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Quantitative Detection of Aflatoxin and Species Identification of *Aspergillus* section *Flavi* Isolates from Peanuts using Molecular Approaches

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ABSTRACT

Totally, 50 *Aspergillus* section *Flavi* were identified isolates having aflatoxin biosynthesis genes on peanut by molecular method and aflatoxin production. Primer pair (IGS-F/R) recognized the aflatoxin biosynthesis gene (*aflJ-aflR*) targeting the intergenic region (IGS) on DNA was amplified by polymerase chain reactions (PCR). The PCR product were restricted by *Bgl*II enzyme within Restriction Fragment Length Polymorphism (RFLP) and obtained from 33 (66%) *Aspergillus flavus* was cleaved into three band sizes of 362, 210 and 102 bp. However, *Bgl*II enzyme generated two band sizes of 363 and 311 bp for 17 (34%) *Aspergillus parasiticus*. An investigation examined DNA sequence data to characterize these isolates and describe the species. Phylogenetic analysis showed that *A. flavus* and *A. parasiticus* have been identified in different groups. All the *A. flavus* and *A. parasiticus* isolates produced aflatoxins. The present study provides a new method on molecular characterization of *A.* section *Flavi* in Turkey.

Key words: Aflatoxin, *Aspergillus flavus, Aspergillus parasiticus,* Molecular approach, Peanut, Phylogenetic analysis.

INTRODUCTION

Aspergillus spp. particularly, *Aspergillus flavus* and *Aspergillus parasiticus* produced aflatoxins (Lavkor, 2019). The identification of *A. flavus* and *A. parasiticus* as morphologically closely related is based on morphological characters, therefore the fungi isolates may have been incorrectly identified. The morphology-based method is reported to be insufficient for determining the aflatoxigenic *Aspergillus* to distinguish between aflatoxigenic and non-aflatoxigenic isolate of *Aspergillus* species, which have been focused on molecular methods based on aflatoxin biosynthesis genes (Khoury *et al.,* 2011). Moreover, PCR-based methods are used on aflatoxin biosynthetic genes to identify aflatoxigenic fungi (Gonzalez-Salgado *et al.,* 2008). PCR-RFLP is widely used to characterise the aflatoxin biosynthetic genes in *Aspergillus* species (Khoury *et al.,* 2011). *A.* section *Flavi* have more than 20 genes involved in aflatoxin biosynthesis (Heperkan *et al.*, 2012). The *aflR* and *aflS* (*aflJ)* are regulatory genes in aflatoxin biosynthesis (Georgianna and Payne, 2009).

Different molecular techniques are used to identify *Aspergillus* species that have aflatoxin biosynthesis genes (Ehrlich *et al*., 2007). Kesmen *et al.* (2014) reported that 66 *A. flavus* isolated from different products were detected using specific primer pairs for structural (*pks* 1) genes by PCR and they reported that this pair of primers could be used successfully in detecting aflatoxigenic *A. flavus*. In a study conducted in India, *A. flavus* isolated in peanut was performed by PCR targeting the *aflR*1 and *aflR*2 genes. As a result of the analysis, positive results were obtained from the PCR products (Manonmani *et al*., 2005). Midorikawa *et al.* (2014) reported that identification of 137 *A.* section *Flavi* isolates from nutshell material in the Amazon region of Brazil were amplified with rDNA ITS, β-tubulin and calmodulin gene regions by PCR. After the amplification, PCR product digested with *Dra*I enzyme and *A.* section *Flavi* was defined as *A. flavus, A. tamarii* and *A. nomius* by PCR-RFLP. This result indicated that PCR-RFLP was defined as *A. flavus, A. nomius* and *A. tamarii* having aflatoxin biosynthesis genes.

In this study, modify molecular method approach was used for the first time for identification of *Aspergillus* section *Flavi* isolated from peanut samples during harvest and storage periods in Adana and Osmaniye. An investigation examined DNA sequence data to characterize 50 different fungal isolates and describe the species. The aim of this study was to to detect aflatoxigenic *A. flavus* and *A. parasiticus* isolates.

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MATERIALS AND METHODS

Aspergillus section *Flavi* isolates and culture conditions

A total of 50 *Aspergillus* section *Flavi* were isolated from peanut samples during harvest and storage periods in Adana and Osmaniye, Turkey. A. section *Flavi* fungal cultures were

maintained on patato dextrose agar (PDA) and incubated at 25°C for 5 days. In order to obtain young mycelium for DNA extraction, fungal isolates were transferred in petri dishes containing 20 ml of modified PDA (For 200 ml: 8 g glicose, 75 g patato, 3 g agar) and incubated at 25°C overnight.

DNA extractions

DNA extractions were performed using some modifications according to the method described by Doyle and Doyle (1987). According to method, the young fungi mycelium was crushed using mortar and pestle with liquid nitrogen solution. The powder mycelium was collected in a 2 ml centrifuge tubes and incubated with 1 ml extraction buffer [2% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) β-mercaptoethanol, 0.1 M Tris/HCl, 20 mM EDTA] for 60 min at 65°C on a thermomixer. Then 1000 µl chloroform/isoamyl alcohol (24:1) added to tubes and centrifuged at 13000 rpm for 15 min 600 µl of the supernatant was transferred into 1.5 ml centrifuge tube, adding 600 µl of cold 2-propanol. Samples were then incubated for 2 h at –20°C and centrifuged for 10 min at 10000 rpm and then the supernatant was discarded. The pellet was washed with 1 ml of 70% ethanol and centrifuged for 10 min at 11000 rpm. Then supernatant discarded. Pellet was air dried for 1-2 hours then dissolved in 100 µl of sterile H_2O. Quantify the DNA samples by measuring the absorbance of the sample using a Nanodrop.

PCR amplification

According to Khoury *et al.* (2011), primer pair IGS-F/R was designed to target the intergenic spacer (IGS) for aflatoxin biosynthesis genes, *aflR-aflJ* (Ehrlich *et al*., 2003, 2007); that corresponded to PCR product of 674 bp. The primers' sequences were as follows: IGS-F, 5′ -AAGGAATTCAGGAA TTCTCAATTG-3′; IGS-R, 5′ -TCCACCGGCAAATCGCC GTGCG-3′. The β-tubulin gene was amplified to a 340 bp fragment on genomic DNA with primers Tub-F (CTCGAGC GTATGAACGTCTAC) and Tub-R (AAACCCTGGAGGCAG TCGC). The amplification was performed in 25 µL reaction volume containing: 12.5 µl buffer (10X Dream Taq Green Buffer), 1 µl genomic DNA, 1 µl of each primer, 9.5 µl ddH_2O. The PCR reaction were modified according to Khoury *et al*., 2011). The β-tubulin reaction conditions were modified: 95°C for 3 min, 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min and extension of 10 min at 72°C. IGS reaction conditions were modified: 94°C for 4 min, 35 cycles of 94°C for 60 s, 57°C for 40 s, 72°C for 1 min and extension of 10 min at 72°C. The PCR amplified products were examined by electrophoresis on 1% w/v agarose gel.

RFLP analysis

The IGS PCR products were digested with restriction enzyme *Bgl*II. The reaction contained an equal volume (2 µl) of enzyme, 10X buffer 0, 10 µl of PCR product and purified water up to 32 µl and incubated for 1-16 hours at 37°C. 2 µl of 6X DNA Loading Blue for every 10 µl PCR product were mixed before 2% agarose gel loading. Then the fragments were separated by electrophoresis on 2% agarose gel for 3 h at 70 V.

DNA sequencing and phylogenetic tree

All sequence data were done by Medsantek. Alignment of the partial β-tubulin gene sequence data were performed using the software package MEGA sequence analysis software version 6.0 (Kumar *et al*., 2012). Multialignment was performed by Clustal W (Thompson *et al*., 1994) alignment program. Phylogenetic tree was conducted using MEGA version 6.0 (Kumar *et al.*, 2012). The neighbour-joining method was constructed phylogenetic tree (Saito and Nei, 1987). The sequence similarities were performed using BLAST searches programs in the National Center for Biotechnology Information (NCBI).

Aflatoxin production

The *Aspergillus* cultures were grown in potato dextrose agar at 28°C for 7 days. The fungal spore suspension within Tween 20 (0.2%) was collected using filter paper into an erlenmeyer. Spores concentration was supplied of $5x10^6$ spores/ml into vials (Abbas *et al*., 2011). The flow rate was 1 ml/min and the injection volume was 100 ml. The Agilent 1100 HPLC system was used. The detection was performed at ex:360 nm and em:440 nm. The mobile phase was a mixture of water: acetonitrile: methanol (600:200:300, v/v/v) with addition of 132 mg KBr and 385 ml HNO_3 (AOAC, 2002).

RESULTS AND DISCUSSION

Agarose gel electrophoresis separated the PCR products from amplification as shown in Fig 1 and Fig 2. PCR conditions for 50 DNA isolates by β-tubulin gene and IGS were amplified the size of 340 bp and 674 bp, respectively. A total of 50 DNA isolates were observed to have positive result in β-tubulin genes and IGS, respectively.

Molecular techniques have also been widely applied in an attempt to differentiate aflatoxigenic and non-aflatoxigenic isolates of *Aspergillus* species. Similar to our study, three (*ver*-1, *omt*-1 and *apa*-2) genes, coding for key enzymes and a regulatory factor in aflatoxin biosynthesis, have been used to detect *A. flavus* and *A. parasiticus* species in peanut, corn and grains. Positive DNA amplification have been obtained only aflatoxigenic *A. flavus* and *A. parasiticus* (Shapira *et al.* 1996). Four different regions in India have been examined to isolate aflatoxin-producing fungi in 80 peanut by targeting the *afl*R *nor*1 and *ver*1 aflatoxin biosynthesis genes. As a result of the study, positive correlation was remained between three genes and reference *A. flavus* isolates in 24 out of 80 samples (Hussain *et al*., 2015). Hence, this finding of the present study concurs with the literatures.

PCR-RFLP-based method was used to identify *aflJ-aflR* intergenic region of *A. flavus* and *A. parasiticus* isolates using aflatoxin biosynthetic genes (Khoury *et al*., 2011; Raphaël *et al*., 2013). PCR products of *aflJ-aflR* intergenic

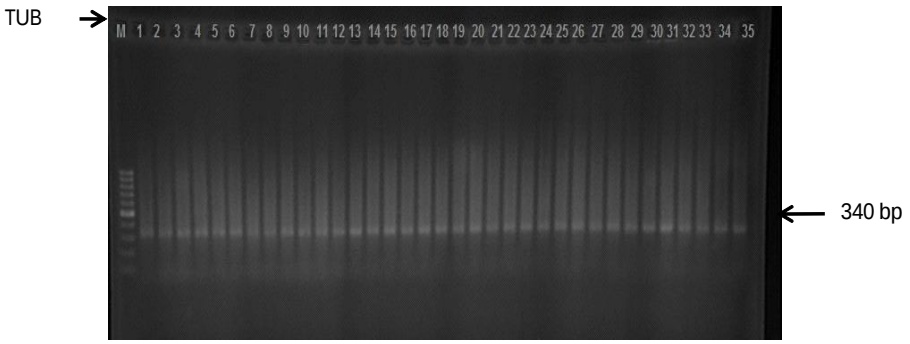


Fig 1: A total of 1% agarose gel electrophoresis showing of 340 bp bands in PCR product amplified with β-tubulin primary pair (TubF/ R). Lane M: 100 bp DNA Marker; Lane 1 to 35: *A.* section *Flavi* isolates.

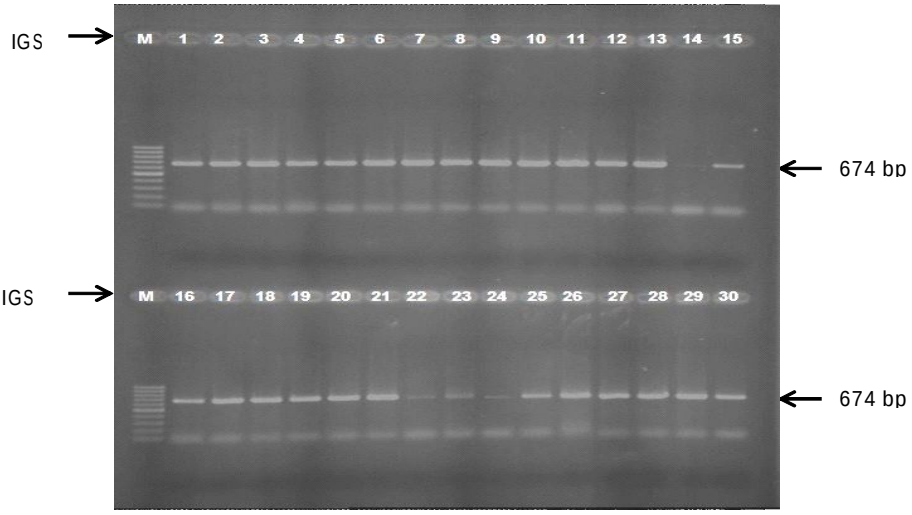


Fig 2: A total of 1% agarose gel electrophoresis showing of 674 bp bands in PCR product amplified with IGS primary pair (IGS F/R). Lane M: 100 bp DNA Marker; Lane 1 to 15*: A.* section *Flavi* isolates; Lane M: 100 bp DNA Marker; Lane 16 to 30: *A.* section *Flavi* isolates.

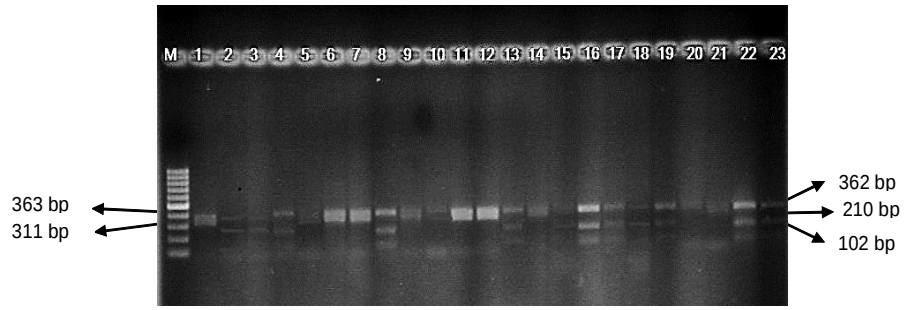


Fig 3: A total of 2% agarose gel electrophoresis showing the restriction profiles of the *aflR-aflJ* intergenic spacer PCR product digested with *Bgl*II. Lane M: 100 bp DNA size marker; Lane 1*: A. parasiticus,* Lane 2 to Lane 4: *A. flavus,* Lane 5 to Lane 7: *A. parasiticus,* Lane 8: *A. flavus,* Lane 9 to Line 12: *A. parasiticus,* Lane 13: *A. flavus,* Lane 14: *A. parasiticus,* Lane 15 to Lane 19: *A. flavus,* Lane 20 to Lane 21*: A. parasiticus,* Lane 22 to Lane 23: *A. flavus.*

region were cleaved into three fragments of 362, 210, 102 bp and 363, 311 bp, respectively, using *Bgl*II restriction enzyme by PCR-RFLP (Fig 3). Similar results were achieved in another study, nine different regions in Serbia have been studied to isolate 10 *Aspergillus* spp. from post-harvest wheat. IGS-F/R primer pairs were targeted by PCR-RFLP analysis method *of aflR-aflJ* intergenic spacer and *Aspergillus* species were determined. All IGS-PCR products were digested with *Bgl*II enzyme. *A. flavus* and *A. parasiticus* having aflatoxin biosynthesis genes were separated fragments 362, 210, 102 bp and 363, 311 bp, respectively (Nikolic *et al*., 2018).

A total of 50 isolates obtained during harvest and storage period were described using sequence analysis of β-tubulin genes (Fig 4). As can be seen on phylogenetic tree, *A. flavus* and *A. parasiticus* have been identified in

Table 1: Occurrence of aflatoxins (µg/L) in *A. flavus and A. parasiticus* isolates analyzed by HPLC.

Fungi	Number of isolate	Range of aflatoxins (µg/L)			
		B_1	B_2	G_1	G_2
A. flavus	33	0.45-4521.64	0.85-62.93	-	-
A. parasiticus	17	23.32-2562.87	0.22-5.52	86.12-651.93	0.34-6.32

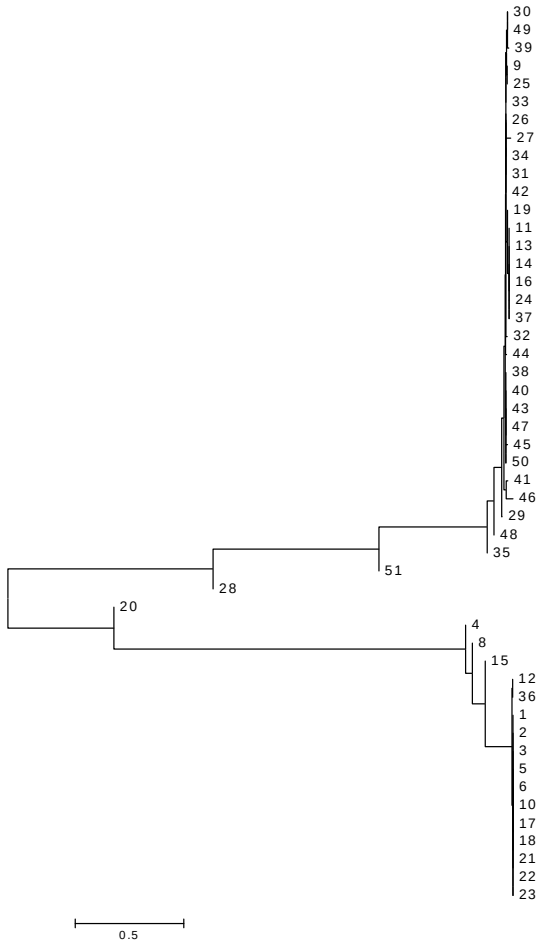


Fig 4: Dendrogram showing the genetic relationship among 50 isolates.

different groups. Analysis of the phylogenetic tree showed that isolate 20 found to be in a different branches. In addition, isolate 28 and isolate 51 were seen dramatically to be different in the group of 31 isolates. Nevertheless, 31 *A. flavus* isolates were high similarity in their group. Similar results were achieved in another study performed the dendrogram, which showed the genetic related within *A.* section *Flavi* isolated from soil and peanut samples from Cordoba region in Argentina, were divided into two groups as A and B. The isolates of 48 *A. flavus* and 34 *A. parasiticus* were included in group A and B, respectively (Barros *et al*., 2006).

In this study, 33 (66%) *A. flavus* and 17 (34%) *A. parasiticus* isolates are indicated aflatoxigenic properties. Aflatoxigenic 33 *A. flavus* isolates are produced AFB_1 and AFB_2 (Table 1). All the *A. parasiticus* isolates are produced B and G group of aflatoxins. In a similar study, total of 70 *A.* section *Flavi* isolated from maize in Italy was analyzed by HPLC. While *A. flavus* was representing 93%, 70% of *A.* section *Flavi* produced aflatoxins. A total of 23% isolates produced <10 ng/g, 10% isolates produced 10-100 ng/g, 12.8% isolates produced 100-1000 ng/g and 24.3% isolates produced >1000 ng/g of medium conditions (Giorni *et al*., 2007). In another similar study, total of 19 *A. flavus* isolated from maize samples were examined the aflatoxin production in liquid medium (8 days at 28°C) using TLC. 12 *A. flavus* (%63) produced aflatoxin B_1 and B_2 between 10 and 700 µg g^{-1} (Lee and Hagler, 1991). Variability in production of aflatoxins, especially among *A. flavus* isolates, has often been reported and discussed (Clevstrom and Ljunggren, 1985).

As many researchers reported aflatoxigenic *A. parasiticus* are widely detected in peanut (Vaamonde *et al*., 2003), peanut and sugarcane (Kumeda *et al*., 2003) growing fields. Totally, 37 *A. parasiticus* (94.6%) isolated from peanut, wheat and soybean was analyzed by HPLC, it produced AFB and AFG. So, describing an aflatoxigenic potential of *A. parasiticus* isolates would be appropriate (Vaamonde *et al*., 2003). In another similar study, it was reported that 18 *A. parasiticus* isolated from almonds in Portugal and all *A. parasiticus* isolated from soil of cornfields in Iran have high aflatoxigenic potential producing both B and G aflatoxins (Razzaghi-Abyaneh *et al*., 2006; Rodrigues *et al*., 2009).

CONCLUSION

In conclusion, 33 (66%) *A. flavus* and 17 (34%) *A. parasiticus* isolates are identified, respectively, as *aflJ-aflR* intergenic spacer for aflatoxin biosynthesis genes. This is the first study that modified molecular method of toxigenic *A. flavus* and *A. parasiticus* isolates from peanut in our country. This approach of differentiating *A. flavus and A. parasiticus* species are very rapid, specific and sensitive compared to traditional methods. All *A. flavus* and *A. parasiticus* isolates are aflatoxigenic *Aspergillus* species from peanut samples. Aflatoxigenic fungi may contaminate peanuts throughout several stages. This study will be provided for the determination of targeting genes that play a role in toxin production in our country. Prevention of preharvest contamination of peanut by aflatoxigenic *Aspergillus* species should be a critical focal point to prevent aflatoxin contamination and exposure.

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