

Exophiala asiatica, a new species from a fatal case in China

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We describe a new species, *Exophiala asiatica*, isolated from an infection of the pharynx in a 20-year-old, immunocompetent woman in Nanjing, China. The infection was initiated by a fishbone prick in the pharynx, soon developed with facial nodules but subsequently seemed to have disappeared. Tonsil ulceration with progressive soreness of the pharynx was observed 3 years later. Dysphagia, headache and paralysis occurred four years after first signs of infection. Hyphae and yeast-like cells were detected in tissue and a black fungus was recovered repeatedly from pharynx tissue. Despite antifungal therapy for more than one year, the patient died of apparent cerebral dissemination of the etiologic agent. On the basis of morphology, nutritional physiology, ribosomal small subunit DNA and ITS sequence data the strain could not be matched with any existing species. A new species, *Exophiala asiatica*, is therefore proposed.

Keywords Black yeast, laryngitis, brain, *Exophiala*, taxonomy

Introduction

Exophiala infection has aroused worldwide attention because of the cerebral and disseminated infections in young, otherwise healthy Asian patients. Due to its progressive, highly mutilating and fatal disease process, *Exophiala dermatitidis* has been considered to be an emerging pathogen [1–5]. Sporadically fatal cases have been reported to be caused by other species of the genus *Exophiala*, such as *E. spinifera*, known from previously reported disseminated infections with cutaneous nodules which ultimately reached the bones [6,7]. *Exophiala jeanselmei* was recently described in a fatal case of a young female from China which started as an orbital infection [8]. Systemic infections by melanized fungi in previously healthy individuals have been recognized in China over the past decades, and given its high degree of morbidity and mortality this kind of disease deserves special attention. The situation is particularly pressing in the light of supposed association of opportunistic

melanized fungi with environmental pollution by xenobiotics [9].

In the present paper we report a new species in the genus *Exophiala* recovered from a fatal case in a previously healthy female student. The presentation involved pain of the pharynx and dysphasia during 3 months, which were both traced back to a fishbone prick and subsequent recurrent episodes of facial nodules 3 years earlier. The infection was caused by a species of *Exophiala* that could not be identified as any of the known species and is therefore introduced as a new taxon. This was the seventh fatal infection with an *Exophiala* species reported from China.

Case report

A previously healthy 20-year-old woman living in Nanjing, P.R. China, presented with pharynx pain and dysphagia of 3 months duration. The patient recalled a severe fishbone prick of the pharynx 3 years prior to admission. Four months after the initial pharynx trauma, nodules appeared on her face, but they disappeared after she was treated for tuberculosis with isoniazid and rifampin. Three years after the infection apparently resolved, the patient reported tonsil ulceration with progressive sore throat, followed

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by dysphagia, progressive headache, and soon afterwards, paralysis of the legs.

A biopsy of tonsil tissue revealed lymphocyte infiltration, septate, brownish hyphae and yeast-like cells. Re-examination of the slides of the facial nodules from her earlier infection showed similar features.

On examination, the patient was dysphasic, febrile, and could feed orally only with liquid nutrients. Submaxillary lymph nodes were enlarged and haphalgesic, her palate was stiff, and her uvula was disfigured. Her right tonsil was absent as a result of a necrotic process. Palate, uvula, left tonsil, and epiglottis were inflamed, necrotic, and covered with a greyish-black pseudomembrane, which was subsequently found to be rich in black hyphae (Fig. 1A). A nodule 0.5 cm in diameter was present on the left pharyngeal recess. Laboratory results were as follows: leukocytes $13.1 \times 10^9/Q$ with 79% band cells, 20% lymphoid cells; erythrocytes $4.55_{1012}/l$, hemoglobin 131 g/l, platelets $284 \times 10^9/l$. Subgroups of lymphocytes were as follows: T4 cells 16.5%, T8 cells 29.5%, T3 80% [10]. Immunoglobulin types were normal and immunological tests for HIV were negative. Liver, kidney and bone marrow functions were normal. Radiography of the sinus revealed osteolysis and dense areas. Bronchofibrosopic examination showed pharyngeal necrosis. Examination of hematoxiline and eosin stain material from the laryngeal biopsy revealed lymphocyte infiltration, septate hyphae, and spores (Fig. 1B). Similar features were seen in the facial nodules. A black fungus was recovered (BMU 195) from the pharynx, and the patient was diagnosed as having a mycosis with cerebral dissemination which was caused by a pigmented mould. The patient was first given amphotericin B (from 2.5 mg/d gradually increasing to 25 mg/d; total amount given was 750 mg) and despite improvement in her condition, this medication was discontinued because of severe side effects and was switched to itraconazole 0.2 g once daily. Two months later, her clinical symptoms began to improve, but another black fungal strain, BMU 15, was

isolated from pharynx tissue. After being discharged from the hospital, her condition continued to aggravate with leg paralysis being noted one year later. Her infection continued and she died soon afterwards.

Materials and methods

Mycology

Tissues from the pharyngeal biopsy were inoculated onto Sabouraud glucose agar (SGA) with chloramphenicol and incubated at 25°C. Strains BMU 00195 (= CBS 122848) and BMU 00015 (= CBS 122847) were isolated from specimens before and 2 months after the introduction of therapy. These isolates were transferred to potato dextrose agar (PDA), corneal agar (CMA), malt extract agar (MEA) and Czapek agar (CZA) and incubated at 25°C for 14 days in darkness to assess growth rate and to assess colonial phenotypic features. Microscopic morphology was assessed by cutting 1×1 cm blocks from a PDA plate, mounting the blocks onto sterile microscope slides and pin-point inoculating them with the two isolates. The blocks were then covered with sterile cover slips and incubated in moist chambers for 14 d at 25°C. The structure and branching pattern of conidiophores were observed at magnifications of $\times 100$, $\times 200$ and $\times 400$ on intact slide cultures under the microscope without removing the cover slips from the agar blocks. For higher magnifications the cover slips were carefully removed and mounted in lactic acid with aniline blue (Fig. 2A, B).

Physiology

API 20C Yeast Identification System (bioMérieux, Marcy l'Etoile, France) was used according to the manufacturer's instructions with minor adaptations to accommodate slow growing black yeasts. Mature (7 day) cultures grown at 30°C on PDA tubes were used to obtain inoculum suspensions in sterile distilled water which had a final turbidity equivalent to McFarland

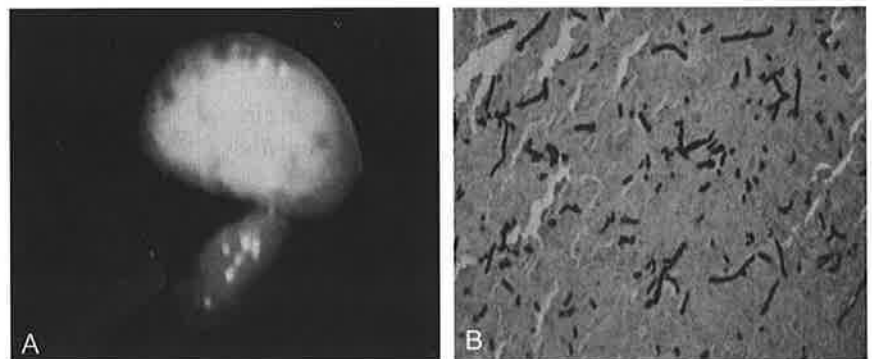


Fig. 1 (A) Palate, uvula, tonsil and epiglottis covered with greyish-black pseudomembrane with inflammation and necrosis. (B) Tonsil tissue biopsy with Grocott silver staining exhibited lymphocyte infiltration, septate hyphae and spores. Magnification $\times 400$.

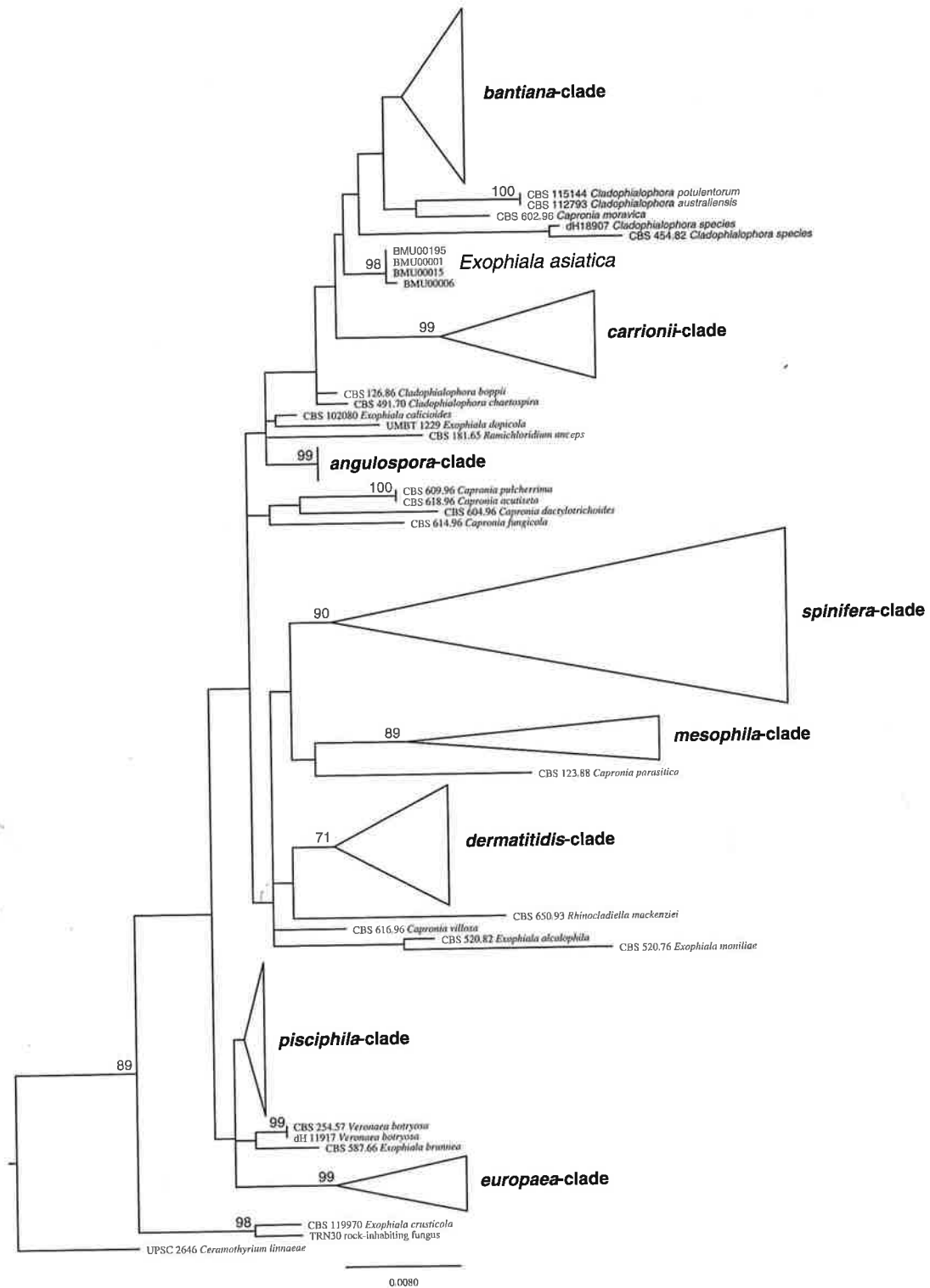


Fig. 2 SSU ML tree of selected members of *Chaetothyriales*. Bootstrapping performed with RAxML after 450 replicates with bootstrapping criterion. Bootstraps >70% are visible at the branches; Pearson average of 100 random splits: 0.992190. *Ceramothyrium linnaeae*, UPSC 2646 was selected as outgroup.

standard #2. API 20C strips were placed in the incubation tray provided by the manufacturer, covered loosely with a lid, and incubated at 30°C for 7 days. Cultures of *Candida glabrata* were used as control. Growth was read daily for 4 days. Thermotolerance was tested by incubating freshly inoculated cultures at 28, 37, 38, 39 and 40°C.

In-vitro susceptibility tests

Antifungal susceptibility tests were performed using the broth microdilution method according to NCCLS (National Committee on Clinical Laboratory Standards) [11] guidelines as described previously [12]. Amphotericin B, ketoconazole, miconazole, itraconazole, fluconazole, flucytocine and terbinafine were obtained commercially and tested separately.

DNA sequencing

About 0.1 g mycelium grown on PDA was transferred to a 2 ml Eppendorf tube containing a 2:1 (w/w) mixture of silica gel and Celite (Merck, Amsterdam, The Netherlands); DNA was extracted according to methods described previously [13]. We designed a pair of primers, NCS1/D12 (NCS1 5'-GTA AGC GCA AGT CAT CAG CTT GCG-3'; D12 5'-GAG CTG CAT TCC CAA ACA ACT CGA C) targeted at the lower end of 18S rDNA, ITS1, 5.8S rDNA, ITS2 and upper end of 28S rDNA to amplify the internal transcribed spacer (ITS) region of rDNA; N1/N9 for small subunit (18S) ribosomal gene [14]. PCR was performed on 50 µl volumes of a reaction mixture containing 10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂·6H₂O, 0.01% gelatin, 200 mM of each deoxynucleotide triphosphate, 25 pmol of each primer, 10–100 ng rDNA, and 0.5 U Taq DNA polymerase (Bioline, GC Biotech, Alphen a/d Rijn, The Netherlands). Thermal cycling parameters were set according to their annealing temperature with the methods described elsewhere. Amplicons were purified using the Wizard DNA clean up kit® (Promega, Madison, USA) or with Sephadex G-50 Superfine to remove remaining primers and dNTPs. Sequencing was performed in Bioasia Co. Ltd, Shanghei, China, using sets of primers, N1/N9 for 18S rDNA and ITS1/ITS4 for ITS rDNA. The D1/D2 regions were sequenced at CBS. Sequence PCR was performed as follows: 95°C for 1 min, followed by 30 cycles consisting of 95°C for 10 sec, 50°C for 5 sec, and 60°C for 2 min. BigDye terminator cycle sequencing RR mix v 1.1 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) was used according to the manufacturer's instructions.

Sequence analysis and taxonomy

Sequences of ITS were aligned in a database using BioNumerics software v. 4.61 (Applied Maths, Kortrijk, Belgium) and 18S sequences were aligned with ARB beta-package (v. 22-08-2003) developed by W. Ludwig *et al.* (2004). ITS trees were reconstructed using neighbor-joining algorithm with Kimura 2 correction with 100 bootstrap replications methods in Treefinder [15]. SSU tree was reconstructed using Maximum Likelihood in RaxML.

Results

Morphology and physiology

The two isolates of the black fungus recovered from the patient's tonsil tissue before and during treatment showed good growth at 25°C. The maximum temperature tolerated was 40°C. Colonies on MEA and PDA grew faster but their morphologic characteristics (below) were similar to those of colonies grown on CMA and CZA. The isolates showed identical cultural and microscopic morphologies and had similar physiological characteristics, except that BMU 15, isolated during therapy, was able to assimilate adonitol, while BMU 00195 could not.

Susceptibilities to antifungal agents

The minimum inhibited concentrations (MIC) against strains BMU 00195/00015 of the tested antifungals were as follows: amphotericin B 0.25/1 µg/ml, ketoconazole 0.25/0.25 µg/ml, miconazole 2/2 µg/ml, itraconazole 0.25/0.5 µg/ml, fluconazole 16/64 µg/ml, 5-flucytocine 4/4 µg/ml, terbinafine 0.03/0.03 µg/ml.

SSU phylogeny and ITS sequence data

PCR-based amplification of SSU rDNA with primers NCS1/D12 yielded products of about 1,500 bp with strains BMU 00015 and 00195, as well as strains BMU 00001 (*E. alcalophila*), IFM 4869 = CDC B-1785 (*Exophiala* sp.) and 00009 (*E. bergeri*) and IFM 40919. Analysis of aligned sequences revealed the presence of 539 bp insertions one base pair upstream of the universal primer ITS1. Amplicons of strains lacking the intron were about 1,000 bp. An SSU tree with collapsed branches was constructed with hundreds of strains (Fig. 3); BMU 00015 and 00195 were identical to each other and could be confidently aligned with members of the order *Chaetothyriales*. The sequences showed 100% identity to strains BMU 00001 (*Exophiala cf. alcalophila*) and 00006 (*Exophiala* sp.). Strains BMU 00015 and 00195 did not belong to any of the

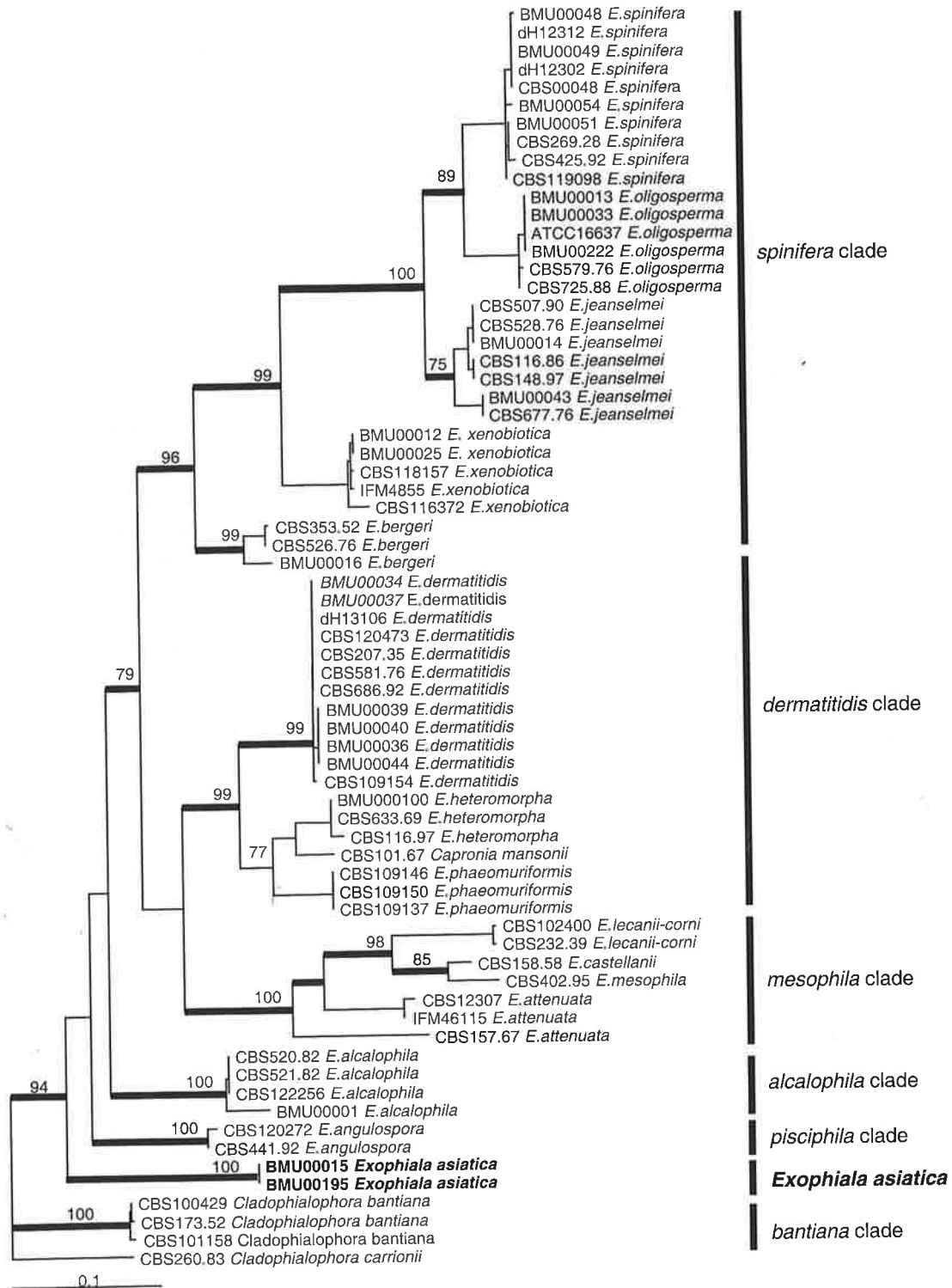


Fig. 3 Phylogenetic tree of *Exophiala asiatica* and allied black fungi based on the completed ITS 1-2 domain including the 5.8S rDNA gene, generated with the Treefinder package using the Neighbor-joining algorithm and Kimura correction. The tree was subjected to 100 bootstrap replications; *Cladophialophora carrionii*, CBS 260.83 was selected as outgroup.

previously recognized chaetothyrialean clades centred around *Cladophialophora bantiana*, *C. carrionii*, *Exophiala angulospora*, *E. spinifera*, *E. mesophila*, *E. dermatitidis*, *E. pisciphila* and *Phialophora europaea*.

Lengths of ITS regions of BMU 00015 and 00195 were 578 bp. ITS rDNA sequences revealed no close match when applying BLASTn in GenBank. The nearest neighbour of strains BMU 00015 and 00195 in a dedicated black yeast data base at CBS containing about 11,000 entries was *Exophiala* sp. IFM 4869 (Fig. 4). The following, sorted by similarity, was found with other *Exophiala* sp.: *E. moniliae* (89.7%), *E. dermatitidis* (86.5%), *E. alcalophila* (86.4%), *E. bergeri* (86.5%) and *E. lecanii-corni* (86.5%). In addition, similarities with species of the genus *Exophiala*, as well as the above mentioned members of *Phialophora*, *Cladophialophora* and *Fonsecaea* never exceeded 80–85%. The species was consequently regarded to represent a hitherto undescribed taxon, which is introduced below.

Exophiala asiatica Dong Ming Li, Ruo Yu Li, de Hoog & Duan Li Wang, *sp. nov.* – MycoBank MB 511857, Figs. 4 and 5.

Coloniae in agar farina avenae confecto (OSD) vel agar PDA dicto 25°C 7 mm diam post 14 dies, primum leves et zymoideae, deinde elevatae, velutinae, griseo-olivaceae; reversum olivaceo-nigrum. Hyphae leves, dilute olivaceo-brunneae, intervallis regularibus septatae, cellulas germinantes, ellipsoideas, 4.5–6.0 × 4–5 µm, proferentes. Mycelium torulosum quasi absens. Conidiophora vix distinguenda, ramosa vel simplicia, terminalia vel intercalaria; nonnumquam cellulae gemmantes etiam conidiogenae. Anneloconidia haud septata, late ellipsoidea, levia, 3.0–4.5 × 1–2 µm, cicatrice basilari vix distinguenda. Temperatura maxima crescentiae 38°C. Teleomorphe ignota.

Ex-type strain CBS 122847 = BMU 15 (dried culture holotype in CBS herbarium CBS-H-20111), isotype culture BMU 195, isolated from tonsil tissue of a patient, China, D.L. Wang.

Cultures grown on oatmeal agar (OA) and potato dextrose agar (PDA) at 25°C attain 7 mm diam. in 14 days, initially smooth and yeast-like, becoming raised, velvety, greyish olivaceous; reverse olivaceous black. Hyphae smooth-walled, pale olivaceous brown, regularly septate, forming ellipsoidal germinating cells measuring 4.5–6.0 × 4–5 µm, especially in young cultures; budding cells similar but smaller. Torulose mycelium nearly absent. Conidiophores poorly differentiated, branched or unbranched, terminal or intercalary; sometimes isolated budding cells functioning as conidiogenous cells are present. Conidiogenous cells bear a terminal or subterminal area from which successive conidia are formed by percurrent growth resulting in a peg-like extension with inconspicuous annellations which are discernible by scanning electron microscopy (Fig. 4J). Anneloconidia non-septate, broadly ellipsoidal, smooth-walled, 3.0–4.5 × 1–2 µm, with inconspicuous basal scars. Maximum growth temperature 38°C. Teleomorph unknown.

CBS 122847 = BMU 15 (dried culture holotype in CBS herbarium CBS-H-20111); CBS 122848 = BMU 195, both isolated from tonsil tissue of a human patient, China, D.L. Wang.

Discussion

We document a fatal *Exophiala* infection characterized by facial nodules, sore pharynx, tonsil necrosis, paralysis and osteolysis accompanied by fever in an apparently immunocompetent college student. The 20-year-old girl had experienced a trauma caused by a fishbone prick of her pharynx 3 years earlier, which is presumed to be the initial cause of the infection. Over a 20-month follow-up period, exacerbations were always accompanied by discontinuing antifungal therapeutic interventions, whereas improvements after therapy were followed by increased antifungal resistance. The MICs to most of the antifungal agents tested proved the species to be susceptible. However, after a period of treatment, strain BMU 00015 showed higher *in vitro* values to amphotericin B (1 µg/ml *vs.* 0.25 µg/ml) and fluconazole (>64 µg/ml *vs.* 16 µg/ml). This suggests that during management of this type of severe fungal infection, antifungal regimens should be adjusted according to broth MIC results and patients' reactions. In the present patient, medication was discontinued several times because of financial problems. This may have contributed to increasing resistance of the etiologic agent to antifungal agents and finally to the death of our patient.

The black fungus recovered from the affected pharynx tissue appeared to be an undescribed species of the genus *Exophiala*. *Exophiala asiatica* is the fourth *Exophiala* species causing fatal infections in China, which includes *E. dermatitidis*, *E. spinifera*, and *E. jeanselmei* [7,8,16,17]. Assignment of the new species to the existing taxonomic system was problematic. Sequences analysis of SSU rDNA classified the fungus in the order *Chaetothyriales*, with close association to the teleomorph genus *Capronia*. Though the anamorph genus *Exophiala* is polyphyletic judging from SSU rDNA phylogeny data, the present species clearly represented a new clade, together with a clinical strain from the USA, *Exophiala* sp. (IFM 4869 = CDC B-1785) and *Exophiala alcalophila* (Fig. 3).

A 539 bp insertion was noticed in the SSU gene on position 1768 as compared to *Saccharomyces cerevisiae*, which is one base upstream of the ITS1 primer location. An intron characteristic for the neurotropic species *Cladophialophora bantiana* was noted at the same location [18,19]. Similar insertion locations were listed by Haase *et al.* [20] in *Exophiala moniliae* and *Fonsecaea pedrosoi*. Though it is unconfirmed whether these insertions play a role in pathogenesis and evolutionary process, their existence and distribution among

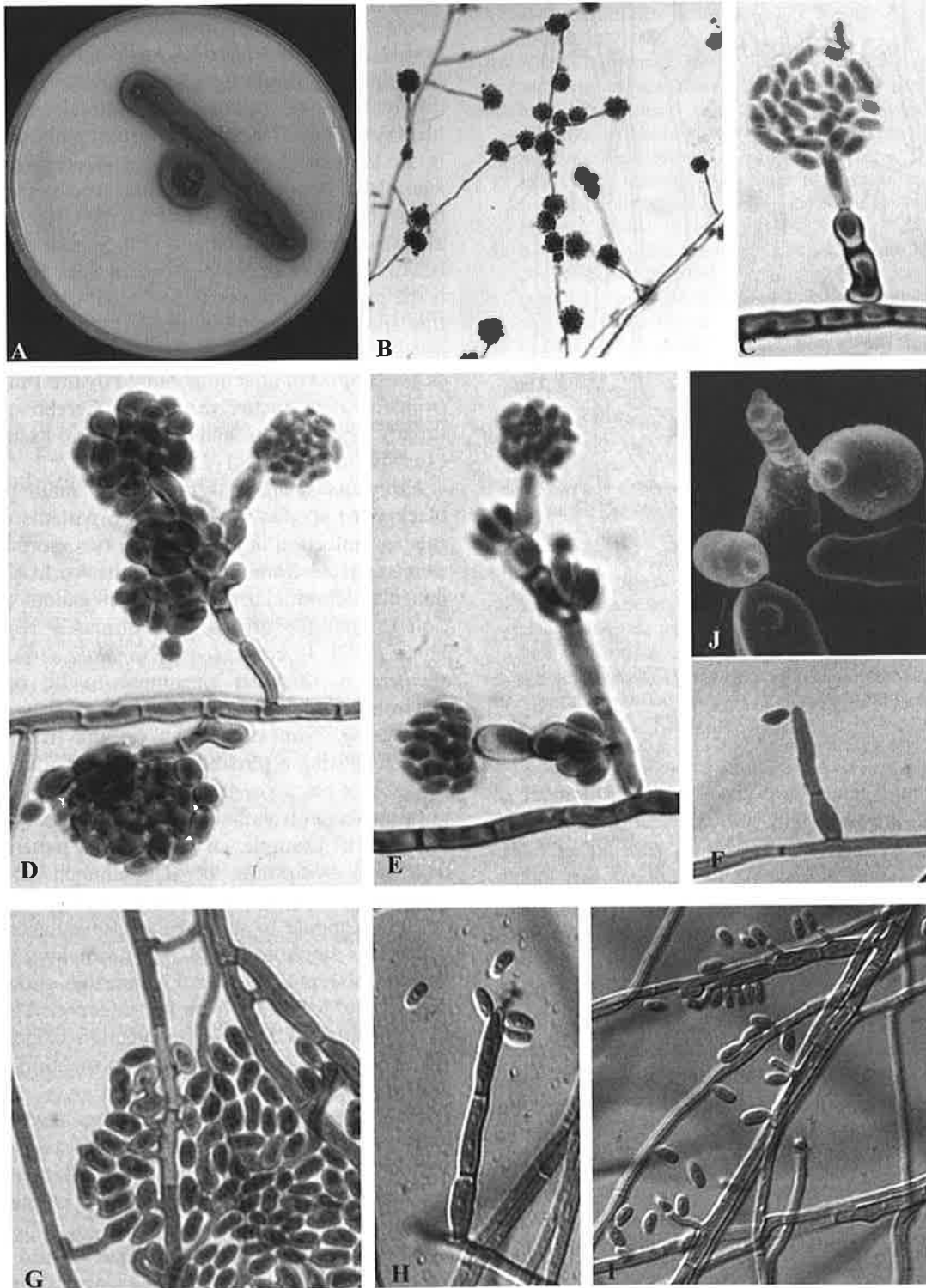


Fig. 4 *Exophiala asiatica*, CBS 122847. (A) Colony on CMA (2 weeks). (B) Conidia and hyphae (magnification $\times 100$). (C–I) Conidiogenous cells and one-celled conidia (magnification $\times 1000$). (J) Scanning electron microscopy of conidiogenous cells showing annellations.

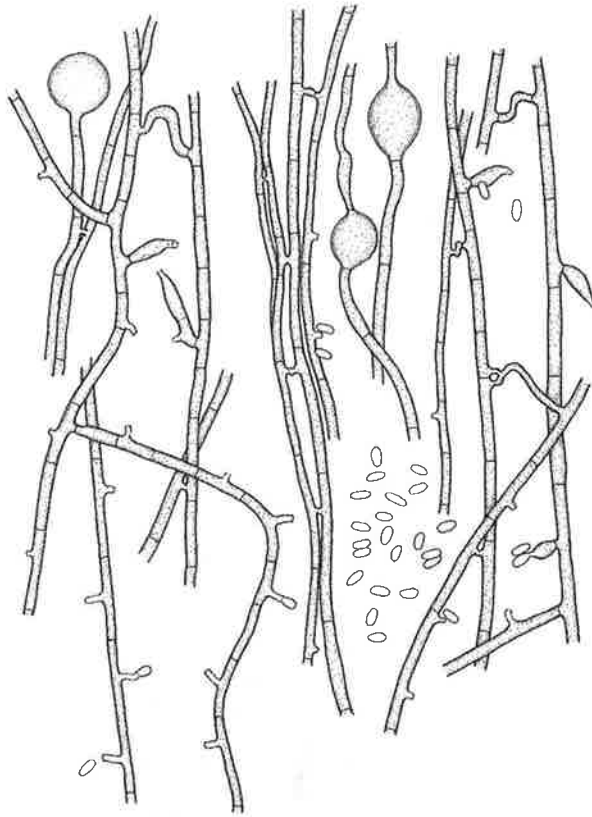


Fig. 5 Microscopic morphology of *Exophiala asiatica*, CBS 122847; slide culture on PDA, 14 d.

organisms and genes have given rise to a number of theories of intron origin and evolution. Homology among introns indicates horizontal transfer in BMU 00001 (*E. alcalophila*), 00003 (*E. xenobiotica*), 00006 (*Exophiala* sp.) and 00009 (*E. bergeri*) and other pathogenic black yeast-like fungi, as is known for other fungal species [21].

The corresponding ITS tree (Fig. 4) of the SSU *E. asiatica* clade showed considerable diversification among species. Its similarity to the nearest described species (*E. alcalophila*) was less than 90%. Comparing the ITS spacer lengths, *E. dermatitidis* (582 bp) is the longest in *Exophiala*, the new species is the second longest (578 bp), while the shortest one is *E. attenuata* (533 bp); the total range is 99 bp. Multiple alignment showed the presence of a CT-rich region in the 5' end of ITS2, which accounts for most of the variation. A distance tree constructed on the basis of ITS sequences with species allowing confident partial alignment shows that BMU 00015/000195 is truly unique, corresponding with the result of SSU analysis with *Exophiala alcalophila* being nearest neighbor at considerable distance.

Molecular identification of 20 clinical black yeasts from China revealed six *Exophiala* species, i.e., *E. dermatitidis* ($n=7$), *E. spinifera* ($n=5$), *E. xenobiotica* ($n=1$), *E. jeanselmei* ($n=2$), and *E. oligosperma* ($n=1$), in addition to *E. asiatica*. Seven isolates, involving four different species, concerned fatal infections in otherwise healthy patients. The most important pathogenic species is *E. dermatitidis*, followed by *E. spinifera* and *E. jeanselmei*. Remarkably, the same species are known to be involved in fungal infections in the United States, but always in debilitated patients [22]. Similar to other East Asian countries, the neurotropic species *E. dermatitidis* is the predominant species in China. Clinical strains of this species belong to a single ITS group within *E. dermatitidis*, referred to as genotype A [24]. The average clinical course of infections caused by this fungus varied from five days to twelve months. Cerebrospinal fluid rapidly changed to black and brain tissue showed extensive necrosis [16,17,23].

Exophiala spinifera is the second main pathogenic black yeast species in China. Two patients died of *E. spinifera* infection in China, while two more fatal cases were reported from the rest of the world [25,26]. All patients had nodular lesions, hematogenous dissemination to multiple organs and ultimately reaching the bones [7,27]. In contrast to *Exophiala dermatitidis*, *E. spinifera* is therefore presumed to be osteotropic. Environmental strains of the species have been reported, e.g., from apple juice [6] and fresh pineapple [24], suggesting a possible route of infection involving food. Since black yeasts are susceptible to most current antifungals, with sufficient medication patient should recover. For example, an Argentinean patient [28] had developed symptoms of disseminated *E. spinifera* infection with lymphadenopathy and fever for 3 years, without response to standard antifungal agents among which was amphotericin B. After switching to posaconazole (800 mg/day, for 13 months), however, she successfully recovered from her infection. The Chinese patient with the *E. asiatica* infection could not even afford itraconazole therapy, not to mention the newer antifungal agents like posaconazole.

Exophiala jeanselmei usually causes mycetoma [29], with systemic infection having been recorded after traumatic inoculation in debilitated patients [30]. A systemic case in an otherwise healthy, pregnant young woman was reported from China [8]. The infection began at the orbital fossa and spread into the brain glossolalia, leading to right leg paralysis and death within three days despite amphotericin B therapy.

Exophiala asiatica is now added to the list of species potentially causing fatal infections. Taking into account the clinical spectrum of black yeasts and the recurrence

of fatal infections in China, it is puzzling why these infections are particularly – and for some species exclusively – found in East Asia, and what kind of preventive measures can be taken. Medical workers in this region should pay more attention to the potentially severe infections of black fungi.

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