PCR - BASED DETECTION METHODS AS A TOOL FOR IDENTIFICATION OF *ASPERGILLUS* SPECIES

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INTRODUCTION

Aspergillus **species are one of the most important producers of aflatoxin that can contaminate wide range of agricultural and food commodities. Molecular methods have been widely applied in the identification of different fungi species. Due of its reproducibility, speed, high sensitivity and specificity, PCR based methods have been used to identify the most important *Aspergillus* species.**

OBJECTIVES

The aim of this study was to validate PCR-RFLP based method to discriminate *Aspergillus* at the species level. PCR were performed to successfully amplify the ITS1-5.8S rDNA-ITS2 region, parts of β tubulin and calmodulin gene. One of the goals of this study was to identify the presence of genes (*afl*S, *afl*R, *afl*D and *afl*Q) in 8 examined *Aspergillus species.*

METHODS

Aspergillus **isolates were identified in the level of species by using molecular methods. Genomic DNA was isolated from mycelia using DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instruction. PCR amplifications were carried out with gene-specific primers. Products of PCR reaction obtained after amplification with primers (ITS1/ITS4) were incubated with restriction enzymes *Hha*I and *Mwo*I, while PCR products obtained after amplification with primer pair (Bt2a/Bt2b) were digested with *Alw*I (*Bsp*PI) restriction enzyme.**

The presence of structural genes (*afl*D and *afl*Q) and regulatory genes (*afl*S and *afl*R) were evaluated by PCR using 4 primer pairs. Amplified PCR products (before digestion) and restriction fragments (after digestion) were separated by agarose gel electrophoresis.

Primer pairs	Primer sequence	Primer annealing (°C)
ITS1 ITS4	F 5'-TCCGTAGGTGAACCTGCGG-3' R 5'-TCCTCCGCTTATTGATATGC-3'	56
Bt2a Bt2b	F 5'-GGTAACCAAATCGGTGCTGCTTTC -3' R 5'- ACCCTCAGTGTAGTGACCCTTGGC -3'	61
Cmd5 Cmd6	F 5'-CCGAGTACAAGGAGGCCTTC-3' R 5'-CCGATAGAGGTCATAACGTGG-3'	59
AflR-F AflR-R	5'-AAGCTCCGGGATAGCTGTA-3' 5'-AGGCCACTAAACCCGAGTA-3'	55
AflS-F AflS-R	5'-TGAATCCGTACCCTTTGAGG-3' 5'-GGAATGGGATGGAGATGAGA-3'	55
AflD-F AflD-R	5'-CACTTAGCCATCACGGTCA-3' 5'-GAGTTGAGATCCATCCGTG-3'	55
AflQ-F AflQ-R	5'-TCGTCCTTCCATCCTCTTG-3' 5'-ATGTGAGTAGCATCGGCATTC-3'	55

Table 1. Primer sequences (F-Forward, R-Reverse) and primer annealing (°C) used for polymerase chain reaction

RESULTS

Universal primer pair ITS1 and ITS4 were able to successfully amplify the ITS1-5.8S rDNA-ITS2 region of all tested *Aspergillus* species. Amplification of the 560-610 bp fragment followed by *Hha*I and *Mwo*I restriction in RFLP analysis produced different patterns of fragments among the examined species, revealing genetic variability.

Using the calmodulin primer pair (cmd5/cmd6), a 475-595 base pair fragment was successfully amplified in tested *Aspergillus* species. Amplification of a part of the β tubulin gene was performed by using the primer pair (Bt2a/Bt2b) and generated PCR product ranging in size from 415 to 580 bp. This PCR product was digested with restriction enzyme *AlwI* (*BspPI*). The RFLP pattern of *AlwI* for tested *Aspergillus* was species-specific and none of the examined species generated fragments with similar sizes. PCR test on β tubulin gene generated unique patterns for eight examined *Aspergillus* species (*Aspergillus flavus*, *A. ochraceus*, *A. nidulans*, *A. versicolor*, *A. candidus*, *A. tamari, A. fumigatus* and *A. niger*).

The presence of genes (*afl*S, *afl*R, *afl*D and *afl*Q) involved in aflatoxin biosynthesis pathway were detected only in *Aspergillus flavus.*

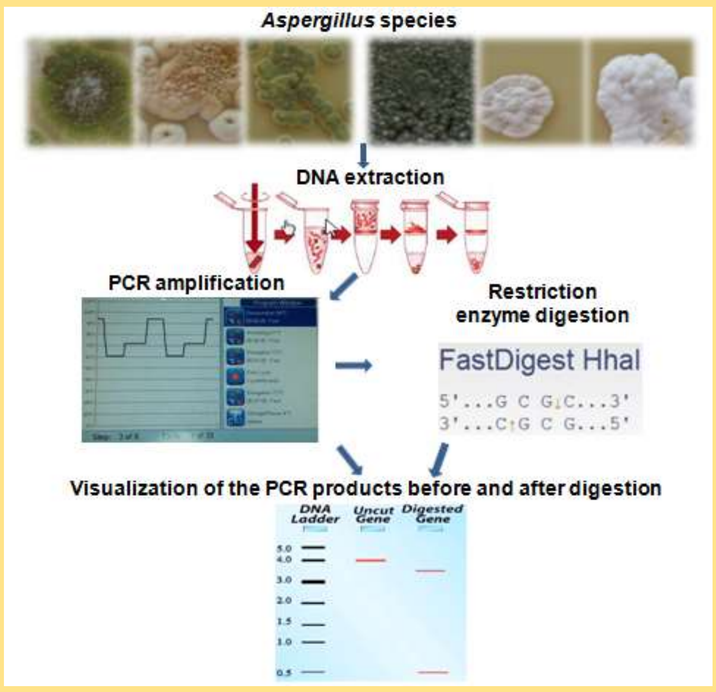

Figure 1. An overview schematic of the molecular identification of *Aspergillus* species by a PCR-RFLP method

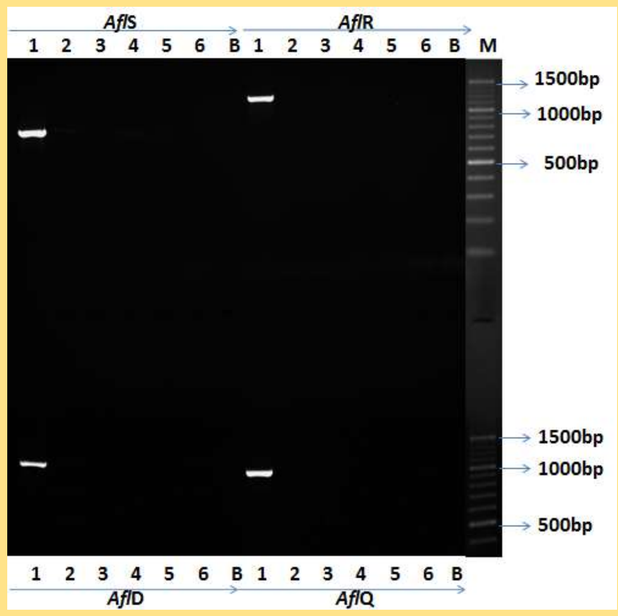

Figure 2. Agarose gel electrophoresis of *afl*S, *afl*R, *afl*D and *afl*Q amplification products of standard *Aspergillus* species. M - O' Range Ruler 100 bp DNA Ladder; B- negative control; Lane 1 *Aspergillus flavus*; Lane 2 *A. ochraceus*; Lane 3 *A. nidulans*; Lane 4 *A. versicolor*; Lane 5 *A. candidus*; Lane 6 *A. tamari*.

CONCLUSION

PCR/RFLP on β tubulin gene provided rapid identification of the most important species of *Aspergillus*. The presence of structural genes (*afl*D and *afl*Q) and regulatory genes (*afl*S and *afl*R) detected in *Aspergillus flavus*, could be considered as a quick and reliable method for the detection of aflatoxigenic *Aspergillus*.

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