

# Fungal keratitis caused by a new filamentous hyphomycete *Sagenomella keratitidis*

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**ABSTRACT.** A previously undescribed hyphomycetous fungus was isolated from keratitis developed in a soft contact-lens wearer. It grows extremely slowly on various culture media. Its phialide-like conidiophores lacking an abrupt inflation and catenate, hyaline ameroconidia lead us to consider the fungus a species of the genus *Sagenomella*.

**Keywords:** Keratitis; Hyphomycetes; *Sagenomella keratitidis*; Taxonomy.

## INTRODUCTION

Use of soft contact lenses has been associated with the potential risk of developing microbial keratitis (Donzis et al., 1987; Wilhelmus, 1987; Gray et al., 1995; Fong et al., 2004). However, previous reports on contact lens associated fungal keratitis showed low prevalence (Yamaguchi et al., 1984; Donzis et al., 1987; Wilhelmus, 1987; Wilhelmus et al., 1988; Kirsch and Brownstein, 1993; Gray et al., 1995; Fong et al., 2004). Here we describe a hyphomycete belonging to the genus *Sagenomella* W. Gams, which is responsible for the keratitis developed in both eyes of a 35-year-old female soft contact-lens wearer.

## MATERIALS AND METHODS

### Fungal isolation, culturing, and observations

A hyphomycete was repeatedly isolated from corneal scraping, contact lens, and storage solution of contact lens of a 35-year-old female. The fungus was inoculated onto 6-cm plastic Petri dishes containing PDYA (39 g/L Difco potato dextrose agar, 10 g/L Difco yeast extract), from which the culture description was made, and incubated at 25°C under 12 h fluorescent light. Colony morphologies on MEA (20 g/L Difco malt extract, 20 g/L Difco agar) and MMCA (Ando et al., 1998; 5 g/L Difco malt extract, 5 g/L Difco corn meal agar, 1 g/L Difco yeast extract, 2 g/L glucose, 5 g/L Difco agar), were also recorded and compared with that on PDYA. Culture was deposited at BCRC (the Bioresource Collection and Research Center, Hsin-chu, Taiwan).

Conidiophores and conidia were examined by light microscopy (LM) and scanning electron microscopy (SEM). Material was mounted in water for examination by LM with a LEICA/LEITZ DMRB microscope equipped with differential interference contrast optics. SEM observations were made by PHILIPS (FEI) QUANTA 200 fitted with Polaron PP2000T cryo-SEM system (Quorum Technologies, UK). Samples for SEM were prepared by cutting blocks of 5 × 5 × 5 mm from one-week-old colonies along with their growing agar media. The samples were placed on a sample holder and deep-frozen in a liquid nitrogen slusher. They were then placed in the preparation chamber where they were subjected to the process of sublimation at -90°C for 15 min in order to eliminate ice crystals from the samples and were subsequently coated with gold at -130°C for 60 seconds with the amperage adjusted at 9-10 mA. Temperature in the preparation chamber was decreased until it reached -160°C before the samples were sent into the SEM chamber for observations.

### DNA extraction, sequencing of various DNA loci, and DNA sequence analyses

Mycelia were grown in 100 mL of malt extract broth (20 g/L Difco malt extract) on a rotary shaker at 120 rpm for one month. They were harvested by filtration through Whatman No. 1 filter paper, freeze-dried, and stored at -20°C. Genomic DNA extraction from mycelium was performed according to Hsieh et al. (2005).

Four nuclear DNA loci were sequenced, including ITS, 18S-rDNA, 28S-rDNA, and TUB2 (see Table 1 for their full name and PCR primer pairs). PCR condition for ITS was as follows: an initial denaturation step at 94°C for 3 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72

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°C for 2 min, and a final extension at 72°C for 10 min. PCR conditions for 18S-rDNA and  $\beta$ -tubulin followed Hsieh et al. (2005), with primer annealing temperatures at 55°C and 50°C, respectively. PCR condition for 28S rDNA followed Søchting and Lutzoni (2003). Reaction components for PCR included approximately 0.1-0.7 ng/ $\mu$ L of total DNA, 0.2  $\mu$ M (28S-rDNA and TUB2) or 2  $\mu$ M (ITS and 18S-rDNA) of each primer, 200  $\mu$ M dNTP, 1.5 mM MgCl<sub>2</sub>, 0.025 U/ $\mu$ L of Taq polymerase (Invitrogen, Carlsbad, CA), and 1x standard PCR buffer supplied with the Taq polymerase. PCR products were cleaned with PCR-M™ clean-up system (Viogene-Biotek Corp., Hsieh, Taipei Co., Taiwan) following the manufacturer's protocol. DNA cloning was then carried out essentially as Sambrook and Russell (2001). Ligated plasmids were used to transform high efficiency competent cells of *Escherichia coli* strain DH5 (Inoue et al., 1990). After incubation overnight at 37°C on LB/carbenicillin/IPTG/X-Gal plates, single transformed white bacterial colonies were picked and transferred into tubes with 6 mL LB/carbenicillin broth and incubated overnight at 37°C in shaking culture. The plasmids were extracted with a plasmid DNA extraction kit (Viogene-Biotek Corp). ABI Big-dye primer sequencing kit (Applied Biosystems) was used for DNA sequencing, and sequencing reactions were electrophoresed on an ABI Prism 377 model DNA sequencer. Purified PCR products were directly sequenced using the same primer pairs as in the PCR reactions, whereas extracted plasmids were sequenced from both directions using T7 and R universal primers.

Similarity values of homology searches in the GenBank database were obtained by using MEGABLAST, with the scores of "match", "mismatch", "existence gap cost", and "extension gap cost" set at 1, -1, 2 and 2, respectively.

## RESULTS

### DNA sequence analyses

The obtained sequences revealed the highest maximum scores with species of the family Trichocomaceae, with the closest sequences of ITS, 18S-rDNA, 28S-rDNA, and TUB2 being AJ519984 from *Sagenomella chlamydospora*

Gené & Guarro, AB024592 from *S. sclerotialis* W. Gams & Breton, NW\_001594105 from *Aspergillus niger* Tiegh., and NW\_00159417 from *A. niger*, respectively (Table 1).

### Taxonomy

***Sagenomella keratitidis*** W.-L. Chen, Y.-M. Ju, H.-M. Hsieh, H.-Y. Lin & F.-R. Hu, sp. nov. Figures 1-2

*Coloniae in PDYA 5-6 mm diameter aetate quatuor hebdomadam attigentes, albida primo, azonata, irregulatim rugosa, rimosa ubi veta, coriacea, marginibus integris vel leviter crenatis, exsudata nulli, cinerascens ubi conidiferae. Phialides hyalinae, laeves, plerumque simplices, ampulliformes, 14-30 × 2-2.5  $\mu$ m, ad apicem angustum gradatim attenuatae. Conidia hyalina, laevia, globosa vel subglobosa, 2.5-3.5  $\mu$ m. Chlamydosporae nullae.*

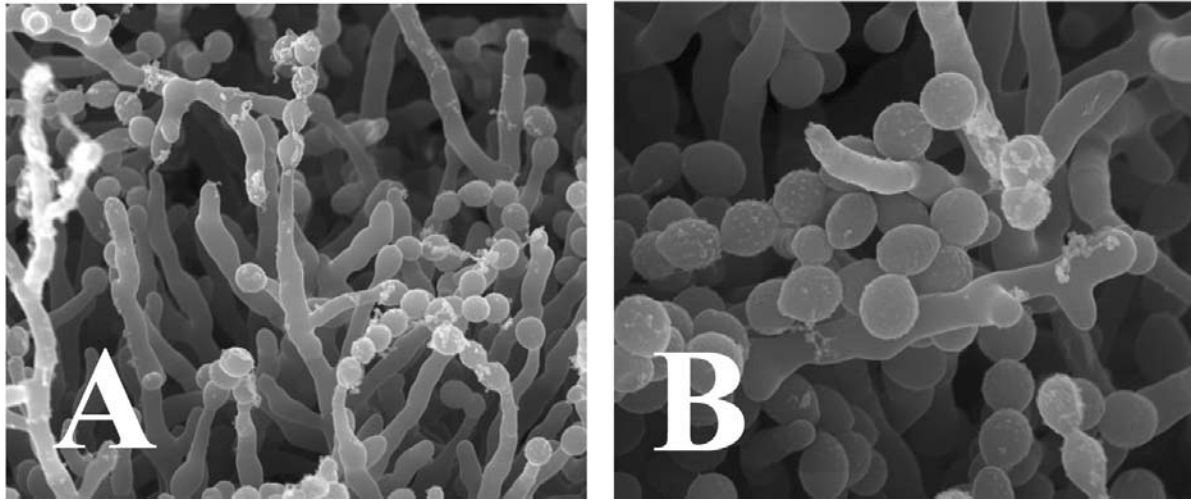
Colonies on PDYA attaining 5-6 mm diam in 4 weeks, whitish first, velvety, azonate, irregularly buckled, cracked in age, leathery, with entire or slightly crenate margins, lacking exudates, becoming light gray with conidial production; reverse light brown. Sporulating regions on the entire surface of colonies. Conidiogenous structures mononematous, unbranched, upright, simple, mostly reduced to single phialides only. Phialides hyaline, smooth, flask-shaped, 14-30 × 2-2.5  $\mu$ m, ca. 1  $\mu$ m broad at apex. Conidia produced enteroblastically in basipetal sequence and remaining in dry chains, hyaline, smooth, globose to subglobose, 2.5-3.5  $\mu$ m. Chlamydospores absent.



Figure 1. Colonies on PDYA in a 6-cm Petri dish at one month.

Table 1. The four nuclear DNA loci of *S. keratitidis* sequenced in the current study.

DNA locus	Abbreviation	Primer pair used	GenBank accession no.	Maximum identity to the sequence in GenBank matched with highest maximum score
Ribosomal internal transcribed spacers	ITS	ITS1/ITS4 (White et al., 1990)	EU140821	89% with AJ519984 from <i>Sagenomella chlamydospora</i>
Ribosomal small subunit	18S-rDNA	NS1/NS4 (White et al., 1990)	EU140822	99% with AB024592 from <i>Sagenomella sclerotialis</i>
Ribosomal large subunit	28S-rDNA	LROR /LR7 (Vilgalys and Hester, 1990)	EU140823	95% with NW_001594105 from <i>Aspergillus niger</i>
$\beta$ -tubulin	TUB2	T1/T22 (O'Donnell and Cigelnik, 1997)	EU140824	86% with NW_001594179 from <i>Aspergillus niger</i>



**Figure 2.** A, Conidiogenous structures mostly reduced into single phialides, which produce conidia in chains; B, Conidia. Scale bars, A = 8  $\mu$ m, B = 4  $\mu$ m. A and B by SEM.

Colonies on MEA and MMCA in general as on PDYA except for growing slightly slower, 4-5 mm diam in 4 weeks and appearing whitish rather than grayish due to less conidial production.

*Etymology.* For the capability of causing keratitis.

*Holotype.* TAIWAN. BCRC 34221, obtained from human keratitis; with isotypes deposited in HAST and National Taiwan University Hospital.

*Notes.* Although this keratitis-causing fungus does not have a known teleomorph, there is of little doubt that it has a close affinity to the ascomycetous family Trichocomaceae, where anamorphs produce conidia from phialides and have connectives between chained conidia in most cases. This is also corroborated by BLAST homology searches performed in the GenBank database by using the four nuclear DNA loci—ITS, 18S-rDNA, 28S-rDNA, and TUB2—that we sequenced. These sequences had highest maximum scores with those from species of the Trichocomaceae. *Sagenomella* and *Torulomyces* Delitsch are the two hyphomycetous genera with affinities to the Trichocomaceae harboring certain *Acremonium*-like fungi. Our fungus fits better in the genus *Sagenomella* mainly because its phialides lack an abrupt inflation, one of the salient features that defines *Torulomyces* (Gams, 1978; Ando et al., 1998). *Sagenomella keratitidis* differs from the other 13 known species in the genus, including the unnamed anamorph of *Talaromyces ocotil* Bills & Heredia (Heredia et al., 2001) in having extremely slow-growing colonies, hyaline, smooth, globose to subglobose conidia, and highly reduced conidiophores (Stolk and Orr, 1974; Gams, 1978; Ueda and Udagawa, 1984; Fassatiová and Pěčková, 1990; Gené et al., 2003). *Sagenomella humicola* (Onions & G.L. Barron) W. Gams and *S. keratitidis* are similar in microscopic features, but the former differs in producing abundant chlamydospores and growing much faster on agar media, with colonies attaining 2 cm diam in 14 days (Onions and Barron, 1967).

## DISCUSSION

Fungal keratitis is uncommon complications in contact lens wearers (Yamaguchi et al., 1984; Donzis et al., 1987; Wilhelmus, 1987; Wilhelmus et al., 1988; Kirsch and Brownstein, 1993; Gray et al., 1995; Fong et al., 2004). Recently, contact lens cleaning solution has been suspected to cause *Fusarium* outbreak from 2005 to 2006 (Chang et al., 2006; Khor et al., 2006). Because no predisposing factors, such as ocular trauma, pre-existing ocular diseases, topical steroid usages or an exposure to Bausch & Lomb ReNu with the MoistureLoc cleaning solution, were involved in this case, poor contact lens hygiene was very likely the reason for this rare fungal infection.

While the majority of *Sagenomella* species are known from soil, *S. bohémica* Fassatiová & Pěčková and *S. sclerotialis* were isolated from peloids of a balneological sample (Fassatiová and Pěčková, 1990) and fodder of ryegrass and lucerne (Gams, 1978), respectively. *Sagenomella oligospora* W. Gams & Luiten (Gams, 1978) has been isolated from diversified substrates, including soil, human nail, and composted chicken manure. The recently described *S. chlamydospora*, however, was identified as causing disseminated mycosis of many internal organs in a dog (Gené et al., 2003). Human corneas, from which this new fungus *S. keratitidis* was isolated, thus represent a unique substrate type known for *Sagenomella*. *Sagenomella keratitidis* grew extremely slowly on common culture media routinely used for culturing hyphomycetes, appearing to sporulate more abundantly on media amended with yeast extract.

The extremely slow growth rates of *S. keratitidis*, which could readily be overgrown by common contaminants, may impose great difficulty on its isolation and purification. It remains to be proven whether the keratitis caused by *S. keratitidis* is simply a rare case or more common than noticed because of the slow-growing

habit of the fungus. In future, this rare pathogen should be suspected in keratitis of contact lens wearers. A longer culturing period, microscopic examinations, and DNA sequences are needed for a proper diagnosis.

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## 由新種絲狀真菌 *Sagenomella keratitidis* 引起之角膜炎

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本研究報告一株由隱形眼鏡配戴者所感染的黴菌性角膜炎病灶上所分離出來、屬於絲菌綱的真菌。此黴菌在各種體外培養基上的生長速度均極為緩慢。我們認為它屬於 *Sagenomella* 屬，主要是因為它的瓶狀分生孢子梗不具一球狀膨大及分生孢子為串生、透明及單細胞。

**關鍵詞**：黴菌性角膜炎；絲菌綱；*Sagenomella keratitidis*；分類學。

