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SHORT COMMUNICATION

Identification of *Scopulariopsis* Species by Partial 28S rRNA Gene Sequence Analysis

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Abstract

The genus *Scopulariopsis* contains over 30 species of mitosporic moulds, which although usually saprophytic may also act as opportunistic pathogens in humans. They have mainly been associated with onychomycosis, and only sporadically reported as a cause of deep tissue infections or systemic disease. Identification of *Scopulariopsis* species still largely relies on phenotype-based methods. There is a need for a molecular diagnostic approach, that would allow to reliably discriminate between different *Scopulariopsis* species. The aim of this study was to apply sequence analysis of partial 28S rRNA gene for species identification of *Scopulariopsis* clinical isolates. Although the method employed did reveal some genetic polymorphism among *Scopulariopsis* isolates tested, it was not enough for species delineation. For this to be achieved, other genetic loci, within and beyond the rDNA operon, need to be investigated.

Key words: *Scopulariopsis* sp., molecular diagnostics, mycosis, sequence analysis, species identification

The genus *Scopulariopsis* accommodates more than 30 species of mitosporic moulds, whose natural habitat is the soil, where they live as saprophytes and are involved in the decay of organic matter. However, some members of the *Scopulariopsis* genus may cause opportunistic infections in humans. Eight species (*S. acremonium*, *S. asperula*, *S. flava*, *S. fusca*, *S. koningii*, *S. brevicaulis*, *S. brumptii*, and *S. candida*) have been reported as causing human diseases. Superficial skin affections, and onychomycosis in particular, are the most predominant clinical manifestations (de Hoog *et al*., 2000). Much rarer are subcutaneous, deep tissue and disseminated infections, most of which occur in immunocompromised individuals and are associated with high mortality (Isidro *et al*., 2006; Beltrame *et al*., 2009; Salmon *et al*., 2010). Treatment of *Scopulariopsis* infections is difficult and usually empirically-based, one reason for this being resistance of *Scopulariopsis* spp. to a broad spectrum of antifungal agents (Cuenca-Estrella *et al*., 2003; Skóra and Macura, 2011).

At present, identification of *Scopulariopsis* species largely relies on the phenotype-based methods, employing both morphological and biochemical criteria. These methods however often produce misleading results. They are also laborious and time consuming, as the fungus has to be obtained in pure culture. Therefore, the use of molecular diagnostic methods is desired, since they provide rapid, unambiguous and highly specific identification of fungal pathogens. However, there are very little data on the molecular diagnostics of pathogenic *Scopulariopsis* spp. in the literature. No information about molecular methods allowing inter- and intra-species differentiation exists.

The purpose of this study was to apply sequence analysis of a fragment of the 28S rRNA gene for the species-level identification of *Scopulariopsis* clinical isolates.

A collection of 44 *Scopulariopsis* sp. isolates was recruited. Within this number, there were 40 clinical isolates of *Scopulariopsis* sp. and 4 reference strains representing *S. brevicaulis* (CBS 112377, CBS 119549, CBS 147.41, CBS 398.54). All clinical isolates (each isolate represented a single patient) were recovered over a 6-year period (2006–2012) from patient skin and nail samples in the Department of Mycology, Chair of Microbiology, Collegium Medicum, Jagiellonian University. The isolates were cultured on potato dextrose agar (PDA; Difco) slants and preliminary species identification was accomplished by conventional morphological analysis including macroscopic and microscopic characteristics (de Hoog *et al*., 2000; Larone, 2002).

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The fungal DNA was extracted using the Fungi&Plant DNA Isolation Kit (EURx, Poland), after the mycelium had been ground to a fine powder in liquid nitrogen. The DNA concentration was measured with the NanoDrop®ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Molecular species identification was performed using a PCR-sequencing assay, as originally described by Monod *et al.* (2006), with some modifications. Briefly, a part of the 28S rDNA was amplified by PCR with universal primers designated LSU1 (5'-GATAGCGMACAAGTAGAGTG-3') and LSU2 (5'-GTCCGTGTTTCAAGACGGG-3'). The reaction mixtures were prepared by using the TopTaq Master Mix kit (QIAGEN, Germany) in a total volume of 25 µl, containing 2 × TopTaq Master Mix (final conc. 1 ×), 0.4 µM each primer, and 1 µl (*ca.* 10–20 ng) of template DNA. The thermal cycling profile was 94°C for 1 min, 30 cycles at 94°C for 30 sec., 58°C for 30 sec., and 72°C for 30 sec., and a final step at 72°C for 10 min. The PCR products were resolved by electrophoresis using 3% agarose gels and were photographed under UV light after ethidium bromide staining. For sequencing, the PCR products were purified using the Clean-Up kit (A&A Biotechnology, Poland). Sequencing was performed on both strands with the primers described above by means of the BigDye ver. 3.1 Terminator Kit (Applied Biosystems) and capillary electrophoresis system ABI 3130xl Genetic Analyzer (Applied Biosystems; Genomed, Warsaw, Poland). Forward and reverse sequences were assembled and edited with ChromasPro ver. 1.7.1 (Technelysium, Australia) and the resulting consensus sequences were searched against the GenBank database of the National Center for Biotechnology Information (NCBI) using the BLASTN algorithm

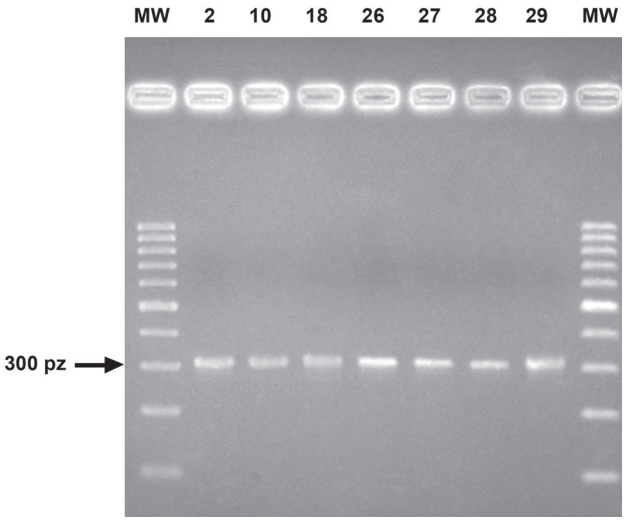

Fig. 1. Results of PCR amplification of a partial 28S rRNA gene for seven selected *Scopulariopsis* strains. MW, molecular weight marker (DirectLoad™ PCR 100-bp Low Ladder, Sigma).
Numbers above the gel lines refer to the strain numbers.

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A     GATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGAAAAGAG
B     GATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGAAAAGAG
      **************************************************

A     AGTTAAAAAGCACGTGAAATTGTTAAAAGGGAAGCGCTTGCGACCAGACT
B     AGTTAAAAAGCACGTGAAATTGTTAAAAGGGAAGCGCTTGCGACCAGACT
      **************************************************

A     TGCGCCCGTCGGATCAACCGTCGCTTGCGGCGGCGCACTCCGGCGGGCTC
B     TGCGCCCGTCGGATCAACCGTCGCTTGCGGCGGCGCACTCCGGCGGGCTC
      **************************************************

A     AGGCCAGCATCAGTTCGTCCGGGGGGGAGAAAGGCGGCGGGAATGTGGCTC
B     AGGCCAGCATCAGTTCGCTCGGGGGGGAGAAAGGCGGCGGGAATGTAGCTC
      ***************  ************************* ****

A     TTCGGAGTGTTATAGCCCGCCGTGTAATACCCTCGGGTGGACTGAGGACC
B     CTCGGAGTGTTATAGCCCGCCGTGTAATACCCTCGGGTGGACTGAGGACC
        **************************************************

A     GCGCGTATGCAAGGATGCTGGCGTAATGGTCGTCAGCGACCCGTCTTGAA
B     GCGCGTATGCAAGGATGCTGGCGTAATGGTCGTCAGCGACCCGTCTTGAA
      **************************************************

A     ACACGGAC
B     ACACGGAC
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Fig. 2. A comparison of two types of sequences ("A" and "B") obtained through direct sequencing of a 308-bp fragment of the 28S rRNA gene.
Nucleotide differences between the two sequences are highlighted in grey.

(http://blast.ncbi.nlm.nih.gov/). Sequence identities of ≥ 99% were used for species identification.

For all the strains tested, a single PCR amplification product of the expected length (308 bp) was obtained (Fig. 1). Two types of sequences of the targeted locus, designated "A" and "B" were detected in 43 (including all four reference strains) and one isolates, respectively. These two sequences differed only at four nucleotide positions: 168 (T or C), 169 (C or T), 196 (G or A), and 201 (T or C) (Fig. 2). A comparative analysis of the sequences obtained with those deposited in the GenBank database revealed that sequence "A" was identical (100% match) with the GenBank sequences derived from 14 strains of *S. brevicaulis* and 4 strains of *Microascus manginii* (teleomorph of *Scopulariopsis candida*). Whereas, sequence "B" was found identical to that of *S. fusca* (10 strains), *S. asperula* (one strain), and *M. manginii* (3 strains) from the GanBank database (Table I).

The results from this study did confirm that the analysed strains belong to the *Scopulariopsis* genus, yet they did not allow to precisely assign the strains to the species level. All but one *Scopulariopsis* strains tested had a partial 28S rDNA sequence identical with that of *S. brevicaulis* and *M. manginii*. Given the fact, that all four reference strains of *S. brevicaulis* were within this group, and that *M. manginii* has not been associated with human infection (in the literature, only one case of an invasive sinonasal infection caused by *S. candida*, the anamorphic form of *M. manginii*, was described in a young female non-Hodgkin's lymphoma patient (Kriesel *et al.*, 1994)), it can be assumed that these strains truly represent the *S. brevicaulis* species. As for one strain, whose partial 28S rDNA sequence

Table I
Comparison of the obtained sequences with the GenBank database

Sequence type[a]	GenBank no.	Strain[b]	Sequence homology
A (1–17; 19–44)	JQ434664.1	*Scopulariopsis brevicaulis* CBS 334.35	100%
	JQ434663.1	*Scopulariopsis brevicaulis* CBS 152.22	
	JQ434662.1	*Scopulariopsis brevicaulis* CBS 119549	
	JQ434661.1	*Scopulariopsis brevicaulis* DTO 145A5	
	JQ434660.1	*Scopulariopsis brevicaulis* DTO 145A3	
	JQ434659.1	*Scopulariopsis brevicaulis* DTO 141I3	
	JQ434658.1	*Scopulariopsis brevicaulis* DTO 145A4	
	JQ434657.1	*Scopulariopsis brevicaulis* LCP 70.3042	
	JQ434656.1	*Scopulariopsis brevicaulis* LCP 83.3363	
	JQ434655.1	*Scopulariopsis brevicaulis* LCP 00.672	
	JQ434654.1	*Scopulariopsis brevicaulis* DTO 148H5	
	JQ434653.1	*Scopulariopsis brevicaulis* DTO 145A2	
	JQ434652.1	*Scopulariopsis brevicaulis* DTO 141I8	
	JN938873.1	*Scopulariopsis brevicaulis* DAOM 225612	
	JQ434650.1	*Microascus manginii* DTO 2B3	
	JQ434649.1	*Microascus manginii* DTO 145B3	
	JQ434648.1	*Microascus manginii* CBS 399.34	
	JQ434651.1	*Microascus manginii* CBS 206.61	
B (18)	JQ434678.1	*Scopulariopsis fusca* CBS 114063	100%
	JQ434677.1	*Scopulariopsis fusca* CBS 289.38	
	JQ434676.1	*Scopulariopsis fusca* CBS 334.53	
	JQ434675.1	*Scopulariopsis fusca* CBS 401.34	
	JQ434674.1	*Scopulariopsis fusca* CBS 668.74	
	JQ434673.1	*Scopulariopsis fusca* CBS 872.68	
	JQ434672.1	*Scopulariopsis fusca* DTO 145A8	
	JQ434671.1	*Scopulariopsis fusca* DTO 145A6	
	JQ434670.1	*Scopulariopsis fusca* LCP 65.742	
	JN938872.1	*Scopulariopsis fusca* DAOM 216340	
	JQ434668.1	*Microascus manginii* MUCL 31465	
	JQ434667.1	*Microascus manginii* MUCL 31574	
	JN938874.1	*Microascus manginii* DAOM 225614	
	JQ434669.1	*Scopulariopsis asperula* CBS 853.68	

[a] Two types of a partial 28S rDNA sequence, designated "A" and "B", found among the 44 *Scopulariopsis* strains tested in this study. Numbers in brackets refer to the numbers of the strains analysed;
[b] Strains, whose partial 28S rDNA sequence were deposited to the GenBank database under the accession number given in the preceding column (GenBank no.).

was found identical to that of *S. fusca*, *S. asperula*, and *M. manginii*, its species identity can only be surmised from the frequencies of perfect sequence alignments. Consequently, the strain could be recognized as *S. fusca*, since a sequence match with that species was most common. Both *S. fusc*a and *S. asperula* have been described, albeit rarely, as the causative pathogens of human onychomycosis (de Hoog *et al.*, 2000).

The results of this study show a highly conserved nature of the large-subunit ribosomal DNA sequence. The high homology of the analysed partial 28S rDNA sequence among different *Scopulariopsis* species precludes its use as a marker for species differentiation. Identification of fungal strains as representing *S. brevicaulis*, based solely on the results from the method applied in this study (Monod *et al.*, 2006; Bontems *et al.*, 2009), is erroneous and is caused by an *a priori* assumption that *Scopulariopsis* strains found in dermatological material most probably are *S. brevicaulis*. However, the latter species is indeed the most common *Scopulariopsis* species observed clinically.

To conclude, to the best of authors' knowledge this is the first report on the molecular identification of *Scopulariopsis* species from Poland. Sequence analysis of partial

28S rDNA, as applied in this study, did show some genetic polymorphism among *Scopulariopsis* strains, but it was not enough, however, for species delineation. For this to be achieved, other genetic loci, not only within the rDNA operon, but also protein-coding genes, such as RPB, TUBB, ATP6, and TEF1α, coding for RNA polymerase, β-tubulin, ATP synthase, and elongation factor EF-1α, respectively, need to be investigated.

There is still a need for a new, diagnostic approach, that would be able to reliably discriminate between *Scopulariopsis* species. The present study only opens the field of research in this direction.

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