

Short communication

***Arachnomyces kanei* (anamorph *Onychocola kanei*) sp. nov., from human nails**

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Five isolates of a slow-growing cycloheximide resistant hyphomycetous fungus were obtained from nail specimens and investigated for their relationship to *Onychocola canadensis* (teleomorph *Arachnomyces nodosetosus*), a known agent of onychomycosis. In one patient diagnosed with superficial white onychomycosis, etiology was confirmed by a nail sample showing atypical filaments in direct microscopy, and by a follow-up specimen yielding cultures of the same fungus. A case of mixed infection with *Aspergillus sydowii* was also confirmed after examination of cultures grown from three successive microscopic-positive hallux nail specimens. For other isolates, etiological significance could not be confirmed by repeat sampling or results of direct microscopy were negative or unknown. Mating experiments yielded setose ascomata containing smooth oblate ascospores typical of *Arachnomyces* species. Phylogenetic analysis of ITS 2 region sequences support the conspecificity of the isolates and their placement within the genus. *A. kanei* sp. nov. (anamorph *O. kanei* sp. nov.) is described.

Keywords *Arachnomyces kanei*, human nails, *Onychocola*, onychomycosis

Introduction

In Ontario, Canada, a large-scale clinical and laboratory investigation of 473 patients with abnormal-appearing nails resulted in confirming of etiological significance for some fungi that might otherwise have escaped routine dermatological diagnosis [1]. A slow-growing cycloheximide-tolerant hyphomycetous fungus producing yellow colonies and slightly swollen intercalary arthroconidia was obtained from two patients, one of whom had been diagnosed with having superficial white onychomycosis (SWO). In this case, the same fungus was obtained from a second specimen taken at a later time and direct microscopy was positive for 'irregular' filaments, that is,

not consistent in morphology with hyphae usually produced by dermatophytes [1,2]. Based on phenotypic similarities, two additional isolates were found among unclassified isolates on deposit at the University of Alberta Microfungus Collection and Herbarium (UAMH), Edmonton, Canada. These were also obtained from nails. Results of direct microscopy were unknown for one of them, whereas another was again associated with irregular filaments but was considered unconfirmed as an etiological agent because a repeat specimen was not obtained. The unusual agent was also later detected in successive samples from the infected nails of an Ontario patient. These had been sent in for routine dermatological analysis, and the samples appeared to contain a mixed infection, which did not involve a dermatophyte.

The slow growth, cycloheximide tolerance and microscopic features of the newly recognized agent suggested a possible affinity to *Onychocola canadensis* Sigler, an

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uncommon agent of distal lateral subungual onychomycosis and of SWO now reported from North America, Europe, Australia and New Zealand [1–9]. Its teleomorph, *A. nodosetosus* Sigler & S. P. Abbott, was discovered when mated strains of *O. canadensis* produced setose cleistothecia and ascospores typical of the genus *Arachnomycetes* [4]. Mating trials and sequence analysis of the ITS 2 region were used to test the hypotheses that the five isolates represent one species and that they are closely related to *O. canadensis*.

Case reports

Case 1

A 78-year-old man from Toronto, Ontario had a 3–4-year history of an abnormally appearing left great toenail (Fig. 1). He had cardiovascular problems, had a pacemaker implanted and was on ranitidine. In September 1996, he presented with a scrotal eruption later diagnosed as tinea cruris, which responded well to topical ketoconazole cream. The lesion on his great toenail (hallux) was typical of SWO, but he was unaware of any toenail abnormalities. Sample collection and laboratory methodology followed the protocols previously outlined [1]. Direct examination of the nail revealed irregular filaments (Fig. 2). Cultures grew a slow-growing yellow fungus. In March of 1997, the patient returned with the same lesion on his left great toenail. Direct examination of the specimen was negative for fungal filaments; however, cultures produced colonies identical to the first isolate. No antifungal medication was indicated. Both isolates were sent to the University of Alberta Microfungus Collection and Herbarium (UAMH) and were designated as UAMH 9022 and UAMH 9024.



Fig. 1 *O. kanei*. Left great toenail of patient 1 with SWO (lesion noted with arrow).

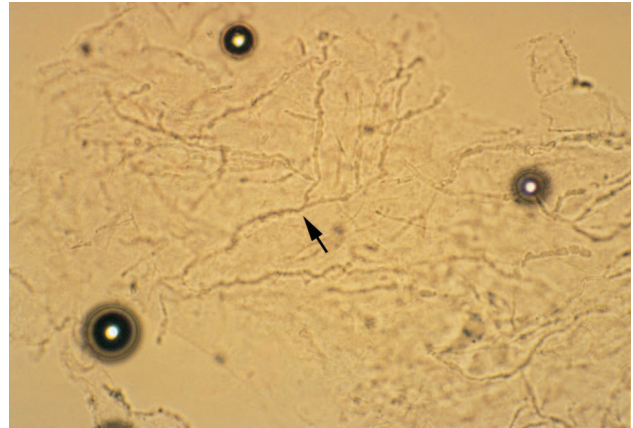


Fig. 2 *O. kanei* irregular filaments (arrow) in direct examination of nail scrapings.

Case 2

The patient was an elderly female who presented with an abnormal left and right great toenail. She was diabetic but experienced no neuropathy in the foot. She was not a farmer or a gardener but she often went to a cottage where she mainly wore sandals. When first examined in July 1997, both great toenails were greatly thickened with whitish plaques on the upper surface suggestive of SWO. Nail scrapings from both toenails were pooled and submitted to the laboratory. These grew 20 or more colonies of a yellow, slow-growing fungus consistent with that seen in Case 1, as well as 20 or more colonies of *Aspergillus sydowii*. Direct microscopy revealed irregular filaments. A follow-up specimen (which nail not specified) obtained in August 1997 yielded only *A. sydowii* and showed regular filaments, that is, consistent with either a dermatophyte or one of several nondermatophytic fungi that are regularly seen in keratinized tissues [2]. In September 1997, a third specimen from the left great toenail yielded 16 colonies of *A. sydowii* as well as six of the undescribed yellow fungus. Direct microscopy again showed irregular filaments. The yellow fungus was compared microscopically and macroscopically with isolates obtained in Case 1 and appeared to be identical; however, subcultures from Case 2 were later lost.

No dermatophytes were obtained from any of these specimens, or from an earlier specimen collected by a different physician and sent to a different laboratory. That specimen was reported as microscopic and culture negative. The patient was not treated with any medication and the hallux nails are still affected. Their thickness presents problems in keeping the nails trimmed. Recently, the patient stubbed her toe badly causing the nail to be further damaged and to fall off. When it grew back,

it demonstrated abnormal regrowth with the same symptoms as previously shown.

Materials and methods

Isolates examined

Three additional isolates were obtained from nails. A 74-year-old male from Toronto, Ontario, presented with SWO on his left fourth toenail. Direct examination was negative for fungal filaments but cultures produced confluent growth of a yellowish mould on Littman oxgall agar (Difco Laboratories, Detroit, MI, USA) and Sabouraud glucose agar + chloramphenicol, cycloheximide and gentamicin [2]. The isolate was deposited as UAMH 9023. UAMH 5908 came from a patient in Toronto. The specimen in this case was heavily positive for irregular filaments, but no follow-up specimen could be obtained to assist in confirming the significance of the unusual isolate. UAMH 9850 (MY-54649) was obtained from a 43-year-old man from Quebec.

Cultural and mating studies

The nail isolates together with ex-type cultures of *A. minimus* (UAMH 7113), *A. gracilis* (UAMH 9756) and *A. nodosetosus* (UAMH 5344) were grown on potato dextrose agar (PDA) (Difco) and oatmeal salts agar (OAT) [2] to observe colony diameters and characteristics and growth rates at 25 °C and 37 °C and were examined at 7, 14 and 21 days. All isolates were tested for urease activity using Christensen's urea broth; requirements for vitamins inositol and thiamine using Trichophyton agars 1–4 (Difco), and for tolerance to cycloheximide at 400 µg ml⁻¹ on Mycosel agar (BBL Microbiology Systems, Cockeysville, MD, USA). Responses on these media and on bromocresol purple–milk solids–glucose agar (BCP-MS-G) [2] were evaluated at 14 days at 30 °C. Characteristics of conidia were examined in slide culture preparations on Pablum cereal agar (CER) [2]. Ultrastructural features were examined with a Hitachi S-2500 scanning electron microscope.

In a preliminary mating experiment, the isolates from the Case 1 patient (UAMH 9022 and 9024) were each paired with UAMH 9023 on OAT and Takashio agar [2]. Using a heavy inoculum from a sporulating culture, an isolate was streaked across the center of the plate. The second strain was streaked perpendicularly to the first. Although no teleomorph was observed within 3 months, plates were retained for 12 months and sparse ascomata were observed in each pairing only on OAT. A second experiment repeated the first crosses and paired remaining strains in all possible combinations, including selfpairings, on OAT. The plates were incubated at 25 °C

exposed to daily room light and observed weekly for 7 weeks and monthly thereafter. Plates without observable signs of ascomatal formation were discarded after the seventh month.

DNA analysis

Cultures were grown on PDA overlaid with a cellophane membrane [10]. DNA extraction followed the method of Cubero *et al.* [11] with some modification. Approximately 100 mg of fresh mycelium was scraped from the surface of the cellophane and placed in a precooled sterile porcelain mortar containing a small amount of acid sterilized sand. Liquid nitrogen was added and the frozen mycelium was ground to a powder. Seven hundred and fifty milliliters of extraction buffer [1% w/v cetyl-trimethyl ammonium bromide; 1 M NaCl; 100 mM Tris; 20 mM EDTA; 1% w/v polyvinyl pyrrolidone] were added to the ground material. The mixture was transferred into a sterile 2 ml screw-capped microcentrifuge tube and incubated for 30 min at 65 °C. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added. The resulting complex was mixed by inverting the tube about 20 times and centrifuged for 15 min at 10 000g at room temperature. The upper layer, which contained crude DNA material, was collected and purified using the QIAquick DNA purification kit (QIAGEN Inc., Mississauga, Ontario) and the purified DNA was stored at –20 °C.

The target DNA region, ITS 2, was amplified using primers ITS 3 and ITS 4 (synthesized by CyberSyn, Aston, PA, USA) [12]. Polymerase chain reaction (PCR) amplification was performed using the following cycling parameters: denaturation at 94 °C for 1 min; annealing at 55 °C for 1 min and extension at 72 °C for 2 min. Initial denaturation was at 94 °C for 2 min, and the final extension was at 72 °C for 7 min. There were 30 cycles. Sequencing of the amplicons was done with the Dyanamic™ ET terminator kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and run on an ABI 377 Automated sequencer (Amersham). Consensus sequences were determined using the Sequencher™ for Windows 4.0.2 (Gene Codes Corp. Ann Arbor, MI, USA) and alignment was done manually using Se-Al v1.0a1 Fat, a sequence alignment program [13]. Phylogenetic analysis was done using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b8 [14] and the robustness of the resultant phylogenetic tree or inferred clades was tested using bootstrap analysis [15] of 1000 resamplings. *Ajellomyces capsulatus* (UAMH 3536; GenBank AF038354) was used as the outgroup taxon.

Results

Mating trials

Ascomata were obtained among five mated isolates on oatmeal agar (Table 1). However, ascomata were sparsely produced in all matings and could not be obtained when the crosses were repeated. No ascomata formed in self-pairings. Infertile ascomata with setae were visible at 4 weeks and ascospores were first observed after 7 weeks. UAMH 5908 and UAMH 9850, as well as the two isolates from the Case 1 patient, UAMH 9022 and UAMH 9024, were designated as (–) mating types. UAMH 9023 was designated as (+) mating type.

Analysis of ITS sequence data

Amplified sequences, ranging from 271 to 302 bp, included a portion of the 5.8S gene, the complete ITS 2 region and a portion of the 28S gene. Alignment was done manually obtaining a total length of 312 characters. Of these characters, 255 were constant, 45 were variable but parsimony-uninformative, and 12 were parsimony-informative. Using the heuristic random sequence stepwise addition search option with gaps treated as missing characters, two most parsimonious trees were obtained, one of which is shown in Figure 3. GenBank accession numbers for the new sequences are noted on the tree. The tree length was 85 steps with a consistency index of 0.941, a retention index of 0.783 and a homoplasy index of 0.059. In the phylogenetic tree, the *Arachnomyces* taxa group together, with four subclusters corresponding to the different species (Fig. 3). The representative nail isolates (UAMH 9024, 9023 and 5908) clustered together with a bootstrap value of 100%, thus strongly supporting their distinction as a new *Arachnomyces* species. Two of the isolates had identical ITS 2 sequences, whereas UAMH 9024 differed at one position. PCR amplification was unsuccessful for one nail isolate (UAMH 9850).

Table 1 Results of mating experiments for *A. kanei* on oatmeal agar at 25 °C

	Plus (+) mating strain UAMH* 9023
Minus (–) mating strains	
UAMH 5908	+
UAMH 9022†	+
UAMH 9024†	+
UAMH 9850	+

*University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta, Canada.

†Isolates obtained from same patient.

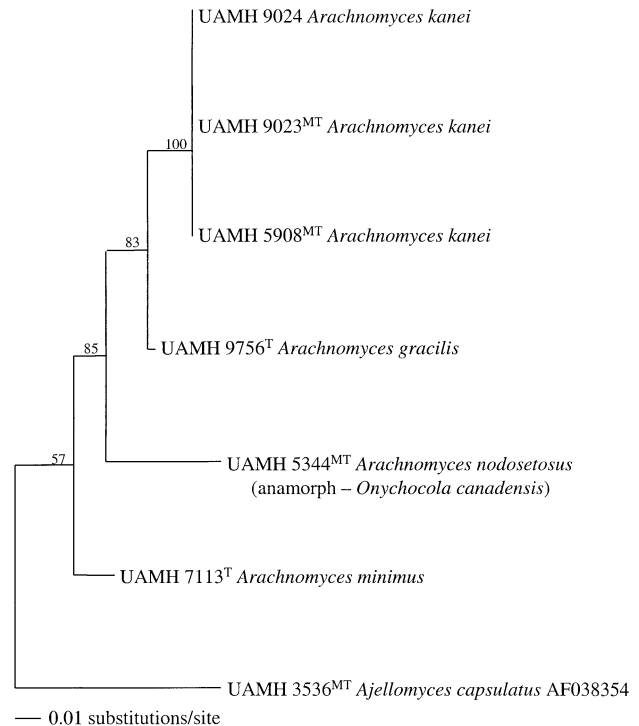


Fig. 3 One of two parsimonious trees for six taxa based on ITS 2 gene sequences of *Arachnomyces* species. The numbers above the branches are percentages of bootstrap values of 1000 resamplings. Numbers before species names refer to UAMH accession numbers and those after species names refer to GenBank accession numbers. (T, ex-type culture; MT, mating type culture).

Taxonomy

Arachnomyces kanei Gibas, Sigler, et Summerbell, sp. nov.

Etymology: *kanei* in honour of Canadian mycologist Dr Julius Kane who contributed greatly to our knowledge of fungi involved in cutaneous infection.

Ascomata cleistothecia, rubro-brunnea, non ostiolata, subglobosa vel globosa, 175–300 µm diam, cum setis 5–8; peridium membranaceum de textura angulari; setulae leves, sparse septatae, apice uncinatae, 6–10 µm diam ad basim, 115–125 µm longae; asci evanescentes, octospori, subglobosi, 7.5 × 6.5 µm; ascosporae leves, oblatae cum umbone polari per mediam partem, pallide brunneae, 3.5–4.5 × 2.5–3 µm; heterothallicae.

Typus UAMH 10027 colonia exsiccata ex cruce UAMH 5908 × UAMH 9023.

Status anamorphosis: *Onychocola kanei* Gibas, Sigler et Summerbell sp. nov.

Conidia sessilia, vel pedicellia, levia vel subtiliter asperulata, clavata 3.5–7 × 3–4 (4.5) µm; conidia intercalaria alterna, cylindricus vel irregularia 3.5–7 (7.5) × 3–4.5 (5) µm.

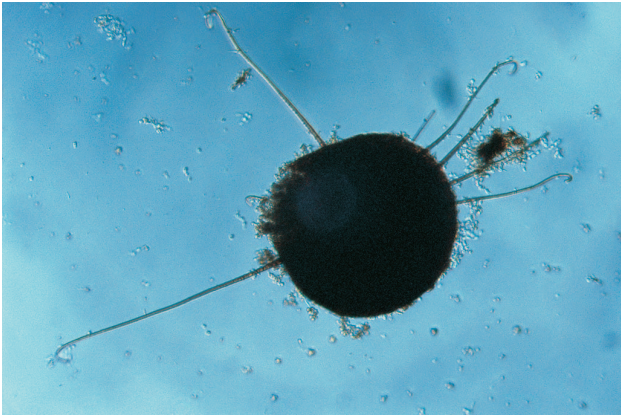


Fig. 4 Ascoma bearing setae, cross of UAMH 9023 \times UAMH 9024, original magnification on slide \times 58.

Typus UAMH 5908, colonia exsiccata et cultura.

Ascomata (cleistothecia) are formed in paired isolates. They are superficial or submerged in mycelia, reddish brown, nonostiolate, subglobose to globose with a diameter of 175–300 μm and bear 5–8 appendages (setae) (Fig. 4). The peridial wall of the ascoma is membranous of the *textura angularis* type. The seta is smooth to intermittently slightly nodose, sparsely septate, uncinata to loosely coiled at the tip, 6–10 μm wide at the base, 4–5 μm along the length and at the tip and 115–125 μm long (Figs 5 and 6). Asci are evanescent, eight-spored, subglobose, 7.5 \times 6.5 μm . Ascospores are smooth, oblate with a polar boss at the center, pale to light brown, and measure 3.5–4.5 \times 2.5–3 μm (Figs 6 and 7). Conidia are sessile or borne on stalks and are smooth to finely asperulate, clavate to pyriform, 3.5–7 \times 3–4 (4.5) μm or are intercalary and alternate,



Fig. 5 Uncinate seta on ascoma produced on OAT after 110 days, cross of UAMH 5908 \times UAMH 9023, magnification \times 1015.

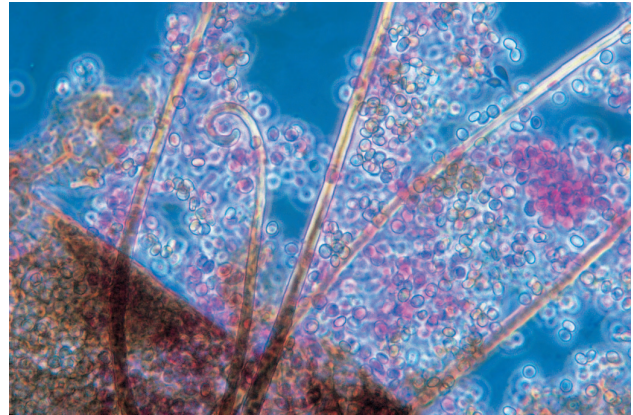


Fig. 6 Part of membranous peridium, setae, ascospores and conidia, cross of UAMH 9023 \times UAMH 9024, original magnification on slide \times 240.

cylindrical or irregular with one or both sides swollen, 3.5–7 (7.5) \times 3–4.5 (5) μm (Figs 8 and 9). Conidia detach by lytic dehiscence (Fig. 9).

Colonies on PDA at 25 $^{\circ}\text{C}$ are white often becoming yellow to grayish yellow, downy, raised, rugose, umbonate to crateriform, attaining a diameter of 15–21 mm in 21 days (Fig. 10). Growth at 37 $^{\circ}\text{C}$ is restricted attaining a diameter of 6–13 mm in 21 days. On OAT at 25 $^{\circ}\text{C}$, colonies are white to grayish white, flat, initially glabrous and then developing downy tufts by 21 days.

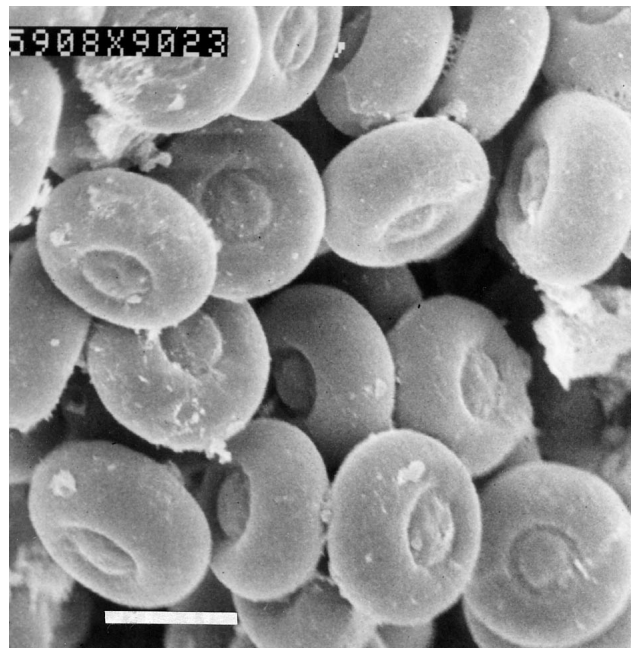


Fig. 7 Oblate ascospores with polar boss at the centre observed by SEM, cross of UAMH 5908 \times UAMH 9023, bar = 2.5 μm .

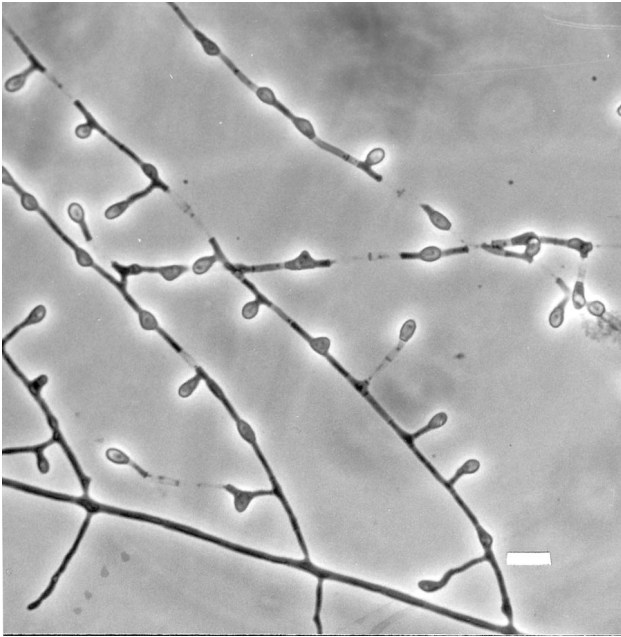


Fig. 8 Solitary aleurioconidia borne on stalks or sessile and cylindrical to swollen arthroconidia of UAMH 9022 in slide culture preparation on CER after 20 days, bar = 10 μ m.

Physiological tests

The *A. kanei* isolates and the ex-type strains of *A. minimus*, *A. gracilis* and *A. nodosetosus* were resistant to cycloheximide and hydrolyzed urea after 14 days. None required specific vitamins for growth. On BCP-MS-G

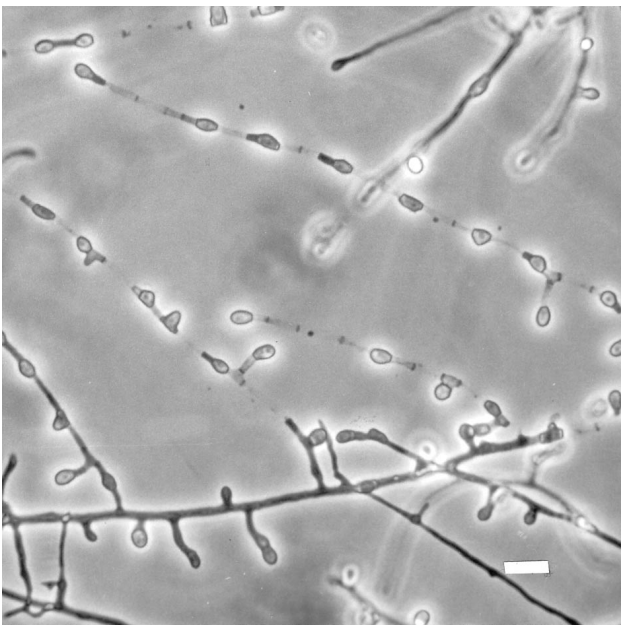


Fig. 9 Aleurioconidia and alternate arthroconidia showing lytic dehiscence, UAMH 9024 in slide culture preparation on CER after 20 days, bar = 10 μ m.

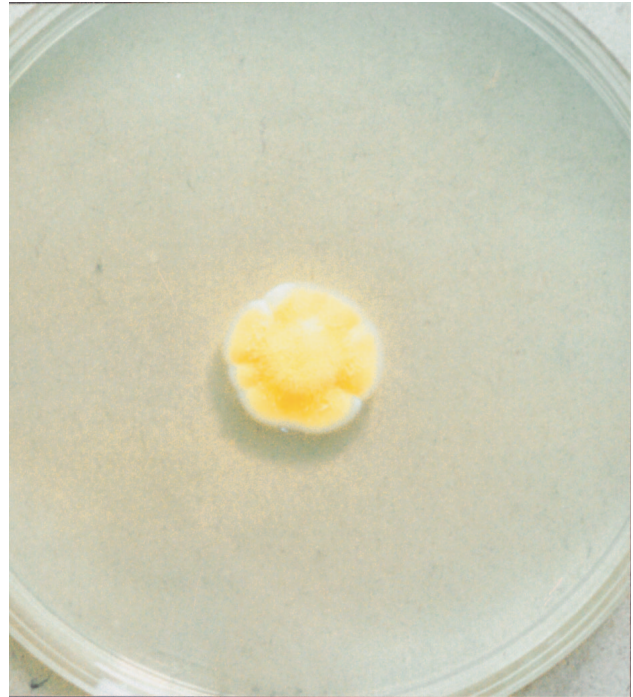


Fig. 10 Colony of *O. kanei*, UAMH 9024, on PDA at 25 °C after 21 days.

medium, all isolates demonstrated the same response. They grew slowly, and did not induce a pH change or clearing of the milk solids by 14 days. Isolates showed slower growth at 37 °C than at 25 °C except *A. gracilis* which failed to grow.

Comments

A. kanei is distinguished from the other species by its setal and anamorph morphology, and colony color. Setae are uncinata to loosely circinate at the tip, and smooth with intermittent small projections or nodose swellings on the surface. It produces aleurioconidia that are sessile or on stalks and intercalary arthroconidia that develop in alternate cells. On PDA, it produces distinctly yellow colonies. Setae of *A. nodosetosus* are also circinate at the tip but are markedly nodose [4]. The anamorph, *O. canadensis*, differs in producing swollen arthroconidia in chains that do not easily break apart [3,4]. Colonies are yellowish white to yellowish gray but rare isolates are strongly yellow. *A. minimus* has setae that are more nodose than those of *A. kanei* and it lacks an anamorph. *A. gracilis* differs in having setae that are straight at the tip and producing nonswollen alternate arthroconidia of the *Malbranchea* type [16].

Discussion

A. kanei is the second *Arachnomyces* species shown to be heterothallic. Although all isolates were successfully

mated, few ascomata were produced in each pairing, they developed slowly, and repeat experiments failed to obtain ascomata. However, the results of the mating experiments combined with the molecular data support our hypotheses that the nail isolates represent one species and that the species belongs in the genus *Arachnomyces*. Bootstrap support for the clade containing representative nail isolates was high (Fig. 3).

Anamorphs are described for two *Arachnomyces* species and they are placed in different genera. The *O. canadensis* anamorph of *A. nodosetosus* is characterized by the production of swollen, one or two-celled arthroconidia that persist in chains. Dehiscence occurs by rhexolysis of thin-walled cells adjacent to, or between, chains of conidia and sometimes by schizolysis of adjacent conidia [3,4] (Fig. 11). The arthroconidia of *A. gracilis* are cylindrical and alternate and they secede by rhexolytic dehiscence. This led Udagawa & Uchiyama (1999) [16] to place the anamorph in *Malbranchea* but they did not provide a species epithet. The anamorph of *A. kanei* is somewhat intermediate between *A. nodosetosus* and *A. gracilis* in forming cylindrical to irregularly swollen alternate arthroconidia but differs in producing solitary aleurioconidia (Figs 8 and 9). These features initially led to the provisional identification of the nail isolates as a *Chrysosporium* species [2,10] and their relationship to *Arachnomyces* species only became apparent with discovery of the teleomorph. Because *Onychocola* is available for disposition of anamorphs of

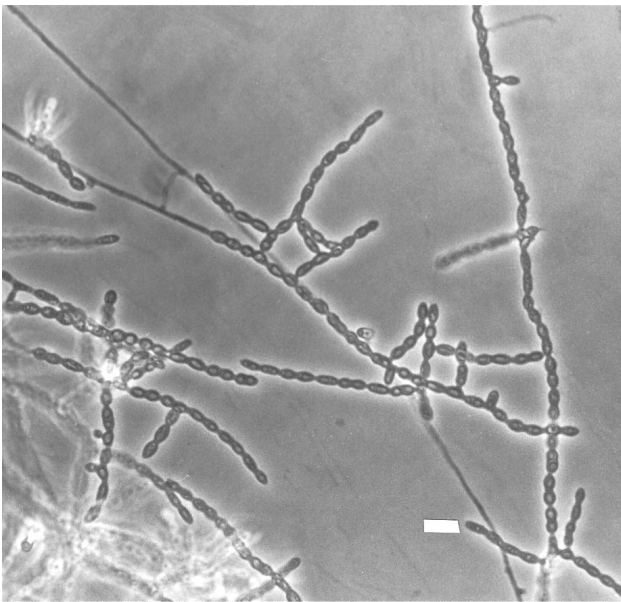


Fig. 11 *O. canadensis* showing swollen arthroconidia in persistent chains, UAMH 5344 in slide culture preparation after 21 days on CER, bar = 10 μ m.

Arachnomyces species, we have given the name *O. kanei* to the anamorph of *A. kanei*.

In the diagnosis of opportunistic onychomycosis, direct microscopy must be positive for fungal filaments potentially consistent with the proposed etiological agent, and repeat isolations are required to show consistency of fungal outgrowth [1,2]. For *O. kanei*, etiology has been confirmed only for case 1 according to strict application of these criteria. Complex, mixed-infection cases such as our case 2 are particularly difficult to interpret without examining a series of specimens deriving from the same nail. For this patient, four separate samplings were taken from one or both nails without recovery of a dermatophyte. Such a record is possible in a true tinea unguium case, but is very rare [17]. *O. kanei* grew from two separate samples both showing microscopic positive for irregular filaments, but *A. sydowii* was isolated also on each occasion. Because specimens from two nails were pooled in the first sampling, and the nail sampled was not recorded in the second, it is difficult to determine retrospectively whether both hallux nails were infected by both isolated fungi, although the left nail did grow both species. The patient's loss of nail and abnormal, hyperkeratotic regrowth is an interesting record for this type of nondermatophyte onychomycosis. Even though we have not been able to reculture the abnormal nail to determine if the abnormality arises from the same causal organism(s), parsimonious interpretation suggests that one or both of the fungi previously repeatedly grown from the nails may be able to persist in the nail bed or paronychia as a dermatophyte would do.

Arachnomyces species in general appear to have potential to cause nail disease, but the stringent requirements for confirming etiology were previously only established for *O. canadensis*. The first report of *O. canadensis* nail infection was based only on three patients but several subsequent reports have substantiated the etiology of this species in both distal lateral subungual and SWO [1–9]. Although *O. kanei* was confirmed as causing SWO in one patient, insufficient data are available in connection with the remaining isolates to ascribe infection categories, or, in some cases, to ascertain etiological causality. Nonetheless, all isolates came from nail specimens. The habitat in nature is not known for *O. kanei* and it is hoped that this description might facilitate not only diagnosis but also the eventual detection of the source of inoculum.

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