PROFESSOR JOCHEN BRASCH (Orcid ID : 0000-0002-8375-9092)
PROFESSOR JOSEPA GENÉ (Orcid ID : 0000-0001-6195-9299)

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A human subcutaneous infection by *Microascus ennothomasiorum* sp. nov.

Jochen Brasch[1], Vera Beck-Jendroschek[1], Isabel Iturrieta-González[2], Karin Voss[1], Josepa Gené[2]

Running head: *Microascus ennothomasiorum*, sp. nov.

[1]Department of Dermatology, University Hospitals of Schleswig-Holstein, Campus Kiel, Kiel, Germany

[2]Unitat de Micologia, Facultat de Medicina i Ciències de la Salut and IISPV, Universitat Rovira i Virgili, Reus, Spain.

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Corresponding author:

Prof. Dr. med. Jochen Brasch

Department of Dermatology, Venerology and Allergology

University Hospitals of Schleswig-Holstein, Campus Kiel

Rosalind Franklin-Straße 7

24105 Kiel

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Germany

Tel.: +49 500 21180

Fax: +49 500 21104

Email: jbrasch@dermatology.uni-kiel.de

Summary

A 60-year-old woman presented with a nodular granulomatous skin lesion on her right thumb. It had developed after inoculation of a splinter of wood. Because it was resistant to various therapies, the nodule was finally excised. Complete healing followed this surgery and a melanized filamentous fungus with scopulariopsis-like morphology was recovered from the dermal tissue. Fitting with no known species, the fungus was subjected to extensive morphological, physiological and genetic investigations. It was characterised by resistance to cycloheximide, growth at 37 ºC, branched conidiophores with cylindrical annellides in brush-like groups producing dark conidia in basipetal chains, and cleistothecia with ellipsoidal to slightly reniform ascospores. Genetically it clustered in a well-supported clade together with *Microascus (M.) brunneosporus*, *M. chinensis, M. intricatus*, *M. longicollis*, *M. micronesiensis* and *M. onychoides*, but formed an independent branch distant from the other *Microascus* species. Based on its unique genetic characteristics and morphological findings, the isolate is proposed as a new species, *Microascus ennothomasiorum.* Morphologically it differs from its phylogenetically closest species by its branched conidiophores and ascomata with a peridium of *textura intricata*. Our observation once again emphasizes that dermal granulomas can be caused by uncommon fungi; diagnostics should therefore include appropriate mycological investigations.

Introduction

Nodular skin lesions without characteristic epidermal alterations are always a diagnostic challenge. Neoplastic tumours must be considered as well as inflammatory processes that lead to proliferative tissue reactions. Nodular lesions that develop on the hand in the sequel of a traumatic penetration of exogenous material may be due to foreign body granulomas.

Furthermore, infectious agents can be inoculated in this way as well and lead to a diversity of dermal infections. Examples for this scenario are atypical mycobacterioses that may hit aquarium owners or subcutaneous fungal infections that are caused by splinter injuries with contaminated wood [1]. Here we report on a case in which after a splinter injury a nodular skin lesion developed on the thumb of a patient. As it turned out, it was caused by an undescribed species of *Microascus*, a genus of ascomycetes which includes several human opportunists able to cause both superficial and deep infections [2].

Case report

A 60-year-old and otherwise healthy woman presented in our hospital with a nodular and ulcerated skin lesion on her right thumb. She reported an injury of this finger while she was cleaning her aquarium six months earlier with inoculation of a splinter of wood. In the following months an indolent swelling developed, and she had herself applied various ointments, including keratolytic ones, and treated the lump with a curette. She recalled that some foreign body was discharged someday, but the swelling persisted. Clinically, we saw a solid nodule with a small ulceration (Fig.1a), but no further signs of inflammation. A treatment trial was performed with topical application of clobetasol proprionate for 2 weeks, but failed to induce a regression. After this, a magnetic resonance imaging (MRT) was performed which revealed a dermal granulomatous reaction and low-grade seroma but no alteration of the bone. The skin tumour was subsequently excised by a hand surgeon and the recovered dermal tissue was tested for mycobacteria and fungi. No mycobacteria were detected microscopically, by DNA-assays and in appropriate cultures (all tests done by the National Reference Center for Mycobacteria, Borstel, Germany). However, numerous colonies of a melanized filamentous fungus with scopulariopsis-like morphology were recovered from cultures of the dermal tissue (see Results). The healing process after this surgery was rapid and complication-free, resulting in an inconspicuous small scar (Fig.1b). Finally, scrapings from stratum corneum were collected 6 months after surgery from the skin area adjacent to the scar for a mycological control. In this control, KOH mountings and mycological cultures were negative.

Materials and Methods

Dermal tissue from the nodular skin lesion on the thumb of the patient was obtained under sterile conditions by a hand surgeon. This tissue was chopped up and used to inoculate Sabouraud agar plates (SGC2; BioMèrieux®, Marcy-l´Etoile, France) without cycloheximide that subsequently were incubated at 26 (±1) and 37 °C. After 2 weeks colonies of the same fungus grew at both temperatures. The fungus was isolated for further characterisation and deposited at the public culture collections of the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherland) and Leibniz Institute-DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) as CBS 144074 and DSM 106608, respectively.

Morphological and physiological assessments

For morphological characterization four-millimetre punches of fresh colonies cut from these SGC2 plates were used for inoculation of the following culture media incubated at 26 (±1) °C and at 37 (±1) °C: potato dextrose agar (PDA; Becton Dickinson Difco, Sparks, MD, USA), oatmeal agar (OA, Becton Dickinson Difco, Sparks, MD, USA) and potato carrot agar (PCA) [2]. These cultures were assessed after 14, 21 and 28 days of incubation. Colour of the colonies was assessed according to Kornerup & Wanscher [3]. PCA and OA cultures were maintained up to two months to ensure ascomata development. Slide cultures for microscopic evaluation were prepared on OA and PCA assessed after 14 days incubation at 26 (±1) °C. Slides were mounted in Shear's solution and photomicrographs were obtained using a Zeiss Axio-Imager M1 light microscope (Zeiss, Oberkochen, Germany) with a DeltaPix Infinity X digital camera.

Additionally, the isolate was incubated in the same culture media at different temperatures (5, 15, 25, 30, 37, 40 and 45 °C) for 14 days in darkness, to define minimum, optimum and maximum temperature for growth. Cycloheximide resistance was also determined with SGC2 agar supplemented with 0.4% of cycloheximide (AppliChem, Darmstadt, Germany). Growth on human hair and the hair perforation test was performed according to Ajello [4]. Growth on human stratum corneum was tested at 26±1 °C. For this purpose, stratum corneum was prepared as described previously [5].

Resistance against common antimycotics was determined at the National Reference Center for Invasive Fungal Infections in Germany (Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie - Hans-Knöll-Institut Jena, NRZMyk) by use of a microdilution test as recommended by The European Committee on Antimicrobial Susceptibility Testing (EUCAST).

All assays and physiological tests were performed in duplicate and included appropriate controls for bacterial contamination according to generally accepted procedures established for molds [6, 7].

DNA extraction, sequencing and phylogenetic analysis

Genomic DNA was extracted from colonies growing on PCA for 7 d at 25 °C and following the protocol of Werner et al. [8] with some modification. Four nuclear DNA regions were amplified and sequenced [i.e., ITS and LSU regions of the rDNA operon, and fragments of the translation elongation factor 1-alpha (EF-1α) and beta-tubulin genes (TUB)], following the criteria of Sandoval et al. [9] for molecular delineation of scopulariopsis-like fungi. The amplification was carried out with the primer pairs ITS5/ITS4 for the ITS region, including the 5.8S gene, NL1/NL4b for the LSU region [10], 983F/2218R for EF-1 α [11] and BT2a/BT2b for TUB [12]. The same pairs of primers were used to obtain the sequences, which were carried out at Macrogen Europe (Macrogen Inc. Amsterdam, The Netherlands). Finally, the sequences generated were assembled and edited using SeqMan v. 7.0.0 (DNAStar Lasergene, Madison, WI, USA) to obtain the consensus sequences. GenBank accession numbers are summarized in Table 1.

Consensus sequences obtained for each locus were aligned with sequences of 34 ex-type and reference strains of *Microascus* species retrieved from GenBank (Table 1), using the ClustalW algorithm [13] under MEGA software v. 6.0 [14], and refined with MUSCLE [15] in the same platform and manually adjusted if needed. Phylogenetic reconstructions by maximum likelihood (ML) and bayesian inference (BI) approaches were performed with MEGA v. 6.0 and MrBayes v. 3.2 [16], respectively. The strain *Pithoascus stoveri* CBS 176.71 was selected as outgroup [9, 17]. The combined analysis of the four phylogenetic markers was tested through Incongruence Length Difference (ILD) implemented in Winclada programme [18].

For the ML analysis of the combined dataset, the best nucleotide substitution model determined by the MEGA program was Tamura Nei with Gamma distribution and Invariant sites (G+I). ML bootstrap values (BML) ≥ 70 % were considered significant. For the BI phylogenetic analysis, the best nucleotide substitution model determined by jModelTest [19] was Hasegawa-Kishino-Yano (HKY) with Gamma distribution and Invariant sites (G+I) for LSU and TUB, Symmetrical model (SYM) with Gamma distribution and Invariant sites (G+I) for ITS, and General time reversible (GTR) with Gamma distribution and Invariant sites (G+I) for EF-1 α . The parameter settings used were two simultaneous runs of 5,000,000 generations, four Markov chains, sampled every 1,000 generations. The 50 % majority rule consensus tree and posterior probability values (PP) were calculated after discarding the first 25% of the samples. A PP value of ≥ 0.95 was considered significant.

Results

Colonies obtained on SGC2 showed a very similar morphology, both macro- and microscopically, and apparently belonged to the same fungus. Based on the presence of branched conidiophores bearing cylindrical annellides in brush-like groups and on the development of small black ascomata (cleistothecia) after 4 weeks, it was morphologically identified as *Microascus* sp..

Colonies on SGC2 reached a diameter up to 16 mm after 14 days at 26(±1) °C. They were beige, elevated at the center and had a rather wooly surface with some irregular folds(Fig. 2a), and the reverse had a brownish shade with a dark center. On PDA the fungus grew a little bit slower than on SGC2 (ca. 12 mm after 14 days), but otherwise looked similar. On OA, the growth rate was like that on SGC2 but the colonies were olive grey (2F2/2E2) to nearly black and wooly with multiple small cleistothecia on the surface (Fig. 2b), and a dark reverse with a green shade (4E2/4D2). On PCA, the colonies reached up to 11 mm after 14 days and were dark green (29F8) to brownish, the reverse being olive at the centre (1E3). The fungus grew at 37(±1) °C in all media tested, but with growth rates markedly slower than at 26(±1) °C throughout. The optimal growth temperature was 25 °C; no growth at temperatures below 15 °C or above 37 °C. Its growth was not suppressed by 0.4% cycloheximide in SGC2 at 26(±1) °C and at 37(±1) °C. It also grew well on human stratum corneum and on human

hair, but the hair perforation test was negative. Cultures were resistant to 5 % NaCl and showed marked proteolysis on bromcresol-purple-casein-glucose agar (BCPCG).

Microscopically, the colonies were composed of smooth-walled septate hyphae that became dark after 14 days on all culture media tested. The fungus showed conidiophores mostly elongated and branched, bearing cylindrical annellides in brush-like groups (Fig. 3a), and producing dark conidia in basipetal chains. The conidia were subspherical to broadly obovoid, smooth- and thick-walled and measured ca. 3 µm in diameter (Fig. 3b). On PDA cleistothecia were present after 14 days with peridium of *textura intricata*, which measured 100 -150 µm diameter (Fig. 3c) and contained thin-walled hyaline asci approximately 6 x 10 µm with hyaline, smooth-walled, ellipsoidal or almost reniform ascospores of ca. 2 x 4 µm (Fig. 3d).

The ILD test showed that the analyses of the four different loci were congruent (P = 0.17) and could be combined. The multi-locus phylogeny of the 36 taxa comprised 2,365 bp (ITS 478 bp, LSU 491 bp, EF-1 α 873 bp and TUB 523 bp) with 554 bp variable (ITS 106 bp, LSU 42 bp, EF-1 α 192 bp and TUB 214 bp) and 425 bp phylogenetically informative (ITS 88 bp, LSU 25 bp, EF-1 α 138 bp and TUB 174 bp). The analysis revealed that the case fungus represented a new lineage in the genus closely related to *M. brunneosporus*, *M. chinensis, M. chinensis, M. intricatus*, *M. longicollis*, *M. micronesiensis* and *M. onychoides* (Fig. 4). Based on its unique genetic characteristics and on its distinct morphological features it is proposed below as *Microascus ennothomasiorum* sp. nov..

Minimal inhibitory concentrations of antifungal agents were as follows: amphotericin B >16 µg/ml, natamycin 4 µg/ml, caspofungin 8 µg/ml, anidulafungin >8 µg/ml, terbinafine 1 µg/ml, voriconazole 1 µg/ml, posaconazole 8 µg/ml, itraconazole >8 µg/ml, isavuconazole 1 µg/ml.

Discussion

Microascus spp. are ubiquitous environmental saprotrophic ascomycetes that are usually isolated from soil or decaying plant material, but can also occur in indoor environments [9, 17]. Traditionally, their asexual morphs were classified as *Scopulariopsis*. However, recent phylogenetic studies demonstrated that both *Microascus* and *Scopulariopsis* were distinct

genera and the species nomenclature was adapted accordingly [9]. Dermatologists are well acquainted with *Scopulariopsis brevicaulis,* which is a common cause of onychomycosis worldwide, but there are several *Microascus* species, less known to clinicians, that are able to cause both superficial and deep human infections especially in immunocompromised patients [2, 20, 21, 22], including necrotizing tracheobronchitis [23]. It is also of note that currently some *Microascus* species, such as *M. appendiculatus, M. brunneosporus*, *M. chinensis*, *M. onychoides*, *M. pseudolongirostris*, *M. restrictus* and *M. verrucosus*, are exclusively known from human clinical specimens [9, 24, 17], although their clinical significance remains unknown.

In the present case, the history of a skin injury that had occurred during cleaning of an aquarium in the first place raised suspicion of an atypical mycobacteriosis, e.g. caused by *Mycobacterium marinum*. In the second place, we considered fungal infections like sporotrichosis because a splinter of wood had presumably remained within the skin for some time. Rotten wood can be colonized by various fungi that are able to induce posttraumatic granulomatous dermal infections. It then became apparent that all tests to prove a mycobacterial infection were negative, but a fungus grew in the mycological cultures at 26 °C and 37 ºC. This fungus had been isolated from dermal tissue obtained surgically under sterile condition, so that a surface contamination could be excluded. Unfortunately, the surgeon who had excised the lesion had only prompted its microbiological investigation but no histology, but the MRT had revealed a granulomatous tissue reaction very well compatible with subcutaneous mycosis. It is plausible that the fungus had been transferred with the wooden splinter. The strain was capable to grow at 26 °C as well as at 37 °C and to digest human keratin. Considering that many *Microascus* spp. can grow on plant material and that their ability to cause deep infections in humans under appropriate conditions is well documented [22, 24], we believe that our isolate was the true pathogen. This idea is supported by the fact that the skin lesion which had persisted for months was cured by the surgical intervention.

Based on its micromorphology and its resistance to cycloheximide, we first thought that our isolate was a *Scopulariopsis* species. Then, after the development of the sexual morph it was distinguishable as a *Microascus* sp.. Only a multi-locus sequence analysis confirmed it to be a *Microascus* species that had not been described before. *Microascus ennothomasiorum* is closely related to a group of species that (Fig. 4), with exception of *M. micronesiensis* [17], have been only isolated from human samples, especially from the

respiratory tract and nails [9, 25]. It can be distinguished from other species of the genus mainly by developing branched conidiophores rather than single conidiogenous cells on vegetative hyphae, and by ascomata with peridium of *textura intricata*. The only species related to *M. ennothomasiorum* with a peridium with such morphology is *M. intricatus,* which morphologically mainly differs in having fusiform ascospores (ellipsoidal to slightly reniform in *M. ennothomasiorum*), mostly globose conidia (subglobose to broadly obovoid in *M. ennothomasiorum*) and by its growth at 40 ºC (maximum temperature for growth in *M. ennothomasiorum* is 37ºC).

Our observation once again illustrates that in the context of a traumatic skin injury with dermal inoculation of exogenous material granulomatous dermal infections may develop that are due to uncommon fungi. Such an infection can even affect individuals without confinement of antimicrobial defence and the spectrum of facultative pathogenic fungi that may be involved under such conditions obviously comprises previously undiscovered species. Diagnostics in cases of unclear granulomatous skin lesions should therefore include appropriate mycological investigations.

Taxonomy

Microascus ennothomasiorum Brasch, Beck-Jendroschek, Voss, Iturrieta-González, Gené, **sp. nov**. MycoBank no.: MB 826957; Fig. 2.

Etymology: The epithet "ennothomasiorum" was chosen in compliment to two renowned dermatologists (Enno Christophers and Thomas Schwarz) who both in their function as heads of the Department of Dermatology at the University Hospitals of Kiel ensured that mycology takes on an important role in this institution.

Typus: Germany, Kiel, from dermal human skin, Oct. 2017, leg. Jochen Brasch (**holotype** CBS H-23646; ex-type cultures CBS 144074, DSM 106608, UKSH 1388-17, FMR 16930).

Colonies on OA attaining 17–18 mm diameter after 14 d at 25 ºC, olive grey (2F2/2E2), slightly raised at the center and granular due to the presence of cleistothecia, aerial mycelium scarce, with submerged mycelium towards the outer zone, margin slightly lobate; reverse brownish grey (4E2/4D2). On PCA at 25 ºC attaining 10–11 mm diameter in

14 d, dark green (29F8), velvety, slightly raised at the centre, aerial mycelium scarce, margin white and regular; reverse olive at the centre (1E3), white at the periphery. *Mycelium* composed of septate, branched, subhyaline, smooth-walled hyphae of 1–2 μm wide. *Ascomata* immersed or superficial, globose to subglobose, 80–160 µm diameter, with a short cylindrical ostiolar neck, dark brown; *peridium* with a *textura intricata*. *Asci* subglobose to ovoidal, 8–10 x 6–7 μm. *Ascospores* ellipsoidal to slightly reniform, 4–6 x 2–3 μm, pale brown, with an inconspicuous germ pore. *Conidiophores* differentiated, mostly branched, bearing terminally a group of 2–4 annellides at each branch, subhyaline, smooth-walled. *Annellides* ampulliform to lageniform, 4–7 x 2–2.5 μm, tapering to a cylindrical annellated zone of 1–1.5 μm wide, subhyaline, smooth-walled, some slightly verruculose. *Conidia* subglobose to broadly obovoid, 3–4 × 2.5–3 μm, with truncate base, 1–1.5 μm wide, brown, smooth- and thick-walled, arranged in chains. Solitary conidia not observed.

Cardinal temperature for growth: Optimum 25 °C, maximum 37 °C, minimum 15 °C.

Physiology: Resistance to 0.4% cycloheximide. Good growth on stratum corneum and hair. Hair perforation test negative.

Differential diagnosis: Microascus ennothomasiorum differs from its phylogenetically closest species by its branched conidiophores and ascomata with a peridium of *textura intricata*.

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Conflicts of interests: None.

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Legends to Figures

Fig.1

Clinical pictures

a) Nodular ulcerated skin lesions on the right thumb of the patient

b) Inconspicuous small scar on the right thumb after surgery

Fig. 2

Macroscopic morphology

a) Colony of *Microascus ennothomasiorum* on SGC2, obverse, after 14 days at 26 °C

b) Colony of *Microascus ennothomasiorum* on OA with cleistothecia after 30 days at 26 ºC

Fig. 3

Microscopic morphology

a) Branched conidiophores with annellides

b) Conidia

c) Cleistothecia with peridium of *textura intricata*

d) Asci and ascospores

Fig. 4

Phylogenetic tree

Maximum Likelihood (ML) tree constructed with ITS, LSU, EF-1 α and TUB sequences of 35 species of the genus *Microascus*. The tree was rooted on *Pithoascus stoveri* (CBS 176.71). Bootstrap support values for ML greater than 70% and Bayesian posterior probabilities greater than 0.95 are given near nodes, respectively. The name of species described here is indicated in bold. Branch lengths are proportional to distance. Ex-type and neotype strains are indicated with ^{T} and ^{NT}, respectively.

Fig.4