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Systematics of the genus Arachnomyces having a predilection to human nail

by

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ABSTRACT

Arachnomyces is a poorly known genus of cleistothecial ascomycetes that has attracted recent attention due to the ability of some species to cause human nail infections. Morphological and molecular characters were used to reevaluate the taxonomic disposition of *Arachnomyces* within the *Ascomycota*, to assess whether species or isolates with suspected affiliation to the genus represent a monophyletic group, and to determine whether anamorphic isolates, provisionally identified as species of *Onychocola* (and in some cases *Malbranchea*) can be assigned to *Arachnomyces* with certainty. Analysis of sequences from the small subunit ribosomal DNA (SSU rDNA or 18S) provided support for the recognition of a new lineage distinct from the *Onygenales* in which the genus had formerly been disposed. A new order and family *Arachnomycetales, Arachnomycetaceae* are described. The genus is shown to be monophyletic and to include anamorphic species within the genus *Onychocola*. Species concepts were assessed using cultural studies, mating tests and analysis of sequences from the internal transcribed spacer region (ITS). Results indicate that variations in anamorph and ascospore morphology and the lack of setae do not have significance above the species level. *Arachnomyces* is emended to include 12 species having setose (appendaged) or nonsetose ascomata, smooth or echinulate ascospores, sclerotia, and anamorphs consisting of arthroconidia, aleurioconidia or lacking. Six species are newly described and include *Arachnomyces aegypticus* (anamorph *Onychocola aegyptica*), *A. caespitosus* (anamorph *O. caespitosa*), *A. glareosus* (anamorph *O. glareosa*), *A. kanei* (anamorph *O. kanei*), *A. parriae*, and *O. gracilis*. *Aphanoascus aciculatus* is transferred

as *A. aciculatus*, and *Malbranchea sclerotica* as *O. sclerotica*. *Arachnomyces peruvianus* is determined as the correct name for *Xanthothecium peruvianum* (*Anixiopsis peruvianus*). This study has broadened the generic concept of *Arachnomyces* species and provided additional characters for recognition of species. *Arachnomyces kanei* and *A. nodosetosus* are confirmed as uncommon agents of nondermatophytic onychomycosis, but many other species appear to have a predilection for human nail. The description of species presented in this dissertation might be of clinical importance not only for the diagnosis of nondermatophytic onychomycosis but also for finding the source of the inoculum.

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CHAPTER 1

INTRODUCTION

The genus *Arachnomyces* Massee & E. S. Salmon was established over one hundred years ago to accommodate *A. nitidus* Massee & E. S. Salmon and *A. sulphureus* Massee & E. S. Salmon (Massee & Salmon 1902). The genus name was derived from the Greek words for 'spider' (*Arachne*) and 'fungus' or 'mushroom' (*mykes*), because of the spider-like appearance of dark brown globose and appendaged cleistothecia that occurred scattered on web-like threads of hyaline mycelia. The taxonomic disposition of the genus has been problematic. The original authors placed it among the powdery mildews because its cleistothecia resembled those of *Erysiphe* R. Hedw. ex DC. and *Uncinula* Lév. in having a membranous peridium, and elongate and sometimes uncinate appendages. Malloch and Cain (1970) isolated and described a third species, *A. minimus* Malloch & Cain, and at the same time moved the genus to the *Onygenaceae* Berk. because of the similarity in peridium and ascospores to *Aphanoascus* Zukal. *Arachnomyces minutus* N Singh & Mukerji and *A. validus* N Singh & Mukerji were described as new coprophilous species in 1978 (Singh & Mukerji 1978). In 1994, a sixth species, *A. nodosetosus* Sigler & S. P. Abbott, was described based on the formation of a teleomorph from mated isolates of *Onychocola canadensis* Sigler. *Onychocola canadensis* was described in 1990 based on a few isolates from specimens of patients with infected nails (Sigler & Congly 1990, Sigler et al 1994). All records of *A. nodosetosus* thus far have been from human nails or skin and this fungus is increasingly being recognized as an uncommon agent of onychomycosis in patients from temperate areas (Sigler & Congly 1990, Campbell et al 1997, Contet-Audonneau et al 1997, Kane et al 1997, Koenig et al 1997, Gupta et al 1998, Sigler & Flis 1998, Llovo et al 2002, Fanti et al 2003, O'Donoghue et al 2003). *Arachnomyces gracilis* Udagawa & Uchiy., from soil in Uganda, became the seventh species in the genus (Udagawa & Uchiyama 1999).

With recognition of its medical importance came a more urgent need for clarification of the placement of *Arachnomyces* in the taxonomic hierarchy and for a better understanding of species within it. In most respects, including the formation of an

arthroconidial anamorph, the production of cleistothecia containing minute ascospores in conglobate clusters of eight, a resistance to cycloheximide, and its association with materials of vertebrate origin, the characteristics of the genus supported placing *Arachnomyces* within the *Onygenales* Cif. ex Benny & Kimbr. A character that was unique and incongruent with other members of the order was the production of elongate coiled gametangia as described for *A. minimus* by Malloch & Cain (1970) who were the first to study an *Arachnomyces* species in culture. On the basis of this character and because *Arachnomyces* did not fit well in any of the four families described within the *Onygenales*, i.e., *Arthrodermataceae* Locq. ex Currah, *Gymnoascaceae* Sacc., *Myxotrichaceae* Locq. ex Currah and *Onygenaceae*, the genus was excluded from the order (Currah 1985). Abbott et al (1996) later argued that the gametangia of *Arachnomyces* were not sufficiently different from other representatives of the *Onygenales* and that the genus could be placed within the *Gymnoascaceae*. This family was deemed appropriate because *Arachnomyces* was not demonstrably keratinolytic and thus not easily placed in the *Arthrodermataceae* or *Onygenaceae*, and because its ascospores were smooth with a polar boss (rather than reticulate or punctate) and discoid-oblate like most other members of the family. However, the membranous nature of the peridium of *Arachnomyces*, when all other *Gymnoascaceae* had hyphal peridia, remained problematic.

A molecular phylogenetic study of *Spiromastix* Kuehn & Orr (*Onygenaceae*), using large subunit ribosomal DNA sequences, included *A. nodosetosus* for comparative purposes (Sugiyama et al 2001). The results showed that *Arachnomyces* probably fits better with (or closer to) taxa within the *Onygenales* than the *Eurotiales* G. W. Martin ex Benny & Kimbr. but nothing else about the placement of the genus could be determined from this work.

In addition to continuing doubts about the correct place of *Arachnomyces* within the *Ascomycota*, there were problems associated with the recognition and delimitation of the seven described species. *Arachnomyces nitidus*, chosen as lectotype of the genus (Abbott et al 1996) and *A. sulphureus* are represented by type specimens, but no living cultures are available. The poor condition of these specimens makes it difficult to conduct morphological comparisons with taxa that are represented by living cultures and

to extract DNA for phylogenetic analysis. Furthermore, neither living nor dried material is available for *A. minutus* and *A. validus*. Thus, at the outset of this project, only three named species, *A. gracilis, A. minimus* and *A. nodosetosus*, were available as living material. These demonstrate tolerance for cycloheximide and similar characteristics in agar culture including slow growth with colonies that are commonly raised and overlaid with tufts of aerial mycelium, and the production of yellow to reddish brown diffusible pigment that strongly color the medium. *Arachnomyces minimus* lacks an anamorph, but *A. gracilis* and *A. nodosetosus* both produce arthroconidia. The anamorph of *A. gracilis* was placed in *Malbranchea* Sacc. and demonstrates regularly cylindrical and alternate arthroconidia (Udagawa & Uchiyama, 1999) while the anamorph of *A. nodosetosus* is a species of *Onychocola*, (i.e. *O. canadensis*) producing chains of persistent arthroconidia that are swollen (Sigler & Congly 1990).

Arachnomyces species also differ from each other in sexual compatibility mechanisms. *Arachnomyces nodosetosus* is heterothallic, i.e. producing ascomata only when opposite mating types are paired (Sigler et al 1994). In contrast, the ex-type, and only known isolate of *A. gracilis*, appears to be homothallic, i.e. self-fertile (Udagawa & Uchiyama 1999). The ex-type culture of *A. minimus* also produces ascomata in culture (Malloch & Cain 1970), but several other strains tentatively identified as this species based on cultural features have not produced a teleomorph and may be degenerate or represent a distinct species.

Additional anamorphic isolates deposited at the University of Alberta Microfungus Collection and Herbarium (UAMH) exhibited cultural features that caused us to suspect that they represented undescribed species of *Arachnomyces*. For example, several isolates recovered from human nail were *Onychocola*-like with swollen conidia, but a relationship with *Arachnomyces* could not be ascertained because they lacked a teleomorph. Other isolates, identified as *Malbranchea sclerotica* Guarro, Gené & De Vroey produced sclerotia resembling the ascomata of *Arachnomyces* species and some relationship was suspected.

The original descriptions of two species, currently disposed in other genera, also suggested a possible relationship to *Arachnomyces*. Malloch and Cain (1970) transferred *Anixiopsis peruviana* Cain as *Arachnomyces peruvianus* (Cain) Malloch & Cain based on

its close resemblance to *A. minimus* and *A. nitidus* when grown in culture. Von Arx and Samson (1973) rejected this and argued that the glabrous ascomata lacking appendages and echinulate ascospores were not typical of *Arachnomyces*. They transferred it to the monotypic genus *Xanthothecium* Arx & Samson. The ex-type culture of a species described as *Aphanoascus aciculatus* Pivkin & Khudyakova and isolated from marine sediments (Pivkin & Khudyakova 2002) was obtained for study because the description of the ascomata and ascospores suggested that it may represent an additional species of *Arachnomyces*.

Given the uncertain position of *Arachnomyces* within the cleistothecial *Ascomycota*, its medical importance, and the number of potentially congeneric anamorphic and teleomorphic taxa, I posed the following research questions: 1. Do the named species of *Arachnomyces*, and the species or isolates with suspected affiliation to this genus, represent a monophyletic group? 2. Is the genus best disposed among others within the *Gymnoascaceae* and *Onygenales*? 3. Can anamorphic isolates, provisionally identified as species of *Onychocola* (and in some cases as species of *Malbranchea*) be assigned to *Arachnomyces* with certainty? I used molecular techniques in conjunction with morphological examination and mating tests to answer these questions.

Molecular sequencing of the nuclear small subunit ribosomal DNA (SSU rDNA) was used to evaluate the phylogenetic position of *Arachnomyces* and assess its relationship with the *Gymnoascaceae*. Sequences of the nuclear internal transcribed spacer (ITS) region were compared to delineate species and to establish a connection between *Arachnomyces* and anamorphic taxa thought to be included in the genus. Mating tests were used to establish teleomorph-anamorph connections between taxa and also to confirm conspecificity.

My results are organized among four chapters. Chapter 2 evaluates the relationship among five strains confirmed to be newly recognized agents of nondermatophytic onychomycosis. Mating trials and sequence analysis of the ITS2 region were used to test the hypotheses that the five isolates represent one species and that they are related to *O. canadensis* (teleomorph *A. nodosetosus*), the first described agent of onychomycosis.

In Chapter 3, SSU rDNA sequences obtained from *Arachnomyces* species were compared with published sequences of onygenalean and other ascomycetes to test the hypotheses that the genus is monophyletic and that it belongs in the *Gymnoascaceae* and also to evaluate possible phylogenetic significance of the variation observed among anamorphs mentioned in Chapter 2.

Mating trials, morphological re-examination, and analysis of ITS sequences were used to demonstrate that the anamorphic taxon *Malbranchea sclerotica* is related to *Arachnomyces* (Chapter 4). Isolates that exhibited cultural characteristics similar to *M. sclerotica* were evaluated for conspecificity. I used similar approaches in Chapter 5 to evaluate the relationship among isolates provisionally identified as *A. minimus*, to assess isolates provisionally assigned to *Arachnomyces* but that could not be referred to any known species, and to test the hypothesis that *A. peruvianus* and *Aphanoascus aciculatus* are species of *Arachnomyces*.

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CHAPTER 2

ARACHNOMYCES KANEI (ANAMORPH *ONYCHOCOLA KANEI*) SP. NOV. FROM HUMAN NAILS[1].

Introduction

In Ontario, Canada, a large scale clinical and laboratory investigation of 473 patients with abnormal appearing nails resulted in confirming of etiologic significance for some fungi that might otherwise have escaped routine dermatological diagnosis (Gupta et al 2001). 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, i.e. not consistent in morphology with hyphae usually produced by dermatophytes (Gupta et al 2001, Kane et al 1997). 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, while another was again associated with irregular filaments but was considered to be unconfirmed as an etiologic 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 dermatologic analysis, and the samples appeared to contain a mixed infection that 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 uncommon agent of distal lateral subungual onychomycosis and of SWO now reported from North America, Europe, Australia and New Zealand (Sigler & Congly 1990, Sigler et al 1994, Campbell et al 1997, Contet-Audonneau et al 1997, Kane et al 1997, Koenig et al 1997, Gupta et al 1998, Gupta et al 2001, Llovo et al 2002). Its teleomorph, *A.*

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[1] A version of this chapter has been published as:
Gibas CFC, Sigler L, Summerbell RC, Hofstader SLR, Gupta AK (2002). *Arachnomyces kanei* (anamorph *Onychocola kanei*) sp. nov., from human nails. Medical Mycology 40: 573–580.

nodosetosus Sigler & S.P. Abbott, was discovered when mated strains of *O. canadensis* produced setose cleistothecia and ascospores typical of the genus *Arachnomyces* (Sigler et al 1994). 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-yr-old man from Toronto, Ontario had a 3-4 year history of an abnormally appearing left great toenail (Fig. 2.1). He had cardiovascular problems, had a pacemaker implanted and was taking 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 (Gupta et al 2001). Direct examination of the nail revealed irregular filaments (Fig. 2.2). 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.

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 Aug. 1997 yielded only *A. sydowii* and showed regular filaments, i.e., consistent with either a dermatophyte or one

of several nondermatophytic fungi that are regularly seen in keratinized tissues (Kane et al 1997). In Sept. 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, nor 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 regrown, 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-yr-old male from Toronto, Ontario presented with SWO on his left 4th toenail. Direct examination was negative for fungal filaments but cultures produced confluent growth of a yellowish mold in this case on Littman oxgall agar (Difco Laboratories, Detroit, MI) and Sabouraud glucose agar + chloramphenicol, cycloheximide, and gentamicin (Kane et al 1997). The isolate was deposited as UAMH 9023. UAMH 5908 came from a patient in Toronto. The specimen 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-yr-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) (Kane et al 1997) to observe colony diameters and characteristics and growth rates at 25 C and 37 C and were examined at 7, 14 and 21 d. 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). Responses on these media and on bromcresol purple-milk solids-glucose agar (BCP-MS-G) (Kane et al 1997) were evaluated at 14 days at 30 C. Characteristics of conidia were examined in slide culture preparations on Pablum cereal agar (CER) (Kane et al 1997). 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 (Kane et al 1997). 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 mo, plates were retained for 12 mo 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 self-self pairings, on OAT. The plates were incubated at 25 C exposed to daily room light and observed weekly for 7 wk and monthly thereafter. Plates without observable signs of ascomatal formation were discarded after the 7th month.

DNA analysis

Cultures were grown on PDA overlaid with a cellophane membrane (Carmichael 1962). DNA extraction followed the method of Cubero et al (1999) with some modification. Approximately 100 mg of fresh mycelium were scraped from the surface of the cellophane and placed in a pre-cooled 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 fifty μl of extraction buffer [1% w/v cetyl-trimethyl ammonium bromide; 1 M NaCl; 100 mM Tris; 20 mM EDTA; 1% w/v polyvinyl polypyrolidone] 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 10,000 x *g* at room temperature. The upper layer, which contained crude DNA material, was collected and purified using the QIAquick DNA purification kit (QIAgen Inc., Mississauga, Ont.) and the purified DNA was stored at -20 C.

The target DNA region, ITS 2, was amplified using primers ITS 3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (synthesized by CyberSyn, Aston, PA) (White et al 1990). 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 Dyenamic™ ET terminator kit (Amersham Pharmacia Biotech, Inc.) and run on an ABI 377 Automated sequencer (Amersham Pharmacia Biotech, Inc.). Consensus sequences were determined using the Sequencher™ for Windows 4.0.2 (Gene Codes Corp. Ann Arbor, MI) and alignment was done manually using Se-Al v1.0a1 Fat, a sequence alignment program (Rambaut 1995). Phylogenetic analysis was done using PAUP* (Phylogenetic Analysis Using Parsimony) v. 4.0b8 (Swofford 2001) and the robustness of the resultant phylogenetic tree or inferred clades was tested using bootstrap analysis (Felsenstein 1985) 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 2.1). However, ascomata were sparsely produced in all matings and could not be obtained when the crosses were repeated. No ascomata formed in self-self pairings. Infertile ascomata with setae were visible at four weeks and ascospores were first observed after seven 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 271-302 base pairs, 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 Fig. 2.3. 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. 2.3). 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 while UAMH 9024 differed at one position. PCR amplification was unsuccessful for one nail isolate (UAMH 9850).

Taxonomy

Arachnomyces kanei Gibas, Sigler *et* Summerbell, sp. nov. **Figs. 2.4 -2.10**

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 x 6.5 µm; ascosporae leves, oblatae cum umbone polari per mediam partem, pallide brunneae, 3.5-4.5 x 2.5-3 µm; heterothallicae.

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

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

Holotypus UAMH 10027, colonia exsiccata e mixtura UAMH 5908 x UAMH 9023.

Ascomata (cleistothecia) are formed in paired isolates. They are superficial or submerged in mycelia, reddish brown, non-ostiolate, subglobose to globose with a diameter of 175-300 µm and bear 5-8 appendages (setae) (Fig. 2.4). The peridial wall of

the ascoma is membranous of the textura angularis type. The seta is smooth to intermittently slightly nodose, sparsely septate, uncinate 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. 2.5, 2.6). Asci are evanescent, eight-spored, subglobose, 7.5 x 6.5 µm. Ascospores are smooth, oblate with a polar boss at the center, pale to light brown, and measure 3.5-4.5 x 2.5-3 µm (Figs. 2.6, 2.7). Conidia are sessile or borne on stalks and are smooth to finely asperulate, clavate to pyriform, 3.5-7 x 3-4 (4.5) µm or are intercalary and alternate, cylindrical or irregular with one or both sides swollen, 3.5-7 (7.5) x 3-4.5 (5) µm (Figs. 2.8, 2.9). Conidia detach by lytic dehiscence (Fig. 2.9). Colonies on PDA at 25 C are white often becoming yellow to grayish yellow, downy, raised, rugose, umbonate to crateriform, attaining a diameter of 15–21 mm in 21 d (Fig. 2.10). Growth at 37 C is restricted attaining a diameter of 6-13 mm in 21 d. On OAT at 25 C, colonies are white to grayish white, flat, initially glabrous and then developing downy tufts by 21 d. HOLOTYPE. UAMH 10027. Dried colony of mixture of UAMH 5908 x UAMH 9023 on OAT.

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 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

Arachnomyces kanei is distinguished from the other species by its setal and anamorph morphology and colony color. Setae are uncinate 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 *Arachnomyces nodosetosus* are also circinate at the tip but are markedly nodose (Sigler et al 1994). The anamorph, *O. canadensis*, differs in producing swollen arthroconidia in

chains that do not easily break apart (Sigler & Congly 1990, Sigler et al 1994). Colonies are yellowish white to yellowish gray but rare isolates are strongly yellow. *Arachnomyces minimus* has setae that are more nodose than those of *A. kanei* and it lacks an anamorph. *Arachnomyces gracilis* differs in having setae that are straight at the tip and producing non-swollen alternate arthroconidia of the *Malbranchea* type (Udagawa & Uchiyama 1999).

Discussion

Arachnomyces 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. 2.3).

Anamorphs are described for two *Arachnomyces* species and they are placed in different genera. The *Onychocola 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 thinwalled cells adjacent to, or between, chains of conidia and sometimes by schizolysis of adjacent conidia (Sigler & Congly 1990, Sigler et al 1994) (Fig. 2.11). The arthroconidia of *A. gracilis* are cylindrical and alternate and they secede by rhexolytic dehiscence. This led Udagawa and Uchiyama (1999) (Udagawa & Uchiyama 1999) 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. 2.8, 2.9). These features initially led to the provisional identification of the nail isolates as a *Chrysosporium* species (Kane et al 1997, Carmichael 1962) and their relationship to *Arachnomyces* species only became apparent with discovery of the teleomorph. Because *Onychocola* is available for disposition of anamorphs of *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 etiologic agent, and repeat isolations are required to show consistency of fungal outgrowth (Gupta et al 2001, Kane et al 1997). 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 (Gentles 1971). *Onychocola 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 non-dermatophytic 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 paronychium 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 superficial white onychomycosis (Sigler & Congly 1990, Sigler et al 1994, Campbell et al 1997, Contet-Audonneau et al 1997, Kane et al 1997, Koenig et al 1997, Gupta et al 1998, Gupta et al 2001, Llovo et al 2002). 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 etiologic 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.

Table 2.1. Results of mating experiments for *Arachnomyces kanei* on oatmeal agar at 25 C.

Minus (-) mating strains	Plus (+) mating strain UAMH[a] 9023
UAMH 5908	+
UAMH 9022[b]	+
UAMH 9024[b]	+
UAMH 9850	+

a - University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta, Canada

b - Isolates obtained from same patient

Figures 2.1-2.2. *Onychocola kanei.*

Fig. 2.1. Left great toenail of patient 1 with SWO (lesion noted with arrow).

Fig. 2.2. Irregular filaments (arrow) in direct examination of nail scrapings.

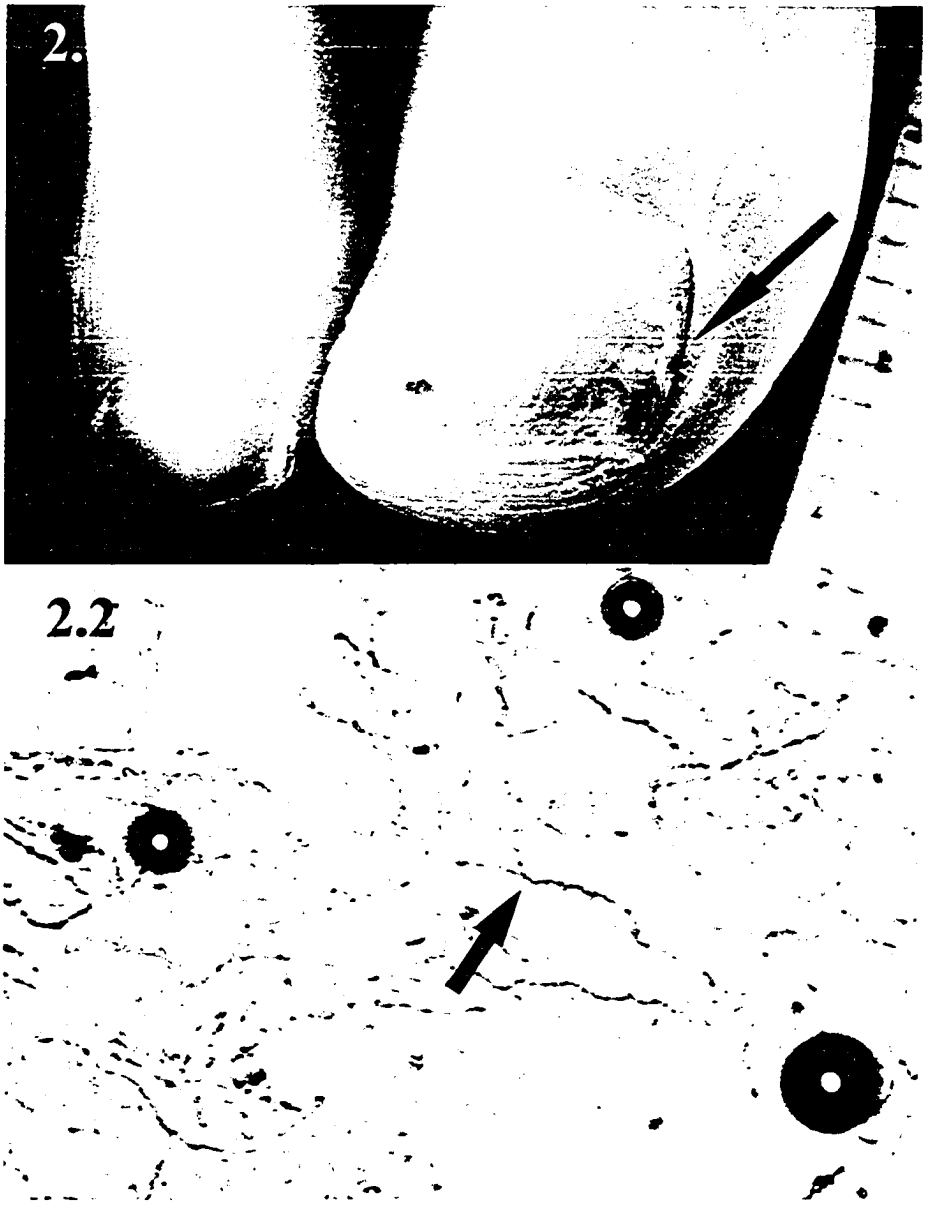
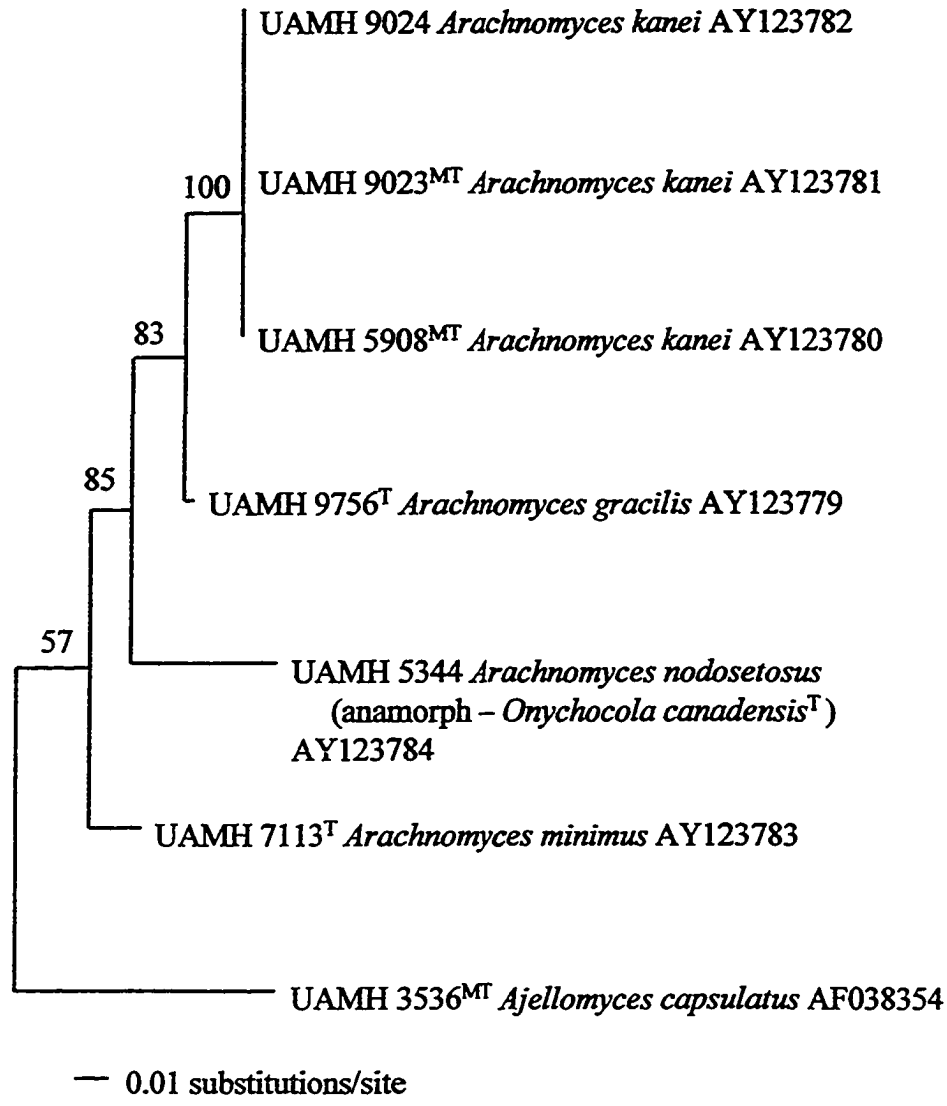


Fig. 2.3. One of two parsimonious trees for six taxa based on ITS 2 gene sequences of *Arachnomyces* species. The numbers above the branches are percentages of bootstrap values of 1000 resamplings. Numbers before species names refer to UAMH accession numbers and those after species names refer to GenBank accession numbers. (T = ex-type culture; MT = mating type culture).



Figures 2.4-2.7. ***Arachnomyces kanei.***

Fig. 2.4. Ascoma bearing setae, cross of UAMH 9023 x UAMH 9024, original magnification on slide x 58.

Fig. 2.5. Uncinate seta on ascoma produced on OAT after 110 d, cross of UAMH 5908 x UAMH 9023, magnification x 1015.

Fig. 2.6. Part of membranous peridium, setae, ascospores and conidia, cross of UAMH 9023 x UAMH 9024, original magnification on slide x 240.

Fig. 2.7. Oblate ascospores with polar boss at the centre observed by SEM, cross of UAMH 5908 x UAMH 9023, bar = 2.5 μm.

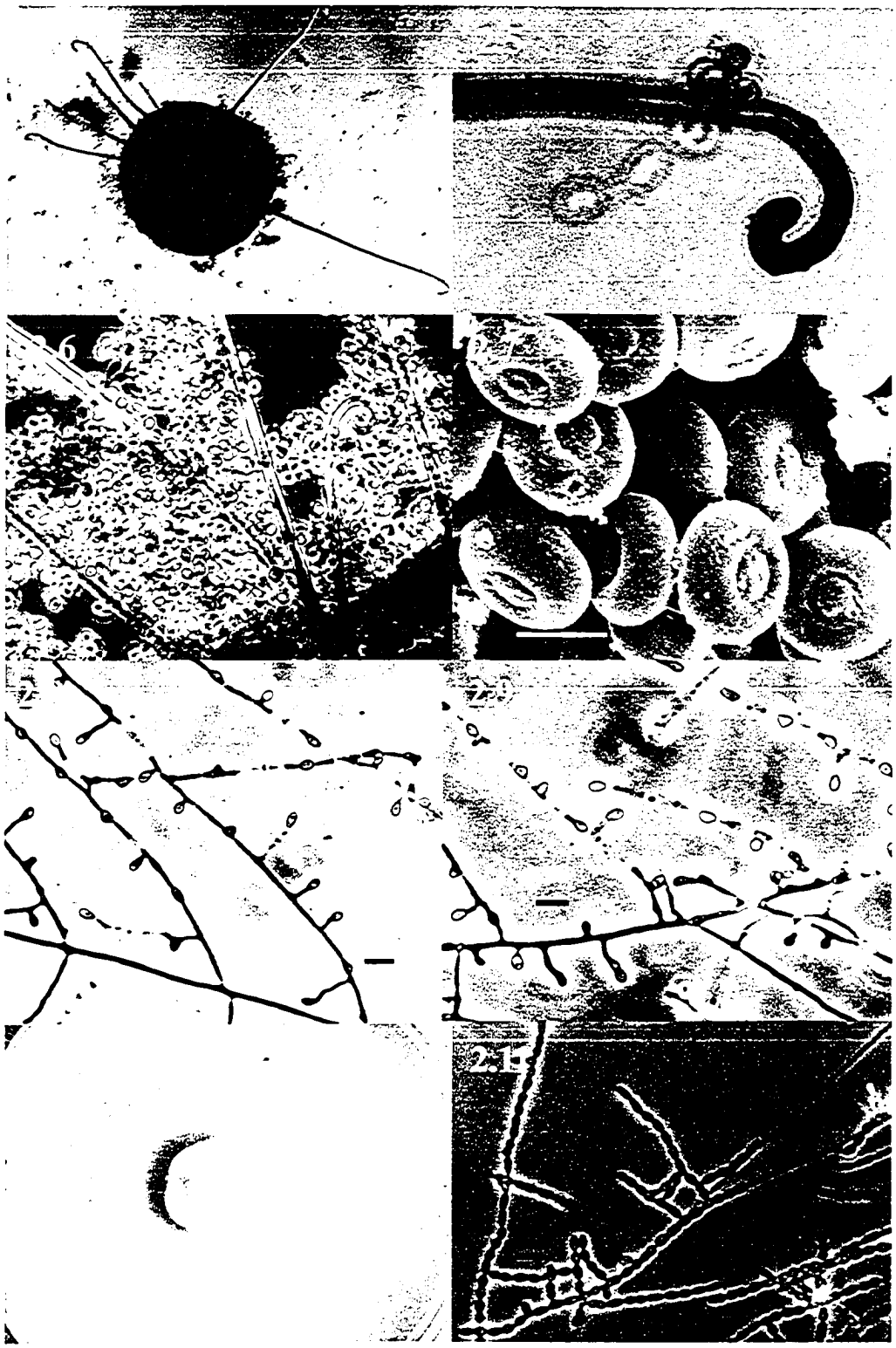
Figures 2.8-2.10. ***Onychocola kanei.***

Fig. 2.8. Solitary aleurioconidia borne on stalks or sessile and cylindrical to swollen arthroconidia of UAMH 9022 in slide culture preparation on CER after 20 d, bar = 10 μm.

Fig. 2.9. Aleurioconidia and alternate arthroconidia showing lytic dehiscence, UAMH 9024 in slide culture preparation on CER after 20 d, bar = 10 μm.

Fig. 2.10. Colony of *O. kanei*, UAMH 9024, on PDA at 25 C after 21 d.

Figure. 2.11. *O. canadensis* showing swollen arthroconidia in persistent chains, UAMH 5344 in slide culture preparation after 21 d on CER, bar = 10 μm



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CHAPTER 3

PHYLOGENY OF THE GENUS *ARACHNOMYCES* AND ITS ANAMORPHS AND THE ESTABLISHMENT OF *ARACHNOMYCETALES*, A NEW EUROTIOMYCETE ORDER IN THE *ASCOMYCOTA*[2]

Introduction

The genus *Arachnomyces* comprises eight species of appendaged cleistothecial ascomycetes producing smooth, oblate, reddish brown ascospores. Massee & Salmon established the genus in 1902 to accommodate *A. nitidus* Massee & E.S. Salmon and *A. sulphureus* Massee & E.S. Salmon. Although the original authors did not select a type species, Malloch & Cain (1970) listed *A. nitidus* as lectotype. Abbott et al (1996) followed this designation when they amended the genus and discussed its taxonomic placement. Although there have been two subsequent reports of *A. nitidus* from Canada and India (Malloch & Cain 1970, Singh & Mukerji, 1978), this species has not been obtained in culture, thus limiting our ability to determine whether it has a conidial state. *Arachnomyces sulphureus* is known only from the original collection from an old bee's nest and the specimen bears only sparse ascomatal structures (Abbott et al 1996). Neither cultures nor holotype material are available for the Indian species, *A. minutus* N. Singh & Mukerji and *A. validus* N. Singh & Mukerji, described from herbivore dung maintained in damp chambers (Singh & Mukerji 1978, Mukerji, pers. comm.). The possible relationship of these fungi with species known in culture is difficult to establish.

The remaining species of *Arachnomyces* exhibit similarities in cultural features, but differences in anamorphs. Features noted for *A. minimus* Malloch & Cain were slow growth, often accompanied by a brownish diffusible pigment, and absence of an anamorph (Malloch & Cain 1970). In 1990, an asexual fungus, *Onychocola canadensis* Sigler, was described on the basis of slow growth, cycloheximide tolerance, and chains of persistent swollen arthroconidia (Sigler & Congly 1990). In culture, it also produced nodose, circinate to loosely coiled setae that suggested potential to produce a teleomorph.

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[2] A version of this chapter has been published as:
Gibas CFC, Sigler L, Summerbell RC, Currah RS (2002). Phylogeny of the genus *Arachnomyces* and its anamorphs and the establishment of *Arachnomycetales*, a new eurotiomycete order in the *Ascomycota*. Studies in Mycology 47: 131–139.

Subsequent mating experiments demonstrated the teleomorph, *A. nodosetosus* Sigler & S.P. Abbott (Sigler et al., 1994). This was the first species of *Arachnomyces* linked to an anamorph. All records of *A. nodosetosus* thus far have been from human nails or skin (Sigler & Congly 1990, Sigler et al 1994, Campbell et al 1997, Contet-Audonneau et al 1997, Kane et al 1997, Koenig et al 1997, Gupta et al 1998, Llovo et al 2002). *Arachnomyces gracilis* was the second species described with an anamorph, but its anamorph was placed in *Malbranchea* by Udagawa & Uchiyama (1999). A third fungus, recovered occasionally from human nail specimens, demonstrated an anamorph with features intermediate between *Onychocola* and *Malbranchea*. A teleomorph identified as *A. kanei* Gibas, Sigler & Summerbell was obtained in mating experiments (Gibas et al 2002). The hyphomycete *Malbranchea sclerotica* Guarro, Gené & De Vroey (Guarro et al 1993) possesses similar cultural attributes (slow growth, brown diffusible pigment, cycloheximide tolerance), and this has led to its inclusion in the present study.

The phylogenetic position of *Arachnomyces* within the *Ascomycota* is unclear and has been the subject of debate as reviewed by Abbott et al (1996). The genus demonstrates morphological convergences with members of the *Onygenales*, but Currah (1985) rejected it as part of the order based on the elaborate ascomatal initials described as long cylindrical coils by Malloch & Cain (1970). Abbott et al (1996) found the initials to be less elaborate, and considered the genus to be placed appropriately within the *Gymnoascaceae* (*Onygenales*) based on the smooth oblate ascospores and an inability to degrade keratin. However, the setose membranous cleistothecia and *Onychocola* arthroconidial anamorph set it apart from other members of the family. In 1978, Lodha transferred species of *Arachnomyces* to *Pleuroascus* Massee & E.S. Salmon described one year prior to the description of *Arachnomyces* (Lodha 1978, Massee & Salmon 1901).

Since the use of morphological criteria has not resolved the disposition of *Arachnomyces*, we obtained sequences of the small subunit region (SSU) of the nuclear ribosomal RNA gene (SSU rDNA) from *Arachnomyces* species and compared these with published sequences of onygenalean and other ascomycetes to test the hypothesis that *Arachnomyces* belongs within the *Gymnoascaceae*, and to evaluate possible phylogenetic significance of the variation observed among anamorphs. Only very recently has a

member of the genus been subjected to molecular scrutiny, and that study (which used sequences of the nuclear large subunit ribosomal DNA) included the single representative *A. nodosetosus* (Sugiyama & Mikawa 2001). Our study includes all species available in culture and results provide more substantial evidence to support the appropriate disposition of the genus *Arachnomyces.*

Materials and methods

Cultures are maintained in the University of Alberta Microfungus Collection and Herbarium (UAMH). Ex-type cultures of *A. gracilis* (UAMH 9756), *A. minimus* (UAMH 7113) and *M. sclerotica* (UAMH 7183) and mating type cultures of *A. kanei* (UAMH 5908) and *A. nodosetosus* (UAMH 6106), were newly sequenced. Sequences of onygenalean taxa and other ascomycete orders and families were obtained from GenBank. *Saccharomyces cerevisiae* Meyen ex E.C. Hansen and *Taphrina deformans* (Berk.) Tulasne were used as outgroup taxa.

Cultures were grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) overlaid with a cellophane membrane (Carmichael, 1962). DNA extraction was done following the method of Cubero et al (1999) with some modification (Gibas et al 2002). Approximately 100 mg of fresh fungal mycelium was scraped from the surface of the cellophane membrane, placed in a pre-cooled sterile porcelain mortar containing a small amount of acid sterilized sand, frozen with liquid nitrogen and ground to a powder. Seven hundred fifty µl of extraction buffer [1% w/v cetyl-trimethyl ammonium bromide; 1M NaCl; 100 mM Tris; 20 mM EDTA; 1% w/v polyvinyl polypyrolidone] 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 tube was inverted about 20 times, and the mixture centrifuged for 15 min at 10 000*g* at room temperature. The upper layer containing crude DNA was purified using the QIAquick PCR Purification Kit (QIAGEN Inc., Mississauga, ON, Canada) and stored at –20 C.

The SSU rDNA was amplified using primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS8 (5'- TCCGCAGGTTCACCTACGGA-3') (synthesized by CyberSyn, Inc., Aston, PA) (White et al 1990). PCR amplification (Perkin Elmer GeneAmp 9700 Thermal cycler, PE Applied Biosystems, Foster, CA) 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. Cycle sequencing was done with the DYEnamic™ ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) using forward primers NS11, NS13, and NS151 developed by K. N. Egger (University of Northern British Columbia, Prince George, B.C., Canada) and reverse primers NS2, NS4, and NS6 (White et al, 1990) and run on an ABI 377 Automated sequencer (Amersham Pharmacia Biotech, Inc.). Consensus sequences were determined using Sequencher™ version 4.0.2 (Gene Codes Corp. Ann Arbor, MI) and alignment was done manually using the sequence alignment program Se-Al v1.0a1Fat (Rambaut 1995). The data matrix was analyzed using PAUP* v. 4.0b8 (Swofford 2001) and the robustness of the resultant phylogenetic tree or inferred clades was tested using bootstrap analysis (Felsenstein 1985) of 500 resamplings.

Characteristics of colonies, ascomata, and anamorph were observed on oatmeal and cereal agar (Kane et al 1997) and on PDA at 25 C. Microscopic observations were done from mounts prepared in lactofuchsin mounting medium (Kane et al 1997). For SEM, materials were fixed in 2.5% glutaraldehyde in Millonig's buffer (Millonig 1961), pH 7.3 and post fixed in 2% osmium tetroxide in the same buffer. After drying to the critical point, the samples were sputter coated with gold and examined with a Hitachi S-2500 (Hitachi, Ltd., Tokyo, Japan).

Results

Sequences of SSU rDNA from four *Arachnomyces* species and for *Malbranchea sclerotica* ranged from 1371 to 1700 bp. Sequences from GenBank ranged from 1095 to 1700 bp in length. Manual alignment yielded a total length of 1836 characters. Of these, 1267 were constant, 227 were variable but parsimony-uninformative, and 342 were parsimony-informative. Using the heuristic random sequence addition search option with gaps treated as missing characters, 18 most parsimonious trees were obtained; one is shown in Fig. 3.1. It had a consistency index of 0.520, a retention index of 0.662, a rescaled consistency of 0.345 and a homoplasy index of 0.480.

The *Eurotiomycetes* clade (bootstrap 94%) included four groups each having strong support (Fig. 3.1). One represented the *Eurotiales* (bootstrap 100%) and another the *Onygenales sensu stricto* (shown as Onygenales I) containing representatives of the families *Arthrodermataceae* (100%), *Onygenaceae* (96%) and *Gymnoascaceae* (98%). Species of *Ajellomyces* grouped together and apart from the other *Onygenales* (80%; shown as Onygenales II). The *Arachnomyces* species and *M. sclerotica* formed a well-supported clade (highlighted clade; 100% support) outside the clade comprising the *Onygenales sensu stricto*. *Malbranchea sclerotica* was shown to be part of the *Arachnomyces* clade grouping in a subclade with *A. minimus* (bootstrap 85%).

Discussion

Some characteristics of the genus *Arachnomyces* suggest a relationship with the *Onygenales*, and this has contributed to the difficulty in classifying the genus within the Ascomycota. These characters include a membranous peridium; lightly pigmented (reddish brown) ascospores without germ pores; vegetative hyphae having ampulliform swellings (i.e. racquet hyphae) and being pale in color; anamorphs of arthroconidia or aleurioconidia having rhexolytic dehiscence, and tolerance to cycloheximide (Malloch & Cain 1970, Currah 1985, Sigler et al 1994, Abbott et al 1996). Sugiyama and Mikawa (2001) included *A. nodosetosus* in their study of LSU rDNA sequence relationships among onygenalean taxa; however, this species was excluded from their overall analysis of the full taxon set and was included only within a reduced taxon set assessing the phylogenetic placement of *Spiromastix* and related taxa. Their results showed that *A. nodosetosus* belongs to a lineage distinct from the clades comprising species of *Ajellomyces* and *Spiromastix*, a finding that agrees with our SSU rDNA sequence data. Our data do not support the hypothesis that *Arachnomyces* is a member of the *Gymnoascaceae* as suggested by Abbott et al (1996) but instead support Currah's (1985) decision to exclude *Arachnomyces* from the *Onygenales*. The phylogenetic tree (Fig.3.1) shows that the genus is not closely related to either the *Onygenales sensu stricto* or the *Eurotiales* but forms an independent well-supported clade (bootstrap 100%). Although the tree shows a sister-group relationship with the *Eurotiales*, it would be difficult to justify extending the concept of the *Eurotiales* to include the *Arachnomyces* clade. The biological differences between these groups are clearly reflected in differences in

phenotypic characters. Our phylogenetic tree also shows that Lodha's decision to transfer *Arachnomyces* species to *Pleuroascus* is incorrect because the latter is placed in the *Leotiomycetes*. *Arachnomyces* species produce arthroconidia or aleurioconidia by fragmentation of a portion of the aerial hyphae, and they are highly cycloheximide tolerant, whereas eurotialean taxa have phialidic or holoblastic anamorphs and exhibit variable, but generally relatively low, cycloheximide tolerance. Our data support the creation of a new order and family placed within the *Eurotiomycetes* (Eriksson 2001).

Taxonomy

ARACHNOMYCETALES, ARACHNOMYCETACEAE Gibas, Sigler & Currah, ord. et fam. nov. **Figs.** 3.2-3.9.

Ascomata cleistothecia, nonostiolata, setulosa, peridium membranaceum de textura angulari; asci evanescentes, octospori; ascosporae oblatae; leviter pigmentatae, foramina germinalia absunt; anamorphoses de arthroconidiis vel aleurioconidiis cum dehiscentia lytica interdum schizolytica.

Ascomata cleistothecia, nonostiolate, with setae (Figs. 3.2, 3.5, 3.6); peridium membranous of *textura angularis* (Fig. 3.3); asci evanescent, eight spored; ascospores oblate, lightly pigmented, lacking germ pores (Fig. 3.4); anamorphs consisting of arthroconidia or aleurioconidia and having lytic dehiscence, sometimes also schizolytic dehiscence (Figs. 3.7-3.9).

Type genus: *Arachnomyces* Massee & E.S. Salmon 1902 emend. Abbott et al 1996

Eight species of *Arachnomyces* have been described, but two are considered *nomina dubia*: holotype specimens of the Indian species *A. minutus* and *A. validus* are lost. There are no living cultures of *A. nitidus* and *A. sulphureus*, and herbarium material of the latter contains few ascomata (Abbott et al 1996). New collections are required to evaluate holomorph concepts for these species.

Arachnomyces minimus, *A. nodosetosus*, *A. gracilis* and *A. kanei*, and the as yet undescribed teleomorph of *Malbranchea sclerotica* (referred to hereafter as *Arachnomyces* sp. I), exhibit strongly similar cultural features, including tolerance to cycloheximide at 400-500 μg/ml, slow growth in culture with colony diameters on PDA

not exceeding 2.5 cm in 21 days and the production of strong diffusible yellowish to reddish brown pigments.

Arachnomyces species grown in culture differ in their sexual systems, setal shape and ornamentation, in their production of sclerotia and in their anamorphs (Table 3.1). The ex-type cultures of *A. minimus* and *A. gracilis* are self-fertile and are assumed to be homothallic. However, some strains putatively identified as *A. minimus* based on growth habit, setal morphology and absence of an anamorph (Sigler et al 1994), do not produce teleomorphs when grown alone, and it is not yet known whether they are conspecific. *Arachnomyces nodosetosus* and *A. kanei* are heterothallic (Sigler et al 1994, Gibas et al 2002), as is *Arachnomyces* sp. I (Gibas et al unpublished results). Setae of *A. gracilis* are straight and taper at the tip; those of other species are circinate to loosely or irregularly coiled (Figs. 3.2, 3.6). Setae are predominantly smooth, but often have intermittent, irregular wall thickenings, being strongly nodose in *A. nodosetosus* (Fig. 3.5) and slightly nodose in *A. minimus, A. gracilis, A. kanei* (Fig. 3.6) and *Arachnomyces* sp. I. In culture, setae resembling those on the ascomata may arise from the vegetative mycelium. Sclerotia are formed only in *Arachnomyces* sp. I.

No anamorph is known for *A. minimus*, and the arthroconidial anamorphs of other species differ, resulting in their disposition in either *Onychocola* or *Malbranchea*. *Onychocola canadensis*, the anamorph of *A. nodosetosus*, produces persistent chains of swollen arthroconidia that appear to separate by a combination of schizolytic and rhexolytic dehiscence (Fig. 3.7). *Onychocola kanei* (teleomorph, *A. kanei*) produces cylindrical to irregularly swollen, alternate arthroconidia as well as aleurioconidia that are sessile or stalked; dehiscence is purely rhexolytic (Fig. 3.8). These features initially suggested a placement within the genus *Chrysosporium* (Gibas et al 2002). The similarly rhexolytically dehiscing arthroconidia of *A. gracilis* differ in being regularly cylindrical and alternate, a characteristic that led Udagawa and Uchiyama to place the anamorph in *Malbranchea* (Fig. 3.4) (Sigler & Carmichael 1976, Udagawa & Uchiyama 1999). Despite these morphological differences, our phylogenetic analysis showed that *Arachnomyces* is monophyletic and that inclusion of *Malbranchea sclerotica* is strongly supported. Our high bootstrap values suggest that these variations in anamorph morphology do not have significance above the species level. Thus, we are placing the

anamorphs in a single genus. When Abbott et al (1996) amended *Arachnomyces*, they referred the anamorph to *Onychocola* and provided the description, "conidia thallic-arthric, barrel-shaped to subcylindrical, hyaline, 0-1 septate, separating by rhexolysis of thin-walled cells or by schizolysis, often persisting in chains." We consider *Onychocola* to be appropriate for the arthroconidial states of *Arachnomyces*, and redispose the anamorph of *A. gracilis* and *M. sclerotica* within the genus. Since Udagawa and Uchiyama (1999) did not provide a binomial for the anamorph of *A. gracilis*, we propose the new species *O. gracilis*.

Onychocola gracilis Gibas, Sigler & Currah sp. nov.

Teleomorph: *Arachnomyces gracilis* Udagawa & Uchiyama. 1999. Mycoscience 40:286

A Latin diagnosis for the anamorph was provided by Udagawa and Uchiyama (1999) as follows:

Hyphae fertiles saepe arcuatae, repetite ramosae. Arthroconidia hyalina, cylindrica vel doliiformia, interdum curvata, 3–8 x 2–3 µm, levia vel asperulata, utrinque vulgo truncata. Arthroconidia ex hyphis principalibus hyalina, cylindrica, 2.5–10 x 1.5–3 µm, levia vel plus minusve asperulata.

Typus: UAMH 9756 colonia exsiccata ex SUM 3046, ex solo Uganda, 1996, isolata S. Uchiyama.

Fertile hyphae arising as lateral branches, often arcuate or sinuous and branching repeatedly to form dense clusters. Arthroconidia hyaline, cylindrical or barrel shaped, sometimes curved, 3–8 x 2–3 µm, smooth-walled to asperulate, truncated at both ends or rounded at one end. Arthroconidia formed on the straight primary hyphae, hyaline, cylindrical, 2.5–10 x 1.5–3 µm, smooth-walled to slightly asperulate (Udagawa & Uchiyama 1999).

Holotype: UAMH 9756, dried colony established from the ex-type strain SUM 3046 (originally isolated from soil in an old termitarium, Uganda, 1996, S. Uchiyama).

Onychocola sclerotica (Guarro, Gené & De Vroey) Gibas, Sigler *et* Currah comb. nov.

Basionym: *Malbranchea sclerotica* Guarro, Gené & De Vroey. 1993. Mycotaxon 98:471.

The distribution and habitat of *Arachnomyces* species are not well understood. There is an association, however, with rotting grasses and with human nails. The habitat of the type species *A. nitidus* is grass stems and rat dung in the U.K. (Massee & Salmon 1902), hay dung compost and dead grass in Canada (Malloch & Cain 1970), and ox dung in India (Singh & Mukerji 1978). *Arachnomyces sulphureus* was also observed on rotten straw from an old bee's nest in the U.K. (Massee & Salmon 1902). *Arachnomyces nodosetosus* in its anamorphic form has been obtained almost exclusively from human nail samples (rarely from skin) where it usually has a role in causing onychomycosis. Cases of infection have been reported from North America, Europe, New Zealand, and Australia (Sigler & Congly 1990, Sigler et al 1994, Sigler & Flis 1998, Campbell et al 1997, Contet-Audonneau et al 1997, Kane et al 1997. Koenig et al 1997, Gupta et al 1998, Llovo et al 2002). *Arachnomyces nodosetosus* is weakly cellulolytic, as measured by its capacity to break down a cellophane membrane, and not keratinolytic as determined by its inability to degrade hairs *in vitro* (Carmichael 1962, Sigler et al 1994, Kane et al 1997). Its niche in nature is unknown, but an association with rotting plant material is suspected because many of the individuals with onychomycosis profess to be keen gardeners. One patient was known to amend her garden with a decomposed straw and manure mixture previously used for mushroom growing (Sigler et al 1994). *Arachnomyces kanei*, which has been confirmed on two occasions as causing onychomycosis and is otherwise only known from nail isolations of uncertain clinical significance (Gibas et al 2002), may be similar in ecology. The ex-type culture of *A. minimus* was obtained from rotting wood. Nonascocarpic isolates thought to represent this species are encountered on rare occasion from nails or skin; to date, only one of these isolates (UAMH 7097) is suspected to have been the cause of an onychomycosis (Sigler et al 1994). Similarly, *Arachnomyces* sp. I (*O. sclerotica*) is a soil species occasionally recovered from cutaneous specimens. Its isolation from at least one nail positive for fungal filaments and its isolation from nails of two other patients suggests the possibility that this species may also play a role in onychomycosis.

The inability of *Arachnomyces* species to degrade keratin does not explain their frequent isolation from nails and their role in causing nondermatophytic onychomycosis. In the case of one patient infected with *A. kanei*, infection recurred after the nail had

fallen off and then regrown, suggesting that the fungus inhabits the nail bed or the paronychium (Gibas et al 2002). Keratin degradation has been assessed only for some isolates of *A. nodosetosus* and *A. kanei* using the in vitro hair digestion assay (Sigler & Carmichael 1976, Kane et al 1997). Detecting keratinolytic abilities in cultures of fungi from nails or other keratin-rich materials relies on the interaction of three variable components, the fungus as it behaves in vitro, the source and condition of the native keratin used in the assay, and the individual who reads and interprets the results. Thus, reports concerning the presence of this ability in cultured fungi can differ (Scott & Untereiner 2004). It should be noted, however, that Scott & Untereiner (2004) did not detect keratin degradation for *A. minimus* using the keratin azure test to assess dye release. The consistent and unequivocal detection of keratin degradation in vitro is useful taxonomically and especially when a strong correlation exists with other characters. Evidence of degradation that is faint or otherwise equivocal, i.e., interpreted as a doubtful or absent, is not by itself definitive for taxonomic purposes and must be weighed against the predictive value of other characters. Repeated isolation of these fungi from nails of living hosts and an inability to demonstrate keratinolysis in vitro, could indicate that the signals necessary for the regulation of the genes responsible are not being provided in culture.

Key to species of *Arachnomyces*

1a. Ascomata produced homothallically (i.e. in single-spore culture); arthroconidial anamorph present or absent 2

1b. Ascocarps not produced in unmated cultures; arthroconidial anamorph present 3

2a. Setae coiled or circinate; anamorph absent or unknown 4

2b. Setae straight and tapered; anamorph of alternate arthroconidia ..... *A. gracilis* (anamorph *O. gracilis*)

3a. Arthroconidia regularly swollen; in persistent chains .....

.....*A. nodosetosus* (anamorph *O. canadensis*)

3b. Arthroconidia alternate; cylindrical to irregularly swollen; readily fragmenting by rhexolysis of thin walled intervening cells 6

4a. Setae slightly nodose; ascospores mostly < 3.5 µm long .....*A. minimus*

4b. Setae smooth; ascospores mostly > 3.5µm long 5

5a. Ascomata 100-300 µm diam .....*A. nitidus*

5b. Ascomata 500-700 µm diam .....*A. sulphureus*

6a. Alternate arthroconidia cylindrical or barrel shaped, stalked conidia absent; sclerotia present .....*Arachnomyces* sp. I (anamorph *O. sclerotica*)

6b. Alternate arthroconidia regularly swollen, sessile and stalked conidia also present, sclerotia absent .....*A. kanei* (anamorph *O. kanei*)

Table 3.1. Differential characteristics of *Arachnomyces* species grown in culture.

	A. gracilis	*A. kanei*	*A. minimus*	*A. nodosetosus*	*Arachnomyces* sp. I (anamorph *O. sclerotica*)
Setal wall	slightly nodose	slightly nodose	slightly nodose	strongly nodose	slightly nodose
Setal tip	straight	circinate or loosely coiled	circinate or loosely coiled	circinate or loosely coiled	circinate or loosely coiled
Ascospore size (µm)	2.8 – 3.2 x 1.5 –2[a]	3.5 – 4.5 x 2.5 – 3[b]	2.8 – 3.5 x 1.5 – 2[c]	4 – 4.5 x 3 – 3.5[d]	3 – 4 x 2.5[e]
Sclerotia	absent	absent	absent	absent	present
Anamorph	alternate cylindrical arthroconidia	stalked or sessile aleurioconidia and swollen alternate arthroconidia	absent	swollen arthroconidia in persistent chains	alternate cylindrical arthroconidia
Thallism	homothallic[f]	heterothallic	homothallic[f]	heterothallic	heterothallic[e]

[a] Udagawa & Uchiyama, 1999

[b] Gibas et al., 2002

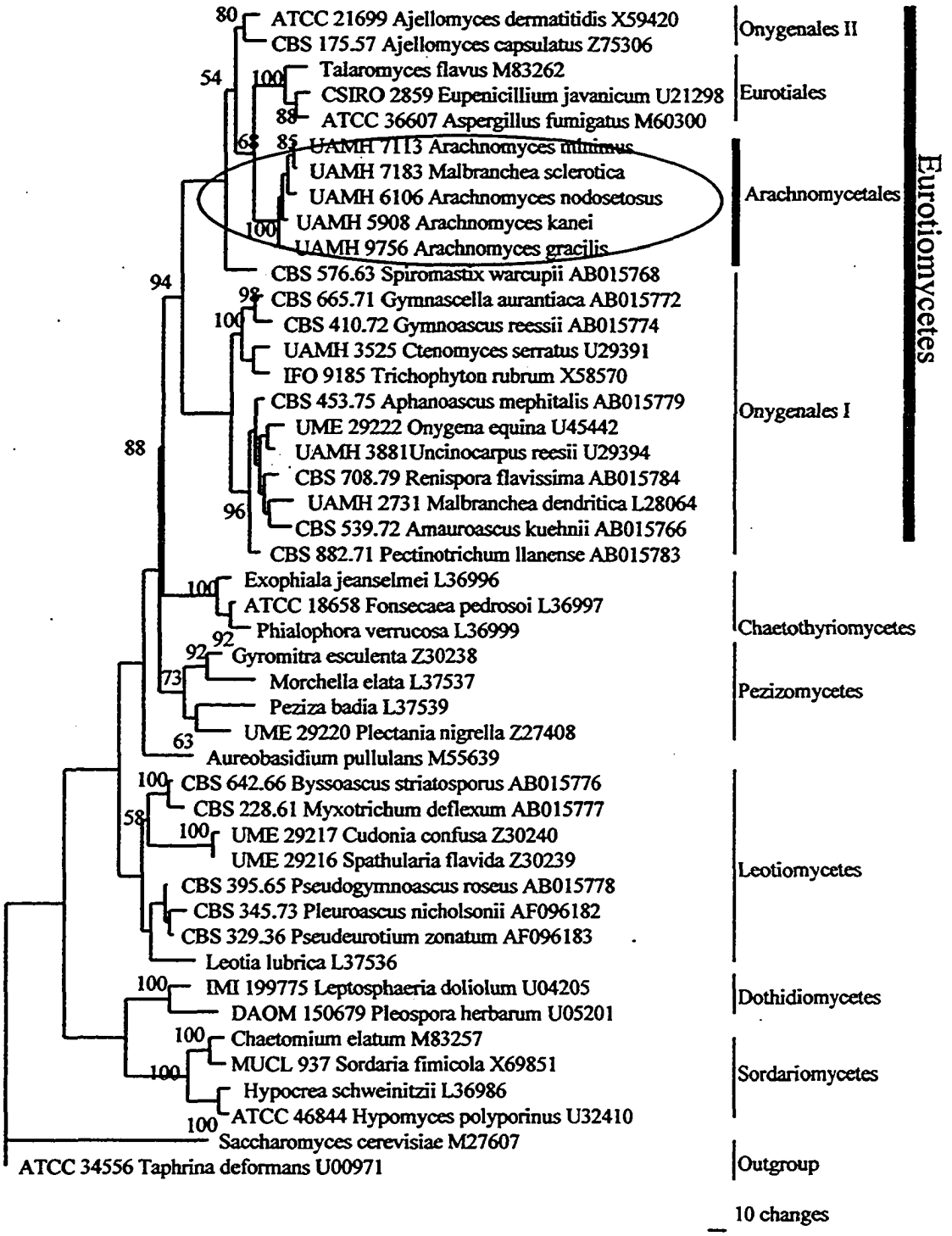
[c] Malloch & Cain, 1970

[d] Sigler et al., 1994

[e] Gibas et al., unpublished results

[f] Based on ex-type strains.

Fig. 3.1. One of 18 most parsimonious trees from analysis of SSU rDNA sequences for *Arachnomyces* species and other ascomycetes. The *Arachnomyces* clade is highlighted. Numbers before species names refer to accession numbers for isolates from culture collections and those after refer to GenBank accession numbers. GenBank accession numbers for the newly sequenced taxa are: *A. minimus* (UAMH 7113) = AF525311; *M. sclerotica* (UAMH 7183) = AF525310; *A. nodosetosus* (UAMH 6106) = AF525309; *A. kanei* (UAMH 5908) = AF525308; *A. gracilis* (UAMH 9756) = AF525307. Numbers above the branches are percentages of bootstrap values of 500 resamplings. Bootstrap values below 50% are not shown. † Sequence L28064 was later shown to be incorrect for *M. dendritica* UAMH 2731, (Sigler et al 2002).



Figures. 3.2. – 3.9. Teleomorphs and anamorphs of *Arachnomyces*.

Fig. 3.2. *Arachnomyces minimus.* Ascomata bearing circinate setae, bar = 200 μm.

Fig. 3.3. *Arachnomyces minimus*. Membranous peridium of *textura angularis*, bar = 100 μm.

Fig. 3.4. *Arachnomyces kanei.* Smooth oblate ascospores observed by SEM (UAMH 5908 x 9023), bar = 2.5 μm.

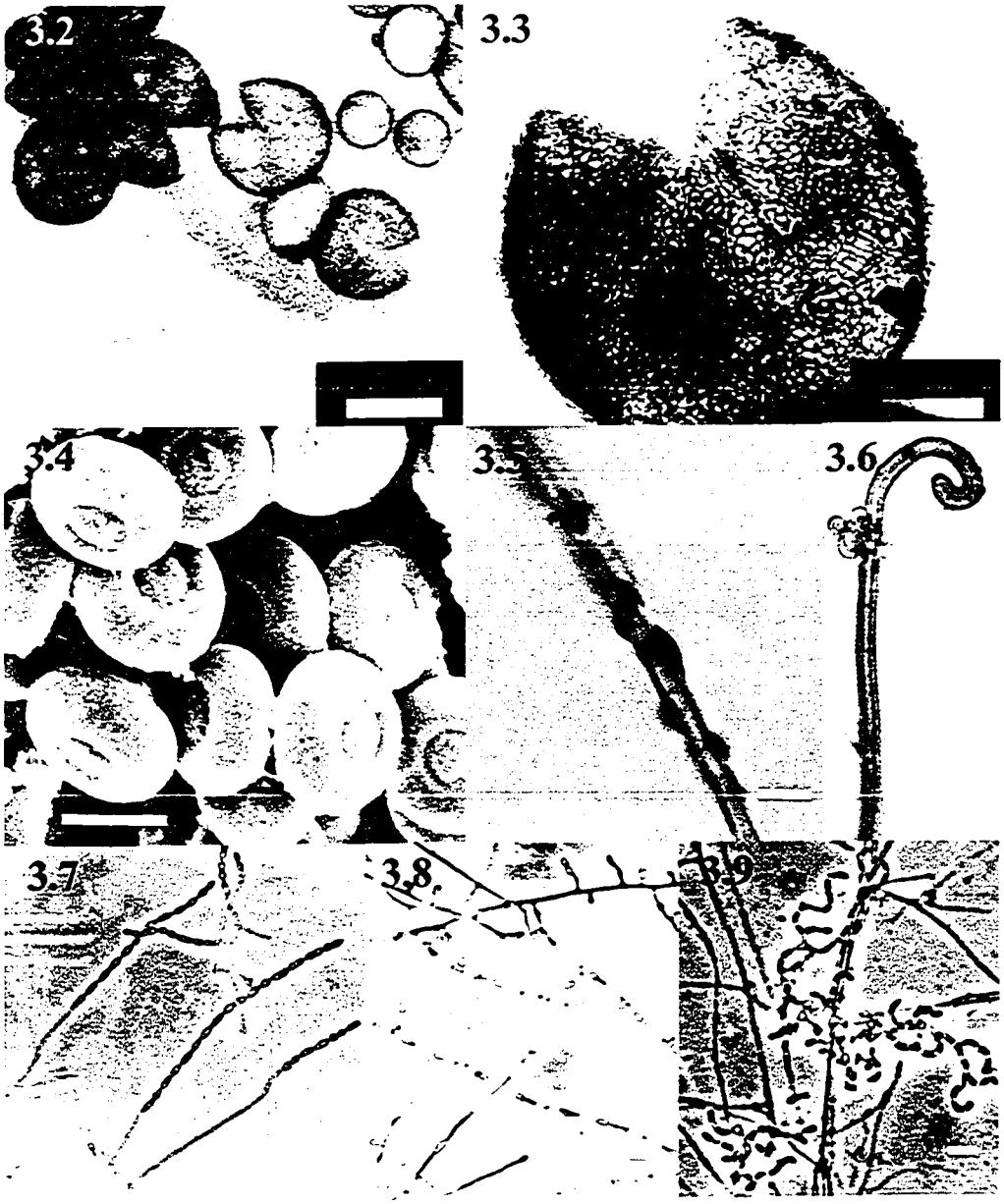
Fig. 3.5. *Arachnomyces nodosetosus.* Strongly nodose seta, bar = 10 μm.

Fig. 3.6. *Arachnomyces kanei.* Slightly nodose seta with circinate tip, bar = 10 μm.

Fig. 3.7. *Onychocola canadensis.* Swollen arthroconidia in persistent chains (UAMH 5344). Bar = 10 μm.

Fig. 3.8. *Onychocola kanei.* Sessile and stalked aleurioconidia and alternate arthroconidia (UAMH 9024), bar = 10 μm.

Fig. 3.9. *Onychocola gracilis.* Cylindrical alternate arthroconidia (UAMH 9756), bar = 10 μm.



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CHAPTER 4

MATING PATTERNS AND ITS SEQUENCES DISTINGUISH THE SCLEROTIAL SPECIES *ARACHNOMYCES GLAREOSUS SP. NOV.* AND *ONYCHOCOLA SCLEROTICA*[3]

Introduction

The genus *Arachnomyces* is a monophyletic assemblage of cleistothecial ascomycetes having anamorphs now assigned to the genus *Onychocola* (Abbott et al 1996, Gibas et al 2002a). Gibas et al (2002a) transferred *Malbranchea sclerotica* Guarro, Gené & DeVroey as *Onychocola sclerotica* (Guarro, Gené & DeVroey) Gibas, Sigler & Currah based on its phylogenetic relationship. In addition, several isolates identified as *O. sclerotica* produced ascomata when mated and the teleomorph was named provisionally as *Arachnomyces* sp. 1. However, the ex-type strain of *O. sclerotica* and one other isolate failed to mate with any of the others. This result suggested that more than one species might be involved. Consequently we re-examined the ex-type and other isolates identified as *O. sclerotica* by conducting more mating trials using progeny from a fertile cross, re-examining morphological characteristics, and comparing nuclear ribosomal internal transcribed spacer (ITS) region sequences. *Arachnomyces* sp. 1 is described here as *Arachnomyces glareosus* Gibas, Sigler & Currah. The anamorph, *O. glareosa* Gibas, Sigler & Currah, bears a strong morphological resemblance to *O. sclerotica* but these taxa are distinct.

Methods

Mating and morphology

In the first mating experiment, 12 strains (Table 4.1), including the ex-type of *O. sclerotica* UAMH 7183 (University of Alberta Microfungus Collection and Herbarium, Edmonton, AB, Canada), were paired in all combinations including self-crosses. All matings were done on oatmeal-salts agar (OAT) (Kane et al 1997) following the method described in Gibas et al (2002b). Two strains that mated to produce fertile ascomata were

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designated as plus (UAMH 7799) and minus (UAMH 8067) mating types. Eight F_1 progeny were obtained from this cross and backcrossed with the parental strains. Two strains, UAMH 7183 and UAMH 10000, that failed to mate in the first experiment were crossed with plus (UAMH 10222) and minus (UAMH 10224) F_1 progeny.

Cultural features of five strains including two non-mating (UAMH 7183 and UAMH 10000) and three mating strains (UAMH 7799, UAMH 10222, and UAMH 10224) were examined on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) at 30 C and 35 C. Tolerance for cycloheximide at 400 µg/mL was assessed by comparing growth on Mycosel medium (BBL Microbiology Systems, Cockeysville, MD, USA) with that on phytone yeast extract agar (BBL) (Kane et al 1997).

Microscopic observations were done from mounts prepared in lactofuchsin. Samples for scanning electron microscopy were prepared as described previously (Gibas et al 2002a) and examined with a Hitachi S-2500 electron microscope (Hitachi Ltd., Tokyo, Japan).

DNA studies

DNA sequences from the ITS ribosomal DNA (rDNA) region, which include ITS 1&2 and 5.8S regions, were determined for one mating strain (UAMH 7799), and two non-mating strains, including the ex-type of *O. sclerotica* UAMH 7183, and UAMH 10000. Sequences of other *Arachnomyces* species were updated to include the ITS1 region since previously published sequences included only a part of the 5.8S and the ITS2 region. Cultures were grown on PDA overlaid with a cellophane membrane (Carmichael 1961). DNA was extracted according to the method described by Cubero et al (1999) with some modifications. The ITS1 region was amplified using primers NSI (5'- GTAGTCATATGCTTGTCTC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al 1990) following the method described in Gibas et al. (2002a). The amplicons were sequenced using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using forward and reverse primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), ITS3 (5'- GCATCGATGAAGAACGCAGC-3'), ITS2 (5'- GCTGCGTTCTTCATCGATGC-3'), and ITS4 (White et al. 1990) and run on an ABI 377 Automated sequencer (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Consensus sequences were generated using Sequencher™ version 4.0.2 (Gene Codes

Corp. Ann Arbor, MI) and alignment was adjusted by eye using Se-Al v1.0a1Fat (Rambaut 1996). Phylogenetic analysis was conducted using PAUP v. 4.0b10 (Swofford 2003) and robustness of the resulting tree was evaluated by bootstrapping (Felsenstein 1985).

Results

Pairings among 10 strains produced setose ascomata and smooth oblate ascospores typical of *Arachnomyces* species within 3 mo (Table 4.2). Five were designated as plus and five as minus mating types. No ascomata were produced in self-self crosses. The eight F_1 progenies derived from UAMH 7799 × UAMH 8067, when backcrossed, were fertile, and mating types segregated equally (Table 4.3). The ex-type strain UAMH 7183 and UAMH 10000 did not cross in the original matings or in crosses with the F1 progeny (Tables 4.2, 4.3).

Aligned sequences had a total of 463 characters, of which 300 were constant, 127 were parsimony uninformative and 36 were parsimony informative. Parsimony analysis generated three equally parsimonious trees with 229 steps. Tree topologies were the same for all trees with regard to the position of the strains of interest. The inferred phylogenetic tree showed the plus mating type strain UAMH 7799 grouping with the non-mating UAMH 10000 in the same cluster with a 97 % bootstrap support and apart from the ex-type of *O. sclerotica*, UAMH 7183 (Fig. 4.1). The two strains clustered with *A. minimus*; although bootstrap support for the relationship was low at < 50 %. Pairwise comparison of ITS sequences between UAMH 7799 and UAMH 10000 showed a high degree (98.8 %) of nucleotide sequence similarity. The 1.2 % difference is due to two base deletions found in the ITS2 region of UAMH 10000. The ex-type of *O. sclerotica* was placed in its own subclade. Although the two groups are clustered together in the same clade, support is low (60 %). Comparison of ITS sequences between UAMH 7799 and UAMH 7183 showed differences in 39 positions. The tree also shows that neither of the two groups is conspecific with any of the *Arachnomyces* species known from culture. Based on mating results and ITS analysis we conclude that *O. sclerotica* is represented currently only by the ex-type strain. All other strains listed in Table 4.1 belong to the new species, *A. glareosus* (anamorph *O. glareosa*).

Taxonomy

Arachnomyces glareosus Gibas, Sigler & Currah, sp. nov. **Figs** 4.2–4.12.

Anamorph: Onychocola glareosa Gibas, Sigler & Currah sp. nov.

Etymology: Latin, *glareosus*, pertaining to the gritty or granular appearance of the colonies due to the presence of abundant sclerotia.

Ascomata cleistothecia, 185–310 diam, rubro-brunnea, subglobosa vel globosa, nonostiolata, setulosa, peridium membranaceum de textura angulari, setulae leves vel leviter nodosae, ordinate septatae, apice uncinatae; asci evanescentes, octospori, subglobosi, 6 × 5 µm, ascosporae leves, oblatae cum umbone polari per mediam partem, 3–3.5 × 1.5–2 µm, heterothallicae.

Typus: UAMH 10234 *colonia exsiccata ex cruce* UAMH 7799 × UAMH 8067.

Onychocola glareosa Gibas, Sigler, & Currah sp. nov.

Hyphae arcuatae; arthroconidia intercalaria, alterna, cylindrica, interdum curvata vel irregularia, levia, hyalina, 2.5-4 × 1.5-2 µm; sclerotia brunnea, subglobosa vel globosa, cortex membranaceus de textura angulari, 45–60 µm diam.

Typus: UAMH 7799, *colonia exsiccata ex cultura.*

Ascomata are cleistothecial 185–310 µm diam, reddish brown, subglobose to globose, non ostiolate, and bear 1 to 5 setae. The *peridial wall* is membranous of the *textura angularis* type. *Setae* are smooth to intermittently nodose, regularly septate, uncinate to loosely coiled or straight at the tip. *Asci* are evanescent, eight-spored and subglobose, 6 × 5 µm. *Ascospores* are smooth, oblate with polar boss, light brown, measuring 3–3.5 × 1.5–2 µm. Heterothallic.

Fertile *hyphae* are arcuate, bearing intercalary alternate arthroconidia. *Arthroconidia* are cylindrical, sometimes slightly curved or irregular with one or both sides swollen, detaching by lytic dehiscence, 2.5–4.5 × 1.5–2 µm. Racquet hyphae are present. *Sclerotia* develop from swollen, thick-walled, light brown cells of the vegetative hyphae; enlarging and aggregating to form a solid tissue, with a membranous rind of textura angularis. Sclerotia are pale brown, subglobose to globose in shape, measuring 45–60 µm diam.

Cultural characteristics: Colonies on PDA at 30 °C are white initially, becoming grayish brown centrally, downy, raised to crateriform attaining a diameter of 18–25 mm in 21 d. All isolates produced abundant sclerotia giving the colony a grainy appearance. Droplets of dark reddish brown exudate occur in areas where sclerotia are produced. A pale brown diffusing pigment starts to appear after 7 d, turning the medium dark reddish brown within 21 d. Growth at 35 °C is restricted with colonies 4–5 mm diam in 21 d. Cycloheximide tolerant.

Notes: UAMH 10000 failed to mate but it is considered conspecific with *A. glareosus* based on high sequence similarity. Pairwise comparison of nucleotide sequences showed 98.8 % similarity with UAMH 7799 and 8.5 % dissimilarity with *O. sclerotica*.

Discussion

Onychocola glareosa and *O. sclerotica* are strongly similar in morphology and this led to the initial identification of all isolates as the latter species. However, the inability of the ex-type strain of *O. sclerotica* (UAMH 7183) to cross with any other strain and the degree of ITS sequence divergence suggest that these are distinct species. Re-examination of *O. sclerotica* revealed only minor morphological differences from *O. glareosa*. Colonies of *O. glareosa* (Figs 4.10–4.12) are dark and grainy due to the formation of abundant sclerotia whereas the colony of the ex-type of *O. sclerotica* is paler, more cottony and less grainy (Fig 4.13). At 35 °C on PDA, growth of *O. glareosa* is strongly inhibited while that of *O. sclerotica* is not (colony diam 20–24 mm in 21 d). Our observations on colony morphology and growth rate at 35 °C are compatible with those reported for *O. sclerotica* in the original description; however, we observed some differences in microscopic features. Guarro et al (1993) described *O. sclerotica* as having arthroconidia measuring 3.5–6.5 × 2–3.5 µm, lacking racquet hyphae and producing irregularly-shaped sclerotia up to 180 µm in diam. We found the arthroconidia to be slightly smaller (2.5–5 × 1.5–3 µm) (Fig. 4.14), racquet hyphae to be present (Fig. 4.15) and the diameter of sclerotia to be up to 60 µm instead of 180 µm (Fig. 4.16). Although we cannot use morphology to reliably distinguish these species, the paler colonies of *O. sclerotica* and better growth at 35 °C may be useful in distinguishing it from *O. glareosa*. Because *O. sclerotica* is currently represented only by the ex-type strain, additional

strains are needed to evaluate whether the observed colonial differences are consistent or whether the more cottony colonies with fewer sclerotia represent a degeneration of the ex-type strain over time.

With the discovery of *A. glareosus* and the placement of *O. sclerotica* within the *Arachnomyces* clade, the production of sclerotia is confirmed as an additional characteristic of the genus. Other defining characters include the production of ascomata with nodose setae that are circinate or loosely coiled or straight, the formation of light brown, smooth, oblate ascospores with a polar boss, and the presence of arthroconidial or aleurioconidial anamorphs featuring lytic or schizolytic dehiscence (Sigler & Congly 1990, Abbott et al 1996, Gibas et al 2002a). Some species lack an anamorph, but when conidia are present, they may consist of swollen arthroconidia in persistent chains (*O. canadensis*), or aleurioconidia that are sessile or stalked, intergrading with cylindrical to irregularly swollen, alternate arthroconidia (*O. kanei*), or cylindrical and regularly alternate arthroconidia (*O. glareosa, O. sclerotica* and *O. gracilis*) (Sigler & Congly 1990, Guarro et al 1993, Abbott et al 1996, Udagawa & Uchiyama 1996, Gibas et al 2002b). These differences in conidial type originally led to the placement of *O. sclerotica* and the anamorph of *A. gracilis* in *Malbranchea*, but we have shown that *Arachnomyces* is monophyletic. We have therefore redisposed all named anamorphs in *Onychocola* (Gibas et al. 2002a). Although *A. glareosus* and *A. gracilis* have similar anamorph morphologies, the latter species lacks sclerotia and its setae are straight and taper toward the distal end. *A. gracilis* is known only from the ex-type strain; single ascospore isolates derived from this strain demonstrated homothallism (Gibas unpublished data).

Our studies suggest that *Arachnomyces* species have a regular but uncommon association with human nails. Two *Arachnomyces* species, *A. nodosetosus* (*O. canadensis*) and *A. kanei* (*O. kanei*), are confirmed as the cause of nondermatophytic onychomycosis (Sigler et al 1994, Gibas et al 2002b). Four isolates of *A. glareosus* were obtained from nail and one from skin scrapings taken from patients for diagnostic purposes (Table 4.1). In two cases, hyphal elements were observed in direct examination of the specimen, but in one of these, a dermatophyte was isolated. For the second case, we have no data on the number of colonies that grew out from the specimen or whether a follow-up specimen was obtained. The latter procedure is required to clarify whether *A. glareosus* has a role

in causing onychomycosis. Further investigation is required to evaluate the possible role of this species in nail infection.

Table 4.1. Source of strains originally determined as *Onychocola sclerotica*.

UAMH no.	Source	Geographic origin	Presence of sclerotia	Mating Type	GenBank No.
7183[T]	Poultry farm soil	Indonesia, Sulawesi	+	did not mate	AY123785
7799	Left thumb nail; DE positive for hyphae	Canada, Southern Alberta	+	+	AY624316
7948	Toenail; DE negative for hyphae	New Zealand, Dunedin	+	+	ND
8067	Skin scraping; DE positive for hyphae	Canada, Southern Alberta	+	–	ND
8204	Cerebrospinal fluid	U.S.A., Montana, St. Louis	+	–	ND
8651	Toenail; DE positive for hyphae; *Trichophyton rubrum* also isolated	Australia, Victoria, Parkville	+	–	ND
8777	*Soil*	Canada, Alberta, Edmonton	+	+	ND
8838	Indoor air of bathroom	Canada, British Columbia, Vancouver	+	–	ND
9025	Skin, left hand	Canada, Ontario	+	+	ND
9713	Toenail; DE negative for hyphae	Canada, Alberta, Lethbridge	+	–	ND
9896	Soil	Canada, Alberta, Edmonton	+	+	ND
10000	Shed dorsal skin, ex Honduran milk snake	U.S.A., Colorado, Pueblo	+	did not mate	AY624315

T = ex-type of *Malbranchea sclerotica*.
DE = direct examination of specimen.
ND = not done.

Table 4.2. Matings among isolates determined as *Onychocola sclerotica*. Results from crosses on OAT after 3 mo at 25 C.

	Plus mating strains						
	7799^{MT}	**7948**	**8777**	**9025**	**9896**	**7183**	**10000**
Minus mating strains						***O. sclerotica* ex-type**	
8067^{MT}	+	+	+	+	+	–	–
8204	+	+	+	+	+	–	–
8651	+	+	+	+	+	–	–
8838	+	+	+	+	+	–	–
9713	+	+	+	+	+	–	–
7183	–	–	–	–	–	–	–
10000	–	–	–	–	–	–	–

MT = designated as mating type strains.

+ = ascomata with ascospores produced.

– = ascomata not formed.

Table 4.3. Results obtained with F1 progeny back-crossed to parental strains and crossed with strains that failed to mate with the same parental strains.

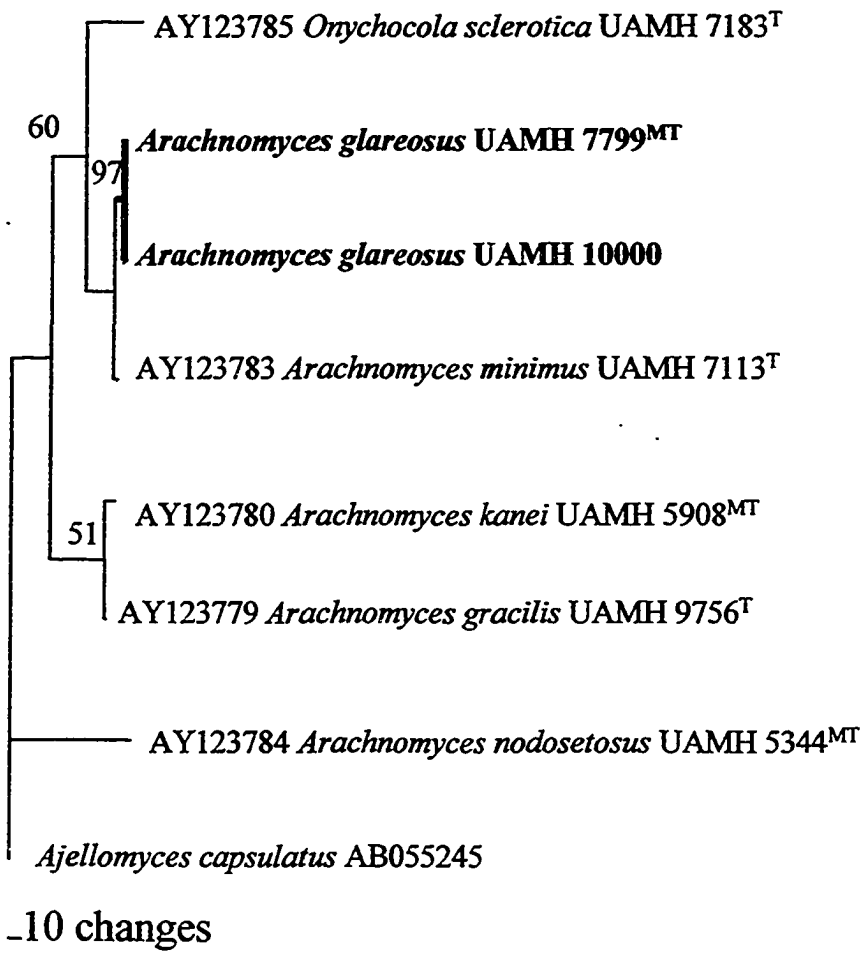
F1 progeny (UAMH No.)	*7799* Plus mating type	8067 Minus mating type	7183 *O. sclerotica* ex-type	*10000*
1 (10222)	+	–	–	–
2	–	+	ND	ND
3 (10223)	+	–	ND	ND
4	+	-	ND	ND
5 (10224)	–	+	–	–
6 (10225)	–	+	ND	ND
7	+	–	ND	ND
8	–	+	ND	ND

+ = ascomata with ascospores produced.

– = ascomata not formed.

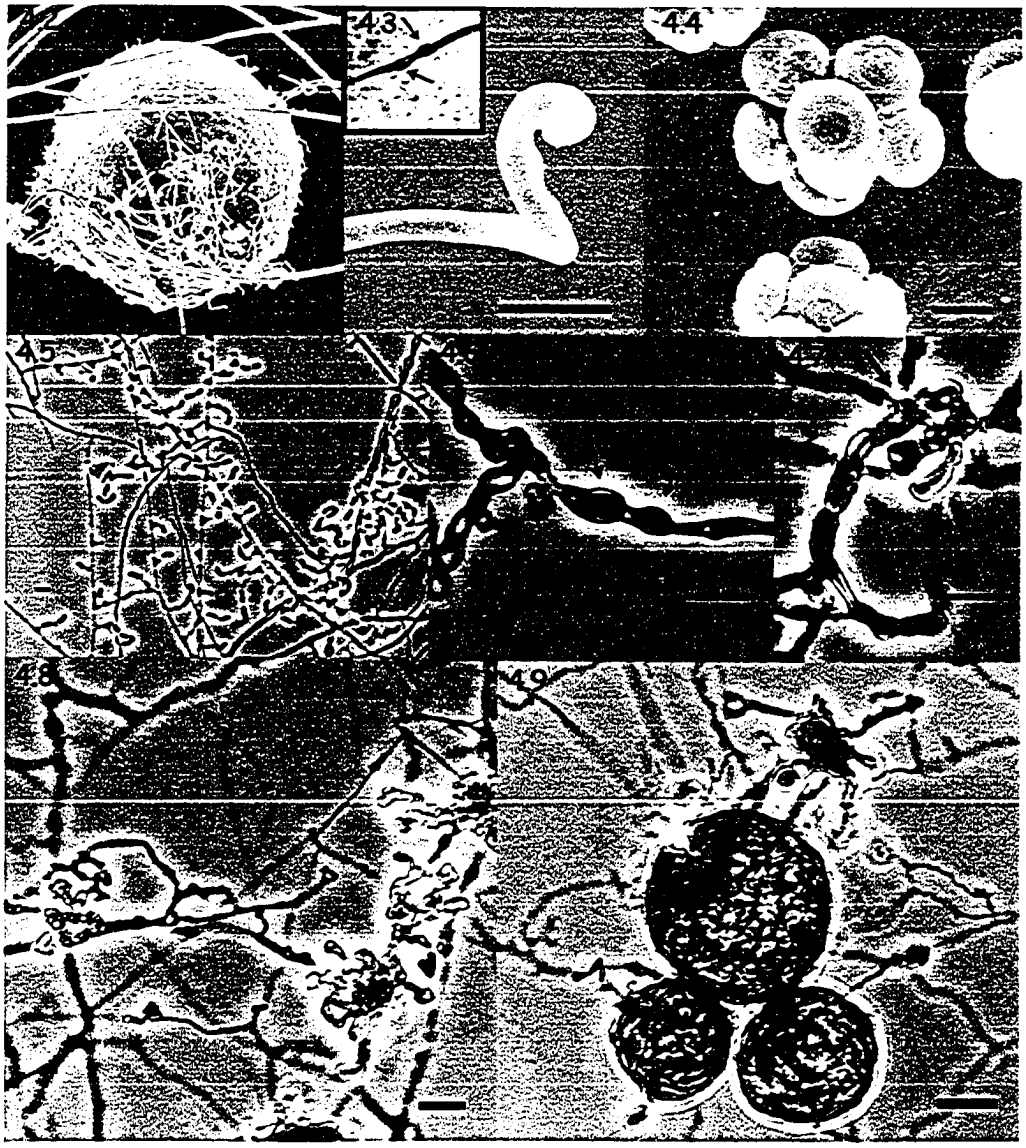
ND = not done.

Fig. 4.1. One of three most parsimonious trees from analysis of internal transcribed spacer (ITS) region ribosomal DNA sequences for *Onychocola sclerotica* strains and related *Arachnomyces* species. Numbers before species names refer to GenBank accession numbers for isolates previously sequenced. GenBank accession numbers for the newly sequenced taxa are listed in Table 4.1. Numbers above the branches are bootstrap percentages for 500 resamplings. Values below 50 % are not shown.



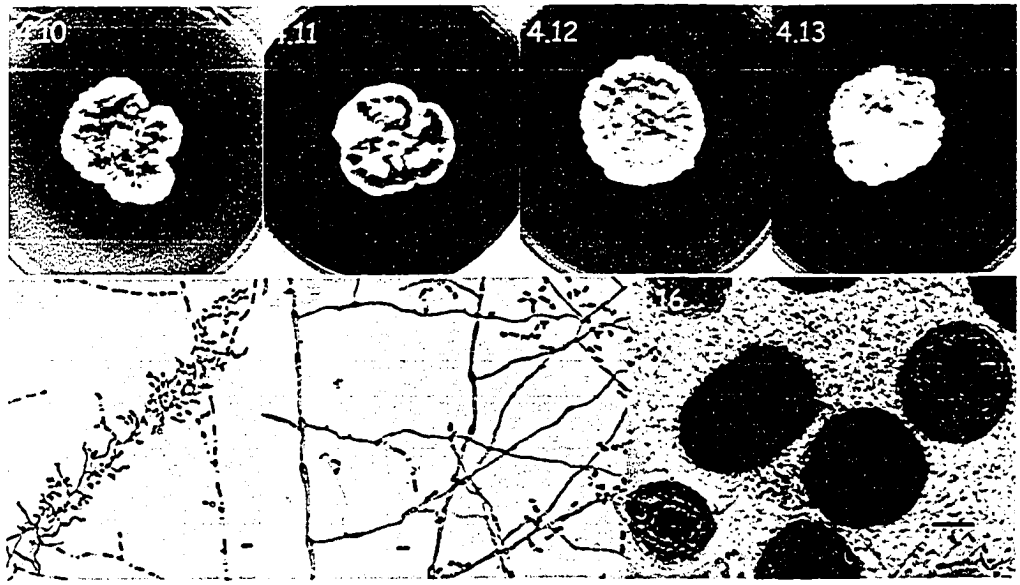
Figs 4.2–4.4. *Arachnomyces glareosus* (UAMH 7799 × UAMH 8067; scanning electron micrographs). 4.2. Ascoma with setae. Scale bar = 50 μm. 4.3. Tip of seta showing loose coil setae. Inset, light micrograph, showing nodose seta (arrows). Scale bar = 10 μm. 4.4. Smooth oblate ascospores. Scale bar = 2.5 μm.

Figs 4.5–4.9. *Onychocola glareosa* (UAMH 7799). 4.5. Alternate cylindrical arthroconidia. Scale bar = 5 μm. 4.6–4.9. Development of sclerotia from vegetative hyphae. Scale bar = 10 μm.



Figs 4.10–4.13. Colonies grown on PDA at 30 C for 42 d. 4.10–4.12. *Onychocola glareosa* UAMH 7799 and UAMH 8067, mating type strains, UAMH 10000, non-mating strain. 4.13. *Onychocola sclerotica* (UAMH 7183).

Figs 4.14–4.16. *Onychocola sclerotica.* 4.14. Alternate cylindrical arthroconidia. Scale bar = 5 μm. 4.15. Racquet hyphae and arthroconidia. Scale bar = 5 μm. 4.16. Sclerotia. Scale bar = 20 μm.



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CHAPTER 5
NEW SPECIES AND COMBINATIONS IN THE GENUS *ARACHNOMYCES* DESCRIBED USING MORPHOLOGY, MATING PATTERNS AND MOLECULAR DATA

Introduction

Arachnomyces Massee & E.S. Salmon is a genus of cleistothecial ascomycetes characterized by setose ascomata containing smooth oblate ascospores (Massee & Salmon 1902, Malloch & Cain 1970, Gibas et al 2002b, Gibas et al 2004). *Arachnomyces* comprises seven species that are associated mainly with rotting grasses and human nails. The position of the genus within the ascomycetes has been uncertain. Phylogenetic analysis of partial small subunit ribosomal DNA (SSU rDNA) revealed that it belongs to a new lineage of ascomycetes (*Arachnomycetaceae* Gibas, Sigler & Currah, *Arachnomycetales* Gibas, Sigler & Currah) Gibas et al 2002b) and that the uncertainty was due to concordance of morphological features with members of the *Onygenales* Cif. ex Benny & Kimbr. Most species demonstrate distinctive arthroconidial or aleurioconidial anamorphs. Some anamorphs were placed originally in *Malbranchea* Sacc. but they are now included in *Onychocola* Sigler based on molecular phylogenies (Gibas et al 2002b). Members of the genus exhibit either homothallism or heterothallism. This has led to problems in identification of isolates lacking ascomata and in assessing relationships among strains. Mating studies have been successful in establishing connections to teleomorphs for several *Onychocola* species (Sigler et al 1994, Gibas et al 2002a, Gibas et al 2004); however, these methods pose problems because some isolates fail to mate or mate only after many months of incubation or under some cultural conditions. Comparison of sequences of the internal transcribed spacer region (ITS) of the rDNA offers an alternative approach to assess the disposition of isolates that fail to mate (Gibas et al 2004).

Analysis of SSU sequence data indicates that *Arachnomyces* is monophyletic and that it includes *A. minimus* Malloch & Cain (Gibas et al 2002b). This is the only species known in culture that has not been connected to an anamorph. Two strains, including the ex-type, produce ascomata but several others identified as this species are infertile. In

some instances characteristic setae are produced but morphology alone is insufficient to determine whether all represent this species.

As part of a continuing study of *Arachnomyces*, I reexamined two species currently disposed in other genera in which the original descriptions suggested a possible relationship to *Arachnomyces*. Malloch and Cain (1970) transferred *Anixiopsis peruviana* Cain as *Arachnomyces peruvianus* (Cain) Malloch & Cain based on its close resemblance to *A. minimus* and *A. nitidus* Massee & E.S. Salmon when grown in culture. This was rejected by von Arx and Samson (1973) who argued that the glabrous ascomata lacking setae and echinulate ascospores of *A. peruvianus* were not typical of *Arachnomyces*. They transferred it to the monotypic genus *Xanthothecium* Arx & Samson. Evidence for close relationship between *Xanthothecium* and *Arachnomyces* has been suggested by preliminary molecular assessment using sequences of the large subunit ribosomal DNA but these studies included few species of *Arachnomyces* (Sugiyama & Mikawa 2001, Sugiyama et al 2002).

The second species, *Aphanoascus aciculatus* Pivkin & Khudyakova, was described as having membranous cleistothecia containing smooth oblate ascospores and an arthroconidial anamorph (Pivkin & Khudyakova 2002). Although the illustrations of the ascospores strongly suggested that this may be an *Arachnomyces* species, ascomata lacked setae. In this study we evaluate the molecular phylogenetic relationship of *X. peruvianum* (Cain) Arx & Samson and *Aph. aciculatus* to *Arachnomyces* and other ascomycetes by comparing SSU rDNA sequences.

We have shown that cultural features, mating tests and ITS sequence comparison are useful for distinguishing *Arachnomyces* species. Analyses of these data led to the recent description of two new species *A. kanei* Gibas, Sigler & Summerbell and *A. glareosus* Gibas, Sigler & Currah (Gibas et al 2002a, Gibas et al 2004) but several other isolates could not be referred to any known species. Here we use the same approaches to evaluate 1) the conspecificity among isolates provisionally identified as *A. minimus* and 2) the relationship among isolates that represent putative new species.

Materials and methods

Fifteen isolates maintained in the University of Alberta Microfungus Collection and Herbarium, Edmonton, AB, Canada (UAMH) were examined (Table 5.1). Group 1

comprised isolates identified as *A. minimus*, including the ex-type culture UAMH 7113, another ascomatal (UAMH 7097) and five non-sporulating isolates. Group 2 included two anamorphic and one ascosporulating strain (UAMH 10365) that expressed strongly similar cultural features. Remaining isolates were ungrouped.

Isolates were grown on oatmeal salts agar (OAT) (Kane et al 1997) and incubated at 22 C to induce ascomata. For Group 1, eight single ascospore isolates were obtained from an ascoma produced by the ex-type of *A. minimus* (UAMH 7113). Non-fruiting isolates from Group 1 were mated in all combinations including self crosses. Mating tests followed the method described by Gibas et al (2002a). Crosses were examined weekly for 28 weeks and then held for 48 weeks before being discarded as negative. Group 2 isolates, UAMH 7392 and UAMH 8604, were mated based on preliminary results of ITS sequence analysis. Eight single ascospore isolates were derived from UAMH 10365. UAMH 1937 and UAMH 8480 produced ascomata on OAT but single ascospore isolations were not done to assess compatibility. UAMH 10248, ex-type culture of *Aph. aciculatus*, was grown on rice medium supplemented with artificial sea water (Synthetic Sea Salt, Instant Ocean, Aquarium Systems, Mentor, Ohio, USA) as described by the original authors as being successful in inducing the teleomorph (Pivkin & Khudyakova 2002), in addition to the standard conditions described above. UAMH 7189 and 10416 were not included in any mating experiment because they appeared culturally distinct from all others.

Colonial and microscopic features were examined for all isolates but details are reported only for those described in the taxonomic section. Isolates were grown on potato dextrose agar (PDA, Difco Laboratories Detroit, Michigan, USA) incubated at 30 C and 35 C and examined weekly for 6 weeks. Colony diameters were recorded at 21 days and photographs were taken at 42 days. Tolerance for cycloheximide at 400 $\mu g\ ml^{-1}$ was assessed by comparing growth on mycosel medium (Mycosel, BBL Microbiology Systems, Cockeysville, Maryland, USA) with that on phytone yeast extract agar (BBL Microbiology Systems). Microscopic observations of conidia were made from slide cultures mounted in lactofuchsin (Kane et al 1997). Ascomata and ascospores were examined from cultures on OAT by light and scanning electron microscopy. Samples were prepared as described by Gibas et al (2002a) and examined with a Hitachi S-2500 scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

DNA sequences of SSU and ITS regions of the nuclear rDNA were obtained using methods described previously but with some modification (Gibas et al 2002a). Crude DNA extracts were used as template for amplification. Primer pairs used for SSU were NS1 (5'- GTAGTCATATGCTTGTCTC-3') and NS8 (5'-TCCGCAGGTTCACCTACGGA-3') (White et al 1990). BMB-CR (5'-GTACACACCGCCCGTCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Lane et al 1985, White et al 1990) were used for the ITS region. Although ITS1 was used previously as the forward primer, BMB-CR was found to provide more consistent amplification of the ITS region. BMB-CR is in position 1624–1640 within the 17S RNA of *Saccharomyces cerevisiae* Meyen ex E. C. Hansen (online at http://www.biology.duke.edu/fungi/mycolab/primers). The amplicons were sequenced by cycle sequencing and run on an ABI 377 automated sequencer (Amersham Pharmacia Biotech Inc. Piscataway, New Jersey, USA). Sequencing primers used for the SSU region were (forward) NS1, NS3, NS11, NS13, NS151 (White et al 1990, Gibas et al 2002b) and (reverse) NS2, NS4, NS6, NS8 (White et al 1990). Sequencing primers used for the ITS region were (forward) BMB-CR, ITS1, ITS3 and (reverse) ITS2 and ITS4. Consensus sequences were generated using Sequencher (Gene Codes Corp., Ann Arbor, Michigan, USA). SSU sequences were newly obtained for two isolates (Table 5.1) and these were compared with sequences previously derived for *Arachnomyces* species and with sequences of onygenalean and other ascomycete taxa. Complete ITS sequences were newly obtained for 14 isolates (Table 5.1). The sequence for UAMH 7113 was published by Gibas et al (2004). Aligned sequences of the ITS region were adjusted manually using the multiple sequence alignment editor Se-Al v2.0a11 Carbon (Rambaut 1996). Sequence analyses for both regions were conducted with PAUP 4.0b10 (Swofford 2002). Heuristic searches were done by setting parsimony as the optimality criterion. Gaps were treated as missing data. Starting trees were obtained at random via stepwise addition with tree-bisection-reconnection as the branch-swapping algorithm. Confidence in the branches of the resulting trees was evaluated by bootstrap analysis (Felsenstein 1985) using 100 replicates. *Saccharomyces cerevisiae* Meyen ex E.C. Hansen M27607 was used as outgroup for the SSU tree and *Aphanoascus canadensis* Currah AJ439690, *Ajellomyces capsulatus* (Kwon-Chung) McGinnis & Katz AB055245 and newly sequenced

Spiromastix grisea Currah & Locquin-Linard UAMH 5409 were used for the ITS analysis.

Results

No ascomata were produced in any pairings among isolates tested within Groups 1 and 2. All eight single ascospores obtained from the *A. minimus* ex-type culture UAMH 7113 were self-fertile. Similarly all single ascospore isolates obtained from Group 2 isolate UAMH 10365 were self-fertile. UAMH 10248 failed to form ascomata on the rice medium with sea water after incubation for twelve months.

SSU rDNA sequences were compared for 45 taxa. A Group 1 intron was found at position 1393 in *A. minimus* UAMH 7113 and *A. gracilis* UAMH 9756 which corresponded to position 1427 in *Saccharomyces cerevisiae* (Gargas et al 1995) but this insertion was not observed in the newly sequenced *X. peruvianum* UAMH 1937 or *Aphanoascus aciculatus* UAMH 10248, or in other *Arachnomyces* species. The insertions were excluded from the phylogenetic analysis. The final alignment of the SSU sequences had a total of 1785 characters of which 1240 were constant, 217 were variable but were parsimony uninformative and 328 were parsimony informative. Heuristic searches using parsimony generated 28 most parsimonious trees, one of which is shown in Fig. 5.1. The score of the best tree was 1285, the consistency index was 0.559, the homoplasy index was 0.441, and the retention index was 0.704. The cladogram shows that *X. peruvianum* and *Aph. aciculatus* are included in the *Arachnomyces* clade with a strong bootstrap support (100%).

Sequences of the ITS region of the nuclear rDNA were compared among 24 isolates. Manual alignment using Se-Al revealed an unalignable fragment 33 bp in length from position 116 to 149 in the ITS1 region of *A. nodosetosus* Sigler & S. P. Abbott. This fragment was excluded from the analysis. The final alignment had a total of 484 characters of which 275 were constant, 93 were parsimony uninformative and 116 were parsimony informative. Six most parsimonious trees were obtained in 470 steps and one is shown in Fig. 5.2. The consistency index was 0.672, homoplasy index was 0.328 and the retention index was 0.638.

Results show that all newly examined isolates are placed within *Arachnomyces* and that none has a high degree of sequence similarity with any known species (Fig. 5.2).

The analysis also reveals a large amount of genetic diversity among isolates originally identified as *A. minimus* and that isolates lacking conidia are not unique to a specific clade. Group 1 isolates are positioned across different branches in the ITS tree. Among these isolates, the ex-type of *A. minimus* and a non-fruiting isolate (UAMH 8967) group in one subcluster (74% bootstrap) and a fruiting (UAMH 7097) and non-fruiting isolate (UAMH 5590) group in another subcluster (79%). There is low bootstrap support (< 50%) for the grouping of the subclusters. Bootstrap support for placement of two other Group 1 isolates (UAMH 8052, 9162) is also low. The remaining isolate UAMH 9728 groups with an ascomatal isolate UAMH 8480 (91%). Group 2 isolates, including an ascomatal isolate, UAMH 10365, and two conidial ones, UAMH 7392 and UAMH 8604, group with 100% suppport. Pair wise comparison of these three ITS sequences show them to be identical. Remaining strains are distributed in other clusters but bootstrap support for the branches is low.

Based on molecular and morphological data, we restrict *A. minimus* to the ex-type strain, propose a new combination for *Aph. aciculatus* and describe three new species of *Arachnomyces*. One new species is described for an isolate (UAMH 7097) formerly identified as *A. minimus*. Members of Group 2 are described as a second species. A third species is described for the ascomatal isolate UAMH 8480. ITS data suggest that other isolates identified originally as *A. minimus* are distinct taxa but these are not described here because they are nonsporulating. These and two other isolates (UAMH 7189 and 10416) are named as *Onychocola* species pending the discovery of additional isolates that may allow their formal description. For *X. peruvianum* we take up the name *A. peruvianus* as proposed by Malloch and Cain and emend the genus to include presence of non setose ascomata, ellipsoidal, echinulate ascospores and sclerotia. *Arachnomyces* species demonstrate strongly similar characteristics; therefore, we begin with a description of the common microscopic and colonial features. In the species descriptions which follow, we focus on the characteristics that distinguish the species and these are summarized for all *Arachnomyces* species known in culture in Table 5.2

Taxonomy

Arachnomyces (Massee et E.S. Salmon) Gibas, Sigler, Currah et S.P. Abbott emend. nov.

Ascomata are cleistothecia, 60-2000 µm in diam, nonostiolate, reddish-brown, subglobose to globose, and commonly setose. Peridium is membranous of textura angularis. Setae are commonly present and are septate, smooth and intermittently nodose, with apices straight to circinate or loosely coiled. Setae sometimes arise from the vegetative mycelium. Asci are evanescent, 8-spored, subglobose. Ascospores lack germ pores and are pale to reddish brown. They are smooth and oblate with polar boss, or are echinulate and ellipsoidal. Anamorphs consist of arthroconidia that are swollen and in persistent chains or that are cylindrical and regularly alternate or of aleurioconidia that are sessile or stalked and that intergrade with irregularly swollen alternate arthroconidia. Dehiscence is mainly lytic but may be schizolytic.

Ascomata form on OAT, but not on PDA. Colonies on PDA are slow growing, rarely exceeding 3 cm in diam in 21 days at 30 C, and are generally raised, downy, velvety to granular, occasionally glabrous, white, yellow, to grayish or greenish yellow on the surface, characteristically producing droplets of colored exudate, and copious yellow to reddish brown diffusible pigments. All species demonstrate resistance to cycloheximide.

Arachnomyces parriae Gibas, Sigler et Currah sp. nov. **Figs.** 5.3-5.4

Etymology: Named in honor of Dinah Parr well known medical mycologist now retired from Auckland Hospital, Auckland, New Zealand.

Holotype: NEW ZEALAND, human nail infection, D. Parr. UAMH 7097 dried colonies and living culture.

Ascomata are cleistothecia 200–510 µm diam, bearing 10–15 setae, nodose, straight at the tip, >500µm long. Setae also found in the vegetative mycelium, arising from a thick walled pigmented swollen cell. Ascospores are smooth oblate with polar boss, 3–4 × 1.5–2 µm. Anamorph absent. Colonies on PDA at 30 C reaching 17 mm diam in 30 d, raised, covered with downy mycelia and droplets of reddish brown exudates, grayish to tan, margin irregular, with yellow to reddish brown diffusing pigment. No growth at 35 C.

Comments

Arachnomyces parriae is distinguished from *A. minimus* by its larger ascomata, straight setae and its inability to grow at 35 C (Table 5.2). *Arachnomyces minimus* has smaller ascomata bearing setae that are slightly coiled or circinate at the tip Fig. 5.5 and 5.6) and it grows at 35 C.

Arachnomyces caespitosus Gibas, Abbott, Sigler et Currah sp. nov. **Figs.** 5.7–5.9

Anamorph: ***Onychocola caespitosa*** Gibas, Abbott, Sigler et Currah sp. nov.

Etymology: Colonies having tufts of yellowish mycelium

Holotype: USA: Nevada, Minden, broom handle, S.P. Abbott. UAMH 10365 dried colonies and living culture.

Ascomata cleistothecia dark brown, 350–2000 µm diam, bearing 15–20 setae 1–2.5 mm long, septate, uncinate to slightly coiled at the tip; ascomatal initials beginning to form at four to five wk, having 6–7 tightly wound coils and reaching up to 9.5 µm long; ascospores oblate, smooth with polar boss, 3.5–4.5 × 2–2.5 µm. Arthroconidia regularly alternate, cylindrical, sometimes irregularly swollen, truncated at both ends or rounded at one end, hyaline, smooth, 3–5.5 × 1.5–2.5 µm. Colonies on PDA at 30 C are 18–20 mm in 21 d, white with pinkish hue initially, developing tufts of light yellow aerial mycelia bearing droplets of reddish brown exudates, mat becoming grayish yellow after 5–6 wks, with radial folds, margin tan to gray, reverse tan to olive brown; diffusible pigments light yellow initially, becoming light reddish brown. No growth at 35 C. Homothallic.

Arachnomyces aegypticus Gibas, Udagawa, Sigler et Currah sp. nov. **Figs.** 5.10–5.11.

Anamorph: ***Onychocola aegyptica*** *Gibas*, Udagawa, Sigler et Currah sp. nov.

Etymology: From geographic location of ex-type culture.

Holotype: EGYPT: Abu Simbel, soil, S. Uchiyama. UAMH 8480 dried colonies and living culture.

Ascomata cleistothecia, 150–250 µm diam bearing 15–22 setae, nodose, 75–200 µm long; ascospores smooth, oblate with polar boss 3.5–4.5 × 2–2.5 µm. Conidia sparse, forming in older cultures, hyphae becoming irregularly swollen, and conidia developing as intercalary, slightly swollen, barrel or irregularly shaped cells; conidia uncommonly

detached. Colonies on PDA reaching 27 mm diam in 21 d at 30 C, white to light yellow, turning brownish gray with age, floccose with loose tufts of aerial hyphae, reverse cream to tan, exudates few and clear, diffusing pigment light yellow. Thermotolerant; colonies attaining 21 mm diam after 21 d at 35 C.

Arachnomyces aciculatus (Pivkin et Khudyakova) Gibas, Sigler et Currah comb. nov. **Fig.** 5.12

Basionym: *Aphanoascus aciculatus* Pivkin & Khudyakova Mycotaxon 131:7. 2002
Anamorph: *Onychocola aciculata* Gibas, Sigler et Currah sp. nov.

We have modified the Latin diagnosis and description provided for the anamorph by Pivkin & Khudyakova (2002) as follows:

Hyphae fertiles irregulariter dichotomae; hyphae primariae 1.5–3 µm diam, ramis fertilibus arthroconidis. Arthroconidia hyalina, levia, cylindracea vel leniter curvula, 3–5 × 1–1.5 µm.

Fertile hyphae arising as straight to slightly undulate; arthroconidia alternate, hyaline, smooth, cylindrical to slightly curved, 3–5 × 1–1.5 µm (4–9(–20) × 1.5–2.2 µm *fide* Pivkin & Khudyakova). Colonies on PDA reaching 14 mm in 21 d, white, raised, floccose, downy; reverse tan. Diffusing pigment light yellow.

Comments

The authentic strain produced abundant elongate ascomatal initials with 3–6 loose to tightly wound coils but did not develop ascomata under any conditions. Pivkin and Khudyakova describe the ascomata as globose, 400–900 µm diam with a peridium of textura angularis. They neither mention nor illustrate ascomatal appendages. Ascospores are oblate with polar depressions, smooth under light microscopy and slightly rugose under SEM, light brown, 2.2–4 × 1.5–2.2 µm. This species is distinct phylogenetically and morphologically in lacking setae. Pivkin and Khudyakova (2002) assigned the anamorph to *Malbranchea* but did not name it. We use *Onychocola* as the anamorph genus for all *Arachnomyces* species. Our measurements of the arthroconidia from slide culture preparations are smaller.

Arachnomyces peruvianus (Cain) Malloch & Cain Can. J. Bot. 48: 840. 1970. **Figs.** 5.13–5.15.

Basionym: *Anixiopsis peruviana* Cain Can. J. Bot. 35: 261. 1957.

≡ *Xanthothecium peruvianum* (Cain) von Arx & Samson Persoonia 7: 377. 1973.

This species has been described in the publications noted above and a brief description is included here. Ascomata are 100–300 µm diam and lack setae. Ascomatal initials are elongate having 6–7 tightly wound coils. Malloch and Cain described the ascomata as glabrous and having a two layered peridium. We have observed that a dark amorphous layer initially covers the peridial wall. The amorphous layer disintegrates at maturity to reveal a membranous inner layer.

Materials examined: UAMH 1937 was examined and sequenced (Table 5.1). UAMH 2907 was compared for ascomatal characteristics.

Discussion

Previous authors (Abbott et al 1996, von Arx & Samson 1973) have rejected the inclusion in *Arachnomyces* of species having non-setose ascomata and echinulate ascospores, but our analysis of SSU rDNA sequences confirm that their inclusion is strongly supported. *Arachnomyces* now includes ten species known in culture and these may be distinguished by cultural and microscopic features (Table 5.2). *Arachnomyces peruvianus* and *A. aciculatus,* having glabrous ascomata, differ from each other by their ascospore wall morphologies (rough vs. smooth) and by their anamorphs. *Arachnomyces peruvianus* lacks an anamorph, while *A. aciculatus* produces narrow, cylindrical, alternate arthroconidia. Among species having setose ascomata, ascospores are invariantly smooth with polar boss and the size range is overlapping. Setal tip morphology is useful in distinguishing *A. gracilis* and *A. parriae,* in which setae are straight and tapered towards the distal end. Species that have circinate or loosely coiled setae are distinguished according to their anamorphs. *A. nodosetosus* produces swollen arthroconidia in persistent chains and *A. kanei* has stalked or sessile arthroconidia and swollen alternate arthroconidia. Three species, including the newly described *A. caespitosus, A. glareosus* and the anamorphic *O. sclerotica,* produce alternate cylindrical arthroconidia, but the latter two species also produce sclerotia. *Arachnomyces aegypticus*

produces a few sparse irregular conidia that are mostly intercalary. Setae of this species are shorter and are predominantly circinate. Anamorphs are absent in *A. minimus* and *A. parriae*. ITS data showed strong evidence of divergence between these two species but they are difficult to distinguish by morphological criteria because they are known only from their ex-type cultures. *Arachnomyces parriae* has slightly larger ascomata and ascospores, straight setal tips and is unable to grow at 35 C.

Results of ITS sequence analysis supported the description of three new teleomorphic species and provided evidence that several additional species of *Arachnomyces* exist. None of the non-sporulating isolates formerly placed within Group 1 could be accommodated in any known species. Although some non-conidial strains clustered with *A. minimus*, the absence of conidiogenesis as a character was not unique to that clade (Fig. 5.2). Non-sporulating strains are here named as *Onychocola* species with the recognition that *Onychocola* was defined originally on the basis of arthroconidia; however, we are applying this name to all anamorphs of *Arachnomyces* species.

Arachnomyces includes both homothallic and heterothallic species, but thallism has not been determined for all species (Table 5.2). The conditions required for expression of the teleomorphs are not yet understood. Among heterothallic species described thus far, some isolates have failed to mate. For example, in the recently described *A. glareosus*, one of 11 isolates did not mate with parental strains or with F1 progeny but ITS sequences showed a high degree of similarity (Gibas et al 2004). *Arachnomyces aciculatus* was described as being teleomorphic, but our culture produced only initials. This isolate may be secondarily homothallic. UAMH 10416, not described here in detail, also produced many initials that failed to develop further. In *A. caespitosus*, one isolate produced ascomata and F1 progeny were self-fertile, but two other isolates having 100% ITS sequence identity, failed to fruit or to mate with each other.

Arachnomyces is known to have an association with human nails. Two species, *A. nodosetosus* and *A. kanei*, are confirmed as agents of nondermatophytic onychomycosis based on criteria of presence of hyphal elements in nail material and isolation of the same fungus from more than one patient sample (Sigler et al 1994, Gibas et al 2002b). Six of eleven isolates of *A. glareosus* were obtained from human nails and skin scrapings; however, due to the difficulty in obtaining follow-up specimens its role in causing

onychomycosis was not clarified (Gibas et al 2004). Similarly, eight of fifteen isolates examined here were obtained from nail and skin specimens (Table 5.1); results of direct examination for six samples were either negative or unknown. Hyphal elements were observed to be present only in the two nail samples from which UAMH 7097 *A. parriae* and UAMH 9162 *Onycochocola* sp. were isolated. Because there are no data on follow up samples, no assessment can be made as to the potential role of these two isolates in causing nail infection. However, species confirmed as agents of onychomycosis demonstrate restricted growth at 35 C, while UAMH 7097 *A. parriae* failed to grow at this temperature.

Table 5.1. List of isolates examined. Groups 1 and 2 represent isolates grouped by morphology.

UAMH number	Original identification	Final identification	Ascomata present	Conidia present	Geographical location	Source
Group 1						
7113[T]	*A. minimus*	*A. minimus*	✓		Dorset, Ontario, Canada	Decayed wood
5590	*A. minimus*	*Onychocola* sp.			New York State, USA	Foot scraping, male 23 yr; [a]DE results unknown
7097	*A. minimus*	*A. parriae*	✓		New Zealand	Yellow nail with nail infection, male; DE+ for hyphae and large arthroconidia
8052	*A. minimus*	*Onychocola* sp.			Dunedin, New Zealand	Right thigh, male; DE- for hyphal filaments
8967	*A. minimus*	*Onychocola* sp.			Lac la biche, Alberta, Canada	Lung of northern flying squirrel
9162	*A. minimus*	*Onychocola* sp.			France	Toenail, DE+ for hyphal filaments
9728	*A. minimus*	*Onychocola* sp.			Ontario, Canada	[b]Hallux, patient 56 yr, suspected [c]DLSO; DE- for hyphal filaments

T – ex type culture
[a] DE – Direct microscopic examination of a nail or skin specimen usually done in dilute alkali solution,
[b] Hallux – great toenail,
[c] DLSO – Distal lateral subungual onychomycosis.

Table 5.1. Continued.

UAMH number	Original identification	Final identification	Ascomata present	Conidia present	Geographical location	Source
Group 2						
7392	*Onychocola* sp.	*A. caespitosus*		✓	Calgary, Alberta, Canada	Skin scraping, groin, male 33 yr; DE results unknown
8604	*Onychocola* sp.	*A. caespitosus*		✓	Quebec, Canada	Skin scraping, boy 10 yr; DE results unknown
10365	*Arachnomyces* sp.	*A. caespitosus*	✓	✓	Minden, Nevada, USA	Broom handle leaning against heat exchanger in restaurant
Miscellaneous						
1937	*Xanthothecium peruvianum*	*A. peruvianus*	✓		Tokyo, Japan	Soil
7189	*Onychocola* sp.	*Onychocola* sp.		✓	Besos Beach, Catalunia, Spain	Marine sediment
8480	*Arachnomyces* sp.	*A. aegypticus*	✓	✓	Abu Simbel, Egypt	Soil
10248[A]	*Aphanoascus aciculatus*	*A. aciculatus*		✓	Dorset, Ontario, Canada	Decayed wood
10416	*Onychocola* sp.	*Onychocola* sp.			New York State, USA	Foot scraping, male 23 yr; [b]DE results unknown

A – authentic

Table 5.2. Differential characteristics of *Arachnomyces* species and their *Onychocola* anamorphs.

	A. aciculatus	*A. aegypticus*	*A. caespitosus*	*A. glareosus*	*A. gracilis*	*A. kanei*
Ascomatal size	400-900	150-250	550-2000	185-310	250-385	175-300
Ascospore wall	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Ascospore size	2.2-4 x 1.5-2.2	4-4.5 x 3-3.2	3.4-4 x 2-2.5	3-3.5 x 1.5-2	2.8-3.2 x 1.5-2	3.5-4.5 x 2.5-3
Setae	Absent	Present	Present	Present	Present	Present
Setal wall		Nodose	Slightly nodose	Slightly nodose	Slightly nodose	Slightly nodose
Setal tip	Circinate or loosely coiled	Circinate or loosely coiled	Circinate or loosely coiled	Circinate or loosely coiled	Straight	Circinate or loosely coiled
Thallism	Not done	Not done?-Homothallic	Homothallic	Heterothallic	Homothallic	Hetero-thallic
Anamorph	*O. aciculata*	*O. aegyptica*	*O. caespitosa*	*O. glareosa*	*O. gracilis*	*O. kanei*
Conidial Type	Alternate cylindrical arthro-conidia	Occasional swollen arthroconidia	Alternate cylindrical arthro-conidia	Alternate cylindrical arthro-conidia	Alternate cylindrical arthro-conidia	Stalked or sessile aleurio-conidia and swollen alternate arthro-conidia
Sclerotia	Absent	Absent	Absent	Present	Absent	Absent
Growth on PDA at 35 C	Restricted	Equivalent to 30 C	No growth	Restricted	No growth	Restricted

Table 5.2. Continued.

	A. minimus	*A. nodosetosus*	*A. parriae*	*A. peruvianus*	*Arachnomyces* sp.
Ascomatal size	100-315	60-300	200-510	100-300	No teleomorph observed
Ascospore wall	Smooth	Smooth	Smooth	Echinulate	
Ascospore size	2.8-3.5 x 1.5-2.2	4-4.5 x 3-3.5	3-4 x 1.5-2	2-3 x 1.5-2	
Setae	Present	Present	Present	Absent	
Setal wall	Slightly nodose	Strongly nodose	Slightly nodose		
Setal tip	Circinate or loosely coiled	Circinate or loosely coiled	Straight		
Thallism	Homothallic	Heterothallic	Not done-?Homothallic	Not done-?Homothallic	
Anamorph	*O. aciculata*	*O. aegyptica*	*O. caespitosa*	*O. glareosa*	*O. gracilis*
Conidial Type	Absent	Swollen arthroconidia in persistent chains	Absent	Absent	Alternate cylindrical arthro-conidia
Sclerotia	Absent	Absent	Absent	Absent	Present
Growth on PDA at 35 C	Restricted	Restricted	No growth	Restricted	Restricted

Fig. 5.1. One of 28 most parsimonious trees from analysis of small subunit ribosomal DNA sequences for *Arachnomyces aciculatus*, *A. peruvianus* and other *Arachnomyces* species. Numbers before species names refer to accession numbers for strains from culture collections. Numbers after species names refer to GenBank accession numbers for strains previously sequenced. Numbers above the branches are bootstrap percentages for 100 resamplings. Values below 50% are not shown.

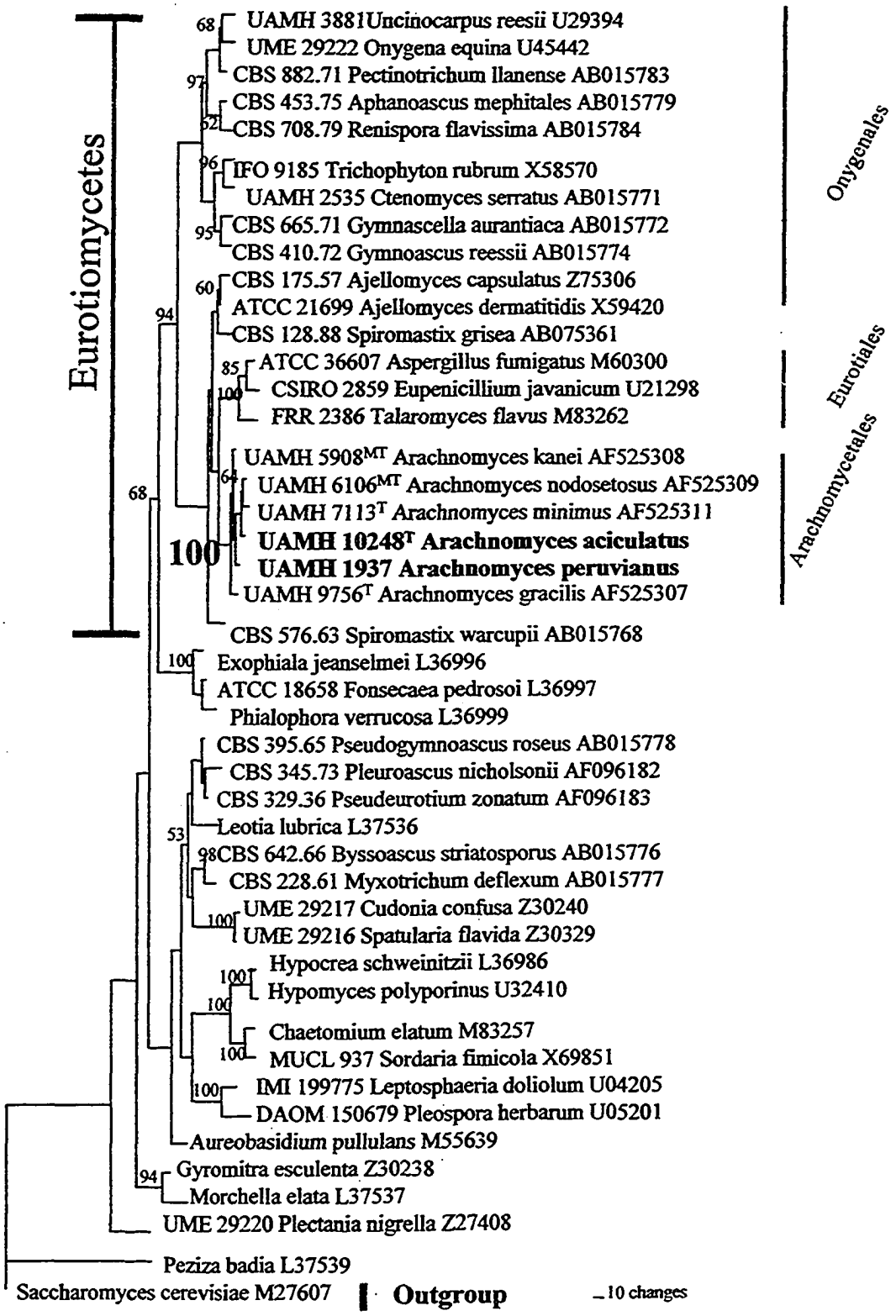
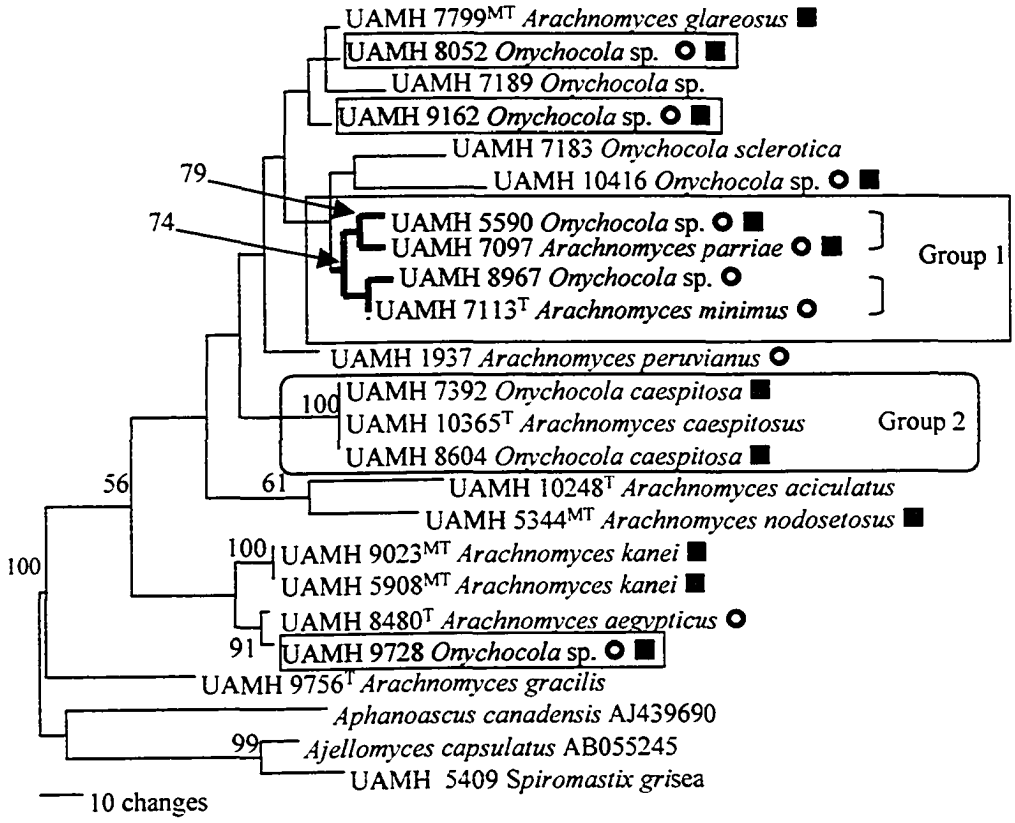


Fig. 5.2. One of 6 most parsimonious trees for 15 strains and related *Arachnomyces* species based on ITS gene sequences. The numbers above the branches are percentages of bootstrap values of 1000 resamplings. Numbers before species names refer to University of Alberta Microfungus Collection and Herbarium accession numbers and those after species names refer to GenBank accession numbers. Values below 50% are not shown. Isolates included in Group 1 and 2 are highlighted.



Legend

☐ Group 1 isolates identified originally as *A. minimus*

O lacking conidia

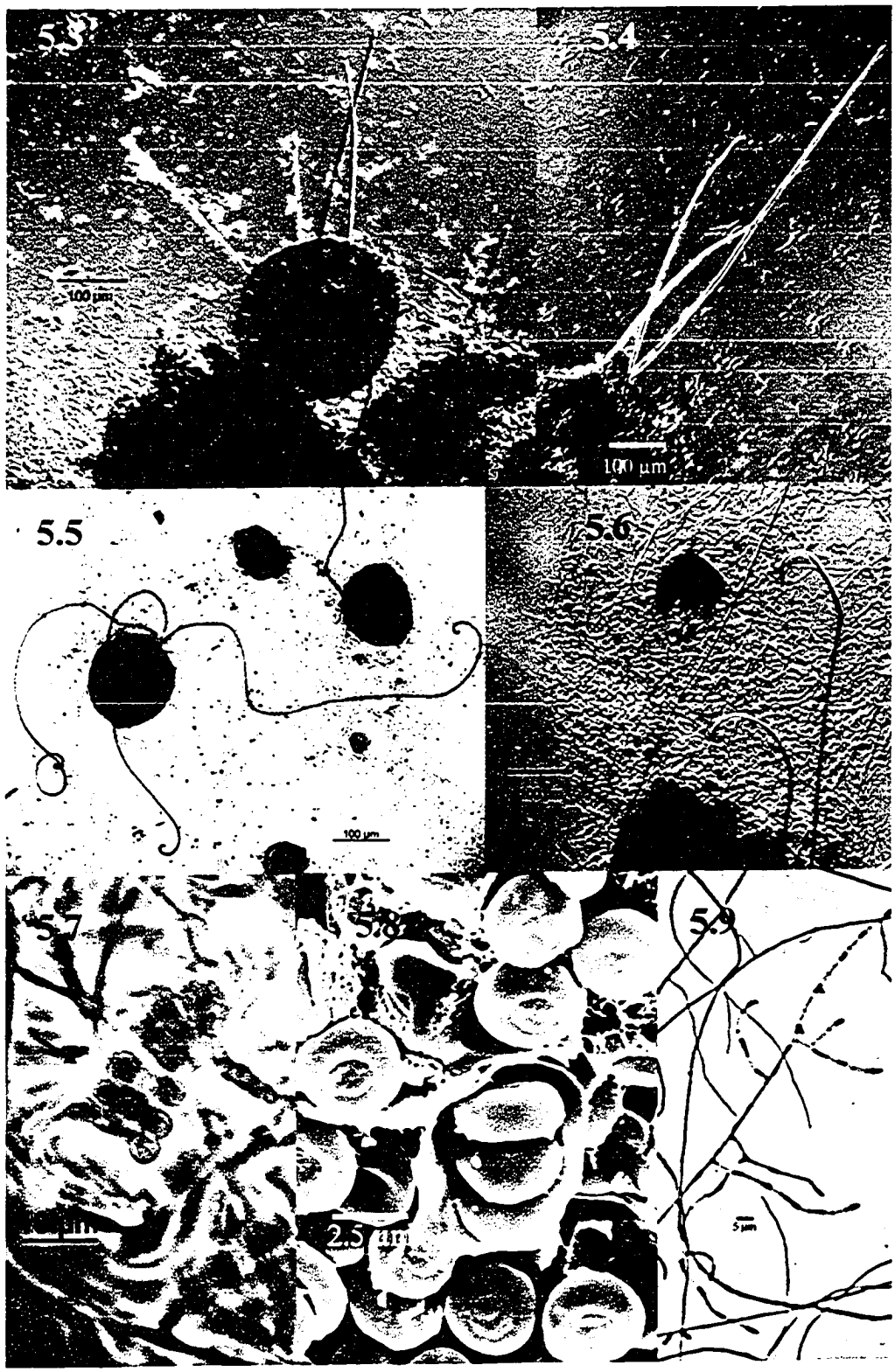
■ associated with human nails or skin

Figures. 5.3–5.9. *Arachnomyces* species

Figs. 5.3–5.4. *Arachnomyces parriae* (UAMH 7097 light micrographs). Ascomata with straight setae. Scale bar = 100 μm.

Figs. 5.5–5.6. *Arachnomyces minimus* (UAMH 7113 light micrographs). Ascomata with circinate setae. Scale bar = 100 μm.

Figs. 5.7–5.9. *Arachnomyces caespitosus* (UAMH 10365). 5.7. Light micrograph showing ascomatal initial. Scale bar = 10 μm. 5.8. Scanning electron micrograph showing smooth oblate ascospores. Scale bar = 2.5 μm. 5.9. Light micrograph showing alternate arthroconidia. Scale bar = 5 μm.

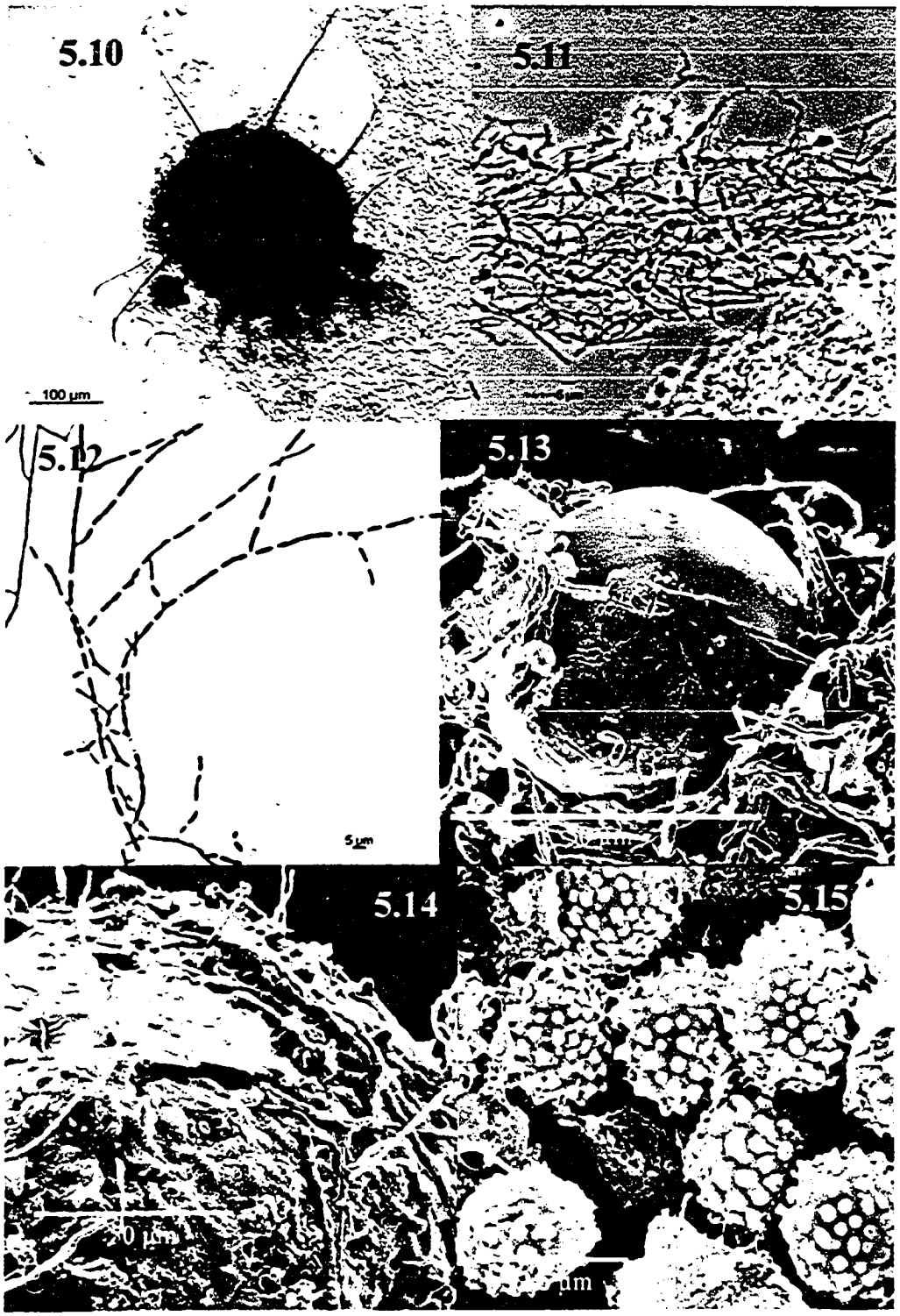


Figures. 5.10–5.15. *Arachnomyces* species.

Figs. 5.10–5.11. *Arachnomyces aegypticus* (UAMH 8480 light micrographs). 5.10. Ascoma bearing circinate setae. Scale bar = 100 µm. 5.11. Irregularly slightly swollen intercalary conidia. Scale bar = 5 µm.

Fig. 5.12. *Arachnomyces aciculatus.* Light micrograph showing alternate regularly cylindrical arthroconidia. Scale bar = 5 µm.

Figs. 5.13–5.15. *Arachnomyces peruvianus* (UAMH 1937 scanning electron micrographs). 5.13. Glabrous ascoma lacking setae. Scale bar 50 µm. 5.14. Disintegrated amorphous outer layer of ascoma revealing membranous inner layer. Scale bar = 50 µm. 5.15. Ellipsoidal echinulate ascospores. Scale bar = 2.5 µm.



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CHAPTER 6

CONCLUSION

At the outset of this project, there were seven species described in *Arachnomyces* Massee & E.S. Salmon including the two original species described 100 years ago and known from dried type material (*A. nitidus* Massee & E.S. Salmon and *A. sulphureus* Massee & E.S. Salmon), two known only from published descriptions (*A. minutus* N. Singh & Mukerji and *A. validus* N. Singh & Mukerji), and three represented by living cultures (*A. gracilis* Udagawa & Uchiy, *A. minimus* Malloch & Cain and *A. nodosetosus* Sigler & S.P. Abbott). The genus was relatively unknown until it was linked to the anamorphic species *Onychocola canadensis* Sigler, a slow growing, cycloheximide tolerant, arthroconidial fungus revealed to be an uncommon agent of human nail infection (onychomycosis) (Sigler & Congly 1990; Sigler et al 1994). The teleomorph, *A. nodosetosus,* was discovered when mated strains of *O. canadensis* produced setose cleistothecia and ascospores typical of the genus *Arachnomyces* (Sigler et al 1994). This finding was significant because this was the first record of an anamorph linked to *Arachnomyces*. Thus, the availability of living material allowed for consideration of the disposition within the taxonomic hierarchy of the cleistothecial ascomycetes, demonstrated a connection to an arthroconidial anamorph, and provided evidence of potential medical importance of members of the genus. However, the placement of the genus within the *Ascomycota* was in dispute, and it was unknown whether production of an anamorph was a unifying characteristic of species within the genus. With availability of living isolates for study, I became aware that *Arachnomyces* species shared common cultural features including slow growth, cycloheximide tolerance and the production of light yellow to reddish brown diffusible pigment. This led to re-examination of a number of non-teleomorphic cultures deposited in the University of Alberta Microfungus Collection and Herbarium (UAMH) that had been tentatively identified as, or were suspected to belong to, the genus based on cultural and/or conidial characters. In this

study, I posed the following questions. 1) Is the genus best disposed within the *Gymnoascaceae* Sacc. (*Onygenales* Cif. ex Benny & Kimbr.) as suggested by Abbott et al (1996)? 2) Can anamorphic isolates, provisionally identified as species of *Onychocola* (and in some cases as species of *Malbranchea* Sacc.), be assigned to *Arachnomyces* with certainty? 3). Is the genus monophyletic? To answer these questions, I used morphological and mating data combined with molecular phylogenetic analysis.

The results of my investigations are set out in four chapters. In the first, I investigated the identity of five anamorphic isolates obtained from nail (Chapter 2). These isolates were similar to *O. canadensis* although conidial morphology was intermediate between the persistent chains of swollen arthroconidia produced by *A. nodosetosus* and the *Malbranchea*-like state of *A. gracilis*. Mating tests demonstrated that these five isolates represented a distinct heterothallic species of *Arachnomyces*. Analyses of sequences from the internal transcribed spacer region (ITS) supported my supposition that they were conspecific and the taxon was named *A. kanei*. This then, became the second species (after *A. nodosetosus*) to be recognized as an agent of nondermatophytic onychomycosis and introduced a third morphological variant to the suite of anamorphs within the genus.

In the next chapter (Chapter 3), I used sequence data from the small subunit ribosomal DNA (SSU rDNA) to test whether species in or assignable to *Arachnomyces* were monophyletic and to confirm that variations in anamorph morphology did not have significance above the species level. Analysis of the sequence data provided evidence that *Arachnomyces* is not a member of the *Gymnoascaceae* and also that it represented a distinct lineage in the *Eurotiomycetes*. A new order *Arachnomycetales* Gibas, Sigler & Currah and new family *Arachnomycetaceae* Gibas, Sigler & Currah were erected to accommodate the genus.

In Chapter 4, molecular analysis, in conjunction with mating tests, were used to establish the relationship of *Malbranchea sclerotica* Guarro, Gené & De Vroey to *Arachnomyces*. Based on molecular data, *M. sclerotica* was transferred to *Onychocola* and renamed *O. sclerotica* (Guarro, Gené & De Vroey) Gibas, Sigler & Currah. Mating tests among 12 isolates showed that the ex-type was genetically distinct from all the other isolates. ITS data supported the recognition of the interfertile isolates and one non-mating

isolate as a species distinct from *O. sclerotica* and a new species, *A. glareosus* Gibas, Sigler & Currah, was described. By pairing strains derived from single ascospores, I demonstrated that *A. glareosus* is heterothallic. The discovery of the teleomorph for this sclerotial species was significant because it added a fourth morphological variant to the suite of anamorphic types and also showed that synapomorphy occurs in the genus.

In Chapter 5, I investigated several taxonomic problems associated with the recognition and disposition of *Arachnomyces* species. In the first instance, I re-examined *Arachnomyces minimus*, a species that has no known anamorph. Mating tests showed that the ex-type strain is homothallic but five other strains that resemble this species did not produce a teleomorph, even when paired. A second teleomorphic strain also exists and is assumed to be homothallic although this was not confirmed by crossing single ascospore isolates. Based on morphological evidence (difference in ascomatal size, setal tip morphology and the ability to grow at 35 C) and results from ITS sequence analysis, the ex-type and the other fruiting isolate are distinct from each other and from all other isolates that had been provisionally identified as this species. Consequently, *Arachnomyces parriae* Gibas Sigler & Currah, another non-anamorphic species in the genus, is recognized. Although molecular data showed that the remaining non-anamorphic and infertile isolates were distinct from the ex-type strains of *A. minimus* and *A. parriae*, there was insufficient support for any of the isolates to be referred to other known species in the genus. In the second instance, I used molecular analysis to resolve the controversy regarding the taxonomic placement of *A. peruvianus* (Cain) Malloch & Cain. SSU rDNA sequences support placing the species in *Arachnomyces* and ITS data confirmed that it is distinct from other species in the genus. However, inclusion of *A. peruvianus* requires some emendation of the morphological criteria that define the genus because it has ellipsoidal, echinulate ascospores and its ascomata lack setae. In the third instance, I redisposed a species that had been described in *Aphanoascus* Zukal. Pivkin and Khudyakova (2002) described *Aphanoascus aciculatus* Pivkin & Khudyakova as having smooth, oblate, ascospores with polar boss. These characters suggested a closer affiliation with *Arachnomyces* than *Aphanoascus* and an analysis of SSU rDNA and ITS sequence data supported my suspicion. My data also indicated that the species was

distinct from others in the genus so it is here renamed *Arachnomyces aciculatus* and is the second species in the genus that lacks ascomatal setae.

ITS sequence data also placed four infertile isolates (Table 5.1) in the phylogenetic framework of *Arachnomyces* and support the hypothesis that the isolates included in the study based on cultural characteristics are monophyletic with *Arachnomyces* species. However, none of the isolates was conspecific with known species of *Arachnomyces*. Two of the four infertile isolates were conspecific with a fertile isolate (Group 2, Table 5.1). Independent production of ascomata by single ascospore isolates confirmed the homothallism in the fertile isolate. *Arachnomyces caespitosus* Gibas Sigler Currah & S.P. Abbott was described for these three isolates. The ITS data were critical in placing these asexual isolates within the genus because without the teleomorph, the number of morphological characters needed for phylogenetic integration were not sufficient.

A major contribution of the research has been to clarify the taxonomic position of *Arachnomyces* and its monophyly. The integration of new taxa using tools such as mating tests and DNA sequence analyses in conjunction with morphological observations has broadened the species and generic concepts of *Arachnomyces* and has provided additional characters for recognition of species. Molecular markers for *Arachnomyces* species have been published and are available for other researchers to use for comparative purposes.

Arachnomyces is emended to include 12 species having setose (appendaged) or nonsetose ascomata, smooth or echinulate ascospores, sclerotia, and anamorphs consisting of arthroconidia, aleurioconidia or lacking. Six species are newly described and include *Arachnomyces aegypticus* (anamorph *Onychocola aegyptica*), *A. caespitosus* (anamorph *O. caespitosa*), *A. glareosus* (anamorph *O. glareosa*), *A. kanei* (anamorph *O. kanei*), *A. parriae*, and *O. gracilis*. *Aphanoascus aciculatus* is transferred as *A. aciculatus*, and *Malbranchea sclerotica* as *O. sclerotica*. *Arachnomyces peruvianus* is determined as the correct name for *Xanthothecium peruvianum* (*Anixiopsis peruvianus*).

Arachnomyces kanei and *A. nodosetosus* are confirmed as uncommon agents of nondermatophytic onychomycosis, but many other species appear to have a predilection for human nail. The ex-type culture of *A. glareosus* and one other isolate under this species were obtained from nail samples that contained hyphal fragments in direct examination. The same is true with *A. parriae* and *Onychocola* sp. UAMH 9162 (Table

5.1). However, follow-up specimens were not obtained and it is not possible to clarify their role in causing nondermatophytic onychomycosis. Future investigators may be able to use the descriptions of the different species and facilitate diagnosis and eventual detection of the source of inoculum.

The occurrence and habitat in nature of *Arachnomyces* species are not well understood. They occur in varied habitat types e.g. human nail or skin, herbivore dung, soil, wood, plant litter and marine sediments (Table 6.1). Five species were recovered from human nail and skin; *A. kanei*, *A. nodosetosus* and *A. parriae* are found exclusively on this habitat type. Two other species, *A. caespitosus* and *A. glareosus*, are found also on wood and soil. *Arachnomyces nodosetosus* is the most frequently isolated species from human substrata followed by *A. glareosus*. Other species are also found on one or two substrata and are rarely seen. *Arachnomyces aegypticus*, *A. gracilis* and *O. sclerotica* were found only in soil, *A. aciculatus* on marine sediment, *A. minimus* on wood and *A. sulphureus* on plant litter. Although *Arachnomyces* species have been isolated from a variety of habitat types and most frequently from human nail and skin, their functional roles in these habitat types are unknown. Challenges in the future will be to determine their true ecological niches as well as the roles these fungi play in nondermatophytic onychomycosis.

Table 6.1. Habitat types for *Arachnomyces* species.

List of Species (No. of Isolates)	Habitat Types[1]					
	Human nail or skin	Herbivore dung	Soil	Wood	Plant litter	Marine sediment
A. aciculatus (3)						●
A. aegypticus (1)			◎			
A. caespitosus (3)	●			●		
A. glareosus (11)	●		◎			
A. gracilis (1)			◎			
A. kanei (5)	●					
A minimus (1)				●		
A. nitidus (3)		●			○	
A. nodosetosus (52)	●					
A. parriae (1)	●					
A. peruvianus (9) *(Xanthothecium peruvianum)*			◎	●		
A. sulphureus (1)					○	
Onychocola sclerotica (1)			◎			
Onychocola sp. (6)	●					●

[1] Based on UAMH records and literature cited.

Literature cited

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