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Original research paper

## PCR-RFLP METHOD IN COMBINATION WITH ON-CHIP ELECTROPHORESIS AS A TOOL FOR DETERMINING OF VARIABILITY BETWEEN IMPORTANT SPECIES OF *ASPERGILLUS*

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**Abstract:** *Aspergillus* species are among the most significant producers of aflatoxins, which can contaminate a wide range of agricultural and food products at any stage of production. The aim of this research was to utilize molecular methods to determine and characterize the variability between isolates of standard *Aspergillus* species. Genomic DNA was isolated from the mycelium of all tested *Aspergillus* isolates. PCR amplifications were performed using gene-specific primers. The PCR method successfully amplified the ITS1-5.8S rDNA-ITS2 region and portions of the  $\beta$ -tubulin and calmodulin genes of all tested *Aspergillus* isolates. PCR products obtained after amplification with primer pairs (ITS1/ITS4 and Bt2a/Bt2b), followed by digestion with restriction enzymes *HhaI*, *MwoI*, and *AlwI* in RFLP analysis, facilitated the identification of variability among the studied *Aspergillus* species. The results of PCR-RFLP analysis on the tested isolates were consistent with those previously obtained through morphological examinations, indicating the effectiveness of this molecular method for identification and determination of variability among important *Aspergillus* species. The presented molecular method based on PCR-RFLP analysis, due to its advantages such as reproducibility, speed, and high sensitivity, represents a valuable tool for monitoring and controlling contamination by *Aspergillus* species in the food supply chain. The method described in this study can be successfully used for rapid identification and determination of variability between isolates of *Aspergillus* species, contributing to improved food safety control and public health.

**Key words:** *Aspergillus* species,  $\beta$  tubulin, Internal Transcribed Spacer, restriction enzymes, Restriction Fragment Length Polymorphism

## INTRODUCTION

*Aspergillus* species pose a significant concern due to their ability to generate aflatoxins, highly toxic secondary metabolites. These fungi are frequently encountered in agricultural products in warm, arid, semi-arid, and tropical regions,

where the prevailing temperature and humidity create an optimal environment for the proliferation of mycobiota and the synthesis of toxins (Cotty & Jaime-Garcia, 2007; Krulj et al. 2016). Toxins can contaminate a wide variety of

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agricultural and food products, posing significant health risks to humans if consumed. While various innovative strategies have been proposed for the reduction of mycotoxins in food/feed (Shanakhat et al., 2018), one of the most commonly employed approaches for their detoxification involves the use of diverse, non-nutritive mycotoxin binders, namely sorbent materials (Bočarov Stančić et al., 2018). Therefore, it is crucial to monitor and control *Aspergillus* contamination in the food supply chain to minimize the adverse health effects associated with these fungi and their toxic metabolites.

In the last two decades numerous molecular techniques have focused on the identification of different fungi species. The use of molecular techniques based on polymerase chain reaction (PCR), significantly reduces the time required for the identification of fungi isolates, compared to culture-based morphological technique (Ahmadi et al., 2015; Raja, Miller, Pearce & Oberlies, 2017; Alshehri & Manikandan, 2020). Given that the pathogenic potential can vary among different species or isolates within the same genus, relying solely on morphological methods for confirming fungal species may be inadequate (Kredics et al., 2007; Kredics et al., 2008; Manikandan et al., 2009). Therefore, the use of molecular techniques for the identification of fungal species becomes crucial.

The development of molecular methods for the genetic differentiation of fungal species has advances their taxonomy due to increased sensitivity and specificity. Due to its reproducibility, speed, high sensitivity and specificity, PCR based test have been used to identify the most important *Aspergillus* species (Ćurčić et al., 2018; Ćurčić, Krulj, Bočarov Stančić & Bodroža Solarov, 2021).

Numerous molecular techniques have focused on *Aspergillus* species identification based on the partial or whole genome sequencing of different gene targets, including internal transcribed spacer (ITS) regions of rDNA and protein encoding genes such as calmodulin and  $\beta$ -tubulin (Wang et al., 2001; Samson et al., 2014; Chalupova, Raus, Sedlarova & Sebela, 2014; Nasri et al., 2015; Krulj et al., 2020). Samson and Varga (2009) recommend the use of at least two genomic sequences (e.g., ITS, calmodulin,  $\beta$ -tubulin, actin) for species description and identification through a multilocus approach. In

this study, molecular analysis was performed by PCR amplification of two genomic regions of the DNA: a part of the internal transcribed region (ITS) rDNA and a part of the  $\beta$ -tubulin gene.  $\beta$ -tubulin is a slowly evolving, conserved gene with a high degree of interspecies variability; consequently it is often used for phylogenetic studies of *Aspergillus* (Balajee et al., 2005). The internal transcribed spacer region is an effective target for phylogenetic analysis in fungi (Henry, Iven & Hinrichs, 2000; Zarrin & Erfaninejad, 2016). The ITS regions are located between the 18S and 28S rRNA genes and offer distinct advantages over other molecular targets, including increased sensitivity due to the existence of approximately 100 copies per genome (Henry et al., 2000). The rRNA gene for 5.8S RNA separates the two ITS regions.

PCR amplification of ITS regions of ribosomal DNA (rDNA) (Criseo, Bagnara & Bisignano, 2001), combined with the sequencing of amplified regions and the analysis of these by comparing them with sequences deposited in GenBank, has been usually employed for the detection of fungal species (Turenne, Sanche, Hoban, Karlowsky & Kabani, 1999; Chen, Tsay, Huang & Chiou, 2002). Zarrin and Erfaninejad (2016) emphasized that variations in a sequence of DNA could be identified using restriction fragment length polymorphism (RFLP), which is able to distinguish minor differences in nucleotides. PCR-RFLP is useful and powerful tool for detecting specific differences in DNA sequences of *Aspergillus* species (Somashekar, Rati & Chandrashekar, 2004, Abastabar et al., 2022) and may be able to identify changes in noncoding regions of DNA. In this study it was shown that the PCR-RFLP method, in combination with a Lab-on-a-chip electrophoresis, can be successfully used to rapidly identify polymorphism among *Aspergillus* species isolates.

## MATERIALS AND METHODS

### Isolates and DNA extraction

All tested isolates were cultivated on PDA (Potato Dextrose Agar) and incubated at 25 °C for 7 days (Fig. 1). Genomic DNA was isolated from mycelia using a DNA Isolation Kit (Agilent Technologies, Santa Clara, CA), according to the manufacturer's instruction. DNA quantity was measured spectrophotometrically by a Nano Drop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific). All

tested *Aspergillus* isolates were analyzed by using PCR-RFLP molecular method.

### PCR and RFLP analysis

PCR amplifications were carried out with gene-specific primers (Table 1) in 25 µl reactions with 1 µl of DNA template, 12.5 µl of 2x PCR Master Mix (Thermo Scientific, Latvia) and 0.6 µM of each primer. All PCR reactions were performed in duplicate.

The specific fragment of the internal transcribed spacer region (ITS1 - 5.8S rDNA – ITS2) was amplified using the universal fungal primers ITS1 and ITS4. PCR was carried out under the following conditions: 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C for 45 sec, extension at 72 °C for 1 min; followed by the final extension at 72 °C for 7 min (Mirhendi et al., 2007). PCR products obtained after amplification with primers ITS1 and ITS4 were incubated for 1h at 37 °C with FastDigest restriction enzymes *HhaI* and *MwoI* (Thermo Scientific, Lithuania). The digestion was performed by incubating a 10 µl aliquot of PCR product with 2 µl of 10xFastDigest buffer and 1 µl of Fast Digest enzyme, in a final reaction volume of 25 µl. To amplify β-tubulin (BenA) and calmodulin (CaM), the primer pairs Bt2a/Bt2b and Cmd5/Cmd6 were used (Table 1).

The beta tubulin gene was partially amplified under the following conditions: 5 min at 95 °C (initial denaturation); 35 cycles for 45 sec at 94 °C, 45 sec at 60 °C (primer annealing), 1 min at 72 °C (primer extension) and 6 min of final extension at 72 °C (Nasri et al., 2015). Products of PCR reaction obtained after amplification with primers Bt2a/Bt2b were digested with *AlwI* (*BspPI*) for 4h at 55 °C. The reaction mixture consisted of 10µl of PCR amplicons, 1µl of *AlwI* (*BspPI*) restriction enzyme, 2µl of 10 x Buffer Tango and 17 µl nuclease free-water.

### Lab-on-a-chip electrophoresis

The Agilent DNA 1000 Reagents (Agilent Technologies, Lithuania) designed for the analysis of DNA fragments from 25 to 1000 base pair (bp) was utilized in this study. This kit served as the final step in the detecting and determining the presence and size of DNA fragments amplified through PCR. Amplified PCR products (before digestion) and restriction fragments (after digestion) with restriction enzymes were separated using Lab-on-a-Chip electrophoresis at 2100 Bioanalyzer system (Agilent Technologies, CA, USA). The system integrates an instrument, data processing 2100 Expert Software, reagents (DNA Ladder, DNA Markers, DNA Dye Concentrate, DNA Gel Matrix) and a microfluidic chip specific for DNA.

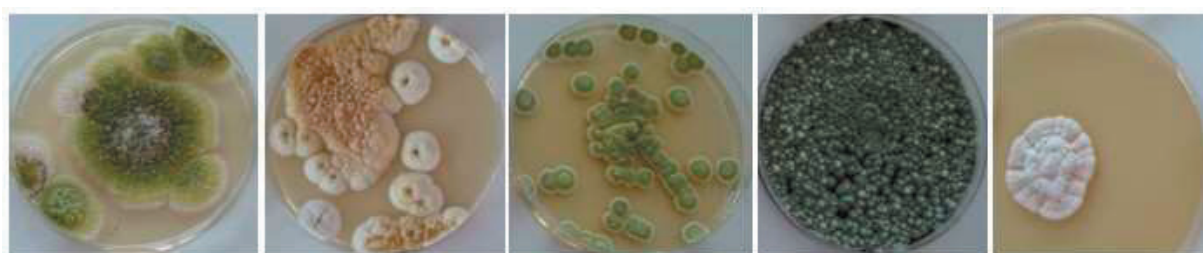


Figure 1. *Aspergillus* species (from left to right): *Aspergillus flavus*, *A. ochraceus*, *A. nidulans*, *A. versicolor* and *A. candidus*

**Table 1.**

Target gene, primer sequences (F-Forward, R-Reverse) and primer annealing (°C) used for polymerase chain reaction

Fragment/Gene	Primer pairs	Primer sequence	Primer annealing (°C)
ITS1-5.8S rDNA-ITS2	ITS1/ITS4	F 5'-TCCGTAGGTGAACCTGCGG -3' R 5'-TCCTCCGCTTATTGATATGC -3'	56
β tubulin (BenA)	Bt2a/Bt2b	F 5'-GGTAACCAAATCGGTGCTGCTTTC -3' R 5'- ACCCTCAGTGTAGTGACCCTTGGC -3'	60
Calmodulin (CaM)	Cmd5/Cmd6	F 5'-CCGAGTACAAGGAGGCCTTC-3' R 5'-CCGATAGAGGTCATAACGTGG-3'	59

Advantages of microfluidics-based automated electrophoresis over traditional gel electrophoresis include dramatically reduced sample (1  $\mu$ L for nucleic acids) and reagent consumption, significantly faster analysis time, and less hands-on activities during sample preparation and data analysis. Sample wells and ladders are filled with marker mix (internal control) to which 1  $\mu$ L of sample or ladder is added. The presence of internal markers including a lower marker at 15 bp and upper marker at 1500 bp in each wells, enabled result comparison. The size of the PCR fragment was determined by referencing the molecular size scale provided on each chip. Quantification of fragments was performed by comparing the peaks with the upper marker measured in each well.

## RESULTS AND DISCUSSION

In the scope of this research, a genetic variability analysis among *Aspergillus* species was conducted. Molecular analysis was performed by PCR amplification of two genomic regions of the DNA: a part of the internal transcribed region (ITS) rDNA and a part of the  $\beta$ -tubulin gene. The goal was to determine whether these regions provided sufficient information to examine the genetic diversity among the studied *Aspergillus* species. Universal primer pairs ITS1 and ITS4 were able to successfully amplify the ITS1-5.8S rDNA-ITS2 region of all tested *Aspergillus* isolates. Amplification of the ITS 1–5.8S–ITS 2 regions from the all *Aspergillus* strains examined in this study generated PCR products ranging in size from 560 to 600bp (Fig. 2).

The results obtained in this study are consistent with the findings of Diba, Mirhendi, Kordbacheh and Rezaie (2014), where PCR amplification of the ITS1–5.8S–ITS2 genomic region generated fragments ranging in size from 565 to 613 bp for six clinically relevant *Aspergillus* species. In this study, the size of the amplified fragment obtained by PCR using the primer pair ITS1/ITS4 for the *Aspergillus flavus* species was 600 bp. The study conducted by Mirhendi et al. (2007) revealed that the size of PCR fragments amplified using ITS1/ITS4 primers before digestion was 595 bp. This aligns with the findings obtained in our current study. The size of this fragment corresponds to the size of the reference strain of *Aspergillus flavus* (Diba et al., 2014), as well as with the

reference strain *A. flavus* ATCC® 9643 (Kruļj et al., 2020).

On the other hand, the size of the fragments obtained after digestion of the PCR products with the *HhaI* restriction enzyme, according to the research of Mirhendi et al. (2007), slightly differs from the results obtained in this study. Differences in the size of fragments obtained using the same enzyme could be attributed to different electrophoresis methods. The same authors (Mirhendi et al., 2007) used agarose gel electrophoresis for the separation of PCR products while in our experiment separation was conducted using chip electrophoresis. Similar discrepancies were also observed in the application of the *MwoI* enzyme when comparing the results of our tests with those of Diba et al. (2014), who investigated the potential of this enzyme for the identification and molecular characterization of medically important *Aspergillus* species. Amplification of the 560-610 bp fragment (Fig. 2) followed by *HhaI* and *MwoI* restriction in RFLP analysis (Fig. 3) resulted in distinct fragment patterns among the examined species.

*HhaI* and *MwoI* restriction enzymes had one, two or more digestion sites for all examined *Aspergillus* species. Digestion of the PCR product using these restriction enzymes produced different patterns of fragments among examined species, with different sizes and number of fragments, revealing genetic variability (Fig. 3). This approach allowed us to determine the variability among the tested *Aspergillus* species, including *Aspergillus flavus*, *A. ochraceus*, *A. nidulans*, *A. versicolor* and *A. candida*.

Using the calmodulin primer pair (cmd5/cmd6), a 475-595 base pair fragment was successfully amplified (results are not shown). Amplification of a part of the  $\beta$  tubulin gene was performed by using the primer pairs (Bt2a/Bt2b) and generated PCR product ranging in size from 405 to 580 bp (Fig. 4), while Nasri et al. (2015), examining clinically significant *Aspergillus* species, obtained PCR products ranging in size from 475 to 564 bp.

These PCR products were digested with restriction enzyme *AlwI* (*BspPI*) who had one or two restriction sites in tested *Aspergillus* species (*Aspergillus flavus*, *A. nidulans* and *A. candida*).

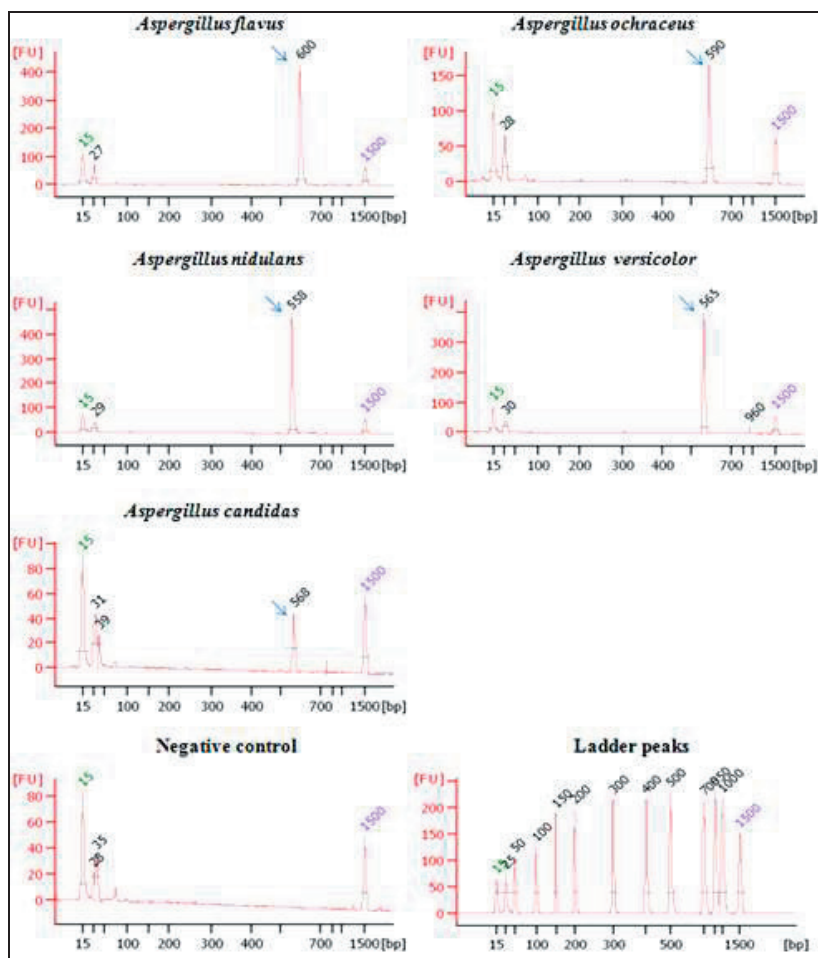


Figure 2. Electropherogram of ITS1-ITS4 PCR amplification products of standard *Aspergillus* species. Peaks of the size 560-600 bp are marked with arrows. Peaks labelled with 15bp and 1500bp are internal calibration markers. Negative control and DNA Ladder peaks of the sizes 25, 50, 100, 150, 200, 300, 400, 500, 700, 850 and 1000 bp are shown

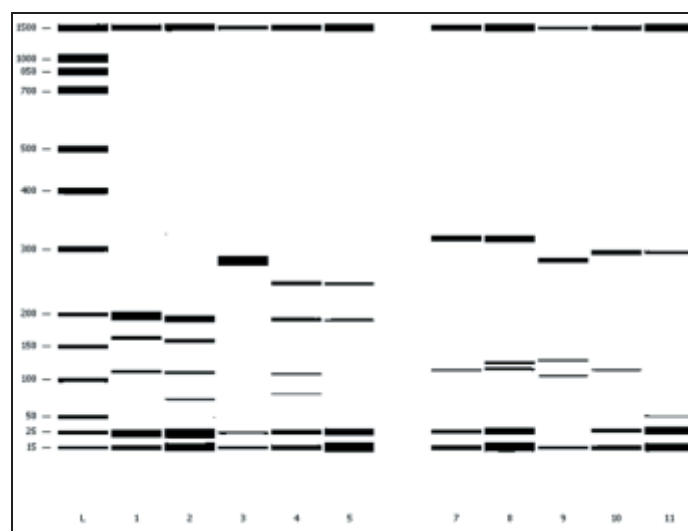


Figure 3. Lab-on-a-chip electrophoresis of ITS1-ITS4 PCR amplification products of standard *Aspergillus* species after digestion with FastDigest restriction enzymes *HhaI* (Lanes 1-5) and *MwoI* (Lanes 7-11); L - 25-1000 bp DNA ladder; Lanes 1 and 7 *Aspergillus flavus*; Lanes 2 and 8 *A. ochraceus*; Lanes 3 and 9 *A. nidulans*; Lanes 4 and 10 *A. versicolor*; Lanes 5 and 11 *A. candidas*; Fragments labelled with 15bp and 1500bp are internal calibration markers

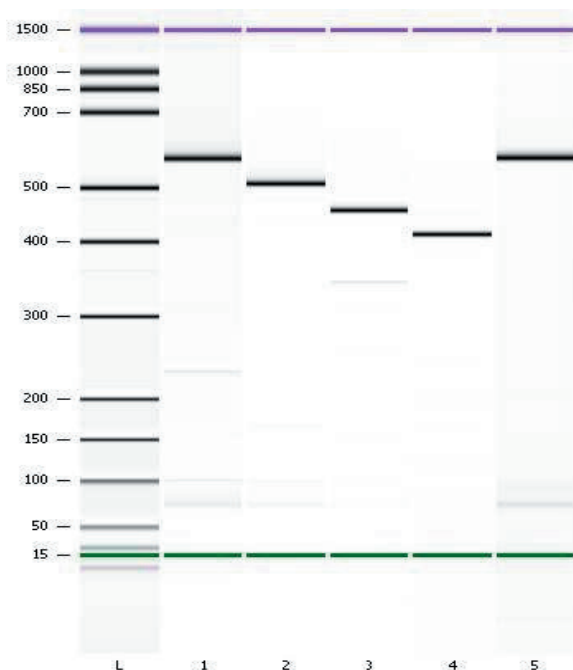


Figure 4. Lab-on-a-chip electrophoresis of  $\beta$ -tubulin amplification products from standard *Aspergillus* species. L - 25-1000 bp DNA Ladder; Lane 1 *Aspergillus flavus*; Lane 2 *A. ochraceus*; Lane 3 *A. nidulans*; Lane 4 *A. versicolor*; Lane 5 *A. candidas*. Fragments labelled with 15bp and 1500bp are internal calibration markers

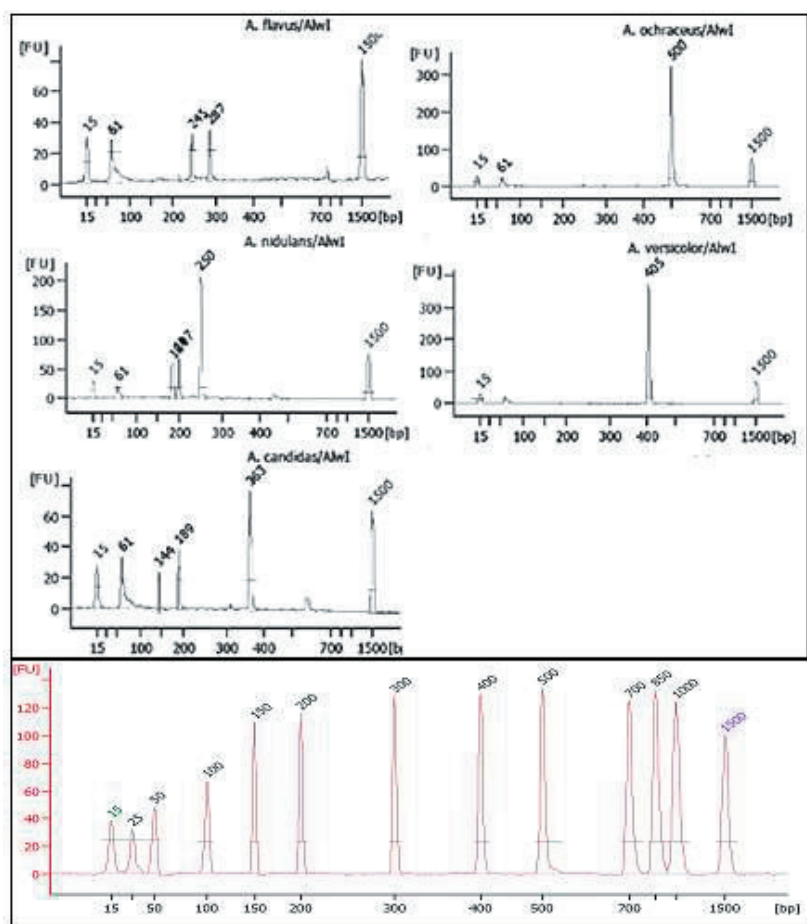


Figure 5. Electropherogram of PCR amplification products of the part of  $\beta$  tubulin gene after digestion with *AlwI* (*BspPI*) restriction enzyme. Peaks labelled with 15bp and 1500bp are internal calibration markers. DNA Ladder peaks of the sizes 25, 50, 100, 150, 200, 300, 400, 500, 700, 850 and 1000bp are shown in the bottom part of the image

Restriction enzyme *AlwI* had no restriction sites for *A. ochraceus* and *A. versicolor*, so PCR amplicons remained undigested (Fig. 5)

Although sequencing methods have advanced, there is still a need to develop alternative methods for locations without widespread access to sequencing laboratories. This study confirms the utility of a simple PCR-RFLP method for identifying important *Aspergillus* species, especially *Aspergillus flavus*, which produces hazardous mycotoxins such as aflatoxins, posing significant health risks to both humans and animals.

## CONCLUSIONS

PCR-RFLP is a valuable molecular tool for screening nucleotide polymorphism among *Aspergillus* species. The PCR amplification pattern of a part of the  $\beta$ -tubulin gene for the tested *Aspergillus* was found to be species-specific, with none of the species generated products of the same sizes. Digestion of the PCR ITS1-ITS4 PCR amplification products using the restriction enzymes *HhaI* and *MwoI* resulted in distinct patterns of fragments among the examined species, showing variations in both size and number of fragment. This revealed genetic variability within the investigated *Aspergillus* species.

The use of molecular techniques based on PCR-RFLP provides significant advantages. The approach described in this paper to distinguish *Aspergillus* species is simpler and more cost-effective than conventional sequencing of PCR products. Additionally, it is faster than morphological identification. The PCR-RFLP method coupled with a Lab-on-a-chip electrophoresis, can be successfully employed for the rapid identification and determination of variability between *Aspergillus* species isolates.

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## PCR-RFLP METODA U KOMBINACIJI SA ELEKTROFOREZOM NA ČIPU KAO ALAT ZA ODREĐIVANJE VARIJABILNOSTI IZMEĐU VAŽNIH VRSTA *ASPERGILLUS*

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**Sažetak:** *Aspergillus* vrste spadaju među najvažnije proizvođače aflatoksina koje mogu kontaminirati širok spektar poljoprivrednih i prehrambenih proizvoda u bilo kojoj fazi proizvodnje. Cilj ovog istraživanja bio je da se primenom molekularnih metoda utvrdi i okarakteriše postojanje varijabilnosti između izolata standardnih vrsta *Aspergillus*. Genomska DNK je izolovana iz micelija svih ispitivanih izolata *Aspergillus* vrsta. PCR amplifikacije su izvedene sa gen-specifičnim prajmerima. PCR metodom je uspešno amplifikovan ITS1-5.8S rDNA-ITS2 region i delovi gena  $\beta$  tubulina i kalmodulina svih testiranih izolata *Aspergillus*. PCR proizvodi dobijeni nakon amplifikacije sa parovima prajmera (ITS1/ITS4 i Bt2a/Bt2b), praćeni digestijom restrikcionim enzimima *Hha*I, *Mwo*I i *Alw*I u RFLP analizi, olakšali su identifikaciju varijabilnosti među proučavanim *Aspergillus* vrstama. Rezultati PCR-RFLP analize na ispitivanim izolatima bili su u skladu sa onima prethodno dobijenim putem morfoloških ispitivanja, što ukazuje na efikasnost ove molekularne metode za identifikaciju i određivanje varijabilnosti među važnim vrstama *Aspergillus*. Prikazana molekularna metoda zasnovana na PCR-RFLP analizi zbog svojih prednosti kao što su reproduktivnost, brzina i visoka osetljivost, čini je dragocenim alatom za praćenje i kontrolu kontaminacije *Aspergillus* vrstama u lancu snabdevanja hranom. Metoda opisana u ovom radu, može se uspešno koristiti za brzu identifikaciju i određivanje varijabilnosti između izolata vrsta *Aspergillus* i doprineti poboljšanoj kontroli bezbednosti hrane i javnom zdravlju.

**Ključne reči:** vrste *Aspergillus*,  $\beta$  tubulin, restrikcioni enzimi, polimorfizam dužine restrikcionog fragmenta

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