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Aspergillus aureoterreus, *A. pseudonomiae*, *A. siamensis*, and *A. flocculosus-ochraceopetaliformis* as Four New Pathogens of Eye Infection in DNA Barcoding Markers

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Background: The use of a morphological method to determine *Aspergillus* species in routine laboratory investigation has always been challenged with low sensitivity and low specificity. Time-consuming specimen preparation and insufficient expertise are the crucial reasons. DNA barcoding markers have been introduced for identification as another gold standard method.

Objective: To investigate DNA barcoding markers in identifying *Aspergillus* strains isolated from patients with eye infection.

Methods: This study extracted 12 DNA samples of 12 *Aspergillus* strains. DNA barcoding genes, i.e., ITS, beta-tubulin (BT2), calmodulin (CAL), and actin (ACT) genes, were amplified and sequenced by polymerase chain reaction and Sanger's sequencing methods. All gene sequences were compared to those of the type or reference species collected from public databases. The species were identified with phylogenetic analyses in free applications from public websites.

Results: ITS, BT2 and CAL, and ACT identified 5, 6, and 3 species and 3, 2, and 1 species complex of *Aspergillus*, respectively. DNA barcoding with multi-locus sequence typing revealed four new pathogens of human eye infection, including *A. aureoterreus*, *A. pseudonomiae*, *A. siamensis*, and *A. flocculosus-ochraceopetaliformis*.

Conclusion: The DNA barcoding markers identified *Aspergillus* species with better specificity than the conventional method. The markers and techniques need to be used as tools for routine *Aspergillus* identification.

Key Words: *Aspergillus* infection, DNA barcoding, Fungal keratitis, New fungal pathogen

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INTRODUCTION

The morphological identification of mold in a routine laboratory investigation always struggles with problems of cultivation, specimen preparation, time consumption, and expertise insufficiency in mycological taxonomy. Molecular techniques, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and DNA identification, have been introduced to resolve those problems. The MALDI-TOF MS appears more appropriate for routine work because of its faster and simpler techniques but its precision and accuracy remain involved in many factors, i.e., specimen preparation, extraction method, library database, etc. Identification with DNA barcoding markers has been another "gold standard method" for fungal identification[1,2]. The method includes polymerase chain reaction (PCR), DNA sequencing, and phylogenetic analyses, which have presently been normalized to perform with a lower cost, less complicated techniques, free software, and an available public database. Consequently, the method should be introduced for routine laboratory use.

Fungal keratitis (FK) and endophthalmitis are serious eye infections characterized by a severe sight-threatening condition. Over 95% of all FK cases are caused by filamentous fungi. Filamentous fungi have caused >50% of all microbial keratitis with culture-positive cases[3,4], and the incidence has increased globally[5-8]. Over 70 filamentous fungal species have been reported as pathogens. *Fusarium* spp. and *Aspergillus* spp. are the most predominant causative agents[9,10]. The patients with *Aspergillus* keratitis demonstrated poorer prognosis than those infected by *Fusarium* and other filamentous fungi[11]. *Aspergillus flavus* has been a dominant species to cause *Aspergillus* keratitis, followed by *A. terreus*, *A. fumigatus*, *A. niger*, *A. nidulans*, *A. ochraceous*[12-14], which are all prevalent species in human environments. However, some unfamiliar species have caused keratitis such as *A. aculeatus*[15], *A. nomiae*[16], *A. viridinutans*[17], and *A. minisclerotigenes*[18]. Many *Aspergillus* species that thrive in human environments were reported infections in humans. The possibility of inducing eye infection by a new pathogenic species needs to be considered. The present study investigated the potentiality of DNA barcoding markers for identifying 12 *Aspergillus* strains isolated from patients with eye infections.

MATERIALS AND METHODS

1. Collection of *Aspergillus* strains, morphological identification, and growth observation

A total of 12 *Aspergillus* strains were isolated from cornea scraping specimens of 12 patients mostly diagnosed with FK. Those patients were admitted to the Srinagarind Hospital, Khon Kaen, Thailand, from January 1, 2022, to December 31, 2022. All *Aspergillus* strains were presumptively determined into species by morphological characteristics. The optimal growths of all strains were examined by inoculating on blood agar (BA) and Sabouraud dextrose agar (SDA) and incubated at 25℃ and 37℃. The growth of each strain was assessed by measuring a colony-diameter. The data were

Table 1. Primers used in this study

Genes	Primers' name	Primer pair sequences (5' to 3')	Ref.
ITS	ITS1 (Fw)	TCCGTAGGTGAACCTGCGG	19
	ITS4 (Rw)	TCCTCCGCTTATTGATATGC	20
	ITS5 (Fw)	GGAAGTAAAAGTCGTAACAAGG	20
	V9G (Fw)	TTACGTCCCTGCCCTTTGTA	19
	LS266 (Rw)	GCATTCCCAAACAACTCGACTC	21
Actin	ACT-512F (Fw)	ATGTGCAAGGCCGGTTTCGC	22
(ACT)	ACT-783R (Rw)	TACGAGTCCTTCTGGCCCAT	22
Beta tubulin	Bt2a (Fw)	GGTAACCAAATCGGTGCTGCTTTC	23
(BT2)	Bt2b (Rw)	ACCCTCAGTGTAGTGACCCTTGGC	23
Calmodulin	cmd5 (Fw)	CCGAGTACAAGGAGGCCTTC	18
(CAL)	cmd6 (Rw)	CCGATAGAGGTCATAACGTGG	18

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processed and analyzed with MS Excel 2020 and Statistical Package for the Social Sciences version 20.0 software. The student *t*-test for continuous variables was conducted to analyze the different radial growth rates between culture media and incubation temperature. A *p*-value of <0.05 indicated a statistical significance.

2. DNA extraction, PCR, and Sanger sequencing

A DNA extraction kit (Vivantis Technologies Sdn Bhd., Selangor Darul Ehsan, MY) was used to DNA-extract all strains. NanoDrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA) was used to quantify DNAs. The internal transcribed spacer (ITS) of the ribosomal gene and partial sequences of actin (ACT), beta-tubulin (BT2), and calmodulin (CAL) genes were amplified by PCR with the primers[18-23] shown in Table 1. The PCR reaction contained 10 μM in 1.5 μl for each primer, 12.5 μl of KOD One™ PCR Master Mix (TOYOBO, JP), and >10 ng of genomic fungal DNA, and added with sterile water to reach a 25 μl of total volume reaction. The PCR condition started at 95℃ for 5 min and was followed by 35 cycles of denaturation at 95℃ for 35 s, annealing at 48℃ for 30 s, extension at 72℃ for 30 s, and amplification in Veriti® Thermal Cycler (Applied Biosystems, CA, USA). The amplification products were checked with 2% gel electrophoresis and submitted to Sanger's sequencing. The Supplementary data file shows the sequences.

3. Identification using phylogenetic analysis of DNA sequences

The sequences of each gene resulted from Sanger's sequencing were edited with BIOEDIT version 7.2.5[24] and blasted against the type materials and some reference strains in GenBank with a megablast in BLAST® blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch). The datasets, including the sequences of the tested strain and those resulting from the megablast, were prepared by BIOEDIT

Table 2. Patients' information and other routine investigations associated to twelve *Aspergillus* strains

No.	Patients' information			Diag.†	Treat‡	Specimen site§	Patho. exam.‖	PCR¶	Fungal cultivation	
	Sex	Age	Under. con.*						Morpho. Ident.**	Strain No.
1	F	60	UD	FK	PKP	Lt	+	+	*A. fumigatus*	FG-026
2	M	52	OT	FK	EV	Lt	ND	ND	*A. flavus*	FG-033
3	M	40	UD	FK	EV	Rt	+	ND	*Aspergillus* sp.	FG-050
4	M	39	OT	FK	EV	Lt	+	ND	*A. fumigatus*	FG-054
5	M	63	UD	FK	PKP	Lt	+	ND	*A. flavus*	FG-060
6	M	67	UD	FK	PKP	Rt	ND	+	*Aspergillus* sp.	FG-069
7	M	44	DM	FK	EV	Rt	+	ND	*A. flavus*	FG-074
8	F	64	UD	FK	PKP	Rt	ND	ND	*A. flavus*	FG-076
9	M	40	OT	ED	EV	Rt	ND	ND	*Aspergillus* sp.	FG-088
10	M	74	UD	FK	EV	Lt	ND	+	*A. flavus*	FG-090
11	M	43	UD	FK	Med.	Lt	ND	+	*A. terreus*	FG-091
12	F	87	UD	FK	PKP	Lt	+	+	*A. terreus*	FG-093

*Underlying conditions: UD=undetermined, OT=ocular trauma, DM=Diabetes Mellitus
†Diagnosis: FK=fungal keratitis, ED=Endophthalmitis
‡Treatment: PKP=Penetrating keratoplasty, EV=Evisceration, Med.=treatment with antifungal drugs but lost the follow up
§Specimen site: Lt=left eye, Rt=right eye
‖ Histopathological examination, ¶PCR of fungal DNA in specimens: + = positive for fungal elements/fungal DNA, ND = not determine
**Morphological identification

version 7.2.5 and aligned with the algorithms, i.e., ClustalW in BIOEDIT version 7.2.5 or Multiple Alignment with Fast Fourier Transform (MAFFT) or MUltiple Sequence Comparison by Log-Expectation (MUSCLE)[25,26] in EMBI-EBI web server (https://www.ebi.ac.uk/jdispatcher/). Phylogenetic analyses for identification had been conducted with MEGA X[27]. The best model and appropriate phylogenetic algorithms were inferred to reconstruct a phylogenetic tree and to estimate percentage similarity.

4. Antifungal susceptibility test

An AST kit, Thermo Scientific™ Sensititre™ YeastOne™ YO10 AST Plate (Thermo Scientific Inc., UK), was used to test 12 *Aspergillus* strains and a reference strain, *A. fumigatus* ATCC-MYA-3626. The inoculum was prepared with a CLSI M38-A2 protocol. The conidial inoculum concentration was approximately 0.4 × 10^4 to 5 × 10^4 CFU/ml. Each strain was tested against 9 antifungal drugs, i.e., anidulafungin (AND), caspofungin (CAS), micafungin (MF), amphotericin B (AB), flucytosine (FC), fluconazole (FZ), itraconazole (IZ), posaconazole (PZ), and voriconazole (VZ). The AST plates were kept at 28-30℃, and the minimal effective concentration (MEC) values were observed after incubation for 24 h by microscopy examination. The minimal inhibitory concentration (MIC) values were observed after 48 h of incubation by a Vizion system, Thermo Scientific™ Sensititre™ Vizion™ Digital MIC Viewing System (Thermo Scientific Inc., UK).

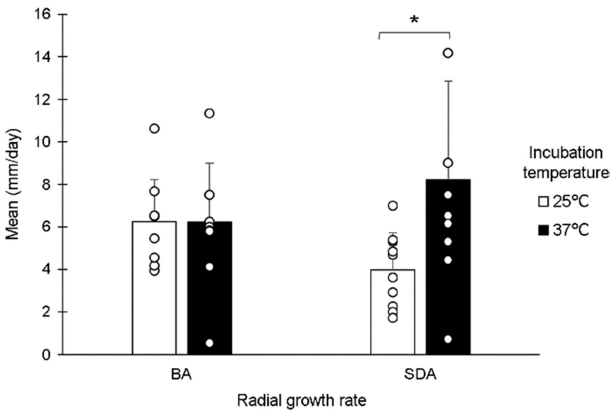

Fig. 1. Comparison of radial growth rates of 12 *Aspergillus* strains on BA and SDA at 25℃ and 37℃

RESULTS

1. Morphological identification and growth characters

Table 2 presents 12 morphologically identified *Aspergillus* strains, together with their associated information. All strains grew at 25℃ and 37℃ and were significantly better on SDA at 37℃ but worse at 25℃ ($p < 0.05$) (Fig. 1). An FG-090 strain demonstrated the fastest growth on BA but an FG-091 strain exhibited the slowest on SDA at 25℃. FG-026, FG-054, and FG-069 strains exhibited the fastest growth on SDA but an FG-088 strain demonstrated the slowest on BA at 37℃ (Fig. 2).

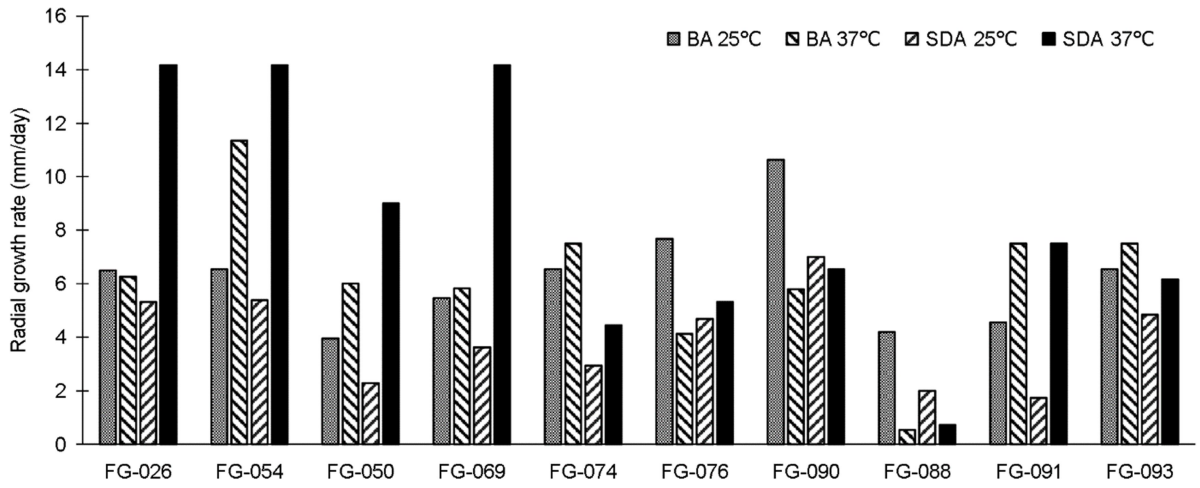

Fig. 2. Radial growth rates of 12 *Aspergillus* strains on BA and SDA at 25℃ and 37℃

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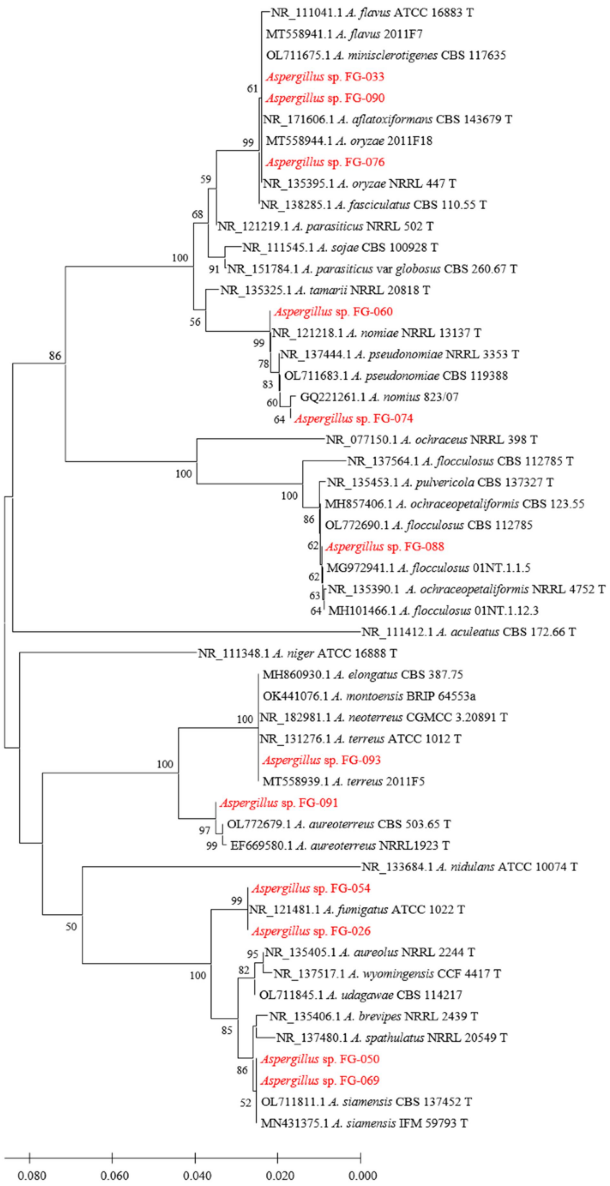

Fig. 3. Phylogenetic tree of 12 *Aspergillus* strains and 41 closely related strains according to a dataset of 53 ITS sequences, the dataset was aligned to 593 positions by MUSCLE and analyzed in MEGAX by the Neighbor-joining method with the *Tamura-Nei* + *G* model having 1,000 bootstrap replications. Number on nodes = % bootstrap support, ≥50% shown, T = type strain

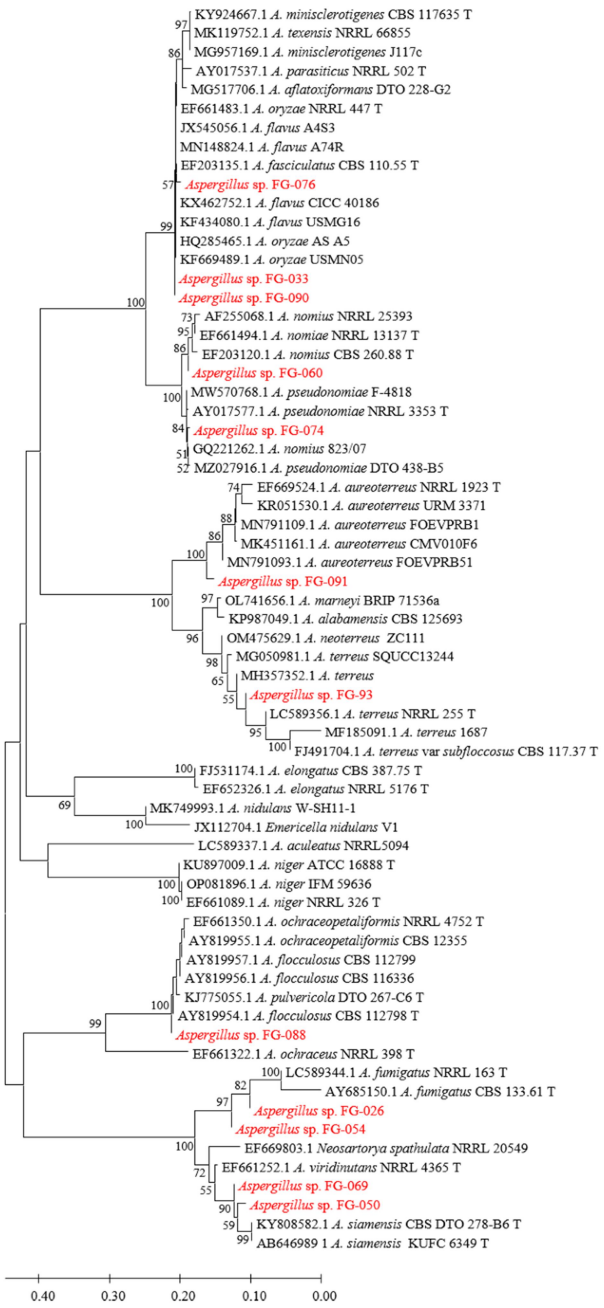

Fig. 4. Phylogenetic tree of 12 *Aspergillus* strains and 54 closely related strains according to a dataset of 66 BT2 sequences, the dataset was aligned to 643 positions by MAFFT and analyzed in MEGAX by the Neighbor-joining method with the *Kimura 2-parameter* + *G* model having 1,000 bootstrap replications. Number on the nodes = % bootstrap support, ≥50% shown, T = type strain

2. DNA barcoding in 12 *Aspergillus* strains

Figs. 3, 4, 5, and 6 show the phylogenetic analyses with ITS, BT2, CAL, and ACT, respectively. ITS, BT2 and CAL, and ACT identified 5, 6, and 3 species and 3, 2, and 1 species complex, respectively. Table 3 concludes all identification results. The multi-locus sequence typing (MLST) identification indicated *A. aureoterreus*, *A. pseudonomiae*, *A. siamensis*, and

Table 3. DNA identification results of twelve *Aspergillus* strains

Strain No.	DNA Identification: the highest similarity (%) comparing to the type or reference materials							
	ITS	Species	BT2	Species	CAL	Species	ACT*	Species
FG-033	100%	*A. flavus* cpx.†	100%	*A. flavus* cpx.‡	97.46~ 97.94%	*A. flavus* cpx.§	99.71% 99.71%	*A. flavus* *A. oryzae*
FG-076	100%	*A. flavus* cpx.†	99.18~ 99.33%	*A. flavus* cpx.‡	97.70~ 98.46%	*A. flavus* cpx.‡	99.71% 99.71%	*A. flavus* *A. oryzae*
FG-090	100%	*A. flavus* cpx.†	100%	*A. flavus* cpx.‡	98.65~ 99.24%	*A. flavus* cpx.‡	99.71% 99.71%	*A. flavus* *A. oryzae*
FG-026	100%	*A. fumigatus*	100%	*A. fumigatus*	99.80%	*A. fumigatus*	99.73%	*A. fumigatus*
FG-054	100%	*A. fumigatus*	100%	*A. fumigatus*	100%	*A. fumigatus*	100%	*A. fumigatus*
FG-088	100% 100%	*A. flocculosus* *A. ochraceo-petaliformis*	100% 100%	*A. flocculosus* *A. ochraceo-petaliformis*	99.67~ 100% 99.83%	*A. flocculosus* *A. ochraceo-petaliformis*	ND	ND
FG-060	100%	*A. nomiae*	99.60%	*A. nomiae*	99.83%	*A. nomiae*	ND	ND
FG-074	99.82%	*A. pseudonomiae*	99.79%	*A. pseudonomiae*	99.60%	*A. pseudonomiae*	ND	ND
FG-050	100%	*A. siamensis*	99.97%	*A. siamensis*	97.67%	*A. siamensis*	95.49%	*A. siamensis*
FG-069	100%	*A. siamensis*	99.98%	*A. siamensis*	97.67%	*A. siamensis*	95.83%	*A. siamensis*
FG-091	100%	*A. aureoterreus*	98.93%	*A. aureoterreus*	98.10%	*A. aureoterreus*	ND	ND
FG-093	100%	*A. terreus* cpx.‖	100%	*A. terreus*	99.13%	*A. terreus*	98.95%	*A. terreus*

*limited number of type or reference materials in a public database
†including *A. aflatoxiformans*, *A. fasciculatus*, *A. flavus*, *A. minisclerotigenes* and *A. oryzae*
‡including *A. fasciculatus*, *A. flavus* and *A. oryzae*
§including *A. fasciculatus*, *A. flavus*, *A. minisclerotigenes* and *A. oryzae*
‖ including *A. terreus*, *A. neoterreus*, *A. elongatus* and *A. montoensis*

Table 4. Antifungal susceptibility test of twelve *Aspergillus* strains

Strain No.	Species	MEC (μg/ml)	MIC (μg/ml)								
		AND	AND	CAS	MF	AB	FC	FZ	IZ	PZ	VZ
FG-033	*A. flavus* cpx.	≤0.015	>8	>8	>8	2	>64	>256	0.50	0.50	1
FG-076	*A. flavus* cpx.	≤0.015	>8	>8	>8	4	>64	>256	0.25	0.25	0.50
FG-090	*A. flavus* cpx.	≤0.015	>8	>8	>8	4	>64	>256	0.25	0.50	1
FG-026	*A. fumigatus*	≤0.015	>8	>8	>8	2*	>64	>256	0.25	0.12	0.50
FG-054	*A. fumigatus*	≤0.015	>8	>8	>8	1	>64	>256	0.25	0.12	0.25
FG-088	*A. flocculosus-ochraceopetaliformis*	≤0.015	>8	>8	>8	8†	16‡	>256	0.50	0.50	0.50
FG-060	*A. nomiae*	≤0.015	>8	>8	>8	2	32	>256	0.50	0.50	2†
FG-074	*A. pseudonomiae*	≤0.015	>8	>8	>8	2	>64	>256	0.25	0.25	0.25
FG-050	*A. siamensis*	≤0.015	>8	>8	>8	2†	>64	>256	0.50	0.50	2†
FG-069	*A. siamensis*	≤0.015	>8	>8	>8	4†	>64	>256	1	0.50	4†
FG-091	*A. aureoterreus*	≤0.015	>8	>8	>8	4	2‡	>256	0.25	0.12	0.25

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Table 4. Antifungal susceptibility test of twelve *Aspergillus* strains (Continued)

Strain No.	Species	MEC (μg/ml)	MIC (μg/ml)								
		AND	AND	CAS	MF	AB	FC	FZ	IZ	PZ	VZ
FG-093	*A. terreus*	≤0.015	>8	>8	>8	2	>64	>256	0.12	0.12	0.12
ATCC-MYA-3626	*A. fumigatus*	≤0.015	>8	>8	>8	2*	>64	>256	0.50	0.25	0.50

MEC=minimal effective concentration, MIC=minimal inhibitory concentration, AND=anidulafungin, CAS=caspofungin, MF=micafungin, AB=amphotericin B, FC=flucytosine, FZ=fluconazole, IZ=itraconazole, PZ=posaconazole, VZ=voriconazole
* = resistant, † = likely resistant (no breakpoint proposed), ‡ = likely sensitive (no breakpoint proposed)

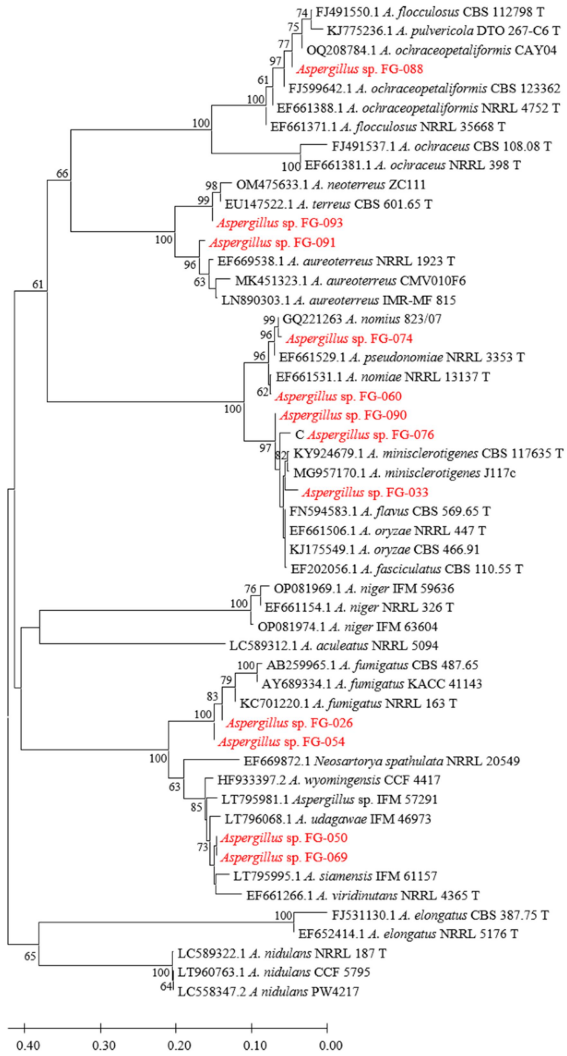

Fig. 5. Phylogenetic tree of 12 *Aspergillus* strains and 40 closely related strains according to a dataset of 52 CAL sequences, the dataset was aligned to 693 positions by MAFFT and analyzed in MEGAX by the Neighbor-joining method with the *Tamura 3-parameter + G* model having 1,000 bootstrap replications. Number on the nodes = % bootstrap support, ≥50% shown, T = type strain

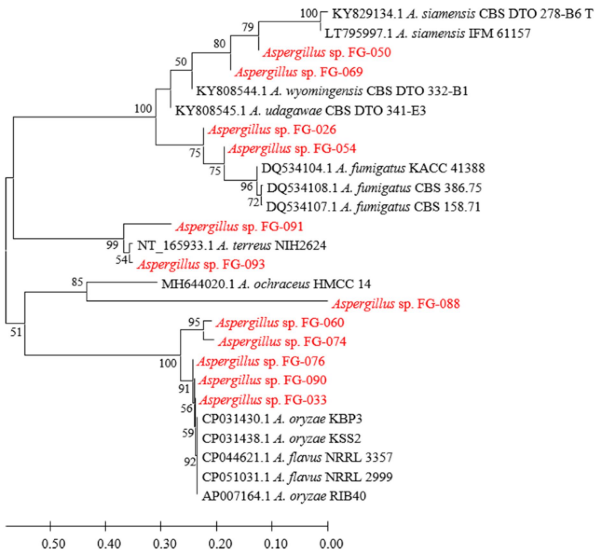

Fig. 6. Phylogenetic tree of 12 *Aspergillus* strains and 14 closely related strains according to a dataset of 26 ACT sequences, the dataset was aligned to 434 positions by MAFFT and analyzed in MEGAX by the Neighbor-joining method with the *Kimura 2-parameter + G* model having 1,000 bootstrap replications. Number on the nodes = % bootstrap support, ≥50% shown, T = type strain

A. flocculosus-ochraceopetaliformis as the four new pathogens causing eye infection in human, and Fig. 7 illustrates their morphological characters.

3. Antifungal susceptibility test

A. fumigatus ATCC-MYA 3626 revealed MIC values of all tested drugs with the AST kit in those ranges recommended by CLSI M61. The MICs of echinocandins and FZ of all tested strains were higher than the test set. However, their other MIC values exhibited differently in AB, FC, and other azoles. Table 4 presents all MEC and MIC values of the 12 strains.

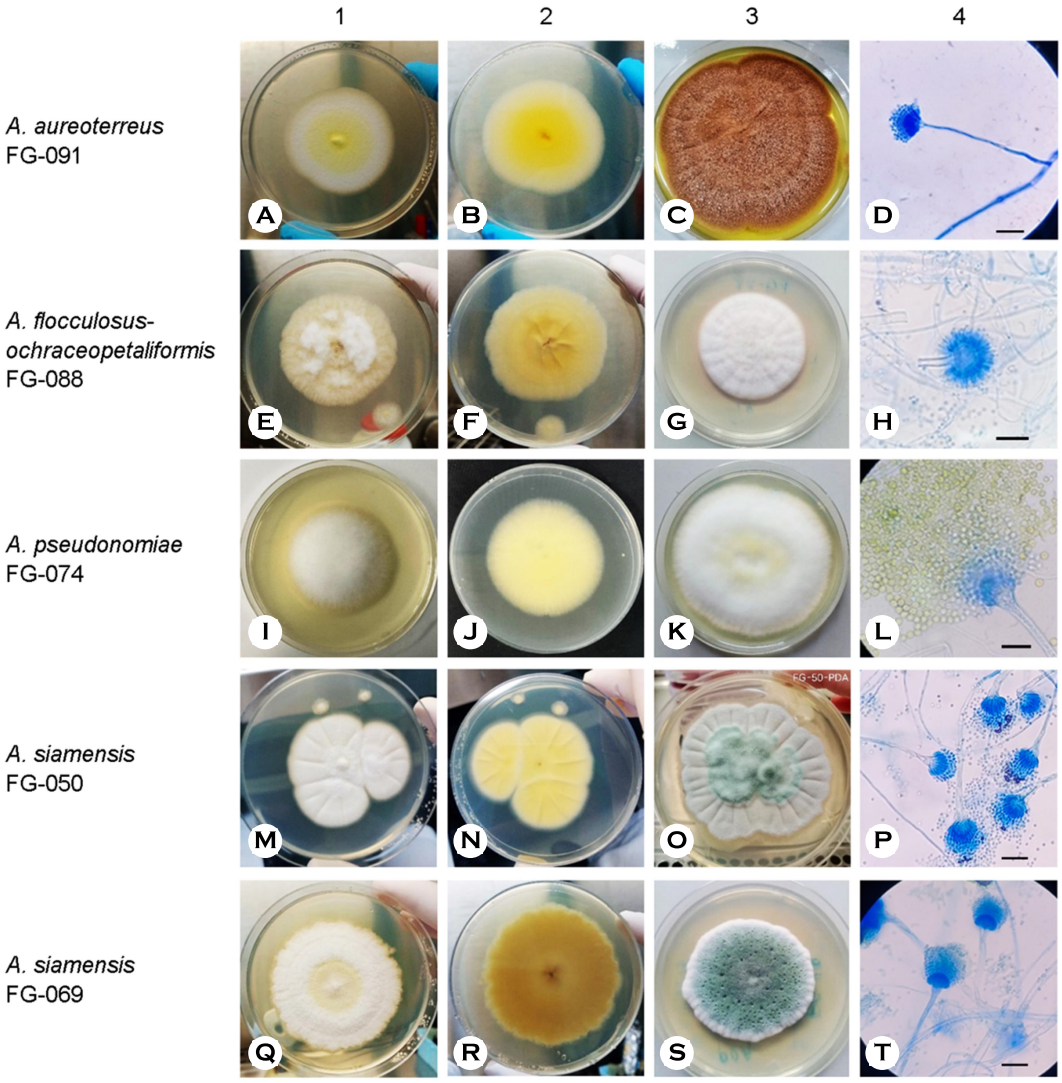

Fig. 7. Colony and conidia morphology of the five new pathogenic *Aspergillus* strains, columns 1 and 2 = colony on SDA at 25℃ for 7 days, 1 = surface, 2 = reverse, column 3 = colony surface on PDA at 25℃ for 7~12 days, column 4 = conidial apparatus on PDA, scale bar = 20 μm.

DISCUSSION

In Thailand, infectious keratitis was annually estimated to occur in 1 per 1,000 people, and approximately 15% of those were FK. Thailand would have an estimated 70,000 patients with infectious keratitis, with approximately 10,500 cases of FK[28]. *Aspergillus* species is one of the most prevalent pathogens of FK[9,10]. A total of 73 FK cases of mold infection were reported in the Srinagarind Hospital, Khon Kaen, Thailand, from January 1 to December 31, 2022, in which 12 (16.44%) cases were caused by *Aspergillus* spp. The incidence is <21.5% and 17.5% of those from India and Ghana[12], respectively. However, the real number of burden cases of *Aspergillus* eye infection is possibly higher than the estimation based on the number of hospitals all around Thailand.

The systematic classification of most fungal species relies on the genealogical species concept with MLST. Genes, such as ITS, and partial sequences of BT2, CAL, and ACT, have been the barcoding markers to identify *Aspergillus* species by DNA sequences[29-31]. ITS has been the primary barcoding marker because of the most available sequence in the public databases. However, it demonstrates less specificity for some *Aspergillus* species such as in *A. flavus* complex and *A. terreus*

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complex. Therefore, the "secondary barcoding markers" viz. BT2, CAL, and ACT have been employed. These markers cannot completely identify the *A. flavus* complex, but they successfully determined *A. terreus*. Presently, these secondary barcoding markers, especially, of some type or reference materials have been unavailable in the public database. All potential barcoding marker sequences of the type and/or reference materials should be generated and submitted into the public database to support the identification of the DNA sequences.

The present study identified 6 *Aspergillus* species and 2 species complex causing eye infections viz. *A. flavus* complex, *A. fumigatus*, *A. terreus*, *A. aureoterreus*, *A. nomiae*, *A. pseudonomiae*, *A. siamensis*, and *A. flocculosus-ochraceopetaliformis* by PCR, Sanger sequencing, and phylogenetic methods. *A. flavus*, *A. fumigatus*, and *A. terreus* have usually been reported as common species[12-15]. *A. nomiae*, a neglected species that causes FK, has been reported[16]. No case of eye infection caused by *A. aureoterreus*, *A. pseudonomiae*, *A. siamensis*, and *A. flocculosus-ochraceopetaliformis* has been previously published. Therefore, this investigation revealed the four new pathogens of eye infection.

Samson, S.W. Peterson, Frisvad & Varga raised *A. aureoterreus* from *A. terreus* var aureus by a yellow pigment producing and a distant genotype from *A. terreus*[32]. ITS, BT2, and CAL are barcoding markers for species identification. *A. aureoterreus* can be found in soil or as a contaminant in wheat flour[32] and spice[34]. The species was mentioned as one isolation from a patient but with no pathological information[15]. Its morphology and growth character are closely similar to those of *A. terreus*. Concurrently, *A. terreus* and *A. aureoterreus* might be misidentified when DNA identification was not available to determine the fungal isolates from many *A. terreus* infection cases. *A. aureoterreus* strain FG-091 (Fig. 7A-D) has presently been the first isolated from a corneal scraping of a patient with keratitis. The fungal DNA could be detected by PCR although the specimens did not reveal fungal elements by pathological examination. The strain demonstrated better growth at 37℃, the same as *A. terreus* FG-093, but with a colony producing a yellow pigment (Fig. 7C). The patient was treated with antifungal drugs but the outcome remains unknown.

Varga, Samson & Frisvad isolated *A. pseudonomiae* from the soil, insect[33], and house dust[35]. ITS and BT2 can be utilized as barcoding markers for this species. The species was proposed because of genetic differences from *A. nomiae*. Similar to *A. nomiae*, it can be morphologically misidentified as *A. flavus*. Manikandan et al.[16] isolated *Aspergillus* sp. strain 823/07 from a keratitis case and identified the fungus as *A. nomiae* by BLASTN search. However, strain 823/07 was later identified as *A. pseudonomiae* by phylogenetic analysis of the concatenated sequence of ITS, BT2, and CAL[35]. Our investigation confirmed the strain 823/07 to be *A. pseudonomiae* by phylogenetic analyses with only a single gene, exhibiting the highest similarities to the type material, i.e., 99.80% of ITS, 99.62% of BT2 and 99.46% of CAL. Consequently, *A. pseudonomiae* strain FG-074 (Fig. 7I-L) is officially the first isolation from the patient with keratitis but unofficially, the second of the reported cases. The strain FG-074 was isolated from the right eye of a patient with diabetes mellitus. Histopathological examination confirmed positive fungal elements. The strain exhibited good growth at 37℃ and morphological characters similar to those of the *A. nomiae* FG-060 strain and the *A. flavus* group. The patient demonstrated a severe prognosis which may be associated with the underlying condition, with evisceration as the outcome.

Manoch, Eamvijarn & Yaguchi[36] first isolated *A. siamensis* from coastal forest soil in Thailand and classified them in *Aspergillus* section Fumigati, with no other habitat being mentioned since then. ITS, BT2, CAL, and ACT are barcoding markers for species identification. The species is closely associated with *A. udagawae* and *A. viridinutans* which have previously been reported their pathogenicity[37-39]. *A. siamensis* first appeared as a pathogen in Japan, causing a fatal disseminated infection in an 18-year-old male patient. The patient received bone marrow transplantation after having chronic granulomatous disease for a long time[40]. Two strains of *A. siamensis*, including FG-050 (Fig. 7M-P) and FG-069 (Fig. 7Q-T), were isolated from two patients with FK. The cases have been the second and the third of the pathogenicity finding but the first of eye infection. The FG-050 strain was isolated from a corneal scraping specimen of a right eye as well as fungal positive by both histopathological staining and PCR for fungal DNA in specimens. The patient was not successfully treated with the medication; hence, evisceration was required. Another strain, the FG-069, was isolated from a cornea specimen of a right eye infection. No histopathological staining was conducted but PCR for fungal DNA was positive. The patient was treated with penetrating keratoplasty. Both FG-050 and FG-069 strains grew better on SDA, especially at 37℃, similar to the type strain[36] and the important pathogens such as *A. fumigatus* and *A. flavus*. The ability to grow well at 37℃ may be a criterion for the pathogenicity prediction of *Aspergillus* species.

Frisvad & Samson classified *A. flocculosus* in the *Aspergillus* section Circumdati or *A. ochraceus* group. It could be isolated from the saltern, soil, and substratum in tropical and temperate areas[41]. The species internally infected grape[35] but

with no reported human infection. Conversely, Batista & da Silva Maia isolated *A. ochraceopetaliformis* from a scalp lesion in a Brazilian man[42] (a type strain CBS 123.55) (https://www.mycobank.org) and reported of causing nail infection in a German woman (strain CBS 123362)[43]. *A. flocculosus* and *A. ochraceopetaliformis* were noted to have been genetically very similar to each other[41,44]. Defining the right taxa of both species is beyond our investigation. Therefore, this study only designated the strain FG-088 (Fig. 7E-H) in a complex species as *A. flocculosus-ochraceopetaliformis*. The strain FG-088 grew slower and did not grow well at 37℃, unlike other *Aspergillus* pathogens. It was isolated from a cornea specimen of the right eye in a patient with eye injury caused by wood puncture and developed endophthalmitis. Histopathological test and PCR for direct detection was not investigated. The patient was treated with enucleation because of a very poor prognosis which may be from severe injury and infection.

The sources of the five strains in the environment around each patient remain unclear. The predisposing factors, such as accidental evidence causing eye injury, trauma, or underlying condition, have to be considered. Notably, the virulence of *Aspergillus* keratitis demonstrated a lower successful treatment rate with slow healing, and a failure of primary treatment was at a higher rate than keratitis by *Fusarium* and other filamentous fungi[12]. The five pathogens exhibited their virulence concordant to the previous investigation.

The MIC-breakpoints of some species and some antifungal drugs proposed by CLSI[45] and EUCAST[46] revealed that only *A. fumigatus* FG-026 was resistant to AB (>1 μg/ml). Conversely, all of them were likely sensitive to IZ (≤1 μg/ml). *Aspergillus* section Flavi, *A. flavus* and *A. nomiae*, seemed to be resistant to PZ (>0.25 μg/ml). Echinocandins with a low MEC (0.015 μg/ml) could affect the growth of all *Aspergillus* strains, but they could not inhibit the growth of all strains at the highest concentration. The similar result was nearly the same as FZ which could not inhibit the growth of all tested strains. Notably, the four new pathogens likely demonstrated distinguished susceptibility profiles. *A. flocculosus-ochraceopetaliformis* strain FG-088 was resistant against AB with the highest MIC (8 μg/ml), but sensitive to FC. Further, the strain was resistant to FZ but sensitive to IZ, as *A. ochraceopetaliformis* mentioned in a previous report[43]. Both *A. siamensis* strains were likely resistant to VZ (>2 μg/ml) with the highest MIC of 4 μg/ml. The phenomenon may be inherited because it is similar to the resistant MICs of other members classified in the sections, Circumdati and Fumigati, that was previously investigated[47]. *A. aureoterreus* strain FG-091 was susceptible to FC with the lowest MIC. FC has barely been used alone to treat *Aspergillus* eye infection but a combination with topical AB demonstrated the synergistic effect in FK with yeast infection[48]. However, the MIC profiles of each strain should have been further assessed, especially associations with the genotypic characters.

In conclusion, the DNA determination with a sequence of a single gene as a barcoding tool for identifying *Aspergillus* species exhibited more satisfactory results than the morphological identification. The method could not determine some closely related members of the *A. flavus* complex. However, because of better precision and accuracy, it remains strongly recommended in routine identification. Moreover, PCR and sequencing facilities have been presently simplified, with a shorter turnaround time. Consequently, the DNA method would be beneficial to appropriately treat a patient, support a high rate of successful treatment, and finally, reliably report the case.

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CONFLICT OF INTEREST

In relation to this article, we declare that there is no conflict of interest.

DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ETHICAL APPROVAL STATEMENT

The study was approved by the Institutional Review Board of (IRB No. HE661018). This study was conducted in accor-

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dance with the principles of the Declaration of Helsinki.

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Supplementary Table. ITS, BT2, CAL, ACT sequences

ITS sequences
>ITS_FG_088 ATCATTACTGAGTGAGGGTTCCTTCGGGGCCCAACCTCCCACCCTTGTATACTGTACCAAGTTGCTTCGGCGGGCCCGCCGTTCGCGCGGCCGCCGGGG GGGAACCCCTCCCCCCGGGCGAGCGCCCGCCGGAGACCCCAACGTGAACACTGTCTGAAGTTTTGTCGTCTGAGTTCGATTGTATCGCAATCAGTTAA AACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAATTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTT GAACGCACATTGCACCCCCTGGTATTCCGGGGGGTATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCCTCGTCCCCCCCG GGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACACCGCTCTCGTAGGCCCGGCCGGCGCTGGC CGACGCTGAAAAGCAACCATTATTTCTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA
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