

## New *Pyrenochaeta* Species Causing Keratitis<sup>∇</sup>

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**We report a new fungus as an agent of fungal keratitis in a diabetic woman. The fungal etiology was established by classic microbiology and PCR following 3 months of antibacterial therapy. The morphological features of the isolate and sequence analysis of the internal transcribed spacer region indicate a new species of *Pyrenochaeta* (*Coelomycetes*).**

### CASE REPORT

A 77-year-old diabetic woman was referred for management of unresponsive microbial keratitis associated with pain and decreased vision in her left eye. She had received several different treatments (antibiotics) over 3 months with no improvement. Slit lamp examination revealed conjunctival hyperemia, peripheral corneal pannus, corneal edema, and a central corneal ulcer 4 mm in diameter with white stromal infiltrates inferiorly (Fig. 1A). After this examination, the condition was thought to be bullous keratopathy with secondary microbial keratitis. Corneal scrapings were taken for bacterial cultures; plated on Columbia agar plates supplemented with 5% sheep blood, Columbia chocolate agar, MacConkey agar, thioglycolate broth, and brain heart infusion broth (all media were from BioMérieux SA, Marcy-l'Étoile, France); and incubated at 37°C. After 24 h, *Streptococcus viridans* grew on brain heart infusion broth. The patient was started on fortified topical cefazolin (5%) together with topical ofloxacin (0.3%) eight times a day. After 2 weeks of treatment, the patient's condition deteriorated and a second corneal scraping was taken for direct examination and direct PCR analysis of the clinical specimen, which consisted of a panfungal PCR that targeted the internal transcribed spacer (ITS) region of the ribosomal DNA gene cluster (6) and cultures. Scrapings were inoculated directly onto Sabouraud dextrose agar with chloramphenicol (Pronadisa, Torrejón de Ardoz, Spain) and incubated at 30°C. Scrapings were also inoculated onto several bacteriological media. Direct examination of Gram and calcofluor white stains of the corneal scrapings revealed the presence of numerous septate and branched hyphae. The panfungal PCR was positive, and with the amplified DNA (530 bp), a BLAST search was performed showing only 86% similarity to a strain of *Leptosphaeria* sp. (GenBank accession no. DQ093683). No growth was observed in any media. The

patient was started on topical amphotericin B (0.15%) hourly, topical ofloxacin (0.3%) eight times a day, and oral fluconazole at 200 mg/day.

After 1 week of treatment, the patient's condition deteriorated. The treatment was therefore discontinued and the next day, a 3-mm corneal biopsy sample was taken from the ulcer edge for performing new direct mounts, panfungal PCR, and cultures. Direct examination with calcofluor white showed a severe fungal invasion of the cornea (Fig. 1B). Again, the amplification and DNA sequence showed 86% homology to the same *Leptosphaeria* strain. After 6 days, a filamentous fungus grew on chocolate agar, showing 100% homology of its ITS DNA sequence with the sequence obtained from the corneal samples. Negative results were obtained with the rest of the bacterial and fungal cultures. New treatment was initiated with topical natamycin (5%) hourly and oral ketoconazole at 200 mg/day. One month later, the lesion improved, but unfortunately it was too late to save the cornea and the patient was programmed for optical penetrating keratoplasty.

For identification, the fungus was subcultured on malt extract agar (Pronadisa, Torrejón de Ardoz, Spain) and oatmeal agar (30 g oat flakes, 1 g MgSO<sub>4</sub>, 1.5 g KH<sub>2</sub>PO<sub>4</sub>, 15 g agar, 1 liter tap water) and incubated at 20°C in the dark. After 1 week of incubation, pycnidia typical of the coelomycetous genus *Pyrenochaeta* developed in all of the media tested but the microscopic features observed in the case strain did not match those of the current accepted species of the genus. Therefore, the isolate was sent to the Centraalbureau voor Schimmelcultures (CBS) in The Netherlands for final identification. The fungus was identified as a new *Pyrenochaeta* species (accession number CBS 121759), which was confirmed by analysis of the ITS sequence of the clinical strain. The sequence (532 bp) (GenBank accession number EU885415), compared with different sequences obtained from the CBS by the ClustalW2 program, showed the highest homology with two strains morphologically identified as *P. unguis-hominis*, 100% with CBS 123295 and 96% with CBS 378.92, differing from the later at 15 bp positions.

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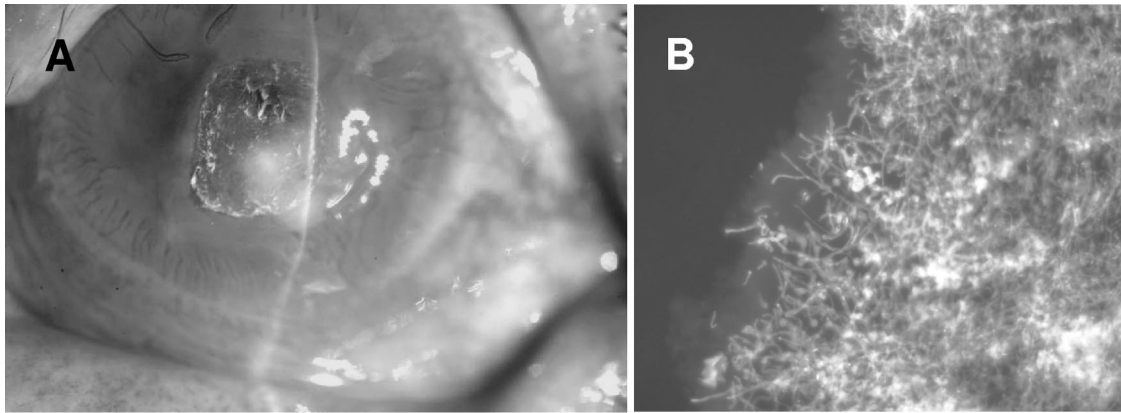


FIG. 1. (A) Clinical picture of the left eye showing conjunctival hyperemia, peripheral corneal pannus, corneal edema, and a central ulcer with white infiltrates. (B) Calcofluor white staining of a corneal biopsy sample showing fungi invading the cornea. Original magnification,  $\times 400$ .

The pycnidia were olivaceous brown to almost black and globose or flask shaped, measuring 100 to 400  $\mu\text{m}$  in diameter, with 1 to 3 ostioles, and with several setae positioned near the ostiole (Fig. 2A and B). The pycnidial wall was of *textura angularis* with dark brown intercellular material (Fig. 2D). The

conidiophores, which emerged from all over the inner surface of the pycnidial wall, were branched at the base, bearing terminal and lateral conidiogenous cells. The conidiogenous cells were phialidic, mostly cylindrical, and 12 to 18  $\mu\text{m}$  long by 2 to 3.5  $\mu\text{m}$  wide (Fig. 2C). The conidia were whitish in mass,

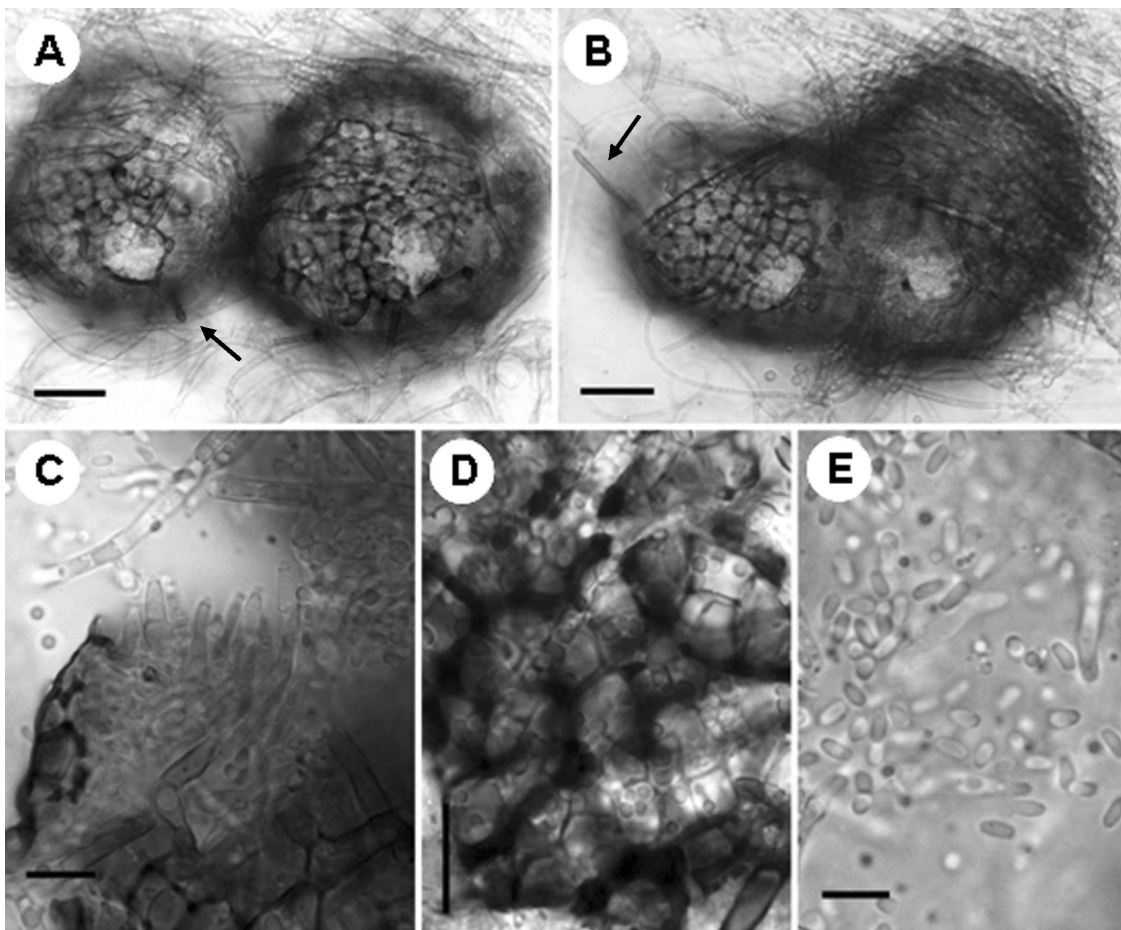


FIG. 2. Light microscopic images of *Pyrenochaeta* sp. (accession no. CBS 121759) on oatmeal agar at 20°C. (A and B) Pycnidia with setae (arrows). Scale bar = 50  $\mu\text{m}$ . (C) Transverse view of the pycnidial wall with conidiophores and conidiogenous cells. Scale bar = 10  $\mu\text{m}$ . (D) Surface view of the conidiomatal wall with dark intercellular material. Scale bar = 10  $\mu\text{m}$ . (E) Conidia from pycnidia. Scale bar = 5  $\mu\text{m}$ .

ellipsoidal, 2 to 4  $\mu\text{m}$  long by 1 to 2  $\mu\text{m}$  wide, straight or slightly curved, hyaline, and smooth walled (Fig. 2E).

Fungal infections of the cornea are caused by a wide variety of fungi, but the vast majority of cases are due to species of *Candida*, *Aspergillus*, and *Fusarium* (10). Many predisposing factors have been mentioned for keratomycosis, including chronic ocular surface disease, dry eye, contact lens wearing, atopic disease, topical steroid use, long-term treatment with broad-spectrum antibiotics, and trauma (particularly with vegetable material or soil). Ulcers caused by yeasts are more common in preexisting corneal diseases, and filamentous fungi are more common following corneal trauma with external material, especially with vegetable material or soil, from which more genera have been reported due to the great diversity of fungi that can colonize these substrates. Therefore, new fungi are frequently added to the list of microorganisms that are able to cause keratitis, such as *Phaeoisaria clematidis* (8), *Alternaria infectoria* (5), or *Sarcopodium oculorum* (7).

The identification of these kinds of fungi is often not easy because they are not part of the spectrum of fungi regularly encountered in clinical samples. The proper diagnosis and identification of the causal agent are crucial for starting adequate treatment and avoiding devastating ocular consequences. In some cases, molecular biology techniques have helped to identify the causal agent (5), but in others, like the one presented here, expert mycologists need to be involved to reach a correct identification. In this case, a very rare fungus was identified as responsible for the infection and must be added to the list of species that can produce keratomycosis. It shows typical features of the genus *Pyrenochaeta*, but none of the accepted species have all of the morphological features observed in this case strain. The color and texture of the colony help to identify the species of the genus (2). The anamorphic genus *Pyrenochaeta* belongs to the group *Coelomycetes* and is related to *Herpotrichia* and *Leptosphaeria*, two ascomycetous genera of the family *Leptosphaeriaceae*. *Pyrenochaeta* contains two species that cause infection in humans, i.e., *Pyrenochaeta romeroi* isolated from cases of black-grain mycetoma (1, 9) and *Pyrenochaeta unguis-hominis* isolated from infected nails on several occasions, although its role in the development of onychomycosis remains questionable (4). Other species of *Pyrenochaeta* are pathogens of various plant species. Identification of the fungi responsible for an infection to the genus or species level is hampered by their frequent failure to produce characteristic diagnostic structures in culture and the lack of reference sequences in the public databases. In addition, some of these sequences correspond to species erroneously identified (3). In particular, the sequences of *Pyrenochaeta* species ob-

tained in the GenBank database when we ran the BLAST program (March 2007) did not match ours. Since new sequences have been added to the GenBank database since that date, a new BLAST performed later allowed us to identify our fungus as a probable novel species of *Pyrenochaeta*. Apart from *P. unguis-hominis*, mentioned above, another close species of *Pyrenochaeta* was *P. nobilis* (CBS 407.76), the type species of the genus *Pyrenochaeta*, which showed 92% homology in the ITS region and differed at more than 35 bp positions. The other strain (CBS 123295), isolated from a Dutch patient with a recurrent nail infection and identified at the CBS as *P. unguis-hominis* on the basis of the morphological characteristics of its fruit bodies, was also sequenced and proved to have an ITS sequence that is 100% homologous to that of our strain. This strain was reexamined morphologically and found to be very similar to the case strain. To our knowledge, the Dutch strain is the only other isolate of this new *Pyrenochaeta* species. The natural habitat of this fungus is unclear.

On the basis of the above-mentioned morphological and molecular studies, we believe that the strain responsible for the infection of our patient is a species of *Pyrenochaeta* not previously described. We are currently preparing the formal proposal of a new species of this fungus.

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We have no proprietary interest in any of the materials described in this article.

#### REFERENCES

1. Andre, M., V. Brumpt, P. Destombes, and G. Segretain. 1968. Fungal mycetoma with black grains due to *Pyrenochaeta romeroi* in Cambodia. *Bull. Soc. Pathol. Exot. Fil.* **61**:108–112.
2. de Hoog, G. S., J. Guarro, J. Gené, and M. J. Figueras. 2000. Atlas of clinical fungi. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
3. Desnos-Ollivier, M., S. Bretagne, F. Dromer, O. Lortholary, and E. Dannaoui. 2006. Molecular identification of black-grain mycetoma agents. *J. Clin. Microbiol.* **44**:3517–3523.
4. English, M. P. 1980. Infection of the finger-nail by *Pyrenochaeta unguis-hominis*. *Br. J. Dermatol.* **103**:91–93.
5. Ferrer, C., J. Montero, J. L. Alió, J. L. Abad, J. M. Ruiz-Moreno, and F. Colom. 2003. Rapid molecular diagnosis of posttraumatic keratitis and endophthalmitis caused by *Alternaria infectoria*. *J. Clin. Microbiol.* **41**:3358–3360.
6. Ferrer, C., F. Colom, S. Frásés, E. Mulet, J. L. Abad, and J. L. Alió. 2001. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. *J. Clin. Microbiol.* **39**:2873–2879.
7. Guarro, J., A. L. Hoffling-Lima, J. Gené, F. D. De, P. Godoy, M. L. Zorat-Yu, L. Zaror, and O. Fischman. 2002. Corneal ulcer caused by the new fungal species *Sarcopodium oculorum*. *J. Clin. Microbiol.* **40**:3071–3075.
8. Guarro, J., L. A. Vieira, F. D. De, J. Gené, L. Zaror, A. L. Hoffling-Lima, O. Fischman, C. Zorat-Yu, and M. J. Figueras. 2000. *Phaeoisaria clematidis* as a cause of keratomycosis. *J. Clin. Microbiol.* **38**:2434–2437.
9. Thammayya, A., M. Sanyal, and N. Basu. 1979. *Pyrenochaeta romeroi* causing mycetoma pedis in India. *J. Indian Med. Assoc.* **73**:66–67.
10. Thomas, P. A. 2003. Fungal infections of the cornea. *Eye* **17**:852–862.