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Diagnostic Difficulties Caused by a Nonclamped *Schizophyllum commune* Isolate in a Case of Fungus Ball of the Lung

LYNNE SIGLER,[1,2]* LUIS M. DE LA MAZA,[3] GRACE TAN,[3] KEITH N. EGGER,[4] AND RICHARD K. SHERBURNE[2]

University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden,[1] and Medical Microbiology & Infectious Diseases, University of Alberta,[2] Edmonton, Alberta, Canada T6G 2E1; Division of Medical Microbiology, Department of Pathology, University of California, Orange, California 92668[3]; and Department of Biology, Memorial University, St. John's, Newfoundland, Canada A1B 3X9[4]

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The presence of clamp connections on hyphae and the development of fruiting bodies in culture are primary characters which allow identification of the basidiomycete *Schizophyllum commune* in cases of human infection. The diagnostic problems presented by a nonclamped, nonfruiting isolate from a dense mass in the right upper lobe of the lung in a female with a past history of pulmonary tuberculosis and diabetes are described. Several features of the isolated fungus, including rapid growth rate and white, dense, cottony colonies, tolerance to the fungicide benomyl at a concentration of 10 μg/ml, and susceptibility to cycloheximide at 400 μg/ml, suggested that it might be a basidiomycete. Transmission electron microscopy showed the presence of a dolipore septum with perforate pore cap characteristic of fungi in the class *Holobasidiomycetes*. However, species identification remained elusive until compatibility tests with known single-basidiospore isolates confirmed the identification of the sterile lung isolate as *S. commune*. Sequence analysis of the 5 internal transcribed spacer region of ribosomal DNA further supported conspecificity.

Fungus ball of the lung, in which a mass of fungal mycelium grows in a preexisting cavity, occurs in patients with underlying pulmonary disorders such as tuberculosis, previous infections caused by systemic fungi, recurrent bacterial pneumonia, lung abscess, or sarcoidosis. The infection is not generally diagnosed by sputum culture since the fungal elements are walled off and are not expelled, but hemoptysis is a common finding. Most cases involve species of *Aspergillus*, most commonly *A. fumigatus*, or *Scedosporium apiospermum* (*Pseudallescheria boydii*) (10). Reports of infection caused by basidiomycetes are rare, and to our knowledge, no basidiomycete has been reported from a case of fungus ball. Other than members of the basidiomycetous yeast genera *Cryptococcus* and *Trichosporon*, probably the best-known basidiomycetous agent of infection is *Schizophyllum commune*. Reports involving the lung include a case of allergic bronchopulmonary mycosis (7) in an otherwise healthy female and repeated isolation of *S. commune* from the sputum of a patient with chronic lung disease (3). Other reports of *S. commune* infection include cases of meningitis (2), sinusitis (1, 8, 13), ulcerative lesions of the hard palate (12), and possible onychomycosis (9) in both immunocompetent and immunosuppressed hosts. In all of the cases reported to date, the fungus has been recognized in tissue and in culture by the presence of clamp connections on the hyphae and by the development of basidiocarps (sexual fruiting bodies) in culture. However, Kamei et al. (7) suggested that diagnosis of infection caused by *S. commune* may be difficult because of (i) the failure to observe clamp connections on hyphae which otherwise appear similar to those of *Aspergillus*, (ii) the absence of fruiting body formation in the dark, and (iii) the possibility of identification only when the fungus is a dikaryon capable of producing basidiocarps. We present a case of necrotizing granuloma of the lung in a patient with a history of tuberculosis and diabetes in whom septate, nonclamped hyphae were observed in histopathologic sections. This report provides an example of the diagnostic problems presented by an isolate which is suspected of being a basidiomycete but which fails to form the characteristic macroscopic and microscopic structures which would allow its identification.

* Corresponding author. Mailing address: University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden, Edmonton, AB, Canada T6G 2E1. Phone: (403) 987-4811. Fax: (403) 987-4141. Electronic mail address: Lynne.Sigler@ualberta.ca.

MATERIALS AND METHODS

Case report. A 53-year-old Vietnamese female was seen in the Emergency Room of the University of California, Irvine, Medical Center in September 1992 complaining of chronic cough and hemoptysis for the previous 7 days. The patient had a history of tuberculosis diagnosed 5 years earlier, and in 1988 she was found to have non-insulin-dependent diabetes. Apparently, she was treated several times over the previous 5 years with antituberculosis drugs. In December 1991, she was started on isoniazid, ethambutol, and rifampin.

A chest X ray and computed tomography scan performed in June 1992 showed a roughly spherical density in the posterior segment of the right upper lobe measuring approximately 2 cm in diameter. Above this level there were many smaller, sharply defined lesions scattered throughout the lung, particularly in the anterior and apical segments. Leading from the larger mass toward the hilum, there were markedly thickened bronchial walls giving the appearance of a tennis racquet, an image usually associated with tuberculosis (Fig. 1). An expectorated sputum specimen collected during this visit was negative for mycobacteria by culture and smear.

A bronchoalveolar lavage was performed in July 1992. A Gram stain showed 4+ erythrocytes but no organisms. Bacterial cultures were positive for a mixed flora. Cultures and direct smears were negative for mycobacteria. Viral cultures were positive for cytomegalovirus and adenovirus. Fungal cultures were negative. The cytopathological analysis was negative for malignancy, and acid-fast bacillus stains were negative.

In October 1992 a right upper lobe lobectomy was performed. The patient had a mild pneumothorax after surgery and was discharged 10 days following surgery.

Histopathology. The pathological report of the frozen intraoperative specimen was described as a necrotizing granuloma, with a fungal form consistent with *Aspergillus* sp. present. Hematoxylin and eosin (H&E)-stained tissue samples from the lobectomy specimen showed histopathological lesions typical of a mycobacterial infection including the presence of caseous calcified granulomas with Langhans cells. Stains for acid-fast bacilli were positive. H&E and the Gomori-methenamine silver (GMS) stains showed that the center of the lesion was occupied by a large mass of hyphae which were present in the cavity but which did not invade the surrounding tissues. The hyphae were septate, varying in width from 1.5 to 5 μm but mostly measuring between 1.5 and 3 μm in diameter,

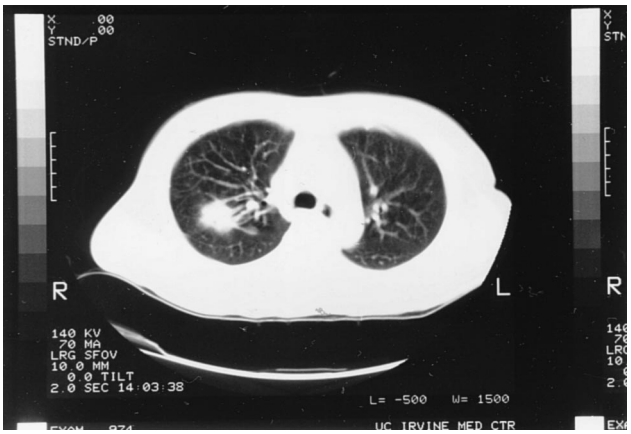

FIG. 1. Computed tomography scan of the chest showing a dense mass in the right upper lobe and thickened bronchial walls giving the appearance of a tennis racket.

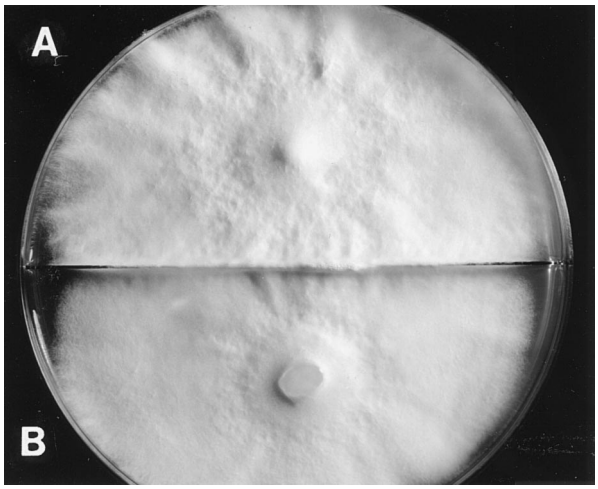

FIG. 3. Colony on unamended SDA (A) and SDA amended with 10 μg of benomyl per ml (B).

sometimes showing irregularly shaped swellings up to 7 μm wide or having short, rounded protrusions, rarely branched at an acute angle or dichotomous. In GMS-stained material, some hyphae showed variation in intensity of staining, with an individual hypha showing short segments of darkly stained areas alternating with lighter-stained areas (Fig. 2).

Mycology. Cultures of the surgical specimen yielded several colonies of a white, rapidly growing fungus on inhibitory mold agar containing ciprofloxacin (Dimed Corporation, St. Paul, Minn.) and on Sabouraud dextrose agar (SDA) containing no antibiotics (BBL/Beckton Dickinson Microbiology Systems, Cockeysville, Md.).

Further studies of the lung isolate were conducted at the University of Alberta. Colonial features were examined by subculture onto phytone-yeast extract agar (PYE) (BBL), Mycosel agar (BBL), 2% malt extract agar (MEA; Difco Bacto malt extract, 25 g; agar, 18 g), potato dextrose agar (PDA; Difco), and Pablum cereal agar (CER) (11) and incubation at 25°C. Growth rates at temperatures of 37 and 43°C were determined with PDA. Incubation was in alternating conditions of light and dark except at 43°C, at which incubation was entirely in the dark. Susceptibility to cycloheximide at a concentration of 400 μg/ml was determined by growing the fungus on Mycosel agar, and tolerance to benomyl was determined by assessing growth rates on unamended SDA compared with those on SDA amended with benomyl at concentrations of 2 and 10 μg/ml as described by Summerbell (15). Additional media were used in attempts to induce sporulation in the sterile isolate, and cultures were incubated for 3 to 4 months under various light conditions. Microscopic observations were made in slide cultures on CER. The isolate from this patient was deposited in the University of Alberta Microfungus Collection and Herbarium as UAMH 7287. The unknown isolate was also compared with known isolates of *S. commune*. A dikaryotic isolate, UAMH 7796 (CDC B-5575), was obtained from a brain abscess; monokaryotic isolates were obtained from the Forest Products Laboratory, U.S. Department of Agriculture, Madison, Wis., as single-basidiospore isolates UAMH 7692 (7599 ss-1), UAMH 7693 (7599 ss-7), UAMH 7694 (FP-74612 ss-2), and UAMH 7695 (FP-74612 ss-3).

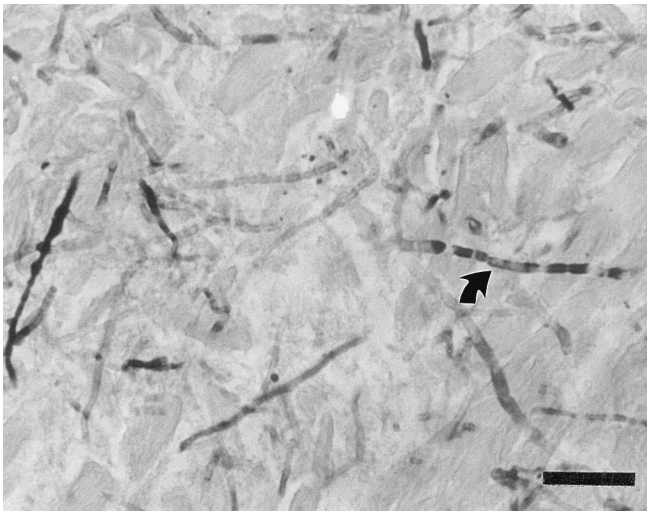

FIG. 2. GMS-stained section showing hyphae of varying widths. Note that some segments also show variations in staining intensity (arrow). Bar, 20 μm.

Additional studies. For transmission electron microscopy (TEM), the sample was prepared by the methodology of Currah and Sherburne (4) and was examined with a Philips model 410 transmission electron microscope. For scanning electron microscopy (SEM), the methodology followed the same procedure described above for TEM except that the sample was rinsed in distilled water following the 1% (wt/vol) osmium tetroxide fixation step and was further incubated with 1% tannic acid in distilled water. The specimen was then rinsed in water and was again incubated in the osmium tetroxide fixative. After a further wash, the sample was dehydrated, dried to the critical point, mounted, and examined in a Hitachi model S 4000 field emission scanning electron microscope at an accelerating potential of 2.5 kV.

To test compatibility between UAMH 7287 and the four monokaryotic strains and among the monokaryotic isolates themselves, the isolates were paired in all possible combinations. A plug of mycelium of 3 mm in diameter was removed from the margin of a colony grown on PDA to one-half of a new PDA plate; a plug from a second isolate was placed on the other half. Microscopic mounts from the contact zone between advancing mycelia were examined for the presence of clamp connections (dikaryotization).

Total genomic DNA was extracted from plugs of mycelium removed from colonies grown on PDA and was freeze-dried for 24 h. The internal transcribed spacer 1 (ITS1) region was amplified with primers ITS1 (17) and ITS10mun (5). The procedures for DNA extraction, amplification, and sequence analysis followed the methodology of Egger and Sigler (5).

Nucleotide sequence accession numbers. Representative sequences are deposited in GenBank under the following accession numbers U21483 (UAMH 7287 and UAMH 7693), U21484 (UAMH 7694), and U21485 (UAMH 7695).

RESULTS

Description of the isolate from this case. Macroscopically, the isolate grew rapidly on all media, reaching diameters of 50 to 60 mm in 7 days on PDA or PYE and 40 mm on 2% MEA. Growth at 37°C was similar, with a colony diameter of 60 mm, but growth was slightly faster at 43°C (diameter, 70 mm) on PDA. Daily growth rates on PDA at temperatures of 25, 37, and 43°C were 8.6, 8.6, and 10.1 mm, respectively. Colonies on PDA or PYE were dense, cottony, white, and slightly raised with a central umbo. The fungus was susceptible to cycloheximide but was tolerant to benomyl at 10 μg/ml (Fig. 3). It produced a strong, unpleasant odor that was easily detectable when the plates were incubated in plastic bags or containers. Microscopically, features of the hyphae in agar culture conformed to those of the hyphae observed in histopathology, demonstrating (i) variation in width, ranging from 1.5 to 5 μm,

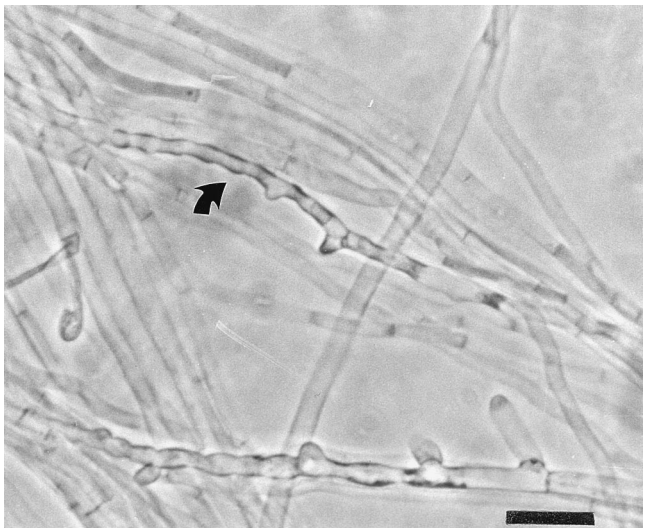

FIG. 4. Slide culture preparation showing segments of hyphae with small, rounded swellings (arrow) and rounded protrusions resembling clamps. Bar, 10 μm.

(ii) the presence of small rounded swellings occurring on some hyphae (crenate hyphae) (Fig. 4) which in the stained sections appeared as variations in staining intensity (Fig. 2), and (iii) short, rounded protrusions somewhat resembling aborted clamp connections (Fig. 4). In slide culture preparations, narrow hyphae often formed loose knots. The isolate failed to produce either sexual or asexual structures on any medium and initially could not be identified. However, the general cultural features and tolerance to benomyl suggested the sterile isolate would be more likely to be basidiomycetous than ascomycetous.

An oblique section of the hypha examined by TEM demonstrated the presence of a dolipore septum with a dome-shaped pore cap showing perforations or openings (Fig. 5). Fracture of a hyphal cell adjacent to the septum and examination by SEM allowed a unique three-dimensional view of the pore cap showing the perforations in the cap (Fig. 6). Lower-magnification

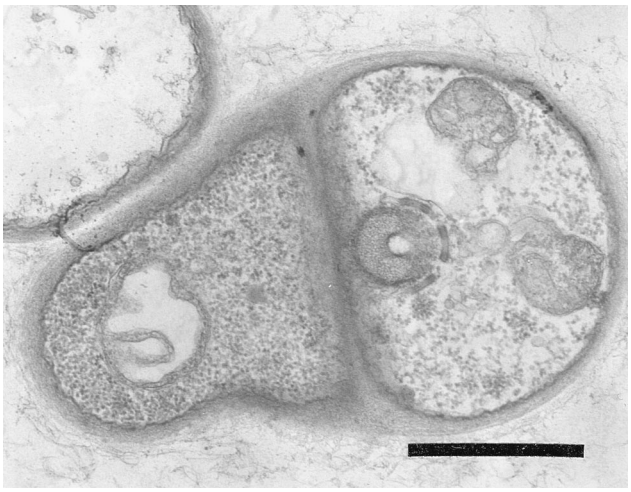

FIG. 5. Transmission electron micrograph of oblique section of hypha showing dolipore septum with perforate pore cap. Bar, 10 μm.

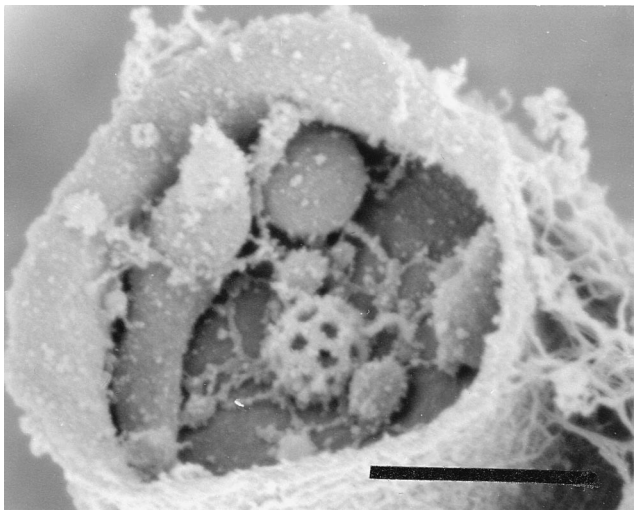

FIG. 6. Scanning electron micrograph showing dome-shaped pore cap with perforations. Bar, 1 μm.

SEM of intact hyphae verified the presence of the distinctive rounded swellings on some hyphae (Fig. 7).

Comparison with *S. commune*. Differences between the dikaryotic (fruiting) isolate and monokaryotic isolates were evident in growth rates and colonial and microscopic morphologies. The dikaryotic isolate (UAMH 7796) grew slightly more slowly at all temperatures, showing daily growth rates of 7.1 mm at 25°C, 5.1 mm at 37°C, and 8.3 mm at 43°C. Colonies were densely woolly and had highly irregular (lobate) margins. By 7 days, several fruiting bodies formed on PDA at 25°C, but not at 37°C, after 2 weeks under conditions of alternating light and dark. Average daily growth rates for the monokaryotic isolates at each temperature were 6.2, 10, and 11.4 mm. However, differences in growth rates were observed between the single basidiospore isolates obtained from the same fruiting body; for example, UAMH 7692 showed daily growth rates of

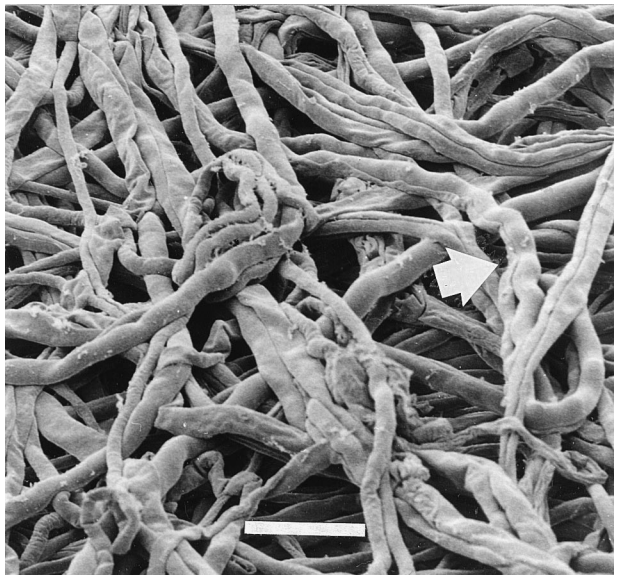

FIG. 7. Scanning electron micrograph of colony showing hyphae with small, rounded swellings (arrow). Bar, 10 μm.

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1   gtaggtgaacctgcggaaggatcattaACGAATCAAACAAGTTCATCTTGTTCTGATCCTGTGCACCTWATGTAGTCCCAAAGCCTTCACGGGCRGCGGT
101 TGACTACGTCTACCTCACACCTTAAAGTATGTTAACGAATGTAATCATGGTCTTGACAGACCYTRAAAMGTTAATacaactttcgacaacggatctcttg
201 gctctcgcatgatgaagaacg
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FIG. 8. Consensus sequence for the ITS1 region of the nuclear-encoded rRNA genes amplified with primers ITS1 and ITS10mun. The 18S rRNA and 5.8S rRNA sequences flanking the ITS1 region are indicated in lowercase letters. Variable positions are indicated by using the standard ambiguity code of the International Union of Pure and Applied Chemistry: R = A or G, Y = C or T, M = A or C, and W = A or T.

6.4, 8.1, and 8.6 mm at each temperature, respectively, compared with respective growth rates of 11.4, 14.3, and 14.3 mm for strain UAMH 7693. Colonies were densely cottony to woolly and, in contrast to the dikaryotic isolate, margins were entire (i.e. smooth and regular). A strong, unpleasant odor was produced by all isolates and was detectable, in some instances, through the closed door of the incubator. Clamp connections were present on hyphae of the dikaryotic isolate; in addition, some hyphae bore short, fine pegs or spinulose projections arising at right angles. This highly distinctive morphologic feature of *S. commune* hyphae was absent from all monokaryotic isolates which also lacked clamp connections. Hyphae of the latter showed considerable variation in width, ranging from 1.5 to 5 μm, and were thin or thick walled, and in slide culture preparations narrow hyphae often formed loose knots. No conidia or chlamydospores were observed.

In compatibility tests, the isolate from the case patient (UAMH 7287) formed clamp connections when it was paired with each of the monokaryotic isolates, thus demonstrating a process of dikaryotization. Compatibility among the monokaryotic isolates occurred only between single basidiospore isolates obtained from different fruiting bodies (i.e., UAMH 7692 formed clamp connections with UAMH 7694 and UAMH 7695 but not UAMH 7693). No pair of isolates formed basidiocarps, and no changes in colonial characteristics occurred in the dikaryotized mycelia.

Alignment of ITS1 sequences indicated that UAMH 7287, UAMH 7693, UAMH 7694, and UAMH 7695 are conspecific. Only five of 221 positions were variable (Fig. 8), for a total variation of 2.26%. No insertions or deletions were observed. The sequences of UAMH 7796 and UAMH 7692 are not reported here. After two attempts, we were unable to obtain an unambiguous sequence for the dikaryotic isolate, and the original isolate of UAMH 7692 sent to K. N. Egger was found to be a basidiomycetous contaminant. Since a subculture of the original isolate demonstrated compatibility in mating tests as reported above, the sequencing was not repeated for the purified isolate UAMH 7692.

DISCUSSION

Macroscopic features of rapid growth and white woolly colonies, tolerance to the fungicide benomyl, and susceptibility to cycloheximide were suggestive initially that the isolate was a basidiomycete. However, since it remained sterile under all conditions, a definite identification was not possible. A diagnosis of aspergillosis could easily have been made in this case since the hyphae in the histopathology section demonstrated only subtle differences and white, sterile isolates of *A. fumigatus* are isolated occasionally from patients with chronic lung disorders. However, the hyphae of the fungus in the necrotic lesion were consistent with those of the isolate in culture, which was proven to be a basidiomycete by ultrastructural evidence of the dolipore septum.

If clamp connections are present, the identification of an isolate as a basidiomycete is simple. However, clamp connections are absent in monokaryotic isolates, and many basidiomycetes lack them completely. Septal ultrastructure has been found to be a useful characteristic in assessing the affinities of an unknown isolate to either the ascomycetes or the basidiomycetes, especially when other characteristics are absent. Most ascomycetous fungi, for example, *A. fumigatus*, have simple, single-pored septa. In contrast, among the basidiomycetes, several different types of septal structure are recognized and are important characters in the taxonomy. The dolipore septum with a multiperforate pore cap confirmed in our isolate by TEM and SEM (Fig. 5 and 6) is typical of members of the class *Holobasidiomycetes* (producing meiospores on nonseptate basidia), which includes the orders *Aphyllophorales* and *Agaricales*.

Among the *Aphyllophorales*, the best-known agent of infection is *S. commune*. Although it is sometimes difficult to evaluate the significance of isolation of *S. commune* from clinical specimens (6, 7, 9), there have been a number of well-documented reports especially involving the nasal musosa, hard palate, and lung (1, 7, 8, 13). To date, all confirmed cases of *S. commune* infection have been based on isolates which form characteristic fruiting bodies in culture. Additional features which allow the identification of an isolate as *S. commune* include clamp connections and narrow hyphal pegs or spicules (1, 2) present on some hyphae. Greer (6) reported that the pegs could also be observed on hyphae growing in tissue. Neither of these distinctive hyphal features was present in the isolate from our case patient or in any of the single basidiospore isolates; moreover, these monokaryotic isolates demonstrated dissimilar colonial features. Since monokaryons are the most common basidiomycetous isolates encountered in the diagnostic laboratory (14, 15), the possibility that our fungus was a monokaryotic *S. commune* isolate was considered but was initially ruled out by these apparent differences. This false-negative result was corrected only when single-basidiospore isolates were obtained for comparison and compatibility tests.

The DNA sequence data confirmed the results of the mating tests. A level of base substitution of 2.26% is well within the range of intraspecific variation commonly observed in fungi (5). The ITS1 region is also highly susceptible to insertion or deletion events. Although insertions or deletions are occasionally found within species, they are particularly common between species. The low level of base substitution and the absence of insertions or deletions indicates that the isolates are conspecific.

Our finding of a monokaryotic isolate of *S. commune* causing infection supports the contention of Kamei et al. (7) that many cases of infection caused by basidiomycetes may be misdiagnosed. Any white, rapidly growing, sterile isolate showing good growth at 37°C with tolerance to benomyl, susceptibility to cycloheximide, and a pronounced odor should be suspected of being *S. commune*. Since compatibility (dikaryotization) between monokaryotic mycelia derived from different fruiting bodies reaches almost 100% (16), the compatibility test, as described in this report, could be used effectively to allow rapid confirmation of the identity of a suspected monokaryotic *S. commune* isolate.

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