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Identification of *Aspergillus* sections *Flavi*, *Nigri*, and *Fumigati* and their differentiation using specific primers

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SUMMARY

Aspergillus species are important in medicine, agriculture and various industries. The sections *Fumigati*, *Flavi*, and *Nigri* are the most important members of the *Aspergillus* genus. This study intended to identify and separate these three *Aspergillus* sections and to differentiate among them using specific primers. A bioinformatics study was initially performed to analyse the sequences of five genes, namely, *beta-tubulin*, *calmodulin*, the pre-rRNA processing protein *Tsr1*, the DNA-replication licensing factor *Mcm7*, and RNA polymerase II second largest subunit (*RPB2*) in the three *Aspergillus* sections using MEGA6 software and the NCBI database. Primers were designed to select genes for each of the *Aspergillus* sections being analysed. A total of 134 environmental and clinical *Aspergillus* species were isolated, purified and initially identified by colony morphology..Subsequently, DNA was extracted using the phenol-chloroform method, specific primers were synthesized, PCR was performed for DNA from all isolates, and the results were compared to morphological characteristics. Of the 134 isolates tested, 56 were *Nigri*, 32 were *Fumigati*, 32 were *Flavi*, and the rest (14 isolates) belonged to other sections. The *beta-tubulin* and *calmodulin* genes were found to be the most suitable for differentiating among these three groups; the *beta-tubulin* gene was used for molecular identification of *Aspergillus* section *Fumigati*, and the *calmodulin* gene for identifying sections *Flavi* and *Nigri*.

Keywords: *Aspergillus*, *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri*, *Aspergillus* section *Fumigati*.

INTRODUCTION

The genus *Aspergillus* belongs to the class Ascomycetes and includes opportunistic and saprophytic fungi that are of importance in industry, medicine, and agriculture, among others. This genus has 8000 species revised to 250 species classified into the nine main sections: *Flavi*, *Fumigati*, *Nigri*, *Udagawae*, *Cricumdati*, *Versicolor*, *Usti*, *Terrei*, and *Emericella* [1-3]. Approximately 40 species are reported to cause human disease. Invasive pulmonary aspergillosis is a severe and lethal infection observed in immune-compromised patients such as those suffering from neutropenia, chronic granulomatous disease (CGD), malignancies, organ transplant patients, long-time use of steroids, and antibiotics and chemotherapy drugs [4].
Due to the life-threatening nature of these infections and drug resistance, the precise identification of the *Aspergillus* species involved is important [5, 6].

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The most important *Aspergillus* species belong to three *Aspergillus* sections, namely, *Fumigati, Flavi,* and *Nigri*, and these, together, account for more than 95% of the pathogenic *Aspergillus* species. It is important to mention here that there has been a ten-fold increase in invasive fungal infections and aspergillosis over the last two decades [7]. The *Aspergillus* section *Fumigati* includes, among others, the species *A. fumigatus*, *A. lentulus*, *A. fumigatiaffinis*, *A. fumisynnematus*, *A. novofumigatus*, and *A. laciniosa* [8].

The *Aspergillus* section *Flavi* is important with respect to the production of aflatoxin (a mycotoxin) and includes the following species, namely, *A. flavus*, *A. parasiticus*, *A. nomius*, *A. oryzae*, *A. sojae*, *A. tamarie*, and *A. pseudotamari* [9]. *Aspergillus* section *Nigri* is economically important, has applications in the industry and includes the following species: *A. niger*, *A. tubingensis*, and *A. carbonarius* [10].

Given the medical and biological importance of *Aspergillus* spp., their spread and diversity, and similarity to each other, precise identification of these sections and species is clearly necessary for any research conducted on the genus *Aspergillus*. The use of morphological criteria for identification of *Aspergillus* species in cancer patients is very difficult,,lacks of accuracy, speed, and sensitivity in many cases, and requires skilled and experienced personnel. Moreover, as environmental factors and conditions influence clinical, morphological, and other methods of identification, accurate methods are needed to identify *Aspergillus* sections and to differentiate them from one another. At present, numerous PCR-based methods are in use, which enjoy attributes such as the required accuracy, requisite test characteristics, and speed [11-13].

Most of the research conducted in Iran and elsewhere on the identification of *Aspergillus* species has used sequence determination techniques and/or PCR-RFLP [14-16]. However, only a few studies have employed universal and/or specific primers to identify *Aspergillus* species or have differentiated among a limited number of *Aspergillus* sections [17].

Thus, this study intended to identify species in the *Aspergillus* sections of *Flavi*, *Nigri*, and *Fumigati*, and to differentiate them from each other employing specific primers that have not been previously used for this purpose.

MATERIALS AND METHODS

Isolation and identification of Aspergillus species

A total of 112 environmental and 22 clinical isolates were collected for purposes of this study. The environmental isolates were taken from air and soil samples. Air samples were plated in chloramphenicol-containing Sabouraud Dextrose Agar medium while soil samples were suspended in a ratio of 3:1 in sterile physiological serum. Clinical isolates were obtained from patients suffering from different clinical forms of aspergillosis including invasive pulmonary aspergillosis, fungal sinusitis and onychomycosis [18, 19]. Three strains of *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger* that were identified using a sequence determination technique in a previous study were used as positive controls [18]. The isolated *Aspergillus* species were studied morphologically (colony characteristics) and microscopically by sporulation method [20, 21].

Bioinformatics study and primer design

The sequences of the five genes *beta-tubulin, calmodulin,* RNA polymerase II second largest subunit (*RPB2*), DNA replication licensing factor *Mcm7(MCM7),* and pre-rRNA processing protein *Tsr1* were first collected and the MEGA6 sequences in the NCBI software related to *Aspergillus* sections *Fumigati*, *Flavi*, and *Nigri,* available in the gene bank were retrieved. Data related to these three sections were compared and analyzed and, finally, the desired gene(s) were selected. The specific primers for each group were designed and synthesized by the Cinnagen Company, Iran. Table 1 shows the characteristics of the primers used.

DNA extraction

The phenol-chloroform method was used to extract DNA, according to Kachuei et al. (2009) [20]. The quality and quantity of the extracted DNA were evaluated using electrophoresis and NanoDrop, respectively.

PCR reaction

The PCR was performed in a total volume of 25 µL in each tube containing 12.5 µL Master mix (buffer, dNTP, Taq DNA polymerase, 2 mM MgCl2; Amplicon III, Denmark), 1 µL of the template DNA, 1 µL of each primer (20 pmol final concentration of

Table 1 - The primers used in the PCR, in order of the sequence and size of their products

Gene name	*Product size*	*Sequence*	*Primer*
Calmodulin	405	5′-GCTGAAGTGTTGGCRTTG3′	FFlv
Calmodulin	205	5′-GTGATTGACTTTTGCCGCCA-3′	FNig
Calmodulin	-	5′-CTCRCGGATCATCTCATC-3″	RFlaNig
B-tubulin	370	5′-CGACCTNTATCCTCCCAAT-3′	FFum
B-tubulin	-	5′-ACCCTCAGTGTAGTGACC-3′	RFum
Calmodulin	450	5′-GCCTCCTCYCTATTCGTRA-3′	FCmd42
Calmodulin	-	5′-GTAAGGAATTCTGGGCAGC-3′	RNig2

Table 2 - Characteristics of the isolated *Aspergillus* species.

Source of Aspergillus\ Name of Aspergillus section	*Nigri*	*Fumigati*	*Flavi*	*Other sections*	*Total*
Environmental isolates	52	26	22	12	112
Clinical isolates	4	6	10	2	22
Total number of isolates	56	32	32	14	134

each primer), and 9.5 µL distilled water. The PCR conditions were as follows. After pre-incubation at 94°C for 5 min, amplification for a total of 35 cycles was carried out that included denaturation at 94°C for 30 s, annealing at 56°C for 45 s, extension at 72°C for 60 s, and a final extension step of 7 min at 72°C. Amplicons were visualized by electrophoresis in 1% agarose gels using the SYBR safe sta

RESULTS

Results of morphological identification

Identification based on morphological characteristics of the isolated *Aspergillus* species indicated that, of the 112 environmental isolates, 52 were *Aspergillus* section *Nigri*, 26 were *Fumigati*, 22 were *Flavi*, and 12 belonged to other *Aspergillus* sections. Of the 22 clinical isolates, 10 were *Aspergillus* section *Flavi*, 6 were *Fumigati*, 4 were *Nigri*, and 2 belonged to other *Aspergillus* sections (Table 2).

Bioinformatic analysis

Gene sequence data showed that the *beta-tubulin* and *calmodulin* genes were suitable for differentiating among the *Aspergillus* sections of *Fumigati*, *Flavi*, and *Nigri*. Based on available information, the specific primers for the *Aspergillus* section *Flavi* could identify 18 of its 22 species, but could not identify the species *A. leporis*, *A. nomius*, *A. pseudonomius*, and *A. onaseus*. Most species in *Aspergillus* section *Nigri* can be detected and identified using the *FNig* and *RFlaNig* genes, and the species *A. japonicus*, *A. fingiensis*, *A. homomorphous*, and *A. aculeatus* can be identified using a second specific primer set for the genes *RNig2*, *FCmd42*. In this study, all the isolates belonging to *Aspergillus* section *Nigri* were amplified using the primers designed for *FNig* and *RFlaNig*.

Results of molecular identification

Figures 1-3 show results of the gel electrophoresis of PCR products for species identified as *Aspergillus* sections *Fumigati*, *Flavi*, and *Nigri*, obtained using specifically designed primers.

A comparison of the results of morphological identification with that of molecular identification showed that five (15.6%) of the species identified as *Aspergillus* section *Fumigati* by the morphological method did not belong to this section, and 2 (6.2%) of the species identified as *Aspergillus* section *Flavi*, based on morphology, were not members of this section. However, morphological identification matched molecular identification for the *Aspergillus* section *Nigri*.

DISCUSSION

As *Aspergillus* sections *Fumigati*, *Flavi* and *Nigri* are among the most important species in the ge-

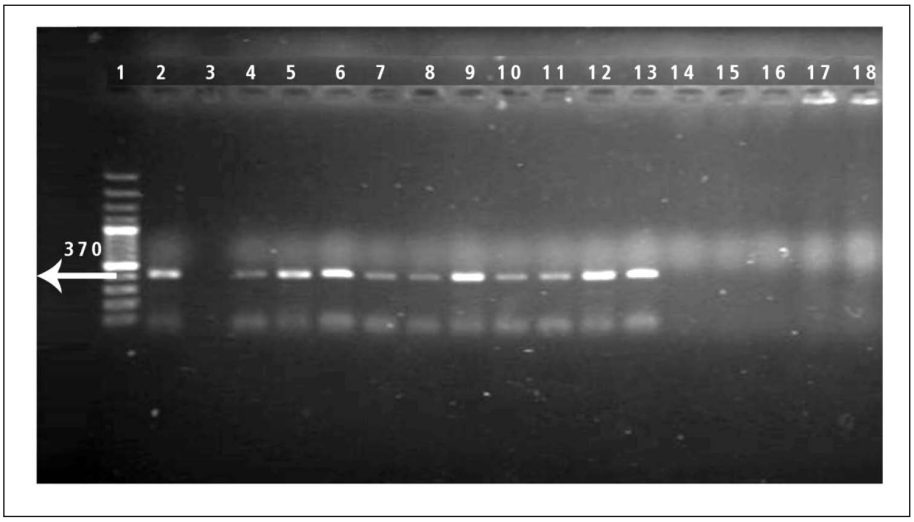

Figure 1 - Gel electrophoresis of PCR products obtained for the species belonging to the *Aspergillus* section *Fumigati* using the specific primers. Lane 1: 100 bp DNA size marker (Fermentas, Germany), Lane 2: positive control (clinical isolate of *A. fumigatus*), Lane 3: negative control, Lanes 4-13: the studied strains of *Aspergillus* section *Fumigati*, Lane 14: *A. niger*, Lane 15: *A. flavus*, Lanes 16-18: the isolates of *Penicillium*, *Fusarium*, and *Trichophyton interdigitale*, respectively.

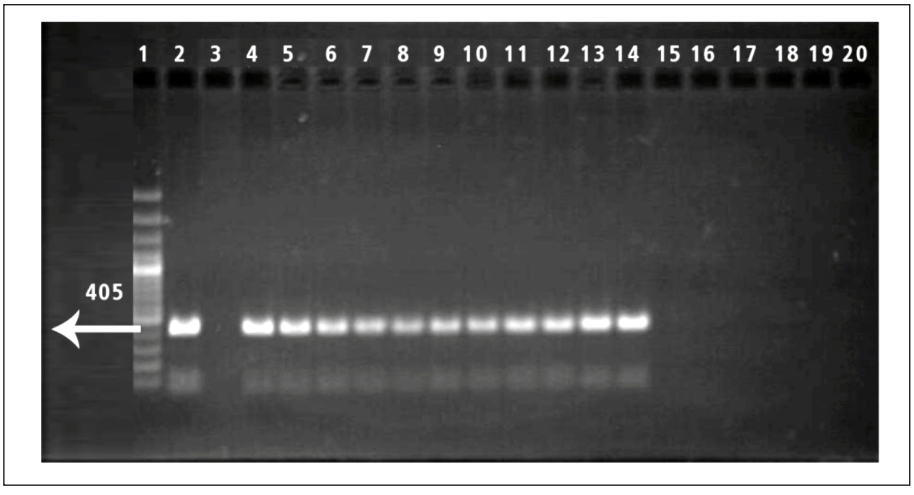

Figure 2 - Gel electrophoresis of PCR products obtained for species belonging to the *Aspergillus* section *Flavi* using the specific primers. Lane 1: 100 bp DNA size marker (Fermentas, Germany), Lane 2: positive control (clinical isolate of *A. flavus*), Lane 3: negative control, Lanes 4-14: the studied strains of *Aspergillus* section *Flavi*, Lane 15: *A. niger*, Lane 16: *A. fumigatus*, Lanes 17-20: the isolates of *Penicillium*, *Fusarium*, *T. interdigitale*, and *T. rubrum*, respectively.

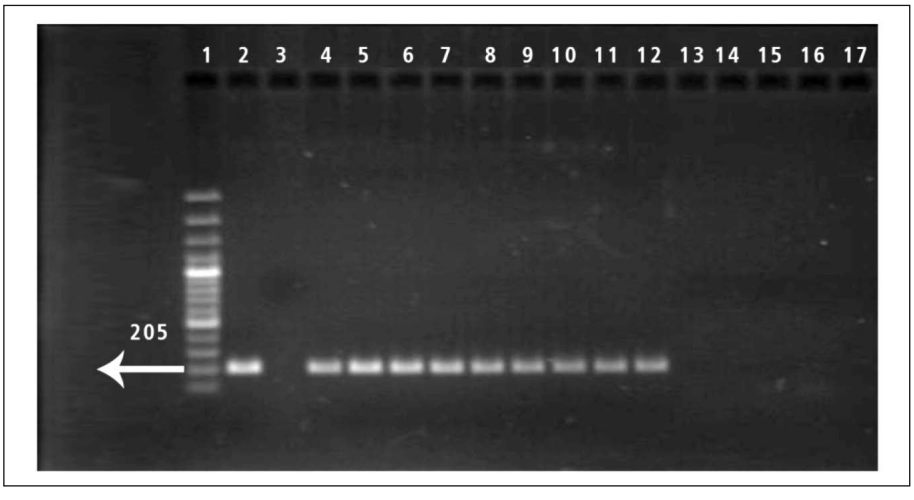

Figure 3 - Gel electrophoresis of PCR products obtained for the species belonging to the *Aspergillus* section *Nigri* using the specific primers. Lane 1: 100 bp DNA size marker (Fermentas, Germany), Lane 2: positive control (clinical isolate of *A. niger*), Lane 3: negative control, Lanes 4-12: the studied strains of *Aspergillus* section *Nigri*, Lane 13: *A. flavus*, Lane 14: *A. fumigatus*, Lanes 15-17: the isolates of *Penicillium*, *Fusarium*, and *T. interdigitale*, respectively.

nus *Aspergillus*, they were selected for this study [3]. Five genes (*beta-tubulin*, *calmodulin*, *Tsrl*, *RBP2*, and *MCM7*) were used to precisely differentiate among the *Aspergillus* isolates collected. Bioinformatic analyses of these genes revealed that *beta-tubulin* and *calmodulin* were ideally suited for differentiating among these three *Aspergillus* sections. Further, *beta-tubulin* was suitable for identification of the section *Fumigati* while *calmodulin* was suited for sections *Flavi* and *Nigri*.

Although Schmitt et al. (2009) demonstrated that segments of the *MCM7* and *Tsrl* genes are useful in establishing phylogenetic relationships between many fungi, especially among Ascomycetes, a bioinformatic search during the course of the present study revealed that little information was available in the NCBI database on *Aspergillus* species and the *Tsrl* and *MCM7* genes [22]. Additionally, the available information was not sufficiently useful in identifying and differentiating among the species, probably due to intraspecific diversity.

In a previous study, *Beta-tubulin* was reported to be important in the identification of *Aspergillus* species [15]. In 2005, Balajee et al. and Hong et al. stated that *beta-tubulin* is important for the identification of *A. lentulus* and in its differentiation from other species in *Aspergillus* section *Fumigati* [14, 15].

Yaguchi et al. (2007) used the *beta-tubulin*, *calmodulin*, and *hydrophobin* genes to identify species in *Aspergillus* section *Fumigati* and, based on these genes, classified the *Aspergillus* section *Nigri* into five clades [23].

In Iran, Shokohei et al. (2011) could identify some *Aspergillus* species based on the *beta-tubulin* gene sequence [24]. In the present study, the primer designed for the identification of the species belonging to *Aspergillus* section *Fumigati* was based on this gene sequence.

In recent years, the *calmodulin* gene has been considered important for the identification of *Aspergillus* species, and some reports have even stated it should be used as the primary gene for identification of *Aspergillus* species [15]. Varga et al. (2011) used this gene to identify species of *Aspergillus* section *Flavi* [9], and Palumbo et al. (2015) could differentiate among four species of *Aspergillus* section *Nigri* (*A. niger*, *A. welwitschiae*, *A. carbonaruis*, and *A. tobingensis*) by designing a group of primers for the *calmodulin* gene [17]. In the current report, the primers employed for the identification of isolates belonging to *Aspergillus* sections *Flavi* and *Nigri* were based on this gene.

We found that a limited number of species in *Aspergillus* sections *Fumigati* and *Flavi* did not respond to the designed primers, while all the analyzed isolates responded to the universal primers ITS1 and ITS4, reiterating the importance of using molecular methods of identification. A review of available literature shows that this is the first study that has used specifically designed primers to identify precisely the isolates belonging to these three *Aspergillus* sections.

CONCLUSION

The present study showed that the *beta-tubulin* and *calmodulin* genes are of greater use in precise identification and that the calmodulin gene is more suitable for *Aspergillus* sections *Flavi* and *Nigri*. We suggest that future studies should endeavor to differentiate further among species in the *Aspergillus* sections of *Fumigati*, *Flavi*, and *Nigri*.

Conflicts of interest. The authors have no conflicts of interest to disclose.

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