

Analysis of the Fungal Flora in Environmental Dust Samples by PCR–SSCP Method

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Abstract Conventional microbiological techniques yield only limited information on the composition of fungal communities in dust. The aim of this study was to establish and optimize PCR–single strand conformation polymorphism (PCR–SSCP) analysis for investigation of fungal diversity in rural dust samples. Three different DNA extraction protocols were tested on 38 fungal cultures. A total of six known universal fungal primer pairs were tested targeting the 18S rRNA gene, the 28S rRNA gene and the ITS region, respectively. Objective evaluation was performed with respect to the following parameters: efficiency to amplify all 38 strains; separation of seven species from different phylogenetic groups on the SSCP gel; additional bands in PCR–SSCP analysis; possibility to classify the amplified gene fragments to species level. Primer ITS1/ITS4 and PowerSoil™ DNA isolation showed the best

performance in most cases and were chosen for further analysis. The detection limit of the developed system was 200 CFU/g dust. Moreover, the reproducibility of the system could be demonstrated, leading to average profile similarities of 94.94 % [SD = 2.51] within gels, 93.03 % [SD = 4.69] between different days and 87.66 % [SD = 6.62] between different gels when testing shed and mattress dust samples. Sequencing allowed identification on species level, in detail: *Alternaria alternata*, *Cladosporium sphaerospermum*, *Cladosporium cladosporioides* as well as the yeasts *Candida cabralensis* and *Candida catenulata*. This demonstrates the adaptability of the method. In this study, a standardized system for fungal community analysis was developed that provides reproducible results applicable for epidemiological purposes.

Abbreviations

ARISA	Automated ribosomal intergenic spacer analysis
CFU	Colony forming unit
CTAB	Cetyltrimethylammoniumbromid
DGGE	Denaturing gradient gel electrophoresis
DSMZ	German collection of microorganisms and cell cultures
EPS	Exopolysaccharide
ITS	Internal transcribed spacer
LSU	Large-subunit
PCR	Polymerase chain reaction
SSCP	Single-strand conformation polymorphism
SSU	Small-subunit

Introduction

During recent years, there has been an increasing interest in the environmental exposure to microorganisms in air and

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dust, especially for health effects [16]. However, the ongoing discussion led to controversial results. Exposure to microbial components seems to be positively related to respiratory diseases like chronic obstructive pulmonary disease [43, 54]. In contrast to occupational exposure, culture techniques showed a potential protective effect of fungal exposure for allergic asthma and atopy early in life [8, 9, 12, 18, 45, 62, 63], which seems to be sustained until adulthood [44]. Not all naturally occurring fungi can be cultivated or identified with common culture conditions [1, 2] and EPS or beta-glucan concentrations do not mirror the total fungal diversity [15]. Therefore, the objective of this study was to apply and evaluate the culture-independent PCR–SSCP method [38, 51].

DNA extraction from fungal spores is challenging due to their complex and sturdy cell wall [60]. A variety of DNA extraction strategies was discussed in literature [30]. In addition, selection of universal fungal barcode primers is important. At the beginning, the dominant target was the highly conserved 18S rDNA [7, 56, 65] in analogy to the bacterial 16S rDNA [57]. Because of higher variability the non-coding internal-transcribed spacer (ITS) region [48] has gained much attention [21, 28, 31, 41, 65, 69] and is now postulated as the fungal barcode [50]. Various universal fungal primers, especially for the ITS region, covering the entire taxonomic range are available [50]. However, some of them are known to co-amplify closely related eukaryotic DNA, especially plant DNA [14, 50]. Moreover, sequencing to species level should be possible [2, 50].

In analogy to Korthals et al. [27] where the focus was based on bacteria, we extended the analysis to detect fungi in environmental dust. In this study universal fungal primers, which were used separately so far, and DNA extraction methods were evaluated for the standardization of fungal community analysis for the first time. Furthermore, we wanted to demonstrate the adaptability of the PCR–SSCP method for the analysis of fungal diversity in environmental dust.

Materials and Methods

Fungal/Yeast Strains and Growth Conditions

A total of 11 fungal strains from the German collection of microorganisms and cell cultures (DSMZ) were selected for PCR–SSCP analysis: *Acremonium strictum* (DSM 3567), *Alternaria alternata* (DSM 1102), *Aspergillus versicolor* (DSM 1943), *Aureobasidium pullulans* (DSM 2404), *Cladosporium herbarum* (DSM 63422), *Eurotium amstelodami* (DSM 62629), *E. chevalieri* (DSM 62630), *E. rubrum* (DSM 62631), *Penicillium chrysogenum* (DSM

844), *Saccharomyces cerevisiae* (DSM 70451), and *Wallenia sebi* (DSM 5329). In addition, a total of 27 fungal strains of the institute's reference stock of the overall most common genera were included: *Alternaria* sp., *Aspergillus clavatus*, *A. flavus*, *A. fumigatus*, *A. versicolor*, *A. niger*, *A. glaucus* Group, *Aureobasidium pullulans*, *Chaetomium* sp., *Chrysosporium* sp., *Cladosporium* sp., *Epicoccum* sp., *Eurotium* sp., *Fusarium culmorum*, *Fusarium* sp., *Moniliella* sp., *Mucor spinosus*, *P. chrysogenum*, *P. brevicompactum*, *Rhizopus peca*, *Stachybotris* sp. strain I, *S. sp.* strain II, *S. chartarum* S1114, *S. chartarum* S1495, *Trichoderma* sp., *Trichothecium roseum*, and *Wallemia* sp.

All reference stocks were cultured by inoculation at room temperature for 7 days on Sabouraud's Agar (Merck, Darmstadt, Germany), until fungal spores sporulated. *Wallenia* species were cultured on DG18 agar (Oxoid, UK) and the DSM strains were cultured on the recommended media (information available at www.DSMZ.de; medium 129: DSM 1943/2404/63422/844; medium 186: DSM 70451; medium 187: DSM 62629/62630/62631/5329; medium 189: DSM 3567; medium 310: DSM 1102). The fungi were harvested with 5 mL sterile water and the spore suspensions were stored at -20°C until further processing.

Extraction of Fungal DNA

DNA extraction was performed with the PowerSoil™ DNA isolation kit (MoBio, USA), with the GenElute™ Plant Genomic DNA Miniprep kit (Sigma-Aldrich, Germany), and with the Cetyltrimethylammoniumbromide (CTAB) method. The commercially available kits were used according to the manufacturer's instructions with 500 μL of fungal spore suspension. Bead beating was applied prior to DNA extraction with the GenElute™ kit for 1 min at 30 Hz with the TissueLyzer II (QIAGEN, Germany) using 0.5 g glass beads with 212–300 μm in diameter (Sigma-Aldrich, Germany). The CTAB method was performed according to Korthals et al. [27] with the following minor modifications: 1 mL of fungal pure culture suspension in sterile water was centrifuged at $10,000\times g$ for 10 min at room temperature and the pellet was resuspended in 500 μL TE buffer (pH 8.0), followed by bead beating.

PCR Amplification

Six primer pairs targeting the 18S rDNA, 28S rDNA and the internal transcribed spacer (ITS) region were tested; each reverse primer was phosphorylated at 5'-end (Table 1). These primer pairs were separately used in fungal community analyses [2, 3, 7, 10, 21, 29, 40, 49, 65]. The PCR reaction was carried out in a total volume of 25 μL for detection analysis and 50 μL for sequencing preparation and

PCR–SSCP analysis. The reaction mix contained 1–5 ng DNA, 0.5 μ M of each primer (0.2 μ M for Fun18sf/ITS4), 1.5 mM $MgCl_2$, 200 mM dNTPs, and 1.25 units HotStarTaq DNA polymerase (QIAGEN, Germany). The amplification programme for primer pairs 0817/1536 (1), NS7/NS8 (2), U1/U2 (3), ITS1/ITS4 (4), and ITS1F/ITS4 (5) was initiated with denaturation at 95 °C for 15 min, followed by 35 (1,2,5), 30 (3), or 36 cycles (4) of denaturation at 94 °C for 45 s, annealing at 50 °C (2,3), 56 °C (1), 60 °C (5), or 62 °C (4) for 60 s and elongation at 72 °C for 60 s, and terminated with final elongation at 72 °C for 10 min. For primer pair Fun18sf/ITS4, amplification was performed according to Pitkäranta et al. [41]. PCR products were visualized by electrophoresis on 1.0 % agarose gels in 0.5 $\times$ TBE buffer and stained with ethidium bromide.

PCR–SSCP Analysis

For polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP), the PCR products were applied as described by Dohrmann and Tebbe [13] and Korthals et al. [27] with some modifications. In detail, the purified ssDNA was diluted with 9 μ L of SSCP loading buffer and denatured for 5 min at 95 °C, immediately cooled on ice and applied in a total volume of 8 μ L per well of a native (non-denaturing), temperature-controlled (20 °C), vertical polyacrylamide gel for SSCP electrophoresis.

The most suitable electrophoretic conditions for SSCP were evaluated with single-strand DNA of seven phylogenetically different fungi including *Wallemia* sp., *A. pullulans*, *Cladosporium* sp., *M. spinosus*, *P. chrysogenum*, *Aspergillus fumigatus*, and *A. glaucus* Group. The selected species are known to occur most commonly in shed and mattress dust samples [6, 19, 32, 36, 37, 46, 64]. The conditions were altered from 31.2 to 45.0 % MDE (MDE® Gel solution, Lonza, Switzerland) in 1 $\times$ TBE gels

and performed for 20–24 h at 400 V and 20 °C. The gels were casted in a PerfectBlue Dual Gel System Twin L (Peqlab, Germany) using 0.4 mm spacers and combs. A total of 400 ng DNA, measured by NanoDrop ND-1000 (Peqlab, Germany), was applied to each lane of the SSCP-gel to increase the comparability of the SSCP profiles. The gels were silver-stained according to Bassam et al. [5] and dried at room temperature for interpretation of the separation results.

Sequencing

Five reference strains—*A. versicolor* (DSM 1943), *C. herbarum* (DSM 63422), *E. amstelodami* (DSM 62629), *P. chrysogenum* (DSM 844), and *W. sebi* (DSM 5329)—were chosen for the evaluation of the ability to identify fungi on species level with the six primer pairs. For sequencing, the PCR products of each primer pair were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany) and quantified with Nanodrop ND-1000 spectrometer (Peqlab, Germany). Sequencing was performed by Sequiserve GmbH (Germany) using the amplification primers as oligonucleotides, with an unphosphorylated reverse primer. The results were compared with the databases of NCBI-BLAST (<http://www.ncbi.nlm.nih.gov/>) and CBS-KNAW (<http://www.cbs.knaw.nl/>). A sequence was regarded as identified on species level if at least one of the two databases provided a correct result. GenBank numbers and strain information were taken from the first database hit.

Objective Evaluation

To identify the primer pair with the best performance, a ranking system was developed with a different weighting of four scenarios: (1) sequencing to species level of five reference strains (50 %), (2) number of additional bands

Table 1 PCR primer pairs used in this study

No	Primer	Sequence (5'–3')	Target region	Product size (bp)	Annealing temperature (°C)	References
1	0817 1536	TTA GCA TGG AAT AAT RRA ATA GGA ATT GCA ATG CYC TAT CCC CA	18S rDNA	762	56	[7]
2	NS7 NS8	GAG GCA ATA ACA GGT CTG TGA TGC TCC GCA GGT TCA CCT ACG GA	18S rDNA	377	50	[65]
3	U1 U2	GTG AAA TTG TTG AAA GGG AA GAC TCC TTG GTC CGT GTT	28S rDNA	260	50	[49]
4	ITS1 ITS4	TCC GTA GGT GAA CCT GCG G TCC TCC GCT TAT TGA TAT GC	ITS	570–590	62	[65]
5	ITS1F ITS4	CTT GGT CAT TTA GAG GAA GTA A TCC TCC GCT TAT TGA TAT GC	ITS	600–630	60	[21]
6	Fun18sf ITS4	TTG CTC TTC AAC GAG GAA T TCC TCC GCT TAT TGA TAT GC	ITS	770–800	50	[41]

detectable after PCR–SSCP gel analysis (20 %), (3) separation of seven fungal species from different phylogenetic groups in the PCR–SSCP gel (20 %), and (4) detection of 38 diverse fungal genera and species by PCR (10 %).

For sequencing incidence (1) grades were assigned from 1.0 to 6.0 (1.0 stood for five time detection and 6.0 stood for no detection on species level). The remaining grades were assigned from 1.0 to 4.0 and calculated as described in Table 2. Evaluation of PCR analysis (4) was based on the number of amplified fungal genera and species. A maximum number of 114 could be achieved (38 fungal pure cultures were included $\times$ three DNA extraction methods; see “[Extraction of Fungal DNA](#)” section).

Finally, allowing for the weighting of the results the average grade for each primer pair was calculated leading to the selected pair.

Detection Limit

For the evaluation of three selected DNA extraction methods, mattress dust was gamma-irradiated with 1.0 MGy (Isotron GmbH, Germany). Dust aliquots of 50 mg were spiked with a suspension of the DSMZ strains *C. herbarum*, *E. amstelodami*, and *P. chrysogenum* from 2.0×10^7 to 2.0×10^2 CFU/g dust (Nos. see “[Fungal/Yeast Strains and Growth Conditions](#)” section; 1:10 dilution per step). The detection limit was determined with primer pair ITS1/ITS4 in a threefold application of DNA extraction and PCR–SSCP analysis. Two negative controls were included each time to insure DNA-free, gamma-irradiated dust.

Reproducibility

The reproducibility of the PCR–SSCP system was demonstrated with the PowerSoil™ DNA isolation kit (MoBio, USA) and primer pair ITS1/ITS4 in three-independent trials. Intragel ($n = 5$), intergel ($n = 5$), and interday ($n = 5$) variances were calculated regarding the band profile similarity. For this purpose, 50 mg dust samples from one swine shed, chicken shed (both from Achselschwang, Germany), cow shed and different farm mattresses (pooled; both from

Thalhausen, Germany) were collected. Dusts from animal sheds were collected with a sterile spatula and a falcon tube; mattress dusts with a hand-held vacuum cleaner as described by Korthals et al. and by Ege et al. [19, 27]. One sample of each dust was applied fivefold to the same gel (intragel variation; all four sample sources) and fivefold to five different gels (intergel variation; without chicken shed). Furthermore, the DNA of the samples was extracted on five different days and applied to the same gel (interday variation; chicken- and swine-shed samples). The profile similarities were examined with the software GelCompar II (Applied Maths BVBA, Belgium) using Pearson correlation coefficients (%) in cluster and average analysis. For normalization of gel to gel differences, a species standard was included using common fungi in house and shed dust air: *P. chrysogenum*, *Au. pullulans*, *A. versicolor*, *Ac. strictum*, *E. amstelodami*, *C. herbarum*, *Al. alternata*, *W. sebi* (all strains from DSMZ (see “[Fungal/Yeast Strains and Growth Conditions](#)” section); index by running distance from above).

Application

To demonstrate the possibility to detect fungi on species level in field samples (shed and mattress dust) after PCR–SSCP analysis, ten dominant-selected bands were carefully excised from the gel by using a sterile, DNA-free scalpel. The DNA was recovered by using the crush and soak procedure as described by Peters et al. [40], reamplified by PCR with the same primer pair, purified with the QIAquick PCR purification kit (Qiagen, Germany) and sequenced by Sequiserve GmbH (Germany) as described above. Three bands from the swine shed and cow shed as well as two bands from the chicken shed and mattress dust profile were chosen. Two double bands were excised, since a separation of the bands was not possible. The sequencing results were compared with cultures of the same samples: 100 mg dust was suspended in 9.9 mL NaCl (pH 7.4), applied to Sabouraud’s (Merck, Germany) and DG18 (Oxoid, Germany) agar in a total volume of 100 μ L and cultured for 7 days at room temperature. Fungi were specified morphologically and by light microscopy. Bandmatching

Table 2 Ranking system for objective evaluation of fungal primers; weighting in brackets

Grades	Identification on species level (50 %)	Additional bands in PCR–SSCP analysis (20 %)	Separation in SSCP gels (20 %)	Amplification efficiency (10 %)
1.0	5 of 5	1–20	Very good	1.0: 114–107; 1.5: 106–99
2.0	4 of 5	21–40	Good	2.0: 98–91; 2.5: 90–83
3.0	3 of 5	41–60	Insufficient	3.0: 82–75; 3.5: 74–67
4.0	2 of 5	61–80	None	4.0: 66–59; 4.5: 58–51
5.0	1 of 5	n/a	n/a	n/a
6.0	0 of 5	n/a	n/a	n/a

analysis was performed with the scanned PCR–SSCP gel by using GelCompar II software (Applied Maths BVBA, Belgium).

Results

Primer Selection and Amplification of Fungal rDNA and ITS Fragments

The selected universal primer pairs (Table 1) amplified products from different regions of fungal ribosomal DNA, including the small-subunit (SSU) 18S rDNA, the large-subunit (LSU) 28S rDNA as well as the total internal transcribed spacer (ITS) region (ITS I and ITS II) between the rDNA subunits incorporating the 5.8S rDNA. As shown in Table 3, PCR amplification of almost all DNA samples extracted from 38 fungal pure cultures was successful with all primer pairs tested. In most cases, PCR products of the expected size appeared as detectable bands on agarose gels with all primer pairs (data not shown). Nevertheless, differences between the primer pairs could be detected in dependence on the used DNA extraction method. At times, detection of different *Stachybotris* sp. and *M. spinosus* failed (Table 3). In contrast to CTAB and GenElute™, the PowerSoil™ method facilitated the detection. Moreover, bead-beating for disruption the fungal cell wall prior to CTAB extraction led to an up to tenfold higher DNA yield (data not shown).

PCR–SSCP Analysis of Fungal Pure Cultures

A total of seven species from different phylogenetic groups were chosen to evaluate the best primer pair regarding the separation in the PCR–SSCP gel. The selected species are known to be the most abundant in shed and mattress dust samples. Substantial differences in separation performance were observed. In particular, the short fragments of 260 bp (U1/U2) and 377 bp (NS7/NS8) precluded differentiation between the phylogenetic groups. Extremely long DNA fragments did not separate in the PCR–SSCP analysis as well. This is particularly the case for the Fun18sf/ITS4 as well as for the 0817/1536 combination even with varying running conditions including temperature, time, and MDE concentration (data not shown). The separation was better than with the other 18S and 28S primers but worse compared to ITS1/ITS4 and ITS1F/ITS4.

ITS1/ITS4 (570–590 bp) and ITS1F/ITS4 (600–630 bp) showed an almost identical separation performance, which led to a wide distribution over the whole gel length. Figure 1 shows different PCR–SSCP analyses performed with ITS1/ITS4.

Even if pure cultures were used, more than one band was observed on the polyacrylamide gel (Fig. 1a, lane 10). Usually, intensity of these bands was different resulting in a dominant band and weak additional bands, but the ratio of the intensity was constant from sample to sample. The additional bands were summed up across the seven included fungi for each primer pair. With the primer pair U1/U2, 19 additional bands were detected, followed by the three ITS region primer pairs with 34 each, 0817/1536 with 46 and NS7/NS8 with 84 bands.

Evaluation of Sequencing to Species Level

A further goal of this study was the detection of fungi on a species level by sequencing. The results of both sequence databases led to a clear identification on genus level in all cases and on species level in many cases (Table 4). Partly, reference strains were identified as another species from the same genus. Hence, these results were not counted as identified on species level for objective evaluation. Usually both databases gave the same results but at times the database CBS-KNAW allowed a better identification (Table 4).

Objective Evaluation

Four parameters were regarded for the comparative analysis of the six primer pairs (see “Objective Evaluation” section). The calculated results are shown in Table 5 leading to ITS1/ITS4 as the primer pair of choice. In an additional calculation with the same weighting, the results were changed in points (3–1), where three points were given for the best primer pair in the respective incidence and one point for the third. The remaining received no points. Again, ITS1/ITS4 was the leading primer pair compared to the others (data not shown) and was chosen for the further analysis.

Detection Limit

Sensitivity in diversity analysis is a crucial point for determining not only the dominant but also the rare species in a particular sample. In our study, the evaluation of the sensitivity was also used for comparing the three DNA extraction methods beside DNA amplification. The PowerSoil™ DNA isolation kit performed best. In all three cases, a detection limit of 200 CFU/g dust could be observed (Fig. 1a), whereas the limit of CTAB and GenElute™ Plant genomic miniprep kit was 200–20,000 and 2,000–20,000 CFU/g dust, respectively (data not shown).

Reproducibility

As shown in Fig. 1b, band separation of the different ssDNA molecules of the examined dust samples (data not

Table 3 PCR amplification results

Fungi	CTAB						PowerSoil™						GenElute™					
	1 ^a	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
<i>Acremonium strictum</i> DSM 3567	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Alternaria</i> sp.	+	+	+	+	+	+	w	+	+	+	+	+	+	+	+	+	+	+
<i>Alternaria alternata</i> DSM 1102	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Aspergillus clavatus</i> .	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Aspergillus flavus</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Aspergillus fumigatus</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Aspergillus glaucus</i> Group	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Aspergillus niger</i>	+	w	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Aspergillus versicolor</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Aspergillus versicolor</i> DSM 1943	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Aureobasidium pullulans</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Aureobasidium pullulans</i> DSM 2404	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Chaetomium</i> sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Chrysomilia</i> sp.	+	+	+	+	w	+	+	+	+	w	w	+	+	+	+	+	+	+
<i>Cladosporium</i> sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Cladosporium herbarum</i> DSM 63422	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Epicoccum</i> sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Eurotium</i> sp.	w	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Eurotium amstelodami</i> DSM 62629	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Eurotium chevalerie</i> DSM 62630	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Eurotium rubrum</i> DSM 62631	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Fusarium</i> sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Fusarium culmorum</i>	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+
<i>Moniliella</i> sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Mucor spinosus</i>	–	+	–	–	–	+	+	+	L	L	+	+	+	+	+	+	+	+
<i>Penicillium chrysogenum</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Penicillium chrysogenum</i> DSM 844	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Penicillium brevicompactum</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Rhizopus peca</i>	–	+	+	+	+	–	+	+	L	L	+	+	+	+	+	+	+	+
<i>Saccharomyces cerevisiae</i> DSM 70451	+	+	+	L	L	L	+	+	+	+	L	L	+	+	+	+	+	+
<i>Stachybotris</i> sp. strain I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Stachybotris</i> sp. strain II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Stachybotris chartarum</i> S1114	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–
<i>Stachybotris chartarum</i> S1495	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
<i>Trichoderma</i> sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Trichothecium roseum</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Wallemia</i> sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Wallemia sebi</i> DSM 5329	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H ₂ O	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–

+ amplification, – no amplification, L Larger fragment, w weak band

^a for key to primer set numbers see Table 1

shown) was investigated for detection of the reproducibility of the PCR–SSCP method.

Figure 2, as an illustrative example, shows the results in cluster analysis for swine shed dust. In the lower part of the gel, a band shift could be observed after gel normalization

due to compression of the band profile on the basis of running distance differences (Fig. 2b). The intragel average band profile similarities varied from 86.11 % [SD = 6.86] for mattress dust to 98.23 % [SD = 0.78] for chicken-shed dust. Intergel similarity comparison showed similarities

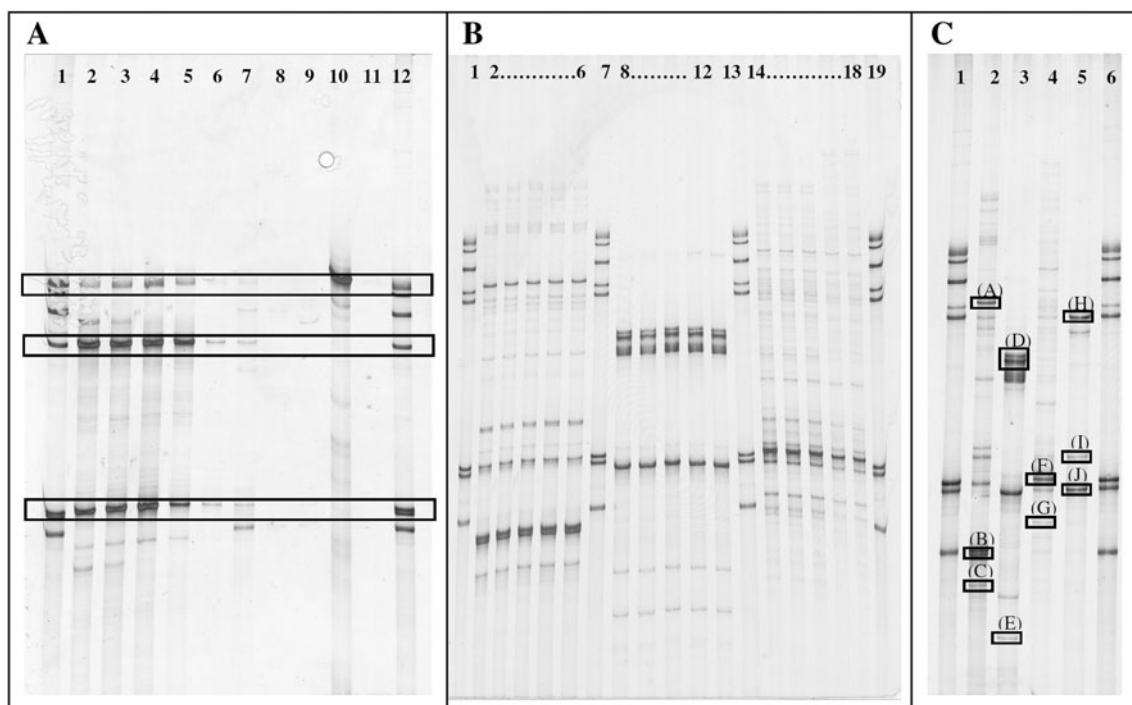


Fig. 1 PCR–SSCP analysis with primer pair ITS1/ITS4 after PowerSoil™ DNA extraction. Lanes A 1/12, B 1/7/13/19, C 1/6: species standard (up to down; DSMZ strains): *Penicillium chrysogenum*, *Aureobasidium pullulans*, *Aspergillus versicolor*, *Acremonium strictum*, *Eurotium amstelodami*, *Cladosporium herbarum*, *Alternaria alternata*, *Wallemia sebi*. **a** PCR–SSCP analysis of gamma-irradiated mattress dust spiked with a suspension of *P. chrysogenum*, *E. amstelodami* and *C. herbarum*. Lanes 2–7: 10-fold dilution series

from 2×10^7 to 2×10^2 CFU/g dust; 8/9: “no DNA” control; 10: positive control from PCR (pure culture of *P. chrysogenum*); 11: negative control from PCR (water). **b** PCR–SSCP analysis of fivefold chicken shed (lanes 2–6), swine shed (lanes 8–12) and farm mattress dust (lanes 14–18). **c** PCR–SSCP analysis of chicken shed (lane 2), swine shed (lane 3), farm mattress (lane 4) and cow shed (lane 5) dust samples

between 81.34 % [SD = 8.64] for mattress dust and 93.51 % [SD = 3.98] for cow shed dust, and the interday band profile similarities were 91.52 % [SD = 4.42] for chicken shed and 94.53 % [SD = 4.95] for swine-shed dust (Fig. 2c). In total, the mean similarities were 94.94 % [SD = 2.51] (intragel), 87.66 % [SD = 6.62] (intergel), and 93.03 % [SD = 4.69] (interday).

Application

In each case PCR–SSCP analysis of shed and mattress dust (field samples) showed a multitude of bands—faint and dominant ones—with different profiles. The goal of the study was to adapt PCR–SSCP analysis for fungal community analysis in environmental dust with specific results on species level. Therefore, ten dominant bands originating from field samples of the reproducibility study were chosen—three from chicken- and cow-shed dust as well as two from swine shed and mattress dust (Fig. 1c). In all cases and with both databases detection on species level was possible demonstrating the functionality of the primer pair ITS1/ITS4. In detail, chicken-shed dust consisted of

Microdochium nivale (band A; 99 %; NCBI-BLAST/CBS-KNAW: DSM 62278) and *Cryptococcus victoriae* (band C; 100 %; NCBI-BLAST: AM160647.1, CBS-KNAW: HB 1221). In the swine-shed dust sample, *Candida cabralensis* (band D; 99 %; only CBS-KNAW: CBS 11679) and *Candida catenulata* (band E; 100 %; NCBI-BLAST/CBS-KNAW: CBS 565) were found. Band F of the pooled mattress dust sample could be specified as *A. alternata* (100 %; NCBI-BLAST: HQ380767.1, CBS-KNAW: DTO 109-E4), even if the running distance was similar to *C. herbarum* of the species standard. The second mattress dust band (band G) included sequences of *Pseudotaeniolina globosa* (99 %; NCBI-BLAST: HQ115663.1, CBS-KNAW: CBS 109889). All three bands of the cow shed dust sample belonged to *Cladosporium* sp.—*Cladosporium sphaerospermum* (band H + I; 98–100 %, NCBI-BLAST: AF455481.1, CBS-KNAW: DTO 117-H2) as well as *Cladosporium cladosporioides* (band J; 100 %; NCBI-BLAST: AF455525.1, CBS-KNAW: DTO 004-C3). The sequencing result was unexpected for band B of chicken shed (*Dermapnyssus gallinae*; poultry bird mite; 99 %; NCBI-BLAST: GQ129212.1, CBS-KNAW: ROL2).

Table 4 Sanger-sequencing results of five DSMZ reference strains amplified by six primer pairs (1–6); comparison of NCBI-BLAST and CBS-KNAW database

Primer pair	1		2		3	
	NCBI-BLAST	CBS-KNAW	NCBI-BLAST	CBS-KNAW	NCBI-BLAST	CBS-KNAW
DMSZ reference strain						
<i>Penicillium chrysogenum</i> (DSM 844)	<i>P. chrysogenum</i> (99 %) (JF178786.1)	<i>P. chrysogenum</i> (100 %) (ATCC 10002)	<i>P. solium/chrysogenum/camemberti</i> (100 %) (JN642222.1/HQ882177.1/GQ458039.1)	<i>P. camemberti/chrysogenum/comune</i> (100 %) (ATCC 10387/ZJ-T2/MA09-AL)	<i>Penicillium. sp./halogiovensel chrysogenum</i> (100 %) (JQ778844.1/JQ434685.1/JQ434684.1)	<i>P. chrysogenum</i> (100 %) (CBS 195.46)
<i>Aspergillus versicolor</i> (DMS 1943)	<i>A. versicolor</i> (99 %) (JN546128.1)	<i>A. versicolor</i> (100 %) (HDJZ-ZWM-16)	<i>A. versicolor</i> (99 %) (AB002064.1)	<i>A. versicolor</i> (99 %) (CBS 583.65)	<i>A. versicolor</i> (100 %) (JN938942.1)	<i>A. versicolor</i> (100 %) (DAOM 222010)
<i>Wallemia sebi</i> (DSM 5329)	<i>W. sebi/muriae</i> (99 %) (AF548108.1/AY741381.1)	<i>W. sebi/muriae</i> (100 %) (ALI 158/MZKI B-952)	<i>W. muriae</i> (100 %) (AY741381.1)	<i>W. muriae</i> (100 %) (CBS 116628)	<i>W. muriae</i> (99 %) (DQ847517.1)	<i>W. muriae</i> (99 %) (CBS 116628)
<i>Cladosporium herbarum</i> (DSM 63422)	<i>Cladosporium sp.</i> (99 %) (JX273057.1)	<i>Cladosporium sp.</i> (100 %) (ATCAJ-13 JERPCR563)	<i>C. bruhnei/ureclinal/cladosporioides</i> (100 %) (AY251093.2/AY251096.2/AY251097.2)	<i>C. cladosporioides/bruhnei/ureclinal</i> (100 %) (CPC 5101/CPC 5390/STE-U 3682)	<i>C. langeronii</i> (100 %) (DQ780380.2)	<i>C. langeronii</i> (100 %) (CBS 101880)
<i>Eurotium amstelodami</i> (DSM 62629)	<i>E. herbariorum</i> (99 %) (EU263606.1)	<i>E. herbariorum</i> (100 %) (NRRL 116)	<i>E. rubrum</i> (100 %) (U00970.1)	<i>E. rubrum</i> (100 %) (no number)	<i>E. chevalierii/amstelodami</i> (100 %) (JN938915.1/JN938912.1)	<i>E. chevalierii/amstelodami</i> (100 %) (DAOM 216346/DAOM 222011)
Summary (identification on species level)	2 of 5	1 of 5	1 of 5	1 of 5	2 of 5	2 of 5
Primer pair	4		5		6	
DMSZ reference strain	NCBI-BLAST	CBS-KNAW	NCBI-BLAST	CBS-KNAW	NCBI-BLAST	CBS-KNAW
<i>Penicillium chrysogenum</i> (DSM 844)	<i>P. chrysogenum</i> (100 %) (JQ015265.1)	<i>P. chrysogenum</i> (100 %) (SL302)	<i>P. chrysogenum</i> (100 %) (JQ015265.1)	<i>P. chrysogenum</i> (100 %) (CBS 112383)	<i>P. dipodomycicola/glandicola/bruceovulum</i> (99 %) (DQ339570.1/DQ339565.1/DQ339557.1)	<i>P. chrysogenum</i> (100 %) (DT0 110-D3)
<i>Aspergillus versicolor</i> (DMS 1943)	<i>A. versicolor</i> (99 %) (JF817276.1)	<i>A. versicolor</i> (100 %) (DT0 090-C2)	<i>Aspergillus sp./versicolor</i> (99 %) (FJ755829.1/FJ878627.1)	<i>A. versicolor</i> (100 %) (DT0 117-G9)	<i>A. versicolor</i> (99 %) (GQ229082.1)	<i>A. versicolor</i> (99 %) (DT0 055-G5)
<i>Wallemia sebi</i> (DSM 5329)	Uncultured/ <i>Wallemia sp.</i> (98/96 %) (FJ820491.1/FJ770080.1)	Uncultured/ <i>Wallemia sebi</i> (98 %) (BF-OTU501/CBS 199.33)	Uncultured/ <i>Wallemia sp.</i> (98/97 %) (FJ820491.1/GU931736.1)	Uncultured/ <i>Wallemia sp.</i> (98 %) (BF-OTU501/DT0 089-G5)	Uncultured/ <i>Wallemia sp.</i> (98/97 %) (FJ820491.1/GU931736.1)	<i>W. muriae</i> (98 %) (DT0 107-F4)

Table 4 continued

Primer pair	4	5	6
DMSZ reference strain	NCBI-BLAST	CBS-KNAW	NCBI-BLAST
<i>Cladosporium herbarum</i> (DSM 63422)	<i>C. cladosporioides</i> (100 %) (JN565298.1)	<i>C. cladosporioides</i> (100 %) (CBS 170.54)	<i>Cladosporium</i> sp./ <i>cladosporioides</i> (100/99 %) (EU167592.1/GQ458030.1)
<i>Eurotium amstelodami</i> (DSM 62629)	<i>E. amstelodami</i> (100 %) (HQ728257.1)	<i>E. rubrum/chevalierii/amstelodami</i> (100 %) (UPM A6/UPM A15/NRRL 90)	<i>E. amstelodami</i> (100 %) (DT0 071-B6)
Summary (identification on species level)	4 of 5	3 of 5	3 of 5

For key to primer set nos. see Table 1
 Similarities (%), GenBank numbers (NCBI-BLAST) and strain numbers (CBS-KNAW) in brackets, correct results are printed in bold

Cultures of the dust samples confirmed the previous mentioned results on genus level in most cases. Dominantly, white yeasts, e.g., *Candida* sp., *Geotrichum* sp., and *Pichia* sp., were detected on both Sabouraud's and DG18 agar in chicken shed dust. In addition, in the swine-shed dust white yeasts grew on DG18 agar among *Mucor* sp. and *Penicillium* sp. On Sabouraud's agar only *Mucor* sp. could be seen because it covered the plate. According to the sequencing results, the dominant fungi in mattress and cow-shed dust were *A. alternata* and *Cladosporium* sp., respectively. Besides, *Cladosporium* sp., *Fusarium* sp., *Eurotium* sp., and *Penicillium* sp. could be found on both agars for mattress dust as well as *Penicillium* sp., *Aspergillus/Eurotium* sp., and *Mucor* sp. for cow-shed dust.

Bandmatching including the band profiles of the species standard (Fig. 2d) led to 35 band classes. At the running distance of the species standards *P. chrysogenum*, *A. pullulans* and *A. versicolor* no bands could be detected. Sequencing results indicated that *A. alternata* as well as *Cladosporium* sp. appeared in two to four of the four samples, whereas the other bands were only present in the respective sample.

Discussion

Our aim was to establish a method for comparative analysis of fungal communities offering the possibility to separate different fungal genera on the basis of their primary DNA sequence. Six primer pairs for 18S and 28S rDNA as well as the fungal ITS region and three DNA extraction methods were compared by objective evaluation in several tests indicating the ITS1/ITS4 primer pair and PowerSoil™ DNA isolation kit as the best and method of choice for fungal community analysis in environmental dust.

Primer and DNA Extraction

At times, detection of some fungi was not possible depending on the used primer pair and DNA extraction method. As reported elsewhere, spores and mycelia could not always be cracked by extraction methods due to their complex and sturdy cell wall [60]. The bead beating method failed partially as described in other studies [35, 56], even if we could also see its usefulness for gaining an increased DNA yield as shown by Roose-Amsaleg et al. and Lakay et al. [30, 47]. In addition, fungal nucleases, polysaccharides, and pigments could also contribute to difficulties in isolating DNA [35]. Wu et al. [66] tested 12 universal primer pairs after DNA extraction with the DNeasy Plant mini kit (Qiagen) followed by bead-beating with zirconia-silica beads. They did not recognize such a

Table 5 Objective evaluation of primer pairs; for ranking system see Table 2

Primer pair	Grades per incidence				
	Sequencing [50 %]	Additional bands in PCR– SSCP analysis [20 %]	Separation in SSCP gels [20 %]	Amplification efficiency [10 %]	Average grade
1	4.0 (2 of 5)	3.0 (46)	2.0 (good)	1.0 (108 of 114)	3.10
2	5.0 (1 of 5)	4.0 (84)	2.0 (good)	1.0 (112 of 114)	3.80
3	4.0 (2 of 5)	1.0 (19)	3.0 (insufficient)	1.0 (111 of 114)	2.90
4	2.0 (4 of 5)	2.0 (34)	1.0 (very good)	1.0 (111 of 114)	1.70
5	3.0 (3 of 5)	2.0 (34)	1.0 (very good)	1.0 (110 of 114)	2.20
6	3.0 (3 of 5)	2.0 (34)	3.0 (insufficient)	1.0 (111 of 114)	2.60

In brackets: [weighting] and (rating); key to primer set nos. see Table 1

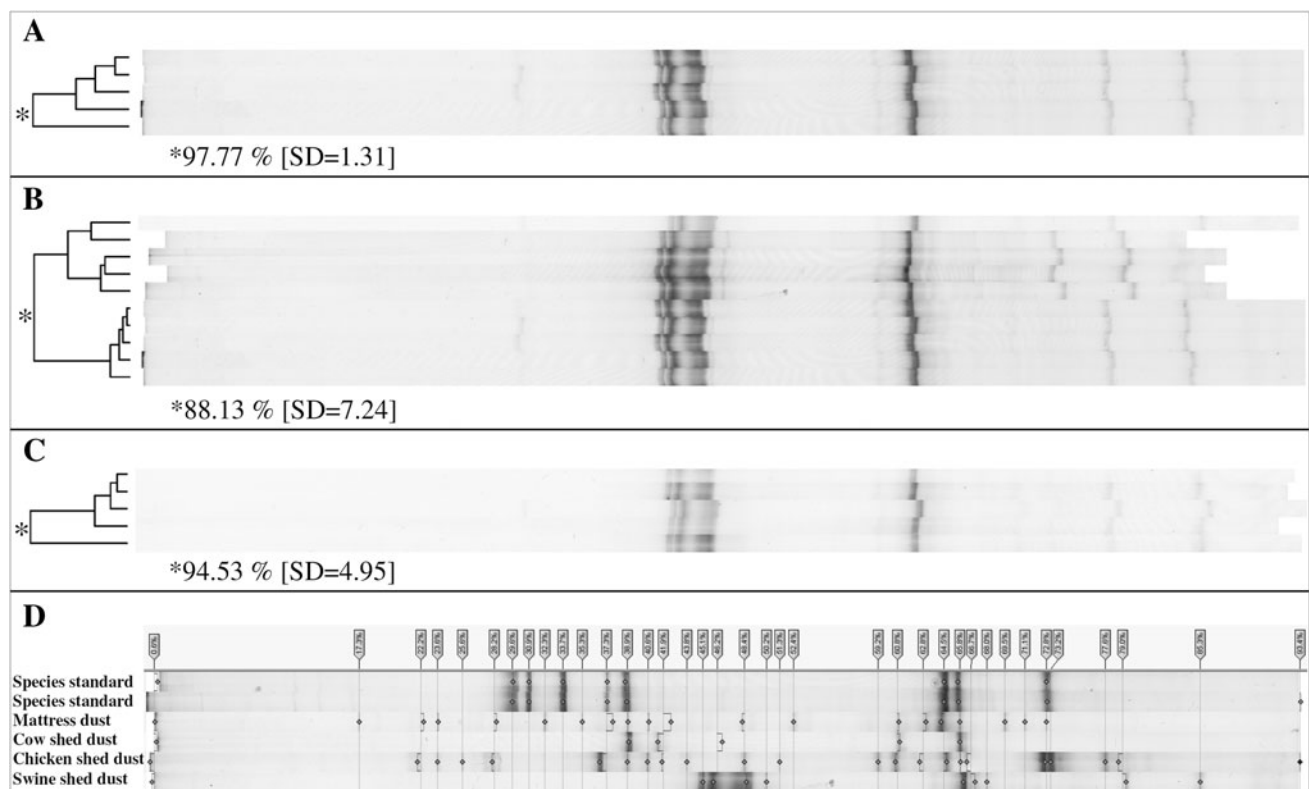


Fig. 2 Band profile similarity cluster analysis (Pearson correlation) and band matching analysis with software GelCompar II **a** Intragel variation. **b** Intergel variation (the below five samples were from **a**).

c Interday variation. **d** Band matching analysis of dust samples. Band classes in percentage due to running distance from the gel start

problem, because *Stachybotris chartarum* could be amplified with used pairs. In addition, Wu et al. and Turin et al. [59, 66] described a lack of amplification of *Aspergillus fumigatus* and *Aspergillus niger* with primer pair ITS1/ITS4. In our study, both species could be detected with all primer pairs, including ITS1/ITS4 [61], after extraction with all DNA isolation methods (Table 3). Wu et al. [66] described a longer PCR fragment in the groups of *Rhizopus* and *Mucor* after amplification with the primer pairs U1/U2 and ITS1/ITS4, which was confirmed in our analysis. In

summary, the choice of the optimal DNA extraction method is the first crucial step in the analysis of fungal communities by a DNA-based method. For this reason, we chose the PowerSoil™ DNA isolation kit for gaining fungal DNA from dust samples.

PCR–SSCP of Fungal Pure Cultures

Separation in the SSCP gel is strongly dependent on the primers. A potential explanation is the low variability

between genera in short fragments. Overall, separation of the bands was hardly possible or worse with primers targeting the highly conserved 18S and 28S rRNA genes compared to the primer pairs amplifying the more variable ITS region [25, 48]. In addition, long fragments could hardly be separated. Because of the limited fragment length variation, a size effect in PCR–SSCP analysis by using ITS primers was not observed in this study.

Sensitivity

We expected a gain of sequence variation between different genera with increasing length of an amplified fragment. Other publications report a decreasing sensitivity with increasing fragment size and an optimum of 150 bp [22–24, 26, 52, 58], whereas others use fragments up to a length of 775 bp [39]. However, in our study sensitivity was secondary to a distinct band profile separation pattern. Additionally, we included three fungal species for detection limit evaluation to mirror the nature better than with only one species, because of a competition of the different fragments in the polymerase chain reaction (Fig. 1a). Detection limit of the system was 200 CFU/g dust which is in the upper range of mold spore concentrations in indoor dust reported by other studies [6, 11]. For this reason, an adequate sensitivity for fungal detection in dust could be assessed.

Band Profile Separation

The multiple bands recognisable on the SSCP gel of fungal pure cultures (Fig. 1a, lane 10) may be attributable to multiple stable single-stranded DNA caused by a high GC-content or unfolded single-stranded DNA due to a lack of renaturation under certain conditions [39, 58]. Moreover, additional bands could be a result of reannealed single strands which led to double-stranded homoduplex DNA [4]. This means the intensity of the different bands on an SSCP gel is not suitable for quantitative purposes.

Reproducibility

To our knowledge this is the first study which demonstrates the reproducibility of the PCR–SSCP method performed with the PowerSoil™ DNA isolation and primer pair ITS1/ITS4. Reproducibility could be shown for four environmental dust samples (Fig. 1b). The values between 88 and 93 % are comparable to other gel-based systems like DGGE [33, 42, 55]. The lower similarity of the intergel comparison might be attributable to a minimal and unavoidable shift in PCR–SSCP conditions—MDE concentration, temperature—which led to small differences in the running distance of the bands (see lower part of the Gel in Fig. 2b) and, therefore, reduced the similarity rate.

Moreover, the band profile similarity decreased with the complexity of the sample (particularly in mattress dust), but the reproducibility was unaffected (Fig. 2a–c).

Sequencing

Culture-independent methods may help to support traditional identification of fastidious fungi [53, 67]. For that reason, we optimized the PCR–SSCP method for the investigation of fungi and could demonstrate primer functionality by sequencing to species level. Previously, investigations of fungal diversity in environmental samples were limited since there was a lack of sequence information in databases [3], even for the highly conserved 18S rDNA region. However, databases are growing. Identification of sequences from our study by CBS-KNAW and NCBI-BLAST provided correct and identical results in most cases (Table 4). However, in some cases the CBS-KNAW database led to a clearer identification on species level. Likewise, Du et al. [17] mentioned misidentification of 66 % of *Morchella* sequences by GenBank (NCBI-BLAST). Therefore, comparison of different databases is still required to obtain meaningful results. The ITS region was postulated as the fungal barcode equivalent to the bacterial 16S rDNA [50]. Accordingly, using ITS sequences provided better results compared to 18S rDNA in our study (Table 4 and “Application” section).

Sequencing of bands was necessary since a definite declaration of profile bands by using the species standard was not possible. Even one base exchange in the analysed sequence might result in a totally different folding structure and, subsequently, in a different running behavior [38]. However, the standard is indispensable for band profile normalization from gel to gel and allows a band profile comparison of large sample subsets to identify cohort-specific dominant bands.

Data Mining

Analysis of the PCR–SSCP band profiles with the band matching tool of the software GelCompar II allowed the comparison of different band profiles and is therefore helpful for investigation of fungal communities using large sample subsets.

Conclusions

New techniques like ARISA or next generation sequencing gained attention in the last years [20, 34, 68]. However, the possibility to standardize the process as shown in this study confirms that PCR–SSCP is still a suitable method to mirror the fungal diversity in epidemiological investigations [19].

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Conflict of interest Authors have no conflict of interest

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