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MULTILOCUS SEQUENCE TYPING OF *SCEDOSPORIUM APIOSPERMUM* AND *PSEUDALLESCHERIA BOYDII* ISOLATES FROM CYSTIC FIBROSIS PATIENTS [§]

Running title: MLST of the *P. boydii* complex from CF patients

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Keywords: *Pseudallescheria boydii*; *Scedosporium apiospermum*; cystic fibrosis; MLST; genotyping

Abstract

Background: Scedosporium and *Pseudallescheria* species are the second most common lung-colonizing fungi in cystic fibrosis (CF) patients. For epidemiological reasons it is important to trace sources of infection, routes of transmission and to determine whether these fungi are transient or permanent colonizers of the respiratory tract. Molecular typing methods like multilocus sequence typing (MLST) help provide this data.

Methods: Clinical isolates of the *P. boydii* complex (including *S. apiospermum* and *P. boydii*) from CF patients in different regions of Germany were studied using MLST. Five gene loci, *ACT*, *CAL*, *RPB2*, *BT2* and *SOD2*, were analysed.

Results: The *S. apiospermum* isolates from 34 patients were assigned to 32 sequence types (ST), and the *P. boydii* isolates from 14 patients to eight ST. The results revealed that patients can be colonized by individual strains for years.

Conclusions: The MLST scheme developed for *S. apiospermum* and *P. boydii* is a highly effective tool for epidemiologic studies worldwide. The MLST data are accessible at mlst.mycologylab.org.

Introduction

In CF patients, respiratory function becomes increasingly affected by fungi during the course of the underlying disease. *Aspergillus fumigatus* is the mould found most frequently and is associated with allergic bronchopulmonary aspergillosis or causes invasive mycosis, especially after organ transplantation. Fungi of the *Pseudallescheria*/*Scedosporium* complex rank second among colonizing hyphomycetes (2-4). This complex includes *S. apiospermum*, *P. boydii*, *S. aurantiacum*, *P. minutispora, S. dehoogii* and the distinct species *S. prolificans.*

The percentage of the respective isolates from CF patients sent to the reference laboratory at the Robert Koch-Institute (RKI) between 1995 and 2010 was 49.4 %, 23.5 %, 9.4 %, 2.4 % and 15.3 %. *S. dehoogii* was not found in clinical samples at all. This complex of hyphomycetes is of special importance because of diagnostic difficulties and a higher resistance to antifungal agents compared to *A. fumigatus*. How patients acquire these fungi is still unclear, especially because *Scedosporium* species are hardly ever detected in indoor air but are commonly found in polluted water and soil (5-7). Some authors reported that CF patients can be chronically colonized by *S. apiospermum* (8-10). Nevertheless, the following issues are as yet unclear: (i) the clinical relevance, (ii) whether patients are colonized by one or more strains, and (iii) the effect of antimycotic treatment on the colonisation with these pathogens. For epidemiological studies it is necessary to exactly identify strains on a subspecies level. MLST has proved to give highly reproducible genotyping results that are comparable worldwide (11-14). Therefore we decided to use MLST in studying *S. apiospermum* and *P. boydii* as the most frequently isolated species in the CF context within the *S. apiospermum* species complex in Germany.

Materials and methods

Fungal isolates

Clinical samples from the respiratory tract of CF patients were cultivated at CF centres from geographically diverse locations in Germany. Phenotypically distinct isolates preliminarily identified as *Pseudallescheria* or *Scedosporium* species were sent to the reference laboratory. Re-identification was performed by sequencing of the internal transcribed spacer (ITS) region of the rDNA (15). The study included all *S. apiospermum* and *P. boydii* isolates received continuously between 2009 and 2010. The number of isolates was increased by including previous isolates from these patients and other previously archived CF isolates, amounting to

a total of 115. Forty-seven isolates from 14 patients were identified as *P. boydii* (Table 1) and 68 isolates from 34 patients as *S. apiospermum* (Table 2). Patients were between four and 41 years of age at the time of the first isolation of the fungus, as documented at the CF centre currently treating the patient; on average, females were 18.9 years old and males 21.3. There was no significant difference between patients with a homozygote and heterozygote mutation of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene and the mean age at the time of the first isolation of the *S. apiospermum* species complex.

Two reference strains for *P. boydii* (CBS 101.22^{T} and CBS 418.73) and three reference strains for *S. apiospermum* (CBS 100392, CBS 100395 and CBS 117407^{T}) were included in the study.

The isolates were stored at -70 °C in Microbank™ tubes (Pro-Lab Diagnostics, Bromborough, U.K.). Before DNA extraction the isolates were grown on Potato Dextrose Agar (PDA) for seven days at 37 °C.

DNA extraction

DNA extraction and purification were performed with the FFPE Tissue LEV DNA Purification Kit designed for the Maxwell® 16 Instrument (Promega, Mannheim, Germany) with minor modification of the manufacturer's protocol. DNA was purified directly from fungal cultures. An inoculating loop of mycelium was suspended in 300 µl of lysis buffer and incubated with shaking at 70 °C for 10 min. The final DNA extraction volume was 120 µl of bidest water.

PCR amplification, sequencing and MLST analysis

Adapted from earlier genotyping studies (16-24), PCR amplification with different primer pairs was attempted for five isolates from each species (*S. apiospermum, P. boydii*) for the following genes: *actin* (*ACT*), *calmodulin* (*CAL*, exon 3-4), *second largest subunit of RNA polymerase II gene* (*RPB2*), *β-tubulin* (*BT2,* exon 2-4), *manganese superoxide dismutase*

(*SOD2*) and *elongation factor 1 alpha* (*EF1α*). To optimise the amplification of *calmodulin* in *S. apiospermum* and *P. boydii* isolates, one primer pair was newly designed based on sequences available from the NCBI website.

The PCR assay (25 µl) included 1 µl of fungal DNA extract, 1 µM of each gene-specific primer (Table 3, TIB MOLBIOL, Berlin, Germany), 0.2 mM of each deoxynucleoside triphosphate (Roche, Mannheim, Germany), 1 x BioTherm™ PCR buffer with 1.5 mM of $MgCL_2$ (Rapidozym, Berlin, Germany) and 1.25 U BioTherm™ *Taq* DNA polymerase (Rapidozym, Berlin, Germany). For *SOD2* a *Pfu* DNA polymerase and 1 × *Pfu* buffer with 2 mM of $MgSO_4$ (Fermentas, St. Leon-Rot, Germany) were used for the PCR reaction.

The amplification for all targeted genes was performed in a T1-Thermocycler (Biometra, Göttingen, Germany) as follows: 5 min of initial denaturation at 95 °C, followed by 35 cycles at 95 °C for 30 sec, gene-specific annealing temperature for 30 sec (Table 3) and 72 °C for 1 min (for *RPB2* the annealing time was 2.5 min). The final extension step was 7 min at 72 °C. The amplification products were electrophoretically resolved on a 1.4 % agarose TBE gel (0.089 M Tris base, 0.089 M boric acid and 0.002 M EDTA) including GelRed™ (0.83 x, Biotium, Hayward, CA, USA) and visualized by UV transillumination (BioDocAnalyze, Biometra, Göttingen, Germany). The products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). For the sequencing of both strands the amplification primers were used. Sequencing was performed according to the BigDye® Termination v3.1 Cycle Sequencing Kit, and the reactions were run on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). Sequencing analysis was performed using the SeqMan pro (Lasergene DNASTAR version 8.1.5) and the BioEdit sequence Alignment Editor (version 7.0.9.0). The sequences were trimmed; starting and endpoints are defined in Table 4. Each variable sequence was classified as a unique allele type. The combination of alleles at each locus defined the sequence type (#ST). All alleles of

each locus were BLAST searched against the NCBI GenBank to prove the specificity of the amplified product.

The data was submitted to the MLST database for pathogenic fungi (http://mlst.mycologylab.org/).

Results

More than one allele type was found for the ITS region of rDNA in both species; however, this was not included in the MLST scheme because it did not provide new information on the ST. For all isolates studied sequence data were obtained for the five MLST loci *ACT*, *CAL*, *RPB2*, *BT2* and *SOD2*. Amplification or sequencing of *EF1α* failed with the primer pairs studied. In total, for the five gene fragments 3,375 bp were analysed. The allele types of each gene locus were clearly distinct between *P. boydii* and *S. apiospermum*.

In *P. boydii* 128 (3.8 %) polymorphic sites were identified among the five gene loci, whereas in *S. apiospermum* 92 (2.7 %) variable nucleotides were found (Table 4). In *P. boydii* analysis of the loci *ACT* and *RPB2* resulted in four alleles, of *CAL* and *BT2* in five and of *SOD2* in six allele types. The variability of nucleotides per locus ranged between 1.6 % (*CAL*) and 14.6 % (*SOD2*). In comparison to *P. boydii*, twelve alleles were observed for the *SOD2* locus in *S. apiospermum*. For the other loci in *S. apiospermum* four to eight alleles were identified. The lowest variability of nucleotides was noted in *CAL* and *ACT* (1.4 %), and the widest one in *SOD2* (6.3 %). Altogether, eight different ST were obtained by combining the five MLST loci in *P. boydii* isolates from 14 patients, while 32 distinct ST were found in the *S. apiospermum* isolates from 34 patients (Table 1 and 2). Twenty-seven out of 34 patients (79.4 %) with *S. apiospermum* were colonized by one or two individual ST. The ST #S1, #S6 and #S14 were shared by two patients, and the sequence type #S31 was found in three patients. The three reference strains CBS 100392, CBS 100395 and CBS 117407^{T} had an

individual ST (#S33 to #S35). In contrast to the high genetic diversity of *S. apiospermum* isolates, only five out of 14 patients harbouring *P. boydii* had a unique ST. The ST #P1 was identified in four, #P7 in three and #P3 in two patients. The type strain CBS 101.22^{T} had a ST distinct from the other *P. boydii* isolates (#P9), but the ST of CBS 418.73 (#P6) was identified also in patient 10. Interestingly, in this patient two different ST (#P1 and #P6) were observed over a nine year period. In five other patients (5, 13, 16, 35 and 38) a unique ST was found in consecutive *P. boydii* isolates from a period of up to ten years (Table 1). In patients colonized by *S. apiospermum* it was only possible to compare isolates from a period of up to three years. Twelve patients had a unique ST. Only in patients 34 and 28 two and three different ST were found, respectively (Table 2).

Discussion

While the life expectancy of patients with CF changed dramatically within the last decade (25), also the spectrum of microorganisms, including fungi, colonizing the respiratory tract became more diverse (7). Therefore, to interpret the clinical relevance of colonizing fungi like *Scedosporium*, reproducible methods for genotyping are required. The MLST scheme developed including five loci turned out to be highly reproducible and effective for genotyping of *P. boydii* and *S. apiospermum* sensu strictu. Again the results confirm the separation of the two species as proposed by Gilgado et al. (23). Primers for the locus *elongation factor 1α* (*EF1α*) which are recommended for molecular typing of *S. auranticaum* and *S. prolificans* (17, 24) turned out to be ineffective in *P. boydii* and *S. apiospermum*. Due to the high discriminatory power of the presented MLST scheme it might be unnecessary to study this gene locus.

The present study confirms the high genetic diversity within the species analysed as described before by a number of different methods (3, 10, 26). The distinction within *S. apiospermum* was higher than the one within *P. boydii*. This seems to conflict with a recent study on a

comparable collective of CF patients from France, where *P. boydii* is more frequently found as *S. apiospermum* and therefore possibly a question of strains studied (10). Nevertheless, nearly all patients studied were colonized by individual strains, five of which harboured two or more of the *P. boydii* complex and *S. prolificans* (data not shown). Mixed colonization with different ST was apparently stable since the ST was detected in sequential samples obtained over a time span of up to nine years. Our results confirm a previous study based on random amplification of polymorphic DNA (RAPD) (3), which found a high genetic diversity in *S. apiospermum* colonizing CF patients and documented the persistence of strains over a period of thirteen months. These authors found that different genotypes from the same patient were usually closely related. In contrast, our MLST data indicate that genotypes within the same patient can be clearly distinct and fungi can permanently colonize the respiratory tract. It is open for discussion whether an antifungal therapy could modify the colonization pattern. Distinct phenotypes regarding the resistance pattern of the identical species can colonize the same patient (26). The aim of longitudinal studies should be to correlate the MLST data with clinical data, including the resistance pattern of isolates as well as risk factors like diabetes mellitus, cortisone and antifungal treatment. A substantial progress would be to analyse the in vitro and in vivo resistance on a molecular level.

Considering the limited number of isolates, there was no striking geographic clustering of genotypes. Nevertheless, three patients with the same genotype of *S. apiospermum* (#S31) were found in Lower Saxony, and two other patients were harbouring an identical ST of *P. boydii* (#P1) in Hamburg. These patients were treated at the same CF centre. Therefore, although to our knowledge they have never met at the ambulatory, a transfer from one patient to another or an exposure to the same source of fungi cannot be excluded completely. To gain new insights on the routes of infection, *Scedosporium* species have to be identified in the surroundings of our patients. Our MLST scheme will allow an informative comparison of environmental with clinical isolates. The prevalence of *P. boydii* and *S. apiospermum* in

patients from European countries varies. While in a set of clinical and environmental strains from Austria, Germany and the Netherlands *S. apiospermum* was the most prevalent one, followed by *P. boydii* (27), in Northern Spain and France *P. boydii* represents the most prevalent species, followed by *S. apiospermum* (10, 26). It would be highly informative to analyse European isolates from different countries with a unique molecular method. The MLST scheme presented here will be a promising tool for further epidemiological studies on fungi belonging to the *P. boydii* complex in Europe and worldwide.

Conflict of interest statement

The authors report no conflicts of interest.

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Table 1

List of *P. boydii* isolates showing the allele types of ITS and the five MLST loci and sequence types (ST).

Table 2

List of *S. apiospermum* isolates showing the allele types of ITS and the five MLST loci and sequence types (ST).

Table 3

Primer oligonucleotides and annealing temperature for ITS and the MLST analysis.

Table 4

Variability between the gene loci.

Table 1

List of *P. boydii* isolates showing the allele types of ITS and the five MLST loci and sequence types (ST).

Patient no.	Age[§] [y]	Sex	CFTR genotype	CF center (federal state)	Isolation year/month	Source	RKI no.	ITS	*ACT*	*CAL*	*RPB2*	*BT2*	*SOD2*	ST
6	30	n.a.	unknown	O (NW)	2001 /07	Sputum	01-0715.01	P2	P1	P1	P1	P1	P1	#P1
47	26	f	delF508/delF508	M (NW)	2010 /09	n.d.	10-0570.01	P2	P1	P1	P1	P1	P1	#P1
				M (NW)	2010 /09*	Sputum	10-0616.01	P2	P1	P1	P1	P1	P1	#P1
				M (NW)	2010 /09*	Sputum	10-0616.02	P2	P1	P1	P1	P1	P1	#P1
38	4	m	delF508/-	P (HH)	2010 /01*	Throat swab	10-0143.01	P2	P1	P1	P1	P1	P1	#P1
				P (HH)	2010 /01*	Throat swab	10-0143.02	P2	P1	P1	P1	P1	P1	#P1
				P (HH)	2010 /01*	Throat swab	10-0144.01	P2	P1	P1	P1	P1	P1	#P1
				P (HH)	2010 /05~	Sputum	10-0343.01	P2	P1	P1	P1	P1	P1	#P1
				P (HH)	2010 /05~	Sputum	10-0344.01	P2	P1	P1	P1	P1	P1	#P1
				P (HH)	2010 /05~	Sputum	10-0345.01	P2	P1	P1	P1	P1	P1	#P1
37	16	f	delF508/-	P (HH)	2010 /01*	Sputum	10-0135.01	P2	P1	P1	P1	P1	P1	#P1
				P (HH)	2010 /01*	Sputum	10-0136.01	P2	P1	P1	P1	P1	P1	#P1
				P (HH)	2010 /02~	Sputum	10-0137.01	P2	P1	P1	P1	P1	P1	#P1
				P (HH)	2010 /02~	Sputum	10-0138.01	P2	P1	P1	P1	P1	P1	#P1
				P (HH)	2010 /03	Throat swab	10-0140.01	P2	P1	P1	P1	P1	P1	#P1
10	15	m	delF508/delF508	K (BY)	2001 /12	Sputum	02-0167.01	P2	P1	P1	P1	P1	P1	#P1
				K (BY)	2002 /01	Sputum	02-0188.01	P1	P2	P3	P3	P3	P5	#P6
				K (BY)	2002 /02	n.d.	02-0363.01	P2	P1	P1	P1	P1	P1	#P1
				K (BY)	2009 /02	Sputum	09-0160.01	P1	P2	P3	P3	P3	P5	#P6

Patient no.	Age[§] [y]	Sex	CFTR genotype	CF center (federal state)	Isolation year/month	Source	RKI no.	ITS	*ACT*	*CAL*	*RPB2*	*BT2*	*SOD2*	ST
				K (BY)	2009 /07	Sputum	09-0504.01	P2	P1	P1	P1	P1	P1	#P1
5	36	f	delF508/delF508	K (BY)	2001 /07*	Sputum	01-0710.01	P1	P1	P1	P1	P1	P2	#P2
				K (BY)	2001 /07*	Sputum	01-0711.01	P1	P1	P1	P1	P1	P2	#P2
				K (BY)	2001 /08	Sputum	01-0719.01	P1	P1	P1	P1	P1	P2	#P2
				K (BY)	2001 /09	Sputum	01-0830.01	P1	P1	P1	P1	P1	P2	#P2
				K (BY)	2009 /12	Sputum	09-0822.01	P1	P1	P1	P1	P1	P2	#P2
				K (BY)	2010 /02	Sputum	10-0165.01	P1	P1	P1	P1	P1	P2	#P2
35	20	m	delF508/delF508	F (LS)	2010 /01	Sputum	10-0049.01	P3	P1	P2	P2	P2	P3	#P3
				F (LS)	2010 /04	Sputum	10-0254.01	P3	P1	P2	P2	P2	P3	#P3
				F (LS)	2010 /09	Sputum	10-0594.01	P3	P1	P2	P2	P2	P3	#P3
13	14	m	delF508/delF508	F (LS)	2007 /07	n.d.	07-0385.01	P3	P1	P2	P2	P2	P3	#P3
				F (LS)	2009 /03	Sputum	09-0257.01	P3	P1	P2	P2	P2	P3	#P3
				F (LS)	2009 /07	Sputum	09-0563.01	P3	P1	P2	P2	P2	P3	#P3
				F (LS)	2009 /08	Sputum	09-0573.01	P3	P1	P2	P2	P2	P3	#P3
				F (LS)	2009 /10	Sputum	09-0730.01	P3	P1	P2	P2	P2	P3	#P3
				F (LS)	2009 /11	Palatine tonsil swab	09-0768.01	P3	P1	P2	P2	P2	P3	#P3
				F (LS)	2010 /02	Sputum	10-0180.01	P3	P1	P2	P2	P2	P3	#P3
				F (LS)	2010 /03	Sputum	10-0238.01	P3	P1	P2	P2	P2	P3	#P3
1	26	m	c.1007T>A/-	B (BE)	1995 /08	Sputum	95-2499.01	P1	P1	P2	P2	P2	P4	#P4
2	16	f	delF508/R553X	N (BB)	1998 /11	Sputum	98-0503.01	P1	P1	P2	P4	P2	P4	#P5
36	19	f	delF508/delF508	F (LS)	2010 /01	Sputum	10-0129.01	P1	P2	P3	P3	P4	P5	#P7
				F (LS)	2010 /04*	Sputum	10-0314.01	P1	P2	P3	P3	P4	P5	#P7
				F (LS)	2010 /04*	Sputum	10-0315.01	P1	P2	P3	P3	P4	P5	#P7

Patient no.	Age[§] [y]	Sex	CFTR genotype	CF center (federal state)	Isolation year/month	Source	RKI no.	ITS	*ACT*	*CAL*	*RPB2*	*BT2*	*SOD2*	ST
23	41	m	delF508/delF508	K (BY)	2009 /03	Sputum	09-0355.01	P1	P2	P3	P3	P4	P5	#P7
20	16	f	unknown	B (BE)	2009 /01	Throat swab	09-0038.01	P1	P2	P3	P3	P4	P5	#P7
				B (BE)	2009 /06	Sputum	09-0443.01	P1	P2	P3	P3	P4	P5	#P7
46	18	f	delF508/c.1521-1523delCTT	Q (RP)	2010 /07*	Sputum	10-0455.01	P1	P3	P4	P4	P5	P6	#P8
				Q (RP)	2010 /07*	Sputum	10-0455.02	P1	P3	P4	P4	P5	P6	#P8
CBS 418.73	n.d.	n.d.	none	n.d.	n.d.	Soil (Tadzhikistan)	-	P1	P2	P3	P3	P3	P5	#P6
CBS 101.22[T]	n.d.	n.d.	none	n.d.	n.d.	Mycetoma (USA)	-	P1	P4	P5	P2	P2	P4	#P9

n.d. – no data; [§] - age at the date of first isolation of *Scedosporium* ssp.; m – male; f – female; CFTR - Cystic Fibrosis Transmembrane conductance Regulator; *,~ - multiple isolates from the same sample; BY – Bavaria; BE – Berlin; BB - Brandenburg; HH - Hamburg; LS – Lower Saxony; NW – North Rhine-Westphalia; RP - Rhineland-Palatinate, CBS – Centraalbureau voor Schimmelcultures

Table 2

List of *S. apiospermum* isolates showing the allele types of *ITS* and the five MLST loci and sequence types (ST).

Patient no.	Age[§] [y]	Sex	CFTR genotype	CF center (federal state)	Isolation year/month	Source	RKI no.	ITS	*ACT*	*CAL*	*RPB2*	*BT2*	*SOD2*	ST
29	21	m	unknown	F (LS)	2009 /08	Sputum	09-0593.01	S1	S1	S1	S1	S1	S1	#S1
39	28	m	delF508/delF508	A (BE)	2010 /02	Sputum	10-0177.01	S1	S1	S1	S1	S1	S1	#S1
32	21	f	unknown	F (LS)	2009 /11	Sputum	09-0800.01	S1	S1	S1	S1	S1	S9	#S2
9	12	f	unknown	B (BE)	2002 /01	Sputum	02-0098.01	S1	S1	S1	S1	S3	S1	#S3
16	19	f	unknown	B (BE)	2008 /12	n.d.	08-0617.01	S1	S1	S1	S1	S5	S1	#S4
30	11	f	delF508/R553X	F (LS)	2009 /09	Sputum	09-0652.01	S1	S1	S1	S3	S1	S4	#S5
				F (LS)	2009 /10	Sputum	09-0752.01	S1	S1	S1	S3	S1	S4	#S5
				F (LS)	2009 /12	Sputum	10-0038.01	S1	S1	S1	S3	S1	S4	#S5
				F (LS)	2010 /04*	Sputum	10-0316.01	S1	S1	S1	S3	S1	S4	#S5
				F (LS)	2010 /04*	Sputum	10-0317.01	S1	S1	S1	S3	S1	S4	#S5
34	13	m	delF508/delF508	F (LS)	2009 /12	Tracheal secretion	10-0037.01	S1	S1	S2	S1	S1	S3	#S6
				F (LS)	2010 /02	Sputum	10-0179.01	S1	S1	S2	S1	S1	S3	#S6
				F (LS)	2010 /06*	Sputum	10-0416.01	S1	S2	S4	S1	S1	S1	#S21
				F (LS)	2010 /06*	Sputum	10-0417.01	S1	S1	S2	S1	S1	S3	#S6
				F (LS)	2010 /09	Sputum	10-0595.01	S1	S1	S2	S1	S1	S3	#S6
45	20	f	delF508/G542X	F (LS)	2010 /06	Sputum	10-0418.01	S1	S1	S2	S1	S1	S3	#S6
28	25	m	delF508/delF508	F (LS)	2009 /07	Sputum	09-0564.01	S1	S1	S2	S1	S1	S9	#S7
				F (LS)	2009 /11	Sputum	09-0751.01	S1	S2	S1	S1	S1	S10	#S14
				F (LS)	2010 /01	Sputum	10-0130.01	S1	S3	S1	S1	S1	S3	#S23

Patient no.	Age[§] [y]	Sex	CFTR genotype	CF center (federal state)	Isolation year/month	Source	RKI no.	ITS	*ACT*	*CAL*	*RPB2*	*BT2*	*SOD2*	ST
				F (LS)	2010 /08	Sputum	10-0593.01	S1	S2	S1	S1	S1	S10	#S14
44	14	f	unknown	G (BW)	2010 /04	Sputum	10-0257.01	S1	S1	S2	S1	S4	S3	#S8
11	18	f	delF508/1525-1G>A	I (BW)	2003 /12	Sputum	03-0533.01	S3	S1	S3	S1	S6	S1	#S9
3	12	f	unknown	C (BY)	2001 /03	Sputum	01-0521.01	S1	S1	S4	S1	S7	S9	#S10
14	33	m	unknown	I (BW)	2007 /11	Sputum	07-0591.01	S1	S1	S4	S1	S1	S8	#S11
				I (BW)	2008 /11	Sputum	09-0021.01	S1	S1	S4	S1	S1	S8	#S11
25	22	f	delF508/1717A	B (BE)	2009 /06*	Sputum	09-0441.01	S2	S1	S4	S2	S5	S8	#S12
				B (BE)	2009 /06*	Sputum	09-0442.01	S2	S1	S4	S2	S5	S8	#S12
				A (BE)	2010 /09	Sputum	10-0577.01	S2	S1	S4	S2	S5	S8	#S12
26	16	f	unknown	L (BY)	2009 /06	Sputum	09-0450.01	S1	S2	S1	S1	S1	S1	#S13
40	19	m	delF508/delF508	A (BE)	2010 /03	Sputum	10-0208.01	S1	S2	S1	S1	S1	S10	#S14
48	19	m	delF508/delF508	F (LS)	2010 /09	Sputum	10-0637.01	S1	S2	S1	S1	S1	S11	#S15
8	23	f	unknown	B (BE)	2002 /01	n.d.	02-0042.01	S1	S2	S1	S1	S3	S1	#S16
31	22	f	unknown	F (LS)	2009 /09	Sputum	09-0653.01	S1	S2	S1	S2	S1	S5	#S17
				F (LS)	2009 /09	Sputum	09-0654.01	S1	S2	S1	S2	S1	S5	#S17
7	29	f	unknown	K (BY)	2001 /03	Sputum	01-0718.01	S1	S2	S2	S1	S1	S3	#S18
				K (BY)	2001 /11	Sputum	01-0988.01	S1	S2	S2	S1	S1	S3	#S18
24	26	m	unknown	B (BE)	2009 /06	n.d.	09-0422.01	S1	S2	S2	S1	S7	S8	#S19
				A (BE)	2009 /09	Tracheal secretion	09-0706.01	S1	S2	S2	S1	S7	S8	#S19
21	6	m	delF508/delF508	C (BY)	2009 /02	Throat swab	09-0141.01	S3	S2	S3	S1	S6	S1	#S20
19	10	f	R553X/R553X	K (BY)	2008 /10	Sputum	08-0633.01	S1	S3	S1	S1	S1	S1	#S22
				K (BY)	2009 /05	Sputum	09-0382.01	S1	S3	S1	S1	S1	S1	#S22

Patient no.	Age[§] [y]	Sex	CFTR genotype	CF center (federal state)	Isolation year/month	Source	RKI no.	ITS	*ACT*	*CAL*	*RPB2*	*BT2*	*SOD2*	ST
				K (BY)	2009 /08	Sputum	09-0539.01	S1	S3	S1	S1	S1	S1	#S22
				K (BY)	2009 /12	Sputum	09-0823.01	S1	S3	S1	S1	S1	S1	#S22
33	15	m	delF508/R533X	H (BW)	2009 /11	Sputum	09-0821.01	S1	S3	S1	S1	S6	S8	#S24
43	15	f	unknown	C (BY)	2010 /04	Throat swab	10-0256.01	S1	S3	S1	S2	S6	S1	#S25
42	36	m	delF508/delF508	F (LS)	2010 /03	Sputum	10-0253.01	S1	S3	S2	S4	S1	S7	#S26
				F (LS)	2010 /06	Sputum	10-0471.01	S1	S3	S2	S4	S1	S7	#S26
4	19	f	unknown	D (BW)	2001 /05	Tracheal secretion	01-0602.01	S2	S3	S3	S2	S2	S5	#S27
				D (BW)	2001 /05	BAL	01-0603.01	S2	S3	S3	S2	S2	S5	#S27
17	11	m	delF508/delF508	B (BE)	2008 /12	n.d.	08-0619.01	S1	S3	S3	S3	S1	S1	#S28
18	14	f	delF508/2146delT	B (BE)	2008 /12	n.d.	08-0620.01	S1	S3	S4	S1	S2	S8	#S29
27	22	f	delF508/delF508	E (BW)	2009 /06*	Sputum	09-0475.01	S2	S4	S1	S2	S6	S6	#S30
				E (BW)	2009 /06*	Sputum	09-0475.02	S2	S4	S1	S2	S6	S6	#S30
				E (BW)	2010 /03	Sputum	10-0222.02	S2	S4	S1	S2	S6	S6	#S30
41	10	f	delF508/delF508	F (LS)	2010 /03	Palatine tonsil swab	10-0252.01	S2	S4	S3	S2	S2	S2	#S31
				F (LS)	2010 /09	Throat swab	10-0638.01	S2	S4	S3	S2	S2	S2	#S31
12	17	f	delF508/delF508	F (LS)	2007 /04	n.d.	07-0291.01	S2	S4	S3	S2	S2	S2	#S31
				F (LS)	2008 /01	BAL	08-0042.01	S2	S4	S3	S2	S2	S2	#S31
				F (LS)	2008 /04	Ethmoid	08-0196.01	S2	S4	S3	S2	S2	S2	#S31
				F (LS)	2008 /04	BAL	08-0197.01	S2	S4	S3	S2	S2	S2	#S31
22	33	m	unknown	F (LS)	2009 /02	Sputum	09-0169.01	S2	S4	S3	S2	S2	S2	#S31
				F (LS)	2009 /07	Sputum	09-0561.01	S2	S4	S3	S2	S2	S2	#S31
				F (LS)	2009 /07	Bronchus	09-0562.01	S2	S4	S3	S2	S2	S2	#S31

Patient no.	Age[§] [y]	Sex	CFTR genotype	CF center (federal state)	Isolation year/month	Source	RKI no.	ITS	*ACT*	*CAL*	*RPB2*	*BT2*	*SOD2*	ST
				F (LS)	2009 /11	BAL	09-0770.01	S2	S4	S3	S2	S2	S2	#S31
				F (LS)	2009 /11	Tissue	09-0789.01	S2	S4	S3	S2	S2	S2	#S31
15	36	f	R347P/1078delT	B (BE)	2008 /03	Sputum	08-0118.01	S4	S5	S2	S2	S1	S1	#S32
				B (BE)	2008 /12	n.d.	08-0618.01	S4	S5	S2	S2	S1	S1	#S32
				A (BE)	2010 /07	Sputum	10-0054.01	S4	S5	S2	S2	S1	S1	#S32
CBS 100395	56	m	none	none	1993	BAL (HTX; Gemany)	-	S1	S2	S1	S1	S1	S3	#S33
CBS 100392	63	m	none	none	1993	Biopsy leg (Hungary)	-	S1	S3	S1	S5	S1	S12	#S34
CBS 117407[T]	n.d.	n.d.	none	none	n.d.	Keratitis (Brazil)	-	S2	S3	S1	S2	S8	S8	#S35

n.d. – no data; [§] - age at the date of first isolation of *Scedosporium* ssp.; m – male; f – female; CFTR - Cystic Fibrosis Transmembrane conductance Regulator; *,~ - multiple isolates from the same sample; BW - Baden-Württemberg, BY – Bavaria; BE – Berlin; LS – Lower Saxony; CBS – Centraalbureau voor Schimmelcultures

Table 3

Primer oligonucleotides and annealing temperature for ITS and the MLST analysis.

Gene	PCR product size [bp]	PCR and sequence primer [5' – 3']	Annealing temp. [°C]	Ref.
ITS	~ 650	**ITS5 -** GGAAGTAAAAGTCGTAACAAGG **ITS4 -** TCCTCCGCTTATTGATATGC	55	(15)
ACT	~ 1,000	**ACT-1 -** TGGGACGATATGGAIAAIATCTTGCA **ACT-4R -** TCITCGTATTCTTGCTTIGAICTCCACAT	57	(18)
CAL	~ 650	**CAL-FW -** GACTATTCACTAACAACGCTGTG **CAL-RW -** GTCTAGTATAATCAAATCGTTAGAG	55	this study
*RPB2**	~ 1,300	**RPB2-5F -** GAYGAYMGWGATCAYTTYGG **RPB2-7R -** CCCATRGCTTGYTTRCCCAT	55	(19)
BT2	~ 650	**BT2a -** GGTAACCAAATCGGTGCTGCTTTC **BT2b -** ACCCTCAGTGTAGTGACCCTTGGC	58	(16)
SOD2	~ 550	**SOD2-F3 -** TCACCACGATAAACACCACC **SOD2-R3 -** CGTCGATACCCAAGAGAGGA	52	Unpublished, from W. Meyer

R: G or A; **W**: A or T; **Y**: C or T; **M:** A or C

*For *RPB2* sequencing additional primer *RPB2*-6F 5'-TGGGGKWTGGTYCCTGC-3', *RPB2*-6R 5'-GCAGGCCARACCAWMCCCCA-3' was used.

Amplification/sequencing failed for: *EF1α* (**EF-1** ATG GGT AAG GAR GAC AAG AC and **EF-2** GGA RGT ACC AGT SAT CAT G; **EF-1H** ATG GGT AAG GAR GAC AAG AC and **EF-2T** GGA AGT ACC AGT GAT CAT GTT (21), **EF1-7(28) F** CAT CGA GAA GTT CGA GAA GG **and EF1-9(86) R** TAC TTG AAG GAA CCC TTA CC (22)

Table 4

Variability between the gene loci.

Locus	Sequence start [5' - 3']	Sequence end [5' - 3']	Size [bp]	*P. boydii*			*S. apiospermum*		
				Alleles [n]	Polymorphism [n]	[%]	Alleles [n]	Polymorphism [n]	[%]
ACT	ATCAAC	GCGAAA	782	4	18	2.3	5	11	1.4
CAL	TTAAAG	TATCCC	579	5	9	1.6	4	8	1.4
RPB2	TAAGCT	TCCCAA	1,092	4	19	1.7	5	17	1.6
BT2	GACGAC	CAGTCC	526	5	24	4.6	8	31	5.9
SOD2	TCTCCA	GCGCGA	396	6	58	14.6	12	25	6.3
Sum/Total			3,375		128	3.8		92	2.7