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Short communication

Internal Transcribed Spacer (ITS) of rDNA of appendaged and non-appendaged strains of *Microsporum gypseum* reveals *Microsporum appendiculatum* as its synonym

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Abstract

Recently a new taxon of geophilic dermatophytes was established as *Microsporum appendiculatum* Bhat and Mariam, based on the presence of appendaged macroconidia. However, such appendages are already known in the related species *Microsporum gypseum*. We conducted a survey of soil in central India as a part of a microbial biodiversity project and obtained two strains of *M. gypseum* with appendaged macroconidia. Using phenotypical characterization in combination with sequencing and restriction fragment length polymorphism (RFLP) of the Internal Transcribed Spacer (ITS) region of rDNA, we found that all strains of appendaged species are identical. Therefore *M. appendiculatum* is regarded as a synonym of *M. gypseum*.

Introduction

During the isolation of geophilic, keratinophilic fungi from different localities in Madhya Pradesh, India we obtained two dermatophytes which were striking by the presence of long, thin appendages on some of the macroconidia. They were morphologically consistent with the recently described species *Microsporum appendiculatum* Bhat & Miriam (Miriam and Bhat 1997). This taxon was separated from the common soilborne dermatophyte *Microsporum gypseum* (Bodin) Guiart & Grigorakis exclusively on the basis of the presence of such appendages. The present study was undertaken to evaluate the validity of *M. appendiculatum* by the use of molecular in addition to morphological methods. Direct examination of the fungus was not possible because there exist no type culture of *M. appendiculatum* since the original authors were unable to culture the fungus in question. Moreover, the only existing type material (dried goat dung) was unavailable for study.

Materials and methods

Strains studied

Three hundred and sixty-five soil samples were collected (as a part of a microbial diversity project of the Department of Biotechnology [DBT], Govt of India) in sealed polythene bags from various

districts of Madhya Pradesh and Chattisgarh with sterile spatula and brought to the laboratory for processing. Keratinophilic fungi were selectively isolated with a hair-baiting procedure (Ajello and Padhye 1974), with the following modification. Sterile distilled water containing 250 mg/l chloramphenicol was added to the hair-baited plate instead of physiological saline. Plates were incubated for 3 weeks at 28 °C in the dark. Two strains with appendaged macroconidia (FGCC K215 and FGCC K282) and an Indian isolate without appendages (FGCC K250) were deposited in the Mycological Herbarium, Department of Biological Sciences, Rani Durgavati University, Jabalpur. The neotype strain of *Arthroderma gypseum* CBS 258.61 and the type strain of *Arthroderma incurvatum* CBS 174.64 (*M. gypseum* is the anamorphic species of both teleomorphs) were used as reference strains.

Microscopy

Observations were made in lactophenol cotton blue mounts using a Nikon Eclipse E800 microscope.

DNA extraction

Fungal strains were maintained on Sabouraud's dextrose agar (SDA) slants. DNA was extracted from cultures grown on SDA plates for 2–3 weeks at room temperature, following the procedure described by Gräser et al. (1999).

PCR and RFLP analysis

For ITS-PCR the universal primers LSU266 (5′-gca ttc cca aac aac tcg act c) and V9D (5′-tta cgt ccc tgc cct ttg ta) amplifying a DNA fragment of about 1000 bp of the gene was used (de Hoog and Gerrits van den Ende 1998). The PCR mixture contained reaction buffer (10 mM Tris-HCl pH 8.0–50 mM KCl–1.5 mM $MgCl_2$), 200 μM of each deoxynucleoside triphosphates (Amersham Pharmacia Bioteh Inc., Piscataway, NJ, USA), 50 pmol each of primers, 2 U of Taq polymerase (AmpliTaq, Applied Biosystems), 10% Dimethylsulfoxide (DMSO) and 50 ng of template DNA. Samples were overlaid with sterile light mineral oil and amplified through 30 cycles in a thermocycler (Perkin Elmer 9600) as follows: initial denaturation for 5 min at 95 °C, denaturation for 1 min at

Table 1. Strains of fungi used in the present study

Strain	Reference #	Status	Source	Accession #
Arthroderma species				
A. borellii (*M. amazonicum*)	CBS 967.68	ST	Hair of spiny rat	AJ877220
A. cajetanum (*M. cookei*)	CBS 228.58	AUT	–	AJ970145
A. cookiellum (*M.* anamorph)	CBS 101.83	HT; MT–	Soil	AM000034
A. corniculatum (*M.* anamorph)	CBS 364.81	HT; MT+	Soil	AJ970143
A. fulvum (*M. fulvum*)	CBS 287.55	T	Human	AJ000627
A. gloriae (*Trichophyton gloriae*)	CBS 663.77	MT–	Soil	AJ877209
A. grubyi (*M. gallinae*)	CBS 243.66	T; MT+	Dog	AJ000612
A. gypseum (*M. gypseum*)	CBS 258.61	NT	Soil	AJ970141
A. gypseum (*M. gypseum*)	FGCC K250	–	Soil	AJ970150
A. gypseum (= *M. appendiculatum*)	FGCC K282	–	Soil	AJ970151
A. incurvatum (*M. gypseum*)	CBS 174.64	T	Human	AJ970153
A. obtusum (*M. nanum*)	CBS 322.61	T; MT–	Human	AJ970149
A. racemosum (*M. racemosum*)	CBS 424.74	–	Soil	AJ970146
A. persicolor (*M. persicolor*)	CBS 468.74	MT–	–	AJ000615
Anamorphic species				
M. duboisii	CBS 349.49	T	Human	AJ970142
M. gallinae (= *M. vanbreuseghemii*)	CBS 300.52	–	–	AJ000620
M. praecox	CBS 288.55	AUT	Human	AJ970148
M. fulvum (= *M. ripariae*)	CBS 529.71	T	Bird nest	AM000035

AUT – authentic strain; HT – holotype strain; MT – mating type strain; NT – neotype strain; ST – syntype strain; T – type strain; CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; FGCC – Fungal Germplam Collection Centre, Department of Biological Sciences, RD University, Jabalpur, India.

95 °C, annealing for 1 min at 55 °C and extension for 1 min at 72 °C. This was followed by final extension step for 1 min at 72 °C. Restriction enzyme analysis of the PCR products was performed using *Mva*I. Resulting fragments were electrophoresed through 2% MetaPhor agarose gels (BioWhitaker Molecular Applications Inc, Rockland Mine, USA) for 2.5 h at 100 V. As reference strains, *A. gypseum* and *A. incurvatum*, were used for comparisons.

Sequencing

PCR products were cleaned with QIAquick PCR purification kit (Qiagen GmbH, Germany) and sequenced using internal primers ITS4 and ITS5 (White et al. 1996) on an automated sequencing system (Beckman-Coulter, Fullerton, U.S.A.).

Alignment and tree construction

Sequences of close related dermatophyte species (Table 1) were aligned using CLUSTAL X (DKFZ, Heidelberg, Germany). Phylogenetic analysis was performed by Parsimony using PAUP (v. 4.0b10) (Swofford 2000) under the Kimura 2 parameter model. The robustness of the branches was assessed by bootstrap analysis with 1000

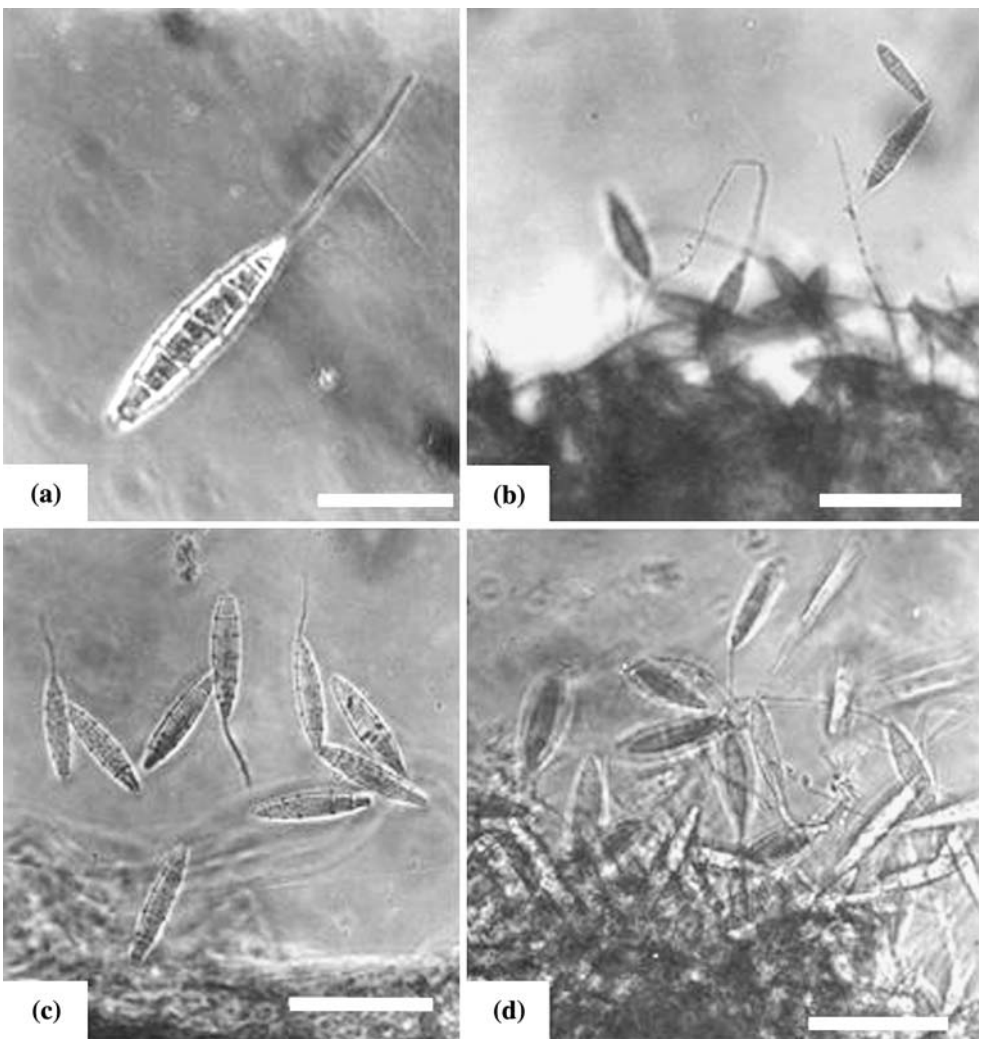

Figure 1. (a) *Microsporum gypseum*: One appendaged macroconidium (PH) (400×). bar = 30 µm. (c) Macroconidia with and without appendages (PH) (200×), bar = 60 µm. (b and d) Long macroconidial appendages tangled together at the periphery of the macroconidial cluster (200×), bar = 60 µm.

replicates. *Trichophyton gloriae* (AJ877209) was used as an outgroup.

Results

Six of the samples analyzed yielded isolates that were unambiguously identified as *Microsporum gypseum* on the basis of morphology and molecular tools. Incubation of hair-baited plates at 28 °C in the dark for more than 2 months resulted in desiccation of soil and fungal growth on hair. The hair was then almost completely digested by the fungus. Strains provisionally identified as *M. gypseum* revealed clusters of 3–8 celled macroconidia which were fusiform, echinulate, $25-60 \times 8.5-15\,\mu m$. Two of the strains isolated from remote sample locations, viz. RS/S/215 (burrow soil near Bandhavgarh National Park, M.P., India, 16.VI.2001) and RS/S/282 (public place, Padhar, M.P., India, 19.I.2002), had macroconidia mostly bearing aseptate, unbranched terminal appendages 45–120 µm in length and 1.0–1.5 µm thick (Figure 1a–c).

PCR-amplification of genomic DNA of *M. gypseum* CBS 258.61, of an Indian *M. gypseum* soil isolate, and of the appendage-forming strain FGCC K282 with primer pair V9D-LSU266 resulted in a nearly 1000 bp amplicon. Restriction digestion of these amplicons revealed identical banding patterns (Figure 2). The complete ITS1, ITS2 and 5.8S region of the appendage-forming isolate was subsequently compared with 17 sequences of strains of closely related geophilic *Arthroderma* species with *Microsporum* anamorphs and two geophilic anamorphic *Microsporum* species, with a geophilic *Trichophyton* species as outgroup (Table 1, Figure 3). A tree was constructed by PAUP 4.0b10 with Parsimony analysis using the heuristic search option. The appendaged isolate (FGCC K282) was 100% identical to an Indian isolate of *M. gypseum* (without appendages; FGCC K250) and about 99% identical with the ex-neotype strain of *A. gypseum* (CBS 258.61). The bootstrap value of the cluster was 100% (Figure 3).

Discussion

M. gypseum strains occasionally have appendaged macroconidia (Vanbreuseghem 1951; Stockdale 1963; El-Ani 1969). Miriam and Bhat (1997) recently introduced a separate species, *M. appendiculatum* based only on the presence of macroconidial appendages for such isolates. Macroconidia were described as 4–6-septate, fusiform, echinulate, 10–60 µm × 10–15 µm, which is consistent with those of *M. gypseum* (de Hoog et al. 2000). Unfortunately no type culture of *M. appendiculatum* exists because the original authors were unable to culture the fungus. Moreover the only type material available (Herb. GUFH No. 010, on partially decomposed goat dung) was not sent upon request. Judging from the original illustrations, macroconidia of *M. appendiculatum* lack appendages as long as they are attached to the conidiophores. A particular percentage of macroconidia develops appendages after dehiscence. El-Ani (1969) also noted that the appendages in strains ascribed to *M. gypseum* are formed only after the macroconidia become liberated from the conidiophores. The dimensions of appendages were also comparable. These phenomena were also observed in our appendaged strains. We therefore consider all strains with appendages on macroconidia to be

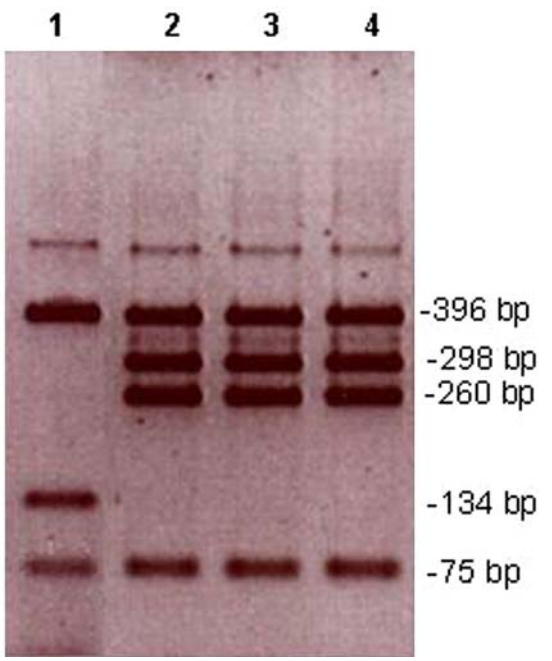

Figure 2. PCR-RFLP patterns of strains of *M. gypseum* from Central India in comparison to reference strains. Lane 1, *Arthroderma incurvatum* CBS174.64; Lane 2, *A. gypseum* CBS258.61; Lane 3, *M. gypseum* FGCCK282 (with appendages); Lane 4, *M. gpseum* FGCCK250 (no appendages).

consistent with the description of *M. appendiculatum*. The function of appendages apparently is to aggregate the macroconidia (Figure 1b, d), preventing excessive desiccation after detachment. El-Ani (1969) supposed enhancement of conidial mass dispersal.

PCR-RFLP profiles of ITS rDNA domains of strains with and without appendages were identical to that of the reference strain CBS 258.61 of *A. gypseum* which is also a macroconidial appendage forming strain studied by Stockdale (1963). The second known teleomorph of *M. gypseum, A. incurvatum*, proved to be different (Figure 2). This was confirmed after ITS sequence comparison of FGCC K282 (appendage-forming strain) with related *Microsporum* species and teleomorphs (Figures 3). The strain clustered with *M. gypseum* at 100% similarity with the non-appendaged Indian strain of *M. gypseum* (FGCC K250) and at nearly 99% with the ex-neotype strain (CBS 258.61) of *Arthroderma gypseum* (Figure 3). This provides convincing evidence that appendaged strains should be referred to *Microsporum gypseum*, and that *M. appendiculatum* should be reduced to synonymy.

In general, sexually reproducing species of geophilic *Microsporum* and *Trichophyton* clades, inclusive of asexual species (e.g., *Microsporum*

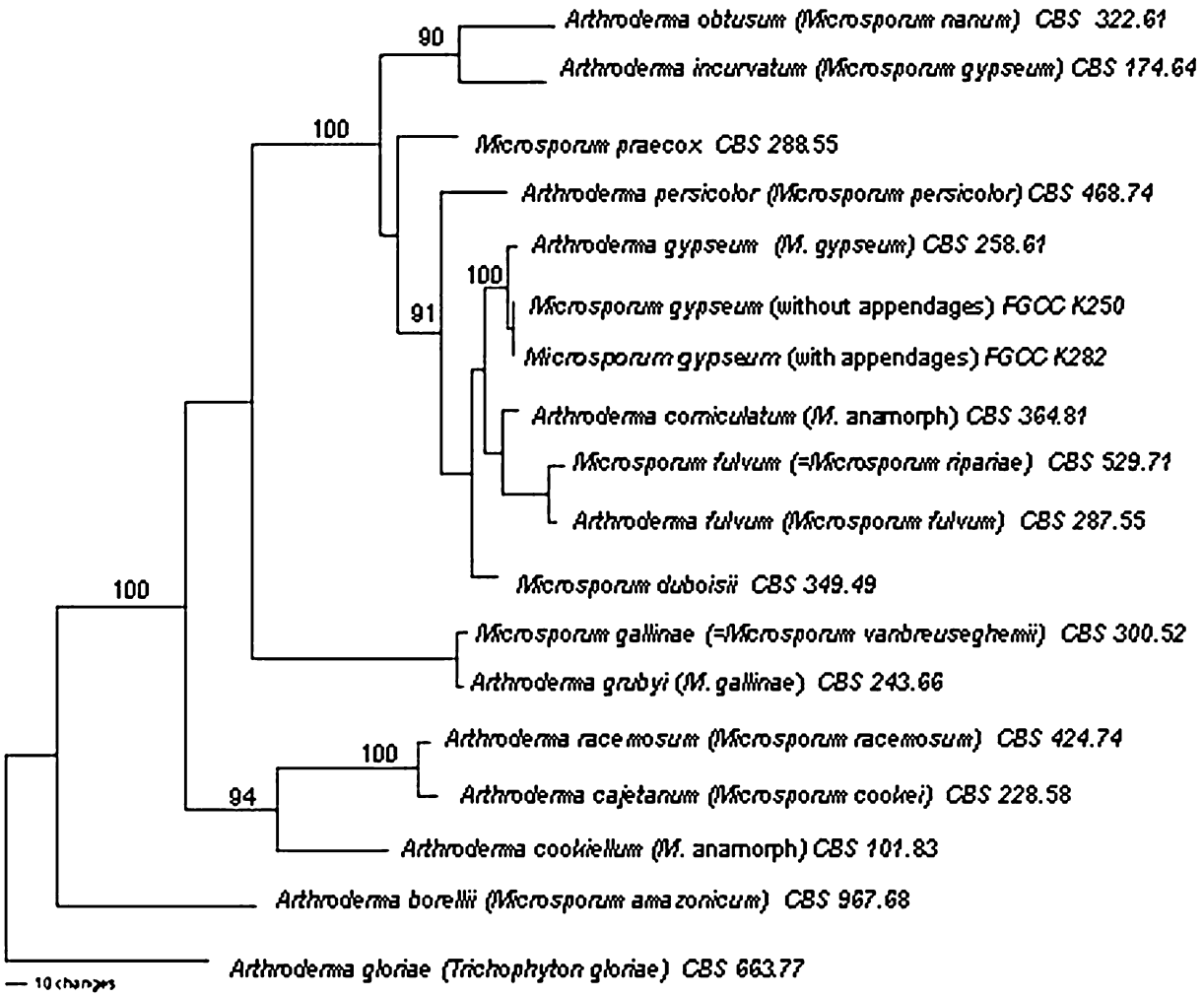

Figure 3. Phylogenetic relationship of the appendaged isolate compared to 14 closely related geophilic dermatophyte species. *A. gloriae* was used as outgroup. The phylogenetic tree was obtained using ITS rDNA sequences and Parsimony algorithm with Kimura correction. Bootstrap values above 90% are shown. Two hundred ninety nine out of the 663 characters included were parsimony informative. The tree was 988 steps. The consistency index was 0.68.

praecox, Microsporum duboisii (Figure 3), *Trichophyton thuringiense* and *Trichophyton phaseoliforme*; Gräser et al. 2000; Brasch and Gräser 2005) are at least 3% distant from each other. The only exceptions are *Arthroderma racemosum* and *Arthroderma cajetanum*, which have more than 97% ITS similarity to each other. The synonymy of the geophilic dermatophyte *Microsporum ripariae* [Hubalek] to *Microsporum fulvum* was confirmed, the ITS sequence of the ex-type strain, CBS 529.71, being highly similar (98%) to that of the morphologically similar *Microsporum fulvum* (Gräser et al. 2000). The ITS sequences of *Microsporum gallinae* and *Microsporum vanbreuseghemii* were found to be 100% identical, contradicting the apparently profound morphological and mating-competence divergences between these fungi (Gordon and Little 1968). *Microsporum ripariae, Microsporum gallinae* and *Microsporum appendiculatum* all showed more than 97% similarity to their respective nearest neighbors.

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