS(J)* intergenic region was constructed using Clustal Omega (Okoth et al. 2018) to illustrate

Table IV
Nucleotide variations in *aflR-aflS(J)* intergenic region sequences of *Aspergillus flavus* isolates.

Nucleotide position	Nucleotide variation	Isolate
103, 385, 478	C with T (substitution)	FZM1: *Aspergillus flavus* strain FZM1 *aflR-aflS(J)* intergenic region, partial sequence
161	Insertion of G	
380, 477, 508	T with C (substitution)	
475	C with G (inversion)	
479–481	Insertion of ACA	
482	A with G (substitution)	
483	G with T (substitution)	
544	A with T (inversion)	
50	T with C (substitution)	FAK45: *Aspergillus flavus* strain *FAK45 aflR-aflS(J)* intergenic region, partial sequence
103	C with T (substitution)	
3	C with T (substitution)	FDY50: *Aspergillus flavus* strain *aflR-aflS(J)* intergenic region, partial sequence
5, 28	G with A (substitution)	

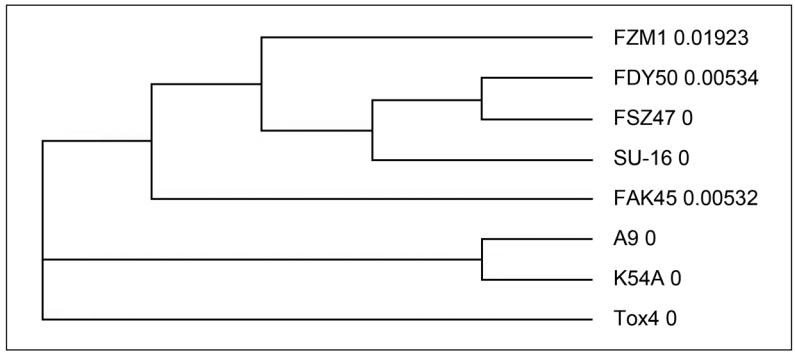

Fig. 5. Maximum Likelihood phylogenic tree showing the relationships between the examined *A. flavus* isolates and reference strains, based on *aflR/aflS(J)* intergenic sequence.

the relationships of isolates among *A. flavus* species. The phylogenetic tree obtained clearly shows that the *A. flavus* strains clustered into three major clades. In addition, several sub-clades indicate a wide variation among *A. flavus* species (Fig. 5). FSZ47 and FDY50 isolates were clustered in one clade and were found to be closely related to *A. flavus* strain SU 16 forming a mixed cluster. In contrast, FAK45 and FZM1 isolates were found to form distinct sub-clades. Variations exist among *A. flavus* isolates; those with SNPs share a closer relationship clustered with *A. flavus* strain SU16; while the isolate FAK45 clustered with a distinct sub-clade. These data agree with the previous finding reported by different groups (Krimitzas et al. 2013; Chiba et al. 2014). Recently, new direct detection methods of *A. flavus* through AF-B1 and Zearalenone by Raman spectrometry (Yang et al. 2021) or AF-B1 alone using a ortable Raman spectrometer combined with colloidal Au nanoparticles (Wang et al. 2022) were reported. This latter rapid technique provides quantitative measurement of AF-B1 in grain crops.

Conclusions

The four diagnosis methods (phenotyping, PCR, PCR-RFLP, and amplicon sequencing) have shown different sensitivity levels of fungal characterization. Phenotypic tools were limited to *A.* section *Flavi* identification; PCR alone failed to distinguish *A. parasiticus* from *A. flavus*; PCR-RFLP in this study was able to detect *A. flavus* only. However, it failed to distinguish between different strains of *A. flavus*. Sequencing and sequence analysis was able to distinguish between the *A. flavus* strains. These data established the selection criteria of the methods to use depending on the segregation level of fungal strains. New methods based on Raman spectrometry to measure AF-B1 alone or in combination with other fungal components might provide additional help for the detection of the pathogenic *A. flavus* in grain crops.

Acknowledgements

This work was supported by the UTHB Algeria and both UGA and INRAE (PAVAL Lab.) in France.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Abriba C, Lennox JA, Asikong BE, Asitok A, Ikpoh IS, Henshaw EE, Eja ME. Isolation of aflatoxin producing species of *Aspergillus* from foodstuffs sold in Calabar markets, Cross River state, Nigeria. J Microbiol Biotechnol Res. 2013;3:8–13.

Adhikari BN, Bandyopadhyay R, Cotty PJ. Degeneration of aflatoxin gene clusters in *Aspergillus flavus* from Africa and North America. AMB Express. 2016 Dec;6(1):62. https://doi.org/10.1186/s13568-016-0228-6

Ahmad MM, Ahmad M, Ali A, Hamid R, Javed S, Abdin MZ. Molecular methods for detecting pathogenic fungi. In: Gupta VK, Tuohy M, Gaur RK, editors. Fungal biochemistry and biotechnology. Saarbrücken (Germany): Lambert; 2010. p. 154–169.

Al-Wadai AS, Al-Othman MR, Mahmoud MA, Abd El-Aziz ARM. Molecular characterization of *Aspergillus flavus* and aflatoxin contamination of wheat grains from Saudi Arabia. Genet Mol Res. 2013; 12(3):3335–3352. https://doi.org/10.4238/2013.September.3.10

Bagyalakshmi R, Lily Therese K, Madhavan HN. Nucleotide polymorphisms associated with Internal Transcribed Spacer (ITS) regions of ocular isolates of *Aspergillus flavus*. J Microbiol Methods. 2007 Jan;68(1):1–10. https://doi.org/10.1016/j.mimet.2006.05.021

Bailly S, Mahgubi A, Carvajal-Campos A, Lorber S, Puel O, Oswald I, Bailly JD, Orlando B. Occurrence and identification of *Aspergillus* section *Flavi* in the context of the emergence of aflatoxins in French maize. Toxins (Basel). 2018 Dec 07;10(12):525. https://doi.org/10.3390/toxins10120525

Bothast RJ, Fennell DI. A medium for rapid identification and enumeration of *Aspergillus flavus* and related organisms. Mycologia. 1974 Mar;66(2):365–369. https://doi.org/10.1080/00275514.1974.12019615

Chang PK, Abbas HK, Weaver MA, Ehrlich KC, Scharfenstein LL, Cotty PJ. Identification of genetic defects in the atoxigenic biocontrol strain *Aspergillus flavus* K49 reveals the presence of a competitive recombinant group in field populations. Int J Food Microbiol. 2012 Mar;154(3):192–196. https://doi.org/10.1016/j.ijfoodmicro.2012.01.005

Chang PK, Horn BW, Dorner JW. Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. Fungal Genet Biol. 2005 Nov;42(11): 914–923. https://doi.org/10.1016/j.fgb.2005.07.004

Chiba T, Takahashi Y, Sadamasu K, Nakama A, Kai A. [Discrimination of *Aspergillus flavus* group fungi using phylogenetic tree analysis and multiplex PCR] (in Japanese). Food Hyg Saf Sci (Shokuhin Eiseigaku Zasshi). 2014;55(3):135–141. https://doi.org/10.3358/shokueishi.55.135

Daghriri SH, El-Bebany AF, Alrumman SA, Hesham A. Molecular characterisation and biological control of *Aspergillus flavus* isolates from Saudi Arabia. Arch. Phytopathol Plant Prot. 2018;51(9–10): 445–460. https://doi.org/10.1080/03235408.2018.1480262

Donner M, Atehnkeng J, Sikora RA, Bandyopadhyay R, Cotty PJ. Molecular characterization of atoxigenic strains for biological control of aflatoxins in Nigeria. Food Addit Contam, Part A. 2010 May; 27(5):576–590. https://doi.org/10.1080/19440040903551954

Ehrlich K, Montalbano BG, Cotty PJ. Sequence comparison of *afl*R from different *Aspergillus* species provides evidence for variability in regulation of aflatoxin production. Fungal Genet Biol. 2003 Feb; 38(1):63–74. https://doi.org/10.1016/S1087-1845(02)00509-1

Ehrlich KC, Kobbeman K, Montalbano BG, Cotty PJ. Aflatoxin-producing *Aspergillus* species from Thailand. Int J Food Microbiol. 2007 Mar;114(2):153–159. https://doi.org/10.1016/j.ijfoodmicro.2006.08.007

El Khoury A, Atoui A, Rizk T, Lteif R, Kallassy M, Lebrihi A. Differentiation between *Aspergillus flavus* and *Aspergillus parasiticus* from pure culture and aflatoxin-contaminated grapes using PCR-RFLP analysis of *aflR-aflJ* intergenic spacer. J Food Sci. 2011 May; 76(4):M247–M253. https://doi.org/10.1111/j.1750-3841.2011.02153.x

Fakruddin M, Chowdhury A, Hossain MN, Ahmed MM. Characterization of aflatoxin producing *Aspergillus flavus* from food and feed samples. SpringerPlus. 2015 Dec;4(1):159. https://doi.org/10.1186/s40064-015-0947-1

Frisvad JC, Hubka V, Ezekiel CN, Hong SB, Nováková A, Chen AJ, Arzanlou M, Larsen TO, Sklenář F, Mahakarnchanakul W, et al. Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. Stud Mycol. 2019 Jun 01; 93(1):1–63. https://doi.org/10.1016/j.simyco.2018.06.001

Gbashi S, Madala NE, De Saeger S, De Boevre M, Adekoya I, Adebo OA, Njobeh PB. The socio-economic impact of mycotoxin contamination in Africa. In: Njobeh PB, Stepman F, editors. Mycotoxins – impact and management strategies. London (UK): IntechOpen; 2018. https://doi.org/10.5772/intechopen.79328

Georgianna DR, Payne GA. Genetic regulation of aflatoxin biosynthesis: from gene to genome. Fungal Genet Biol. 2009 Feb;46(2): 113–125. https://doi.org/10.1016/j.fgb.2008.10.011

Godet M, Munaut F. Molecular strategy for identification in *Aspergillus* section *Flavi*. FEMS Microbiol Lett. 2010 Mar;304(2):157–168. https://doi.org/10.1111/j.1574-6968.2009.01890.x

Hamsa TAP, Ayres JC. A differential medium for the isolation of *Aspergillus flavus* from cottonseed. J Food Sci. 1977 Mar;42(2):449–453. https://doi.org/10.1111/j.1365-2621.1977.tb01520.x

Healy M, Reece K, Walton D, Huong J, Shah K, Kontoyiannis DP. Identification to the species level and differentiation between strains of *Aspergillus* clinical isolates by automated repetitive-sequence-based PCR. J Clin Microbiol. 2004 Sep;42(9):4016–4024. https://doi.org/10.1128/JCM.42.9.4016-4024.2004

Hossain MN, Talukder A, Afroze F, Rahim MM, Begum S, Haque MZ, Ahmed MM. Identification of aflatoxigenic fungi and detection of their aflatoxin in red chilli (*Capsicum annuum*) samples using direct cultural method and HPLC. Adv Microbiol. 2018; 08(01):42–53. https://doi.org/10.4236/aim.2018.81004

Hua SST, Palumbo JD, Parfitt DE, Sarreal SBL, O'Keeffe TL. Development of a droplet digital PCR assay for population analysis of aflatoxigenic and atoxigenic *Aspergillus flavus* mixtures in soil. Mycotoxin Res. 2018 Aug;34(3):187–194. https://doi.org/10.1007/s12550-018-0313-6

Ibrahim N. Molecular identification and evaluation of genetic variability among some aflatoxigenic *Aspergillus flavus* strains isolated from fish feed in Egypt. Egypt J Microbiol. 2019;54(1):103–115. https://doi.org/10.21608/ejm.2019.16611.1114

Kana JR, Joselin Gnonlonfin BG, Harvey J, Wainaina J, Wanjuki I, Robert A, Skilton RA, Teguia A. Mycobiota and toxigenecity profile of *Aspergillus flavus* recovered from food and poultry feed mixtures in Cameroon. J Animal Poult Sci. 2013;2(4):98–107.

Kim HJ, Lee MJ, Kim HJ, Cho SK, Park HJ, Jeong MH. Analytical method development and monitoring of aflatoxin B1, B2, G1, G2 and ochratoxin A in animal feed using HPLC with fluorescence detector and photochemical reaction device. Cogent Food Agric. 2017 Jan 01;3(1):1419788. https://doi.org/10.1080/23311932.2017.1419788

Klingelhöfer D, Zhu Y, Braun M, Bendels MHK, Brüggmann D, Groneberg DA. Aflatoxin – publication analysis of a global health threat. Food Control. 2018 Jul;89:280–290. https://doi.org/10.1016/j.foodcont.2018.02.017

Krimitzas A, Pyrri I, Kouvelis VN, Kapsanaki-Gotsi E, Typas MA. A phylogenetic analysis of Greek isolates of *Aspergillus* species based on morphology and nuclear and mitochondrial gene sequences. BioMed Res Int. 2013;2013:260395. https://doi.org/10.1155/2013/260395

Krulj J, Ćurčić N, Stančić AB, Kojić J, Pezo L, Tukuljac LP, Solarov MB. Molecular identification and characterisation of *Aspergillus Flavus* isolates originating from Serbian wheat grains. Acta Aliment. 2020 Nov 07;49(4):382–389. https://doi.org/10.1556/066.2020.49.4.3

Lavkor I. Quantitative detection of aflatoxin and species identification of *Aspergillus* section *Flavi* isolates from peanuts using molecular approaches. Legume Res. 2020;43(2):195–199. https://doi.org/10.18805/LR-530

Lee CZ, Liou GY, Yuan GF. Comparison of the *aflR* gene sequences of strains in *Aspergillus* section *Flavi*. Microbiology. 2006 Jan 01;152(1):161–170. https://doi.org/10.1099/mic.0.27618-0

Levy L, Castlebury LA, Carris LM, Meyer RJ, Pimentel G. Internal transcribed spacer sequence-based phylogeny and polymerase chain reaction-restriction fragment length polymorphism differentiation of *Tilletia walkeri* and *T. indica*. Phytopathology. 2001 Oct; 91(10):935–940. https://doi.org/10.1094/PHYTO.2001.91.10.935

Liao J, He Z, Xia Y, Lei Y, Liao B. A review on biosynthesis and genetic regulation of aflatoxin production by major *Aspergillus* fungi. Oil Crop Sci. 2020 Oct;5(4):166–173. https://doi.org/10.1016/j.ocsci.2020.11.001

Martínez-Culebras PV, Ramón D. An ITS-RFLP method to identify black *Aspergillus* isolates responsible for OTA contamination in grapes and wine. Int J Food Microbiol. 2007 Jan;113(2):147–153. https://doi.org/10.1016/j.ijfoodmicro.2006.06.023

Martinez-Miranda MM, Rosero-Moreano M, Taborda-Ocampo G. Occurrence, dietary exposure and risk assessment of aflatoxins in arepa, bread and rice. Food Control. 2019 Apr;98:359–366. https://doi.org/10.1016/j.foodcont.2018.11.046

Mohankumar M, Vijayasamundeeswari A, Karthikeyan M, Mathiyazhagan S, Paranidharan V, Velazhahan R. Analysis of molecular variability among isolates of *Aspergillus flavus* by PCR-RFLP of the ITS regions of rDNA. J Plant Prot Res. 2010 Jan 1;50(4): 446–451. https://doi.org/10.2478/v10045-010-0075-4

Nicholson P, Simpson DR, Weston G, Rezanoor HN, Lees AK, Parry DW, Joyce D. Detection and quantification of *Fusarium cul-*

morum and *Fusarium graminearumin* cereals using PCR assays. Physiol Mol Plant Pathol. 1998 Jul;53(1):17–37. https://doi.org/10.1006/pmpp.1998.0170

Nikolic M, Nikolic A, Jaukovic M, Savic I, Petrovic T, Bagi F, Stankovic S. Differentiation between *Aspergillus flavus* and *Aspergillus parasiticus* isolates originated from wheat. Genetika. 2018;50(1): 143–152. https://doi.org/10.2298/GENSR1801143N

Okoth S, De Boevre M, Vidal A, Diana Di Mavungu J, Landschoot S, Kyallo M, Njuguna J, Harvey J, De Saeger S. Genetic and toxigenic variability within *Aspergillus flavus* population isolated from maize in two diverse environments in Kenya. Front Microbiol. 2018 Jan 26;9:57. https://doi.org/10.3389/fmicb.2018.00057

Paterson RRM. Identification and quantification of mycotoxigenic fungi by PCR. Process Biochem. 2006 Jul;41(7):1467–1474. https://doi.org/10.1016/j.procbio.2006.02.019

Pitt JI, Hocking AD, Glenn DR. An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. J Appl Bacteriol. 1983 Feb; 54(1):109–114. https://doi.org/10.1111/j.1365-2672.1983.tb01307.x

Pitt JI, Hocking AD. Fungi and food spoilage. New York (USA): Springer New York; 2009. https://doi.org/10.1007/978-0-387-92207-2

Price MS, Yu J, Nierman WC, Kim HS, Pritchard B, Jacobus CA, Bhatnagar D, Cleveland TE, Payne GA. The aflatoxin pathway regulator AflR induces gene transcription inside and outside of the aflatoxin biosynthetic cluster. FEMS Microbiol Lett. 2006 Feb; 255(2):275–279. https://doi.org/10.1111/j.1574-6968.2005.00084.x

Rao KR, Vipin AV, Venkateswaran G. Molecular profile of non-aflatoxigenic phenotype in native strains of *Aspergillus flavus*. Arch Microbiol. 2020 Jul;202(5):1143–1155. https://doi.org/10.1007/s00203-020-01822-1

Rodrigues P, Venâncio A, Kozakiewicz Z, Lima N. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi* isolated from Portuguese almonds. Int J Food Microbiol. 2009 Feb;129(2):187–193. https://doi.org/10.1016/j.ijfoodmicro.2008.11.023

Singh P, Orbach M, Cotty P. *Aspergillus texensis*: a novel aflatoxin producer with S morphology from the United States. Toxins (Basel). 2018 Dec 03;10(12):513. https://doi.org/10.3390/toxins10120513

Stanković S, Lević J, Nikolić M, Krnjaja V, Jauković M, Nalaz P. Prvi Nalaz *Aspergillus parasiticus* u Proizvodnji Kukuruza u Srbiji. Paper presented at: Proceedings of the XIII Savetovanje o Zaštiti Bilja; 2015 October 23–27; Zlatibor, Serbia. p. 32–33.

Wang H, Liu M, Zhang Y, Zhao H, Lu W, Lin T, Zhang P, Zheng D. Rapid detection of *Aspergillus flavus* and quantitative determination of aflatoxin B_1 in grain crops using a portable Raman spectrometer combined with colloidal Au nanoparticles. Molecules. 2022 Aug 18; 27(16):5280. https://doi.org/10.3390/molecules27165280

Wilson DM, Mubatanhema W, Jurjević Z. Biology and ecology of mycotoxigenic *Aspergillus* species as related to economic and health concerns. In: DeVries JW, Trucksess MW, Jackson LS, editors. Mycotoxins and food safety. Advances in experimental medicine and biology. Boston (USA): Springer; 2002(504). p. 3–17. https://doi.org/10.1007/978-1-4615-0629-4_2

Yang XQ, Yu HC, Yin Y, Yuan YX, Wu H, Li H. [Detection of aflatoxin B_1 and zearalenone in maize by Raman spectroscopy] (in Chinese). J Nucl Agric Sci. 2021;35(1):159–166. https://doi.org/10.11869/j.issn.100-8551.2021.01.0159

Yin Y, Lou T, Yan L, Michailides TJ, Ma Z. Molecular characterization of toxigenic and atoxigenic *Aspergillus flavus* isolates, collected from peanut fields in China. J Appl Microbiol. 2009 Dec;107(6):1857–1865. https://doi.org/10.1111/j.1365-2672.2009.04356.x

Yu J, Chang PK, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP, Bennett JW. Clustered pathway genes in aflatoxin biosynthesis. Appl Environ Microbiol. 2004 Mar;70(3):1253–1262. https://doi.org/10.1128/AEM.70.3.1253-1262.2004

Yu J. Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. Toxins (Basel). 2012 Oct 25;4(11):1024–1057. https://doi.org/10.3390/toxins4111024

Zarrin M, Erfaninejad M. Molecular variation analysis of *Aspergillus flavus* using polymerase chain reaction-restriction fragment length polymorphism of the internal transcribed spacer rDNA region. Exp Ther Med. 2016 Sep;12(3):1628–1632. https://doi.org/10.3892/etm.2016.3479