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Short Communication

Molecular Affirmation of *Aspergillus luchuensis a* Niger Group Species as a Non-Ochratoxigenic Fungus

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Abstract

Amongst the various species of *Aspergillus* section *Nigri*; *Aspergillus luchuensis*, associated with food and beverage fermentation in East Asia, is evaluated by multiple researchers as non-ochratoxigenic based on the extrolite analysis. Beyond extrolite analysis, in the present study, *A. luchuensis* strain was analyzed by a rapid Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique. The Internal Transcribed Spacer (ITS), Intergenic Spacer (IGS) and β-tubulin gene region were PCR amplified from *A. luchuensis*. The digestion of amplicons ITS and β-tub with *Rsa* I resulted in uncut and two bands (420 and 130 bp) respectively, while IGS with *Hinf* I produced three bands (250, 80 and 60 bp). The restriction profile obtained, resembled the profile of strains which lack the ability to produce ochratoxin A (OTA). A clear correlation between the extrolite analysis and PCR-RFLP technique is established, confirming the usability of PCR-RFLP method for the rapid affirmation of non-ochratoxigenic property of *A. luchuensis.*

Keywords: *Aspergillus luchuensis*; Non-Ochratoxigenic; PCR-RFLP; Ochratoxin A.

Introduction

Aspergillus section Nigri (black molds) strains are distributed worldwide and found to grow upon wide variety of substrates. Some of them are widely used and studied for industrial applications [1,2,3]. Besides their industrial importance, black aspergilli are known to produce a unique combination of mainly polyketide derived secondary metabolites and other compounds of mixed biosynthetic origin. There is a possibility that harmful mycotoxins like ochratoxin A (OTA) might contaminate the compounds used in biotechnological processes for food application [4]. Nevertheless, the ability to produce OTA is not present in all the strains of the same species: i.e., around 60– 80% of *A. carbonarius* strains [5,6] and 20–30% of *A. niger* aggregate (*A. niger* or *A. awamori*) [7] are reported to produce OTA. These species were identified using only morphological methods. However, the difficulties in their recognition suggest that it is necessary to carry out more sensitive and adequate molecular characterization to define potential toxicological risks.

A. luchuensis belonging to *Aspergillus* section *Nigri* group, associated with food and beverage fermentation in East Asia has been analyzed for production of mycotoxin. Extrolite from strains of *A.luchuensis* was analyzed by Hong et al. [8] which showed absence of mycotoxins production and therefore was considered safe for food and beverage fermentations. Earlier several researchers have employed molecular methods in an attempt to distinguish between OTA producer and non-producer strains within *Aspergillus* section Nigri [9,10,11]. The possibility to detect the presence of the genes involved in OTA expression has been investigated, but it did not give reliable results [12]. There are methods which are based on PCR-RFLP, which can differentiate the OTA producing strains from the non-producing strains [13].The aim of the study was to confirm and correlate the extrolite analysis based non-ochratoxigenic property of *A.luchuensis* using rapid PCR-RFLP method. This is the first report establishing correlation between the two methods for detection of non-ochratoxigenic strains belonging to the section Nigri. The ITS and β-tub region were utilized as genetic locus for phylogenetic analysis and species recognition of *Aspergillus* species [14], while IGS region was utilized by Carbone and Kohn [15]. Hence, in the present study, three genetic locus ITS, IGS and β-tub region were considered for affirmation of the non-ochratoxigenic property of the strain *A. luchuensis* compared to the ochratoxigenic strain *A.carbonarius* based on the PCR-RFLP profile.

Material and Methods

Fungal Strains and Culture Condition

The fungal isolates strain ASNSC from Advanced Enzymes Technologies Limited (AETL) culture collection identified by CBS, Netherland as *A. luchuensis* (designated as *Aspergillus niger* aggregate by CABI, UK) *and A. carbonarius* from AETL culture collection (identified at AETL) was used in the study as non-ochratoxigenic and ochratoxigenic strains respectively. The fungal strains were grown in 250 ml Erlenmeyer flask containing 50 ml of

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Potato Dextrose Broth (BD, Difco) at 30°C for 48 h at 180 rpm on orbital shaker.

Genomic DNA Isolation and PCR Amplification

Genomic DNA of fungal strains was extracted using Nucleos pin Plant II kit (Macherey-Nagel Gmbh& CO., Germany) according to the manufacturer protocol. The quality and quantity of genomic DNA were estimated spectrophotometrically (Nanodrop ND-1000, Thermo Scientific).

PCR amplifications were performed with the primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCG G-3') and ITS4 (5'-TCCTCCGCT-TATTGATATGC-3') [16], IGS12a (5'-AGTCTGTGGATTAGTG-GCCG-3') and NS1R (5'-GAGACAAGCATATGACTAC-3') [15], BT2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and BT2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') [17] for the ITS, IGS and β-tubulin regions, respectively.

PCR was performed in a 50 µl final volume containing 2 µl of genomic DNA template (50 ng), 25 µl of 2X KODFX buffer (KFX-101, Toyobo, Japan), 10 µl of 2 mmol l^{-1}dNTP's, 1 µl of KODFX DNA Polymerase (1.0 U μl^{-1}) and 1 µl of 10 pmol μl^{-1} each primer. The PCR amplification included an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 98°C for 10s, 50°C (ITS, IGS) / 60°C (β-tub) for 30s and 68°C for 45s followed by final extension at 68°C for 7 min. Amplification was performed in a Veriti thermal cycler (Applied Biosytems). The amplification products were resolved on 1% (w/v) agarose gel stained with ethidium bromide. The positive PCR products were extracted from the agarose gel using Qiagen Gel extraction kit (Qiagen) and further processed for restriction digestion reaction.

Restriction Digestion Profiles

The PCR products were digested as follows: ITS and β-tub region with *Rsa* I (NEB, England) and IGS region with *Hinf* I (TaKaRa Bio). The restriction enzyme digested products were electrophoresed on 2% (w/v) agarose gel stained with ethidium bromide at 100 V for 2 h in 1 X TAE buffer. Gene ruler 100 bp DNA ladder (#SM 0323, Thermo Scientific) molecular weight markers were used to calculate the dimension of each band.

Results and Discussion

Several molecular methods have been employed in an attempt to distinguish between OTA producer and non-producer strains within *Aspergillus* section Nigri [9]. Dao et al. [10] designed two sets of specific primers for polyketide synthase (PKS) gene from *A.ochraceus* NRRL 3174 for detection of OTA producing fungi, which further requires validation. Storari et al. [11] utilized degenerate primers to detect PKS gene fragments and their role in OTA biosynthesis in black aspergilli. The possibility to detect the presence of the genes involved in OTA expression has been investigated but it did not give reliable results [12]. Hence, a rapid and reliable approach for discrimination and identification of ochratoxigenic and non-ochratoxigenic capability by PCR-RFLP method was utilized.

A. luchuensis (black koji mold) extrolite analysis of species showed that they do not produce mycotoxins and therefore can be considered safe for food and beverage fermentation [8].The methodology for carrying our physiological characterization (extrolite analysis) of toxigenic fungi is generally very time-consuming. Also, the detection procedures involve variable detection limit for OTA. Therefore, there is need of rapid, sensitive, specific and reliable molecular method (PCR-RFLP) for routine confirmation of ochratoxigenic fungi [18,19]. Hence, in continuation with extrolite analysis by Hong et al. [8], PCR-RFLP based molecular method was utilized to confirm *A.luchuensis* as non-ochratoxigenic. In the present study; ITS, IGS and β-tub region were amplified using gene specific primers from *A.luchuensis* and *A.carbonarius* as described in material and methods. The amplicon sizes were ~600, 440 and 550 bp for ITS, IGS and β-tub region respectively from *A. luchuensis* (Figure 1) and *A. carbonarius* (Figure 2). The amplicons

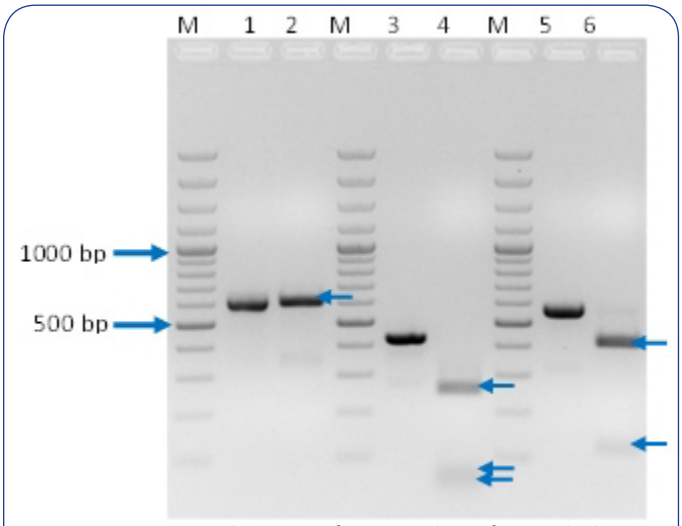

Figure 1: Restriction digestion of PCR products from *A.luchuensis*. Lane 1-2 undigested and digested ITS region with *Rsa*I, Lane 3-4 undigested and digested IGS region with *Hinf*I, Lane 5-6 undigested and digested β-tub gene with *Rsa*I respectively, Lane M: 100 bp DNA molecular size marker.

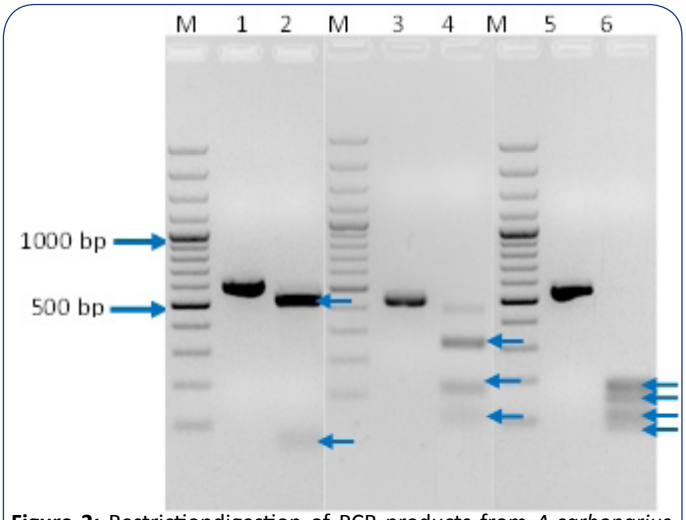

Figure 2: Restrictiondigestion of PCR products from *A.carbonarius*. Lane 1-2 undigested and digested ITS region with *Rsa*I, Lane 3-4 undigested and digested IGS region with *Hinf*I, Lane 5-6 undigested and digested β-tub regionwith *Rsa*I respectively, Lane M: 100 bp DNA molecular size marker.

for ITS, IGS and β-tub region were consistent with the dimensions reported by Varga et al. [20], Carbone and Kohn [15] and Glass and Donaldson [17] respectively.

The discrimination between ochratoxigenic and non-ochratoxigenic strains of *Aspergillus niger* aggregate has been previously studied by PCR-RFLP of ITS, IGS and β-tub region [21]. They have reported discriminative restriction digestion profile type for positive ochratoxigenic strain *A.carbonarius* and non-ochratoxigenic strains of *Aspergillus* section Nigri as described in Table 1 by Zanzotto et al. [21]. Similarly, the restriction enzyme digestion of ITS and β-tub region with *Rsa* I and IGS region with *Hinf* I from *A.luchuensis* and *A.carbonarius* were carried out for differentiation of OTA non-producer strains from OTA producers. As shown in figure 1 for *A. luchuensis*, the restriction digestion profile with *Rsa* I remained uncut for ITS region and produced two bands of approximately 420 and 130 bp for β-tub region, while *Hinf* I produced three bands of approximately 250, 80 and 60 bp for IGS region. The restriction digestion profile observed for *A. luchuensis* for amplicons ITS, IGS and β-tub region was T-C-D respectively (Figure 1) was similar to profile type of non-ochratoxigenic *A. niger* aggregate strains (Table 1). Whereas, the restriction digestion profile for *A.carbonarius* as shown in figure 2, *Rsa* I produced two bands of approximately 510 and 90 bp for ITS region and three bands for β-tub region of around 250 bp, 115 bp and 75 bp, while *Hinf* I produced four bands for IGS region of around 190, 170, 100 and 90 bp. *A.carbonarius* considered as positive ochratoxigenic species gave the expected restriction digestion profile (N-B-A, Fig. 2) as described by Zanzotto et al. [21]. The difference in the profile type observed between *A.luchuensis* and *A.carbonarius* asserts *A.luchuensis* as non-ochratoxigenic species. In conclusion, PCR-RFLP profile of ITS, IGS and β-tub regions from *A.luchuensis* using restriction enzymes *Rsa* I and *Hinf* I allied with the extrolite analysis data as described by Hong et al. [8]. This confirms that *A.luchuensis* is a non-ochratoxigenic species and the PCR-RFLP method can be adopted for a rapid determination of non-ochratoxigenic property of the same.

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Table 1: Restriction digestion profile for ochratoxigenic and non-ochratoxigenic species in *A. niger*aggregate

Digestion profile type	ITS with *Rsa*I	IGS with *Hinf*I	β-tub with *Rsa*I
N	510 / 90 bp	-	-
T	No cutter	-	-
B	-	250/115/75 bp	330/120/90 bp
C	-	250/80/60/50 bp	250/190/110 bp
D	-	-	420/130 bp
Profile type described for *A.niger* aggregate			
Ochratoxigenic species	N	B	B
Ochratoxigenic species (*A. carbanorius*)	N	B	A
Non-Ochratoxigenic species	T	B/C	C/D

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