Saudi Journal of Biological Sciences 27 (2020) 751–756

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Evaluation of molecular identification of *Aspergillus* species causing fungal keratitis

Bader Alshehri [a,1], Palanisamy Manikandan [a,b,1,*]

[a] Department of Medical Laboratory Sciences, College of Applied Medical Sciences, Majmaah University, Al Majmaah 11952, Saudi Arabia
[b] Greenlink Analytical and Research Laboratory India Private Limited, Coimbatore 641014, India

ARTICLE INFO

Article history:
Received 22 November 2019
Revised 6 December 2019
Accepted 19 December 2019
Available online 26 December 2019

Keywords:
Keratitis
Aspergillus
ITS
DNA sequencing
Calmodulin

ABSTRACT

Fungal keratitis caused by the species of *Aspergillus* is a common and leading problem in developing countries like India. In this study, a total of 135 isolates from *Aspergillus* keratitis were studied by sequence analyses of the internal transcribed spacer (ITS) region performed by nucleotide-nucleotide BLAST analysis followed by the initial identification of the isolates based on conidial and colony morphology. The sequence analysis revealed several unusual species which were never reported in eye infections such as *A. tamrii, A. tubingensis, A. braslliensis, A. nomius*, *A. pseudonomius*, *A. sydowii*, *Eurotium amstelodami*. The sequence analysis of the ITS region; the β-tubulin and calmodulin genes brought out the genetic diversity among the isolates as the study intended to locate a more sensitive target sequence to study genetic diversity among a set of test fungal isolates. The PCR amplified sequences of the test isolates of the study as well as sequences belonging to section Flavi obtained from Genbank database were compared and analyzed along with three standard isolates by phylogenetic tree (Neighbor-joining) as to find out a target region/gene that could produce a better resolution to differentiate the isolates. Accordingly, the calmodulin gene had provided better resolution compared to ITS and β-tubulin to study the diversity among the test *Aspergillus* species isolated from fungal corneal ulcer.

1. Introduction

The use of molecular techniques based on polymerase chain reaction (PCR) offers a significant reduction in time required for an accurate diagnosis/identification of isolates, when compared with culture-based morphological methods (Kredics et al., 2015, 2007; Manikandan et al., 2013, 2009; P. Manikandan et al., 2008). PCR technique enables early initiation of specific antifungal therapy as well as it could rapidly amplify and detect fungal DNA even in minute quantities of ocular samples (Thomas, 2009). Though microscopic and colony morphology-based diagnoses of *Aspergillus* keratitis are employed (P. Manikandan et al., 2008), the causative *Aspergillus* strains are mostly identified and reported at the genus level only. As the pathogenic potential may vary between different species/isolates of the same genus and confirmation of fungal species based on morphological methods could be nonstandard, their identification at the species level using molecular techniques would be of great importance (Kredics et al., 2008, 2007; Manikandan et al., 2010, 2009; Palanisamy Manikandan et al., 2008). Further, morphology-based identification misleads where some of the species of *Aspergillus* are not expected as keratitis pathogens due to the lack of literature data about their involvement in corneal infections (Kredics et al., 2007). In addition, molecular studies re-identify certain isolates that are actually reported as 'others' by routine methods. Through the conventional morphological identification system, most of the studies on *Aspergillus* keratitis frequently report only the commonest species and using molecular techniques, there are recent publications (Hassan et al., 2016; Kredics et al., 2015, 2007; Manikandan et al., 2013, 2010, 2009; P. Manikandan et al., 2008) on other members of the genus that are less frequently occurring in keratitis. Deploying a comparative sequencing-based strategy that involves the ribosomal internal transcribed spacer (ITS) region and protein-coding loci, such as the β-tubulin and Calmodulin regions,

* Corresponding author at: Department of Medical Laboratory Sciences, College of Applied Medical Sciences, Majmaah University, Al Majmaah, Saudi Arabia.
E-mail address: m.palanisamy@mu.edu.sa (M. Palanisamy).
[1] Contributed equally to this work.
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2019.12.030
1319-562X/

for the identification of species within the complex of *Aspergillus* sp. are also being recommended widely (Tam et al., 2014). Therefore, the present study identified the test *Aspergillus* at species level using PCR amplified sequences of the ITS regions 1 & 2 and β-tubulin and calmodulin genes phylogenetically in order to genotype to determine the species/clonal nature of the isolates precisely and to examine the genetic diversity among the test *Aspergillus* isolates from fungal corneal ulcer/keratitis cases. Also, to find out a better gene target to identify *Aspergillus* species isolated from fungal keratitis.

2. Material and methods

2.1. Aspergillus strains

One hundred and thirty-five morphologically identified *Aspegillus* strains isolated from fungal corneal ulcer (Department of Microbiology, Aravind Eye Hospital and Postgraduate Institute of Ophthalmology, Coimbatore, India) belonged to September 2005 to August 2008 duration and were included in the study. Microbial cultures were considered positive only if growth of the same organism was observed on two or more solid media; or there was semiconfluent growth at the site of inoculation on one solid medium associated with the identification of the organism of appropriate morphology and staining characteristics on Gram stained corneal smears. After the completion of macroscopic and microscopic characterization, the study isolates were stored in 0.85% saline at 4 °C for further processing.

Cultivation and harvesting of *Aspergillus* species (Kredics et al., 2008, 2007; Manikandan et al., 2010, 2009; P. Manikandan et al., 2008).

The *Aspergillus* isolates were cultivated separately in 20 ml of YPD medium in Erlenmeyer flasks for 2 days at 25 °C on a rotary shaker (120–150 rpm). After cultivation, the mycelia were harvested from a 10 ml culture by transferring the culture to a vacuum filtration. The harvested mycelia were rinsed with several volumes (original culture volume) of 0.1 M $MgCl_2$ and thoroughly dried under vacuum. The dried material was transferred to a chilled mortar and ground into a powder in the presence of liquid N_2. The processed fungal material was stored under −20 °C for further cell wall lysis procedure.

2.2. Extraction and analysis of Aspergillus DNA

DNA extraction was performed using MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the instructions of the manufacturer.

2.3. Amplification of ITS, β-tubulin and calmodulin sequences in aspergilli

The ITS regions 1 & 2 of the ribosomal RNA (rRNA) gene complex, incorporating ITS1, the 5.8S rRNA gene and ITS2 of the *Aspergillus* DNA samples were amplified using primers ITS1 (Forward primer) and ITS4 (Reverse primer) (White et al., 1990). The segment of the β-tubulin gene of the fungal DNA was amplified using primers bT2a and bT2b (Glass and Donaldson, 1995). Similarly, a region of the calmodulin gene of the fungal DNA was also amplified using the primers cmd5 and cmd6 as described by Hong et al. (2006).

2.4. Isolation of amplified fragments of ITS regions, β-tubulin and calmodulin genes

The amplified *Aspergillus* DNA products/amplicons (ITS regions, β-tubulin and calmodulin genes) were fractionated and analyzed using agarose gel and the separation was carried out based on recommended electrophoretic methods. Further, amplicons/fragment isolation from the agarose gel was performed by Simga-Aldrich© GenElute™ Minus EtBr Spin Column according to the manufacturer's instructions.

2.5. Sequencing of the ITS regions, β-tubulin and calmodulin gene amplicons

The DNA sequences of ITS regions 1 & 2, β-tubulin and calmodulin genes were determined using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, CA, USA) and an ABI 3100 DNA sequencer. Both strands of each fragment were sequenced. The resulting sequences were deposited in the GenBank database. Sequence analyses were carried out by BLASTN similarity search at the website (http://www.ncbi.nlm.nih.gov/BLAST) of the National Center for Biotechnology Information and the findings were critically evaluated.

2.6. Phylogenetic analyses of the species of Aspergillus

DNA sequences representing ITS regions, β-tubulin and calmodulin genes were aligned using ClustalX and visually edited inGenedoc, version 2.6 (Nicholas et al., 1997). MEGA (MAC version 6) program was used to generate and edit the phylogenetic trees (Tamura et al., 2007) and the similarity/dissimilarity amongst the sequences of various aspergilii was studied. Following minimum essential standards, the findings were preliminarily analysed and considered for the phylogenetic assessment.

3. Results

The initial identification of the *Aspergillus* isolates based on colony and conidial morphologies was compared with the outcomes of ITS sequencing and a majority of the isolates were also confirmed by ITS sequence analyses. Upon sequencing and comparing the Internal Transcribed Spacer (ITS) regions 1 and 2, β-tubulin and calmodulin genes, a majority of the isolates (90; 67.4%) were confirmed to be *A. flavus* followed by *A. fumigatus* (17; 12.6%), *A. terreus* (8; 5.9%) and *A. tamrii* (8; 5.9%). The study also revealed several uncommon isolates such as *A. nomius* (1; 0.7%), *A. pseudonomius* (1; 0.7%), *A. sydowii* (1; 0.7%) and *Eurotium amstelodami* (1; 0.7%).

3.1. Sequence comparison of ITS regions 1 and 2 among aspergilli

The sequence sizes of amplified products (ITS regions) of the isolates ranged between 550 and 600 bp. The genetic diversity of the isolates (n = 135) was examined based on the sequence analyses of the ITS regions. The initial identification of the isolates based on colony and conidial morphologies of two [*A. fumigatus* (17;12.6%), and *A. terreus* (8; 5.9%)] genera was also confirmed by sequence analyses of the ITS regions performed by nucleotide-nucleotide BLAST analysis. The single exception was *A. flavus* in which, of the 102 originally included isolates (76% of 135) for ITS sequence comparison, only 94 (70% of 135) were re-confirmed as *A. flavus* and the remaining 8 (5.9% of 135 and 7.8% of 102) isolates were originally proved to belong *A. tamarii* based on ITS-based identification.

3.2. ITS regions and phylogenetic evaluation among A. flavus

The phylogenetic positions of isolates belonging to *A. flavus* (n = 53) and *A. tamarii* (n = 5) based on an aligned region of 504 nucleotides were assessed. A total of 46 *A. flavus* isolates belonged to an ITS genotype corresponding to the reference isolates ATCC

20043, NRRL 4822, NRRL 4998, NRRL3751, NRRL 458, NRRL 4818 and NRRL 3518 that were considered for the evaluation. However, 8 *A. flavus* (1945/05, 564/06, 567/06, 903/06, 1460/06, 328/07, 621/07 and501/07) showed only 64% similarity when compared with the sequences of standard isolates as well as other study isolates and were clustered separately. The multiple sequence alignment for 7 of 8 *A. flavus* (isolates 1945/05, 564/06, 567/06, 903/06, 1460/06, 328/07, and 621/07) revealed that the isolates differed from the rest of 46 isolates due to a nucleotide polymorphism pertaining to single base substitutions (base 'A' substituted with base 'G') leading to less similarity with the remaining 46 isolates. Similarly, *A. flavus* 501/07 differed from the remaining isolates because of a single base difference in which, 'A' substituted the base 'G'.

3.3. Calmodulin gene and ITS regions sequence comparison among A. flavus

The calmodulin gene/ITS regions 1 and 2 sequences of 28 study isolates of *A. flavus* were also subjected to phylogenetic analysis so as to compare between ITS and calmodulin gene approaches. It was found that the calmodulin based phylo tree had higher resolution than ITS based evaluation. Specifically, all the 28 isolates were grouped in to only four clusters based on the comparison among ITS sequences. Whereas the same group of isolates (n = 28) was differentiated into 11 clusters by calmodulin sequence comparison. The single exception was strain 2342/05, which was originally identified as *A. flavus* but confirmed to belong to *A. tamarii* based on ITS-based identification. The genetic diversity of the isolates was examined based on the sequence analysis of the ITS region. Fig. 1a shows the phylogenetic positions of the examined isolates within the genus based on an aligned region of 304 nucleotides. Most of the *A. flavus* isolates belonged to an ITS genotype corresponding to the reference strain ATCC 20043. Sequences of the *A. tamarii*, *A. terreus* and *A. fumigatus* isolates were identical with those of the reference strains NRRL 20818, ATCC 1012 and ATCC 9197, respectively. A total of 54 datasets from the findings of the comparative phylogenetic analysis for ITS regions and calmodulin gene are shown in Fig. 1a and 1b respectively.

3.4. Sequence comparison of β-tubulin gene among black aspergilli

A segment of β-tubulin gene was amplified (376-bp product) using primers bT2aand bT2b and sequenced for the re-confirmation of eight black aspergilli (*A. niger*) isolates that were earlier identified using morphological features. Based on the Genbank blast search of the β-tubulin sequences, only four isolates were re-confirmed as *A. niger* and the remaining were turned out to be *A. tubingensis* (n = 2) and *A. brasilliensis* (n = 2).

3.5. Sequence comparison of ITS regions among A. fumigatus

When the usage of ITS to determine the genetic diversity among *A. fumigatus* was evaluated, it was found that all the isolates belonged to an ITS genotype corresponding with the reference isolates ATCC 9197 and ATCC 16907 and showed 100% similarity with standard isolates except the strain 1439/06 which had only 49% similarity as a nucleotide polymorphism was identified pertaining to single base substitution. Analysis based on multiple sequence alignment of the 77 nucleotide sequence revealed that the substitution of the base C with G led to the difference in the strain 1439/06 from other isolates. It was also found that the strain was

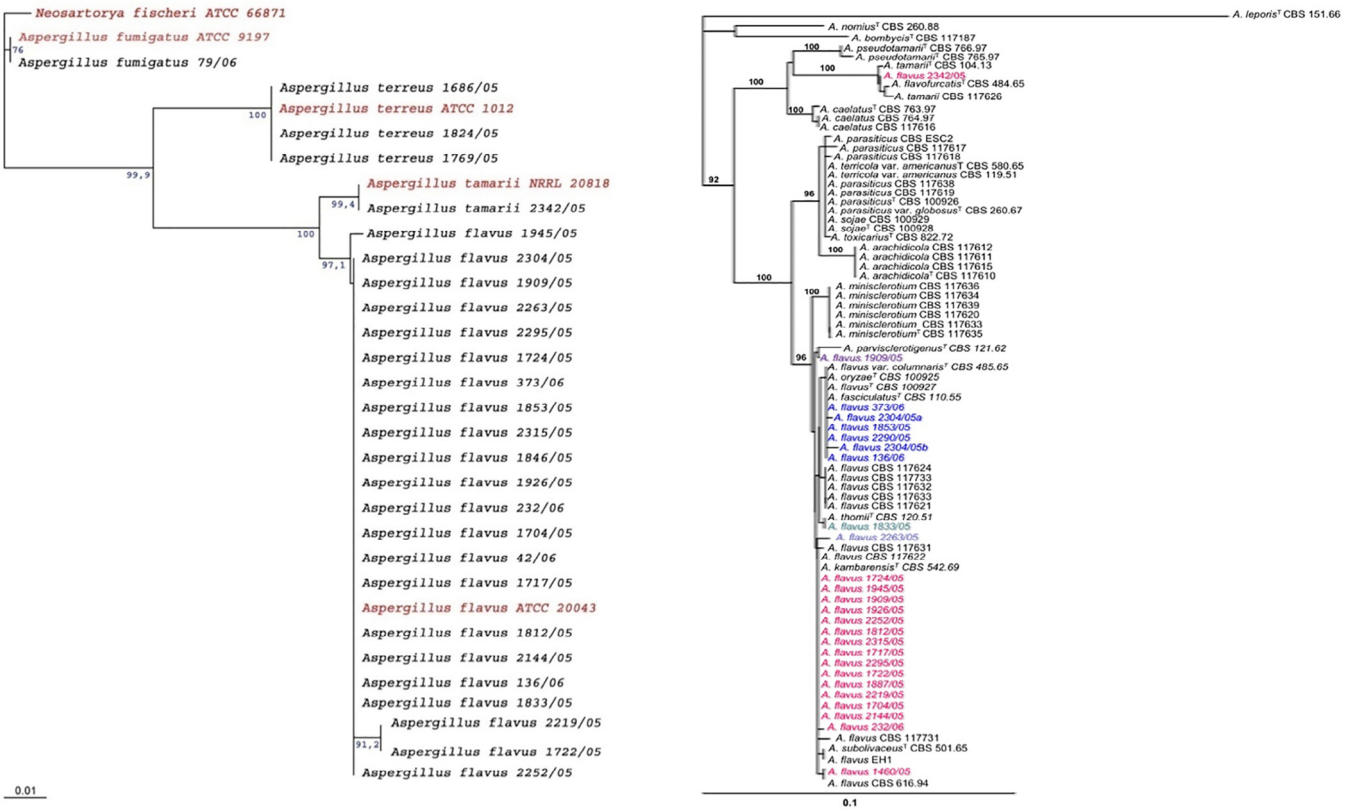

Fig. 1. (a) Genetic diversity of *Aspergillus* strains isolated from corneal ulcers based on the sequence analysis of the ITS region. Scale bar represents genetic distance. Numbers below branches indicate the percentages with which the given branch was supported by bootstrap analysis. (b) Neighbour-Joining tree showing genetic diversity of *A. flavus* isolates based on the sequence analysis of the calmodulin sequence data. Scale bar represents genetic distance.

highly similar to the sequence of *A. fumigatus* (WM 06.99) isolated from clinical source in Australia.

3.6. Sequence comparison of ITS regions, β-tubulin and calmodulin genes among the isolates of A. tamari, A. terreus, A. tubingensis, A. brasilliensis and A. nomius

ITS sequences of the *A. tamarii* (n = 8) and *A. terreus* (n = 7) isolates were identical with those of the reference isolates NRRL 35454 and ATCC 1012 respectively. All the isolates of *A. tamarii* and *A. terreus* isolates were grouped under separate clusters and showed respectively 66 and 100% similarity among the respective group. However, the ITS, β-tubulin, and calmodulin sequences of *A. tamarii* (2342/05) isolates were proved to be similar to the matching sequences of *A. tamarii* strain NRRL 25565. Similarly, the β-tubulin sequence of *A. tubingensis* isolate 39/07 was completely identical with the corresponding sequence of the *A. tubingensis* type strain CBS 134.48, whereas the isolate 620/07 of *A. tubingensis* differed from it in a single 'T' deletion. The sequences of the two isolates of *A. brasilliensis* were proved to be completely identical to each other as well as to the corresponding sequence of CBS 101740, the type strain of *A. brasiliensis*.

4. Discussion

Based on the molecular identification techniques involving ITS regions, β-tubulin and Calmodulin genes characterization, 16 isolates were independent and grouped under seven uncommon/rare species, which were previously identified as various other species using conventional mold identification techniques. Remarkably, all these uncommon species were yet to be reported in eye infections and hence were published as case studies between 2007 and 2010 (Table 1) (Kredics et al., 2009, 2008, 2007; Manikandan et al., 2010, 2009) by the authors of the study.

In this study, to evaluate the usefulness of sequencing ITS regions, β-tubulin and calmodulin genes so as to assess the genetic diversity amongst aspergilli, a computational analysis was also carried out using appropriate sequence data (ITS, β-tubulin and calmodulin genes) of three standard isolates [*A. pseudonomius* NRRL 3353 (AF338643), *A. pseudonomius* NRRL 6552 (EF661528) and *A. pseudocaelatus* CBS 117616T (EF409242)]. Precisely, the sequences of isolates belonging to section Flavi were obtained from Genbank database, compared and analyzed as stated earlier to assess the isolates. Based on the analysis, it was found that targeting the calmodulin gene had provided better resolution compared to ITS and β-tubulin. In particular, clade wise grouping of aspergilli of Flavi section revealed more resolution amongst calmodulin sequences than others.

Accurate identification of pathogenic species and prompt diagnosis of the disease are important for epidemiological purposes particularly in the context of serving as a guide to clinical management/control pathogens (Marr et al., 2002; Steinbach et al., 2004; Steinbach and Stevens, 2003) and several tools and methods have been developed over the years for this purpose. In particular, the improvements in DNA analysis and the role of Polymerase Chain Reaction (PCR) in recent years including their cost- effectiveness attribute a significant utility for molecular approaches towards accurate and prompt identification of fungal pathogens (Bagyalakshmi et al., 2008) compared with culture-based morphological identification. While use of molecular identification/ typing methods would enable skipping of conventional culture methods of pathogen's as their DNA could be isolated and purified employing suitable tools directly from collected specimens.

In the absence of such molecular techniques, most of the *Aspergillus* strains isolated from keratomycosis in ophthalmic diagnostic laboratories could unfortunately be identified and reported at the genus level only, although their accurate identification at the species level would be of great importance in the context of an effective/dynamic therapy (P. Manikandan et al., 2008). More importantly, the recent reports of reduced antifungal drug susceptibilities among some species of *Aspergillus* (Kontoyiannis et al., 2002; Lass-Flör et al., 2008; Sutton et al., 1999), reinforce the use of molecular methods for the timely and accurate identification of aspergilli to the species or strain/clonal level (Ellis, 2002).

Molecular strain typing methods are the powerful tools and several different gene targets for molecular identification of *Aspergillus* species have been investigated such as various ribosomal RNA, DNA topoisomerase, mitochondrial cytochrome *b*, beta-globulin, etc. Of which the most effectively used target to date have been the 5′ end of the large subunit rRNA gene (D1-D2 region), the Internal Transcribed Spacer (ITS) regions 1 and 2 and β-tubulin and calmodulin gene sequences (Bagyalakshmi et al., 2008). In particular, sequencing of the ITS regions, β-tubulin and calmodulin gene and their evaluation have several specific advantages to identify the *Aspergillus* in to genus and section level species (Balajee S A, 2008).

The present study investigated the usefulness of molecular identification methods (ITS regions, β-tubulin and calmodulin gene sequences) over conventional microbiological methods so as to study the aspergilli caused keratitis more precisely and rapidly. Critical analyses of ITS sequences were done for all the isolates in general forre-conforming the morphological identification. Further, the uncommon species identified by ITS analyses were in turn reconfirmed through specific evaluation of other gene loci such as β-tubulin for black aspergilli and calmodulin for A. nomius and *A. tamari*.

Of the 135 aspergilli identified initially based on conidial and colony morphology, only 88 isolates were noted to be in

Table 1
Identification and sequence details of uncommon species of *Aspergillus* isolated from corneal ulcer reported previously.

Species	Numbers deposited	GenBank accession numbers/gene target	Reference
A. tubingensis	2	EU600388 (*β-tubulin*) EU600389 (*β-tubulin*)	Kredics et al. (2009)
A. brasiliensis	2	EU600387 (*β-tubulin*) EU600386 (*β-tubulin*)	Manikandan et al. (2010)
A. nomius	1	GQ221261 (*ITS*) GQ221262 (*β-tubulin*) GQ221263 (*calmodulin*)	Manikandan et al. (2009)
A. tamarii	1	EF525554 (*ITS*) EF525555 (*β-tubulin*)	Kredics et al. (2007)
A. tamarii	6	*ITS, β-tubulin, calmodulin*	Homa et al. (2019)
A. sydowii	1	*ITS, β-tubulin, calmodulin*	Unpublished
A. pseudonomius	1	*ITS, β-tubulin, calmodulin*	Unpublished
Eurotium amstelodami	1	*ITS, β-tubulin, calmodulin*	Unpublished

concurrence with the identification results generated based on sequence analysis of ITS regions performed by nucleotide-nucleotide BLAST analysis. Remarkably, the remaining 12% of the isolates was identified as uncommon/rare species. Precisely, 8 (6%) isolates were confirmed *as A. tamarii* which were initially identified as *A. flavus* by microscopic and macroscopic features and they formed a separate group as there was single nucleotide/base polymorphism of substitution. Further, 57% (4 of 7isolates) of the isolates from section Nigri was also noted to belong two different species such as *A. brasilliensis* and *A. tubingensis*. Of the eight *A. niger*earlier identified using morphological features, only four were re-confirmed as *A. niger* and the remaining were turned out to be *A. tubingensis* (n = 2) and *A. brasilliensis* (n = 2) based on the Genbank blast search of the β-tubulin sequences. However, morphologically confirmed isolates of *A. terreus* and *A. fumigatus* were 100% in consensus with the ITS based identification.

In this study, the ITS regions 1 and 2 based evaluations also brought out that *A. fumigatus* had high intraspecies similarity except the isolate 1439/06which had single nucleotide substitution and the sequence of this isolate had 100%identity with the strain *A. fumigatus* WM 06.99 isolated from clinical source in Australia (unpublished data from Genbank). Kanbe et al. (2002) applied nested PCR with a mixture of specific primers to the DNA topoisomerase II gene for the specific identification of five medically important species of *Aspergillus* such as *A. flavus*, *A. fumigatus*, *A. niger*, *A. nidulans* and *A. terreus*. Such identification approaches enable the detection of a wider range of species -including most of the keratitis pathogenic aspergilli and point out the potential to be adapted for the fast identification of the causative agents of *Aspergillus* keratitis at the species level. These scrutinization suggested that simplified PCR kits could have the potential to provide highly sensitive and specific diagnostic capabilities for detecting ocular pathogens in the laboratory. Real-time PCR may also be promising for this purpose (Goebes et al., 2007). However, the main limitations of the widespread use of PCR-based techniques are the expertise and cost, which are essential and more than that of classical culture-based methods.

In another study, ITS1 targeted PCR identification system was described for the three major human pathogenic species of *Aspergillus* which included *A. flavus*, *A. fumigatus*, and *A. niger* and subsequent, separate PCR reactions or nested PCR with species-specific primers (Sugita et al., 2004). de Aguirre et al. (2004) successfully designed DNA probes directed to the ITS2 region of rDNA to differentiate seven medically important *Aspergillus* species, *A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *A. terreus*, *A. ustus* and *A. versicolor* from one another and from other opportunistic molds and yeasts by using an amplification and detection system based on PCR-enzyme immunoassay. Similarly, Bagyalakshmi et al. (2008) analyzed the genetic similarity among ocular isolates of *A. flavus* by PCR-RFLP and DNA sequencing of the ITS region, and reported a novel pattern of nucleotide polymorphisms due to inversions and substitutions as well as both single-nucleotide differences and short lengths of sequence diversity due to insertions or deletions.

Conclusively, the phylogenetic analyses comprising the target sequences of the study among *A. flavus*, calmodulin-based evaluation brought out a higher resolution. In particular, this would assist in typing/categorizing the fungal isolates leading to unambiguous identification of the fungal pathogen and in estimating the incidence of *Aspergillus* species in corneal ulcers from an area. As the increasing context of *Aspergillus* keratitis is one of the major causes of blindness across the globe, frequent studies focusing on the epidemiological particulars of the disease and diversity facts of the pathogen are very significant. Remarkably, enhancing conventional methods of *Aspergillus* isolation with interventions through cost-effective molecular methods-based identification would increment the genetic diversity studies of *Aspergillus* isolates as well as the microbiological investigations (for precise and prompt diagnosis) of the fungal keratitis.

Data availability

The collected data used to support the findings of this study are included within the article.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

The Authors would like to thank Deanship of Scientific Research at Majmaah University, Al Majmaah, 11952, Saudi Arabia for supporting this work under the Project Number R-1441-51.

References

Bagyalakshmi, R., Therese, K.L., Prasanna, S., Madhavan, H.N., 2008. Newer emerging pathogens of ocular non-sporulating molds (NSM) identified by polymerase chain reaction (PCR)-based DNA sequencing technique targeting internal transcribed spacer (ITS) region. Curr. Eye Res. https://doi.org/10.1080/02713680701864780.

Balajee, S.A., 2008. DNA sequence based methods for species identification in the genus Aspergillus. Aspergillus species in human keratomycosis. In: Varga, J., Samson, R.A. (Eds.), Aspergillus in the Genomic Era. Wageningen Academic Publishers, The Netherlands, pp. 261–275.

de Aguirre, L., Hurst, S.F., Choi, J.S., Shin, J.H., Hinrikson, H.P., Morrison, C.J., 2004. Rapid differentiation of aspergillus species from other medically important opportunistic molds and yeasts by PCR-enzyme immunoassay. J. Clin. Microbiol. 42, 3495–3504. https://doi.org/10.1128/JCM.42.8.3495-3504.2004.

Ellis, D., 2002. Amphotericin B: spectrum and resistance. J. Antimicrob. Chemother. 49 (Suppl 1), 7–10.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. https://doi.org/10.1017/CBO9781107415324.004.

Goebes, M.D., Hildemann, L.M., Kujundzic, E., Hernandez, M., 2007. Real-time PCR for detection of the Aspergillus genus. J. Environ. Monit. https://doi.org/10.1039/b618937g.

Hassan, A.S., Al-Hatmi, A.M.S., Shobana, C.S., Van Diepeningen, A.D., Kredics, L., Vágvölgyi, C., Homa, M., Meis, J.F., De Hoog, G.S., Narendran, V., Manikandan, P., 2016. Antifungal susceptibility and phylogeny of opportunistic members of the genus fusarium causing human keratomycosis in South India. Med. Mycol. 54. https://doi.org/10.1093/mmy/myv105.

Homa, M., Manikandan, P., Szekeres, A., Kiss, N., Kocsubé, S., Kredics, L., Alshehri, B., Dukhyil, A.A. Bin, Revathi, R., Narendran, V., Vágvölgyi, C., Shobana, C.S., Papp, T., 2019. Characterization of Aspergillus tamarii strains from human keratomycoses: molecular identification, antifungal susceptibility patterns and cyclopiazonic acid producing abilities. Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.02249.

Hong, S.B., Cho, H.S., Shin, H.D., Frisvad, J.C., Samson, R.A., 2006. Novel Neosartorya species isolated from soil in Korea. Int. J. Syst. Evol. Microbiol. https://doi.org/10.1099/ijs.0.63980-0.

Kanbe, T., Yamaki, K., Kikuchi, A., 2002. Identification of the pathogenic Aspergillus species by nested PCR using a mixture of specific primers to DNA topoisomerase II gene. Microbiol. Immunol. https://doi.org/10.1111/j.1348-0421.2002.tb02771.x.

Kontoyiannis, D.P., Lewis, R.E., May, G.S., Osherov, N., Rinaldi, M.G., 2002. Aspergillus nidulans is frequently resistant to amphotericin B. Mycoses. https://doi.org/10.1046/j.1439-0507.2002.00797.x.

Kredics, L., Narendran, V., Shobana, C.S., Vágvölgyi, C., Manikandan, P., Varga, J., Galgóczy, L., Kocsubé, S., Németh, T.M., Papp, T., Homa, M., Baranyi, N., Szekeres, A., Körmöczi, P., Krizsán, K., Anita, R., Revathi, R., Gomathi, P., Hassan, A.S., Singh, Y.R.B., Mythili, A., Selvam, K.P., Dóczi, I., Leitgeb, B., 2015. Filamentous fungal infections of the cornea: A global overview of epidemiology and drug sensitivity. Mycoses. https://doi.org/10.1111/myc.12306.

Kredics, L., Varga, J., Antal, Z., Samson, R.A., Kocsubé, S., Narendran, V., Bhaskar, M., Manoharan, C., Vágvölgyi, C., Manikandan, P., 2008. Black aspergilli in tropical infections. Rev. Med. Microbiol. 19. https://doi.org/10.1097/MRM.0b013e3283280391.

Kredics, L., Varga, J., Kocsubé, S., Dóczi, I., Samson, R.A., Rajaraman, R., Narendran, V., Bhaskar, M., Vágvölgyi, C., Manikandan, P., 2007. Case of keratitis caused by Aspergillus tamarii. J. Clin. Microbiol. 45. https://doi.org/10.1128/JCM.00920-07.

Kredics, L., Varga, J., Kocsubé, S., Rajaraman, R., Raghavan, A., Dóczi, I., Bhaskar, M., Németh, T.M., Antal, Z., Venkatapathy, N., Vágvölgyi, C., Samson, R.A., Chockaiya,

M., Palanisamy, M., . Infectious keratitis caused by Aspergillus tubingensis. Cornea. https://doi.org/10.1097/ICO.0b013e3181967098.

Lass-Flör, C., Mayr, A., Perkhofer, S., Hinterberger, G., Hausdorfer, J., Speth, C., Fille, M., 2008. Activities of antifungal agents against yeasts and filamentous fungi: Assessment according to the methodology of the European committee on antimicrobial susceptibility testing. Antimicrob. Agents Chemother. https://doi.org/10.1128/AAC.00662-08.

Manikandan, P., Dóczi, I., Kocsubé, S., Varga, J., Németh, T.M., Antal, Z., Vágvölgyi, C., Bhaskar, M., Kredics, L., 2008a. Aspergillus species in human keratomycosis. Aspergillus in the Genomic Era. https://doi.org/10.3920/978-90-8686-635-9.

Manikandan, P., Varga, J., Kocsubé, S., Anita, R., Revathi, R., Németh, T.M., Narendran, V., Vágvölgyi, C., Panneer Selvam, K., Shobana, C.S., Babu Singh, Y.R., Kredics, L., 2013. Epidemiology of Aspergillus keratitis at a tertiary care eye hospital in South India and antifungal susceptibilities of the causative agents. Mycoses 56. https://doi.org/10.1111/j.1439-0507.2012.02194.x.

Manikandan, P., Varga, J., Kocsubé, S., Revathi, R., Anita, R., Dóczi, I., Németh, T.M., Narendran, V., Vágvölgyi, C., Bhaskar, M., Manoharan, C., Samson, R.A., Kredics, L., 2010. Keratitis caused by the recently described new species Aspergillus brasiliensis: Two case reports. J. Med. Case Rep. 4. https://doi.org/10.1186/1752-1947-4-68.

Manikandan, P., Varga, J., Kocsubé, S., Samson, R.A., Anita, R., Revathi, R., Dóczi, I., Németh, T.M., Narendran, V., Vágvölgyi, C., Manoharan, C., Kredics, L., 2009. Mycotic keratitis due to Aspergillus nomius. J. Clin. Microbiol. 47. https://doi.org/10.1128/JCM.01051-09.

Manikandan, Palanisamy, Vismer, H.F., Kredics, L., Doczi, I., Marasas, W.F.O., Bhaskar, M., Anita, R., Revathi, R., Narendran, V., 2008b. Corneal ulcer due to Neocosmospora vasinfecta in an immunocompetent patient. Med. Mycol. https://doi.org/10.1080/13693780701625149.

Marr, K.A., Carter, R.A., Crippa, F., Wald, A., Corey, L., 2002. Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. Clin. Infect. Dis. https://doi.org/10.1086/339202.

Nicholas, K.B., Nicholas, H.B., Deerfield, D.W., 1997. EMBNEW. NEWS. EMBNEW. NEWS 4, 14.

Steinbach, W.J., Benjamin, D.K., Kontoyiannis, D.P., Perfect, J.R., Lutsar, I., Marr, K.A., Lionakis, M.S., Torres, H.A., Jafri, H., Walsh, T.J., 2004. Infections due to Aspergillus terreus: A multicenter retrospective analysis of 83 cases. Clin. Infect. Dis. https://doi.org/10.1086/421950.

Steinbach, W.J., Stevens, D.A., 2003. Review of newer antifungal and immunomodulatory strategies for invasive aspergillosis. Clin. Infect. Dis. https://doi.org/10.1086/376523.

Sugita, C., Makimura, K., Uchida, K., Yamaguchi, H., Nagai, A., 2004. PCR identification system for the genus Aspergillus and three major pathogenic species: Aspergillus fumigatus, Aspergillus flavus and Aspergillus niger. Med. Mycol. https://doi.org/10.1080/13693780310001656786.

Sutton, D.A., Sanche, S.E., Revankar, S.G., Fothergill, A.W., Rinaldi, M.G., 1999. In vitro amphotericin B resistance in clinical isolates of Aspergillus terreus, with a head-to-head comparison to voriconazole. J. Clin. Microbiol.

Tam, E.W.T., Chen, J.H.K., Lau, E.C.L., Ngan, A.H.Y., Fung, K.S.C., Lee, K.C., Lam, C.W., Yuen, K.Y., Lau, S.K.P., Woo, P.C.Y., 2014. Misidentification of Aspergillus nomius and Aspergillus tamarii as Aspergillus flavus: Characterization by internal transcribed spacer, β-tubulin, and calmodulin gene sequencing, metabolic fingerprinting, and matrix-assisted laser desorption ionization-ti. J. Clin. Microbiol. https://doi.org/10.1128/JCM.03258-13.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599. https://doi.org/10.1093/molbev/msm092.

Thomas, P.A., 2010. Aspergillus keratitis. In: Aspergillosis: From Diagnosis to Prevention. Springer Netherlands, Dordrecht, pp. 973–998. doi:10.1007/978-90-481-2408-4_57.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols. https://doi.org/10.1016/B978-0-12-372180-8.50042-1.