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Mating patterns and ITS sequences distinguish the sclerotial species *Arachnomyces glareosus* sp. nov. and *Onychocola sclerotica*

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Abstract: Mating patterns among twelve strains of *Onychocola sclerotica* demonstrated that the ex-type was genetically distinct from all other strains. Ten strains, when crossed, produced an *Arachnomyces* state. A re-examination of morphology and an analysis of nuclear ribosomal internal transcribed spacer (ITS) region sequences supported recognizing the interfertile strains and one infertile strain as a species distinct from *O. sclerotica*. *Arachnomyces glareosus* (anamorph *O. glareosa*) *sp. nov*. and *O. sclerotica* are similar in producing a sclerotial synanamorph but they are phylogenetically distinct.

Taxonomic novelties: *Arachnomyces glareosus* Gibas, Sigler & Currah sp. nov. (anamorph *Onychocola glareosa* Gibas, Sigler & Currah sp. nov.)
Key words: *Arachnomyces*, ITS, *Malbranchea sclerotica*, *Onychocola*, sclerotia.

INTRODUCTION

The genus *Arachnomyces* Massee & E.S. Salmon is a monophyletic assemblage of cleistothecial ascomycetes having anamorphs now assigned to the genus *Onychocola* Sigler (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.

MATERIALS AND METHODS

Mating and morphology

In the first mating experiment, 12 strains (Table 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 designated as plus (UAMH 7799 = CBS 116129) and minus (UAMH 8067 = CBS 116128) 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, U.S.A.) at 30 ºC and 35 ºC. Tolerance for cycloheximide at 400 µg/mL was assessed by comparing growth on Mycosel medium (BBL Microbiology Systems, Cockeysville, MD, U.S.A.) 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).

Table 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	+	+	AY624315
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	AY624316

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

DNA studies

DNA sequences from the ITS ribosomal DNA (rDNA) region, which include ITS1, ITS2 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 shown in Fig. 1 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 and ITS4 (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, ITS3, ITS2, 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 2). Five strains were designated as plus and five as minus mating types. No ascomata were produced in self crosses. The eight F_1 progenies derived from UAMH 7799 × UAMH 8067, when backcrossed, were fertile, and mating types segregated equally (Table 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 2, 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. 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.

Table 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 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.

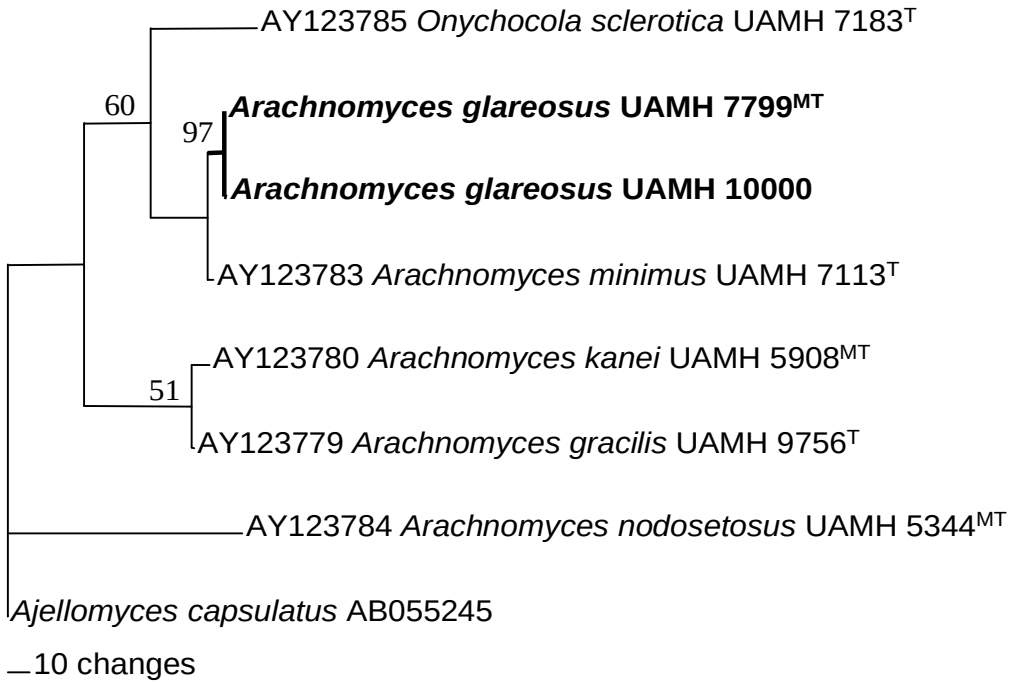


Fig. 1. One of three most parsimonious trees from analysis of internal transcribed spacer (ITS) region ribosomal DNA sequences for *Onychocola sclerotica* strains and related *Arachnomyces* species. Numbers before species names refer to GenBank accession numbers for isolates previously sequenced. GenBank accession numbers for the newly sequenced taxa are listed in Table 1. Numbers above the branches are bootstrap percentages for 500 resamplings. Values below 50 % are not shown.

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 1 belong to the new species, *A. glareosus* (anamorph *O. glareosa*).

Taxonomy

Arachnomyces glareosus Gibas, Sigler & Currah, **sp. nov.** MycoBank MB500151. Figs 2–12.
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 µm diam, rubro-brunnea, subglobosa vel globosa, nonostiolata, setulosa, peridium membranaceum textura angulari, setulae leves vel leviter nodosae, ordinate septatae, ad apicem uncinatae; asci evanescentes, octospori, subglobosi, 6 × 5 µm; ascosporae leves, oblatae, in parte media umbonatae, 3–3.5 × 1.5–2 µm, heterothallicae.
Typus: UAMH 10234 colonia exsiccata ex combinatione cruce UAMH 7799 × UAMH 8067.

Onychocola glareosa Gibas, Sigler, & Currah, **sp. nov.** MycoBank MB500152.

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 textura angulari, 45–60 µm diam.
Typus: UAMH 7799, *colonia exsiccata ex cultura.*

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

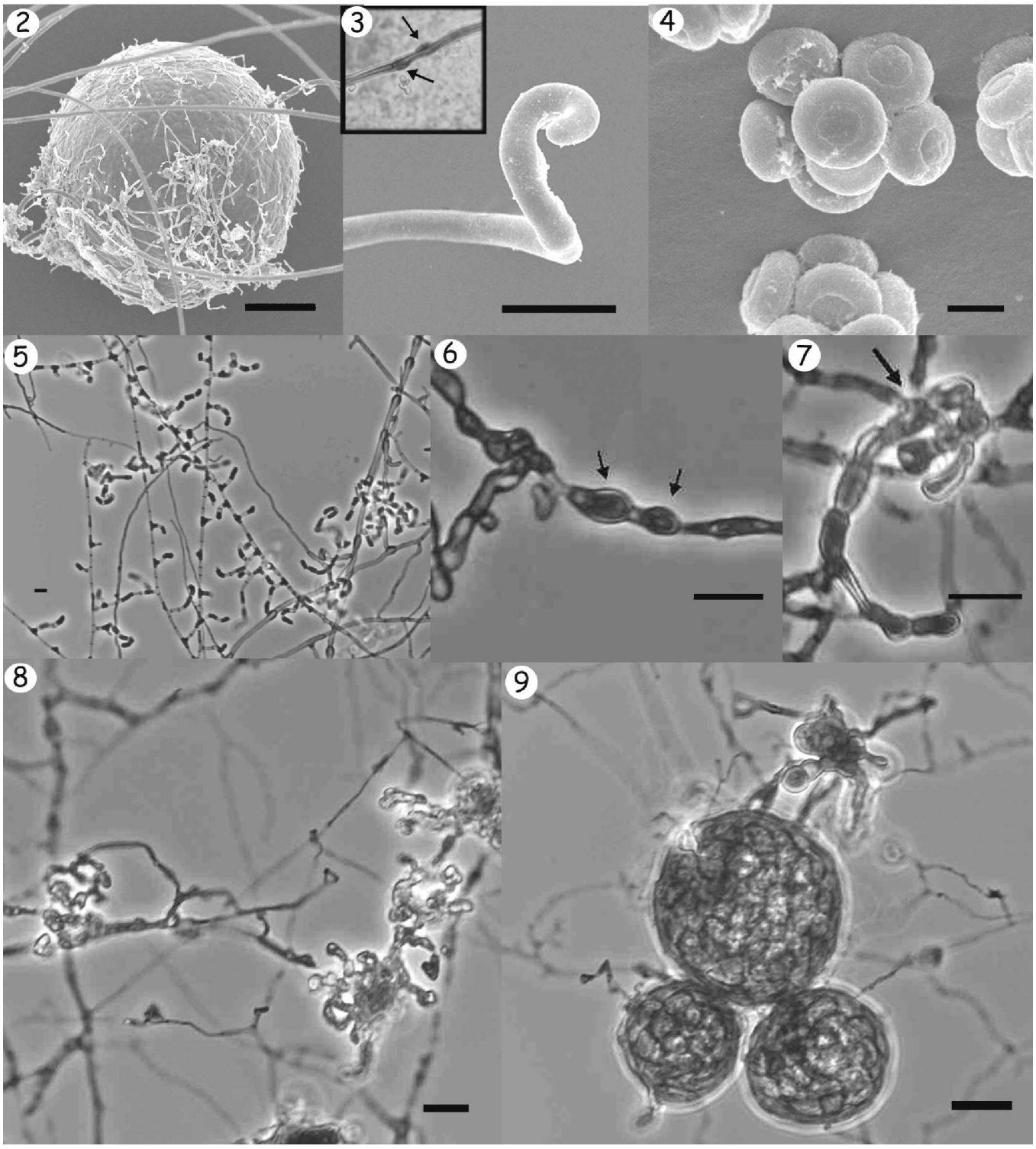
Fertile *hyphae* arcuate, bearing intercalary alternate arthroconidia. *Arthroconidia* cylindrical, sometimes slightly curved or irregular with one or both sides swollen, detaching by lytic dehiscence, 2.5–4.5 × 1.5–2 µm. Racquet hyphae are present. *Sclerotia* develop from swollen, thick-walled, pale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

Cultural characteristics: Colonies on PDA at 30 ºC white initially, becoming greyish brown centrally, downy, raised to crateriform attaining 18–25 mm diam in 21 d. All isolates producing abundant sclerotia giving the colony a grainy appearance. Droplets of dark reddish brown exudate occurring in areas where sclerotia are produced. A pale brown diffusing pigment first appearing after 7 d, turning the medium dark reddish brown within 21 d. Growth at 35 ºC restricted, reaching 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

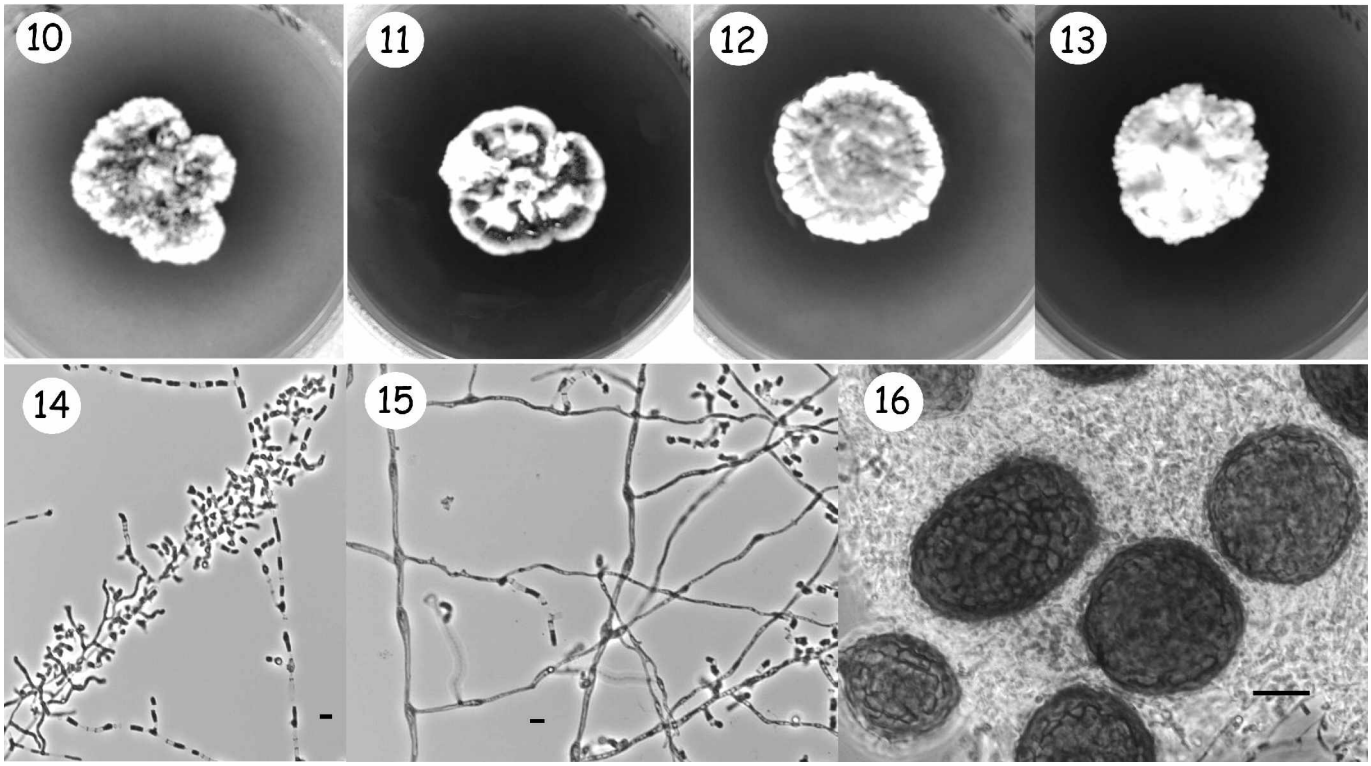
Onychocola glareosa and *O. sclerotica* are strongly similar in morphology and this led to the initial identification of all isolates as the latter species. However, the inability of the ex-type strain of *O. sclerotica* (UAMH 7183) to cross with any other strain and the degree of ITS sequence divergence suggest that these are distinct species. Re-examination of *O. sclerotica* revealed only minor morphological differences from *O. glareosa*. Colonies of *O. glareosa* (Figs 10–12) 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. 13). At 35 ºC 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 ºC 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. 14), racquet hyphae to be present (Fig. 15) and the diameter of sclerotia to be up to 60 µm instead of 180 µm diam (Fig. 16).



Figs 2–4. *Arachnomyces glareosus* (UAMH 7799 × UAMH 8067; scanning electron micrographs). 2. Ascoma with setae. Scale bar = 50 µm. 3. Tip of seta showing loose coil. Inset, light micrograph, showing nodose seta (arrows). Scale bar = 10 µm. 4. Smooth oblate ascospores. Scale bar = 2.5 µm. **Figs 5–9.** *Onychocola glareosa* (UAMH 7799). 5. Alternate cylindrical arthroconidia. Scale bar = 5 µm. 6–9. Development of sclerotia from vegetative hyphae. Scale bar = 10 µm.

Although we cannot use morphology to reliably distinguish these species, the paler colonies of *O. sclerotica* and better growth at 35 ºC 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.



Figs 10–13. Colonies grown on PDA at 30 ºC for 42 d. 10–12. *Onychocola glareosa* UAMH 7799 and UAMH 8067, mating type strains, UAMH 10000, non-mating strain. 13. *Onychocola sclerotica* (UAMH 7183). **Figs 14–16.** *Onychocola sclerotica.* 14. Alternate cylindrical arthroconidia. Scale bar = 5 µm. 15. Racquet hyphae and arthroconidia. Scale bar = 5 µm. 16. Sclerotia. Scale bar = 20 µm.

Other defining characters include the production of ascomata with nodose setae that are circinate or loosely coiled or straight, the formation of pale 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 unpubl. 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 two from skin scrapings taken from patients for diagnostic purposes (Table 1). In three cases, hyphal elements were observed in direct examination of the specimen, but in one of these, a dermatophyte was isolated. For the other cases, we have no data on the number of colonies that grew out from the specimens or whether follow-up specimens were 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.

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