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Evaluation of molecular methods for identification of filamentous fungi in food

Evaluierung von molekularbiologischen Methoden für die Identifizierung von filamentösen Pilzen in Lebensmittel

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Summary

In food microbiology, fungi are an important cause for spoilage. The intake of mycotoxin-contaminated food displays a great risk for animal and human health. To monitor fungal hazard of food contamination, a rapid and reproducible method for the characterization of food-associated fungi is needed. In order to establish a molecular based identification strategy, we applied sequencing of the nuclear rDNA region containing the internal transcribed spacers and the 5.8S rRNA gene (ITS-1 – 5.8 S – ITS-2). A total of 43 fungal strains, representing 38 species from 12 genera that are relevant for food spoilage, were examined. In a second approach, species specific real-time PCR was applied. After evaluating the specificity of the assay, 20 food samples from routine diagnostics were analyzed and the identification results of the real-time PCR were compared to the identification by traditional morphological methods. This work demonstrates the applicability of the real-time PCR assays as a helpful tool for the rapid identification of food associated fungi.

Keywords: internal transcribed spacer, mould identification, food spoilage, real-time PCR

Zusammenfassung

Pilze zählen in der Lebensmittelmikrobiologie zu den wichtigsten Verderbniserregern. Die Aufnahme von mit Mykotoxinen verunreinigten Lebensmitteln stellt ein Gesundheitsrisiko für Tier und Mensch dar. Um die Gefahr, die von Lebensmitteln ausgeht, die mit Pilzen verunreinigt sind, überprüfen zu können, wird eine schnelle und reproduzierbare Methode für die Identifizierung von Lebensmittel-assoziierten Pilzen benötigt. Für die Etablierung einer molekularbiologischen Indentifizierungsmethode erfolgte zunächst eine Sequenzierung der beiden rDNA-Genbereiche der Internen Transkribierten Spacer und die der 5,8S-rDNA-Gensequenz (ITS-1 – 5.8S – ITS-2). Mit dieser Methode wurde eine Gruppe von 43 Pilzstämmen, die 38 Spezies aus 12 Gattungen umfasst, die als Verderber in Frage kommen, untersucht. In einem zweiten Ansatz wurden spezies-spezifische Real-time PCR Untersuchungen vorgenommen. Nach Überprüfung der Spezifität der Methode, wurden 20 Lebensmittelproben aus der Routinediagnostik untersucht und die Ergebnisse mit den Ergebnissen aus der traditionellen Morphologie verglichen. Diese Arbeit zeigt, dass die Real-time PCR ein hilfreiches Werkzeug bei der schnellen Identifizierung von Pilzen in Lebensmitteln ist.

Schlüsselwörter: intern trankribierter Spacer (ITS), Schimmelpilzidentifikation, Lebensmittelverderb, Real-time PCR

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Introduction

Filamentous fungi play an important role in food spoilage. The generation of mycotoxins is the most important aspect of mould spoilage. Mycotoxins cause acute toxic, carcinogenic, mutagenic, teratogenic, immunotoxic, and oestrogenic effects in humans and animals. In the last years many countries established regulations to protect the consumer from mycotoxin contaminated food.

Robust and rapid identification methods for fungi are needed to monitor the food production process and improve food quality and security.

Classical phenotype-based methods are prevalently used for the identification of fungi in food industry. They are time-consuming and laborious and require skilled taxonomists. Also the classical methods are hindered by the unstable and subjective nature of phenotypic characteristics, which are readily influenced by culturing conditions. Some fungi used in food industry even can undergo changes in their phenotypic characteristics. The application of *Penicillium (P.) camemberti, P. nalgiovense* and *P. chrysogenum* as starter culture in food industry for example imposed selection pressure towards reduced pigmentation and sporulation. The loss of these original characteristics makes it difficult to delineate these species by non-specialists.

To date Polymerase Chain Reaction (PCR) and DNA hybridization techniques are promising approaches which have been demonstrated to be sensitive and specific for the detection of fungi. These techniques were applied for the identification of fungi in indoor environments, clinical samples, environment and food.

To establish a molecular based strategy for the identification of moulds we tested ITS sequencing, the "gold standard" for fungal species identification. The method is based on PCR amplification of the nuclear ribosomal internal transcribed spacer (ITS) region (ITS1, 5.8S rRNA, and ITS2) located between the nuclear small- and large-subunit rRNA genes, followed by sequencing of the resulting amplicon. The obtained consensus sequence (~500–600bp) was then analysed with fungal ITS sequence databases for species identification. Furthermore, a TaqMan® probe based real-time PCR assay, patented by the Environmental Protection Agency (EPA) for the detection and identification of indoor air fungi (http://www.epa.gov/nerlcwww/moldtech.htm) was tested for its adaptability to food samples. For the detection of *Fusarium* species the primer and probes published by Waalwijk et al. were applied.

Materials and methods

Fungal strains

A total of 43 reference strains, representing 38 species from 12 genera of food-relevant fungi were included in this study (Tab. 1). They were obtained from the Technical University of Munich (TUM)/Weihenstephan, Germany, the Helmholtz Center Munich, Germany and the Centraalbureau voor Schimmelcultures (CBS), the Netherlands.

Fungal strains were cultivated on Sabouraud-Glucose (2 %)-Agar with Gentamicin and Chloramphenicol (Heipha # 349e) for 4–6 days at 25 ºC.

Species identification in food samples

In routine diagnostics food samples were directly plated on conventional Yeast Extract Glucose Chloramphenicol (YGC)-Agar and incubated for 5 days at 25 °C. Fungal colonies were separated on Sabouraud-Glucose (2 %) agar and cultured for 4–6 days at 25 °C. After morphological species identification, DNA from the genera *Aspergillus* and *Penicillium* was isolated. For identification, DNA was examined by real-time PCR using the appropriate primer/probe sets.

Morphological species identification

Classical morphological analyses were done by the fungal reference centre (PRZ, Jena, Germany) as well as by our own laboratory unit.

Extraction of fungal DNA

DNA was extracted using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, Freiburg, Germany) according to the manufacturer`s instructions. Therefore a mycelial sample of ca. 1 cm^2 was cut out from the culture plate. DNA was eluted in 50 µL Elution Buffer. DNA concentrations were measured by PicoGreen method (Quant-iT PicoGreen ds reagent, life technologies, Darmstadt, Germany) according to manufacturer's instructions and diluted to a working concentration of 1.5 ng/µL for real time PCR.

Amplification and sequencing of the ITS region

For amplifying genes encoding the ITS region the fungi specific primers ITS1 (5`TCC GTA GGT GAA CCT GCG G3`) and ITS4 (5`TCC TCC GCT TAT TGA TAT GC3`) were used. PCR was performed in a total reaction volume of 25 µL including 12.5 µL HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany), 0.4 µM of each primer, 1 µL DNA in solution and 10.5 µL water. Amplification was performed on a thermal cycler with the following programme: an initial denaturation was carried out at 95 °C for 15 min, followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 52 °C for 30 s extension at 72 °C for 1.5 min. Finally an extension step of 10 min at 72 °C was performed. Success of amplification was tested by capillary electrophoresis of the PCR products using Bioanalyzer 1000 DNA Kit (Agilent Technologies, USA).

Data analysis

Data analysis was performed using the basic local alignment search tool (BLAST) from the national Centre for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast) as well as the web-based, commercial SmartGene ITS Fungi Module and its embedded ITS reference database (SmartGene, Lausanne, Switzerland). ITS-based identification applying NCBI BLAST was done by using the best-scoring reference sequence in accordance with the following criteria: score > 700, E – value 0 and an identity ≥ 98 %. The best results were sorted by identities and mismatches. For all sequence-based identifications, the generated sequence chromatograms were first proofread using the SmartGene ITS Fungi Module (SmartGene, Lausanne, Switzerland) applying the embedded ProofReader functionality: an alignment against an automatically selected closely related ITS reference sequence from the embedded database allowed a rapid review of potentially divergent positions in order to generate a meaningful consensus sequence. When the comparison of the resulting consensus sequence yielded more than one result without mismat-

TABLE 1: *Collective of food relevant fungi used in the study analyzed by ITS sequencing.*

Species	Strain	Identification by ITS sequence analysis (% sequence homology, Mismatches) using SmartGene ITS sequencing analysis supplementing NCBI BLAST sequence analysis		Final identification
Alternaria alternata	Helmholtz	*Alternaria alternata* *Alternaria arborescens* *Alternaria gaisen*	(100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Alternaria* spp.
Aspergillus alliaceus	DSM 813	*Aspergillus alliaceus*	(100 %, 0 Mismatches)	*Aspergillus alliaceus*
Aspergillus carbonarius	M 324	*Aspergillus carbonarius*	(100 %, 0 Mismatches)	*Aspergillus carbonarius*
Aspergillus flavus afla–	CBS 119.62	*Aspergillus flavus* *Aspergillus oryzae*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Aspergillus* spp.
Aspergillus flavus afla+	CBS 113.32	*Aspergillus oryzae* *Aspergillus flavus*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Aspergillus* spp.
Aspergillus fumigatus	CBS 113.55	*Aspergillus fumigatus*	(100 %, 0 Mismatches)	*Aspergillus fumigatus*
Aspergillus niger	–	*Aspergillus niger* *Aspergillus awamori* *Aspergillus foetidus*	(100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Aspergillus* spp.
Aspergillus ochraceus	CBS 263.67	*Aspergillus ochraceus* *Aspergillus westerdijkiae*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Aspergillus* spp.
Aspergillus parasiticus	CBS 126.62	*Aspergillus sojae* *Aspergillus parasiticus* *Aspergillus toxicarius*	(100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Aspergillus* spp.
Aspergillus versicolor	DCBS 245.65	*Aspergillus versicolor*	(100 %, 0 Mismatches)	*Aspergillus versicolor*
Cladosporium cladosporioides	CBS 112388	*Cladosporium cladosporioides* *Cladosporium phaenocomae* *Cladosporium funiculosum* *Cladosporium cucumerinum*	(100 %, 0 Mismatches) (100 %, 0 Mismatches (100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Cladosporium* spp.
Eurotium herbariorum	Ringversuch 7/2004	*Eurotium repens* *Eurotium rubrum* *Eurotium niveoglaucum* *Eurotium herbariorum*	(100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Eurotium* spp.
Fusarium avenaceum	DSM 62161	*Fusarium tricinctum* *Fusarium avenaceum*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Fusarium* spp.
Fusarium avenaceum	–	*Fusarium tricinctum* *Fusarium avenaceum*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Fusarium* spp.
Fusarium culmorum	CBS 417.86	*Fusarium asiaticum* *Gibberella zeae* *Fusarium vorosii* *Fusarium aethiopicum* *Fusarium culmorum* *Fusarium lunulosporum* *Fusarium brasilicum*	(100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Fusarium* spp.
Fusarium culmorum	TMW 4.0547	*Fusarium culmorum*	(100 %, 0 Mismatches)	*Fusarium culmorum*
Fusarium equiseti	CBS 406.86	*Fusarium equiseti* *Fusarium chlamydosporum* *Fusarium incarnatum*	(100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Fusarium* spp.
Fusarium graminearum	DSM 4527	*Fusarium graminearum* *Fusarium asiaticum*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Fusarium* spp.
Fusarium poae	DSM 62376	*Fusarium poae*	(100 %, 0 Mismatches)	*Fusarium poae*
Fusarium proliferatum	DSM 62261	*Fusarium proliferatum* *Gibberella moniliformis* *(Fusarium verticilloides)*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Fusarium* spp.
Fusarium sambucinum	CBS 185.29	*Fusarium solani* *Fusarium sambucinum*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Fusarium* spp.
Fusarium solani (var. *coerumleum*)	DSM 62416	*Fusarium cf. solani*	(100 %, 0 Mismatches)	*Fusarium cf. solani*
Fusarium verticilloides	CBS 218.76	*Fusarium verticilloides*	(100 %, 0 Mismatches)	*Fusarium verticilloides*
Geotrichum candidum	CBS 187.67	*Geotrichum candidum*	(100 %, 0 Mismatches)	*Geotrichum candidum*
Mucor mucedo	DSM 809	*Mucor mucedo*	(100 %, 0 Mismatches)	*Mucor mucedo*
Mucor racemosus	Ringversuch 2002 D	*Mucor racemosus*	(100 %, 0 Mismatches)	*Mucor racemosus*

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Penicillium brevicompactum	CBS 256.31	*Penicillium brevicompactum*	(100 %, 0 Mismatches)	*Penicillium brevicompactum*
Penicillium camemberti	CBS 249.77B DSM 1233	*Penicillium camemberti*	(100 %, 0 Mismatches)	*Penicillium camemberti*
Penicillium chrysogenum	CBS 306.48	*Penicillium chrysogenum* *Penicillium commune*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Penicillium* spp.
Penicillium chrysogenum	CBS 112383	*Penicillium clavigerum*	(100 %, 0 Mismatches)	*Penicillium clavigerum*
Penicillium commune	CBS 311.48	*Penicillium commune* *Penicilium camemberti*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Penicillium* spp.
Penicillium commune	CBS 343.51	*Penicillium commune* *Penicillium camemberti*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Penicillium* spp.
Penicillium corylophilum	CBS 768.97	*Penicillium aethiopicum*	(100 %, 0 Mismatches)	*Penicillium aethiopicum*
Penicillium crustosum	CBS 110074	*Penicillium crustosum* *Penicillium commune* *Penicillium griseoroseum*	(100 %, 0 Mismatches) (100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Penicillium* spp.
Penicillium echinulatum	CBS 112289	*Penicillium solitum* *Penicillium echinulatum*	(100 %, 0 Mismatches) (100 %, 0 Mismatches)	*Penicillium* spp.
Penicillium expansum	Ringv. 1/2003 C	*Penicillium expansum*	(100 %, 0 Mismatches)	*Penicillium expansum*
Penicillium roqueforti	CBS 221.30	*Penicillium roqueforti*	(100 %, 0 Mismatches)	*Penicillium roqueforti*
Penicillium solitum	CBS 288.36			mixed sequence
Penicillium verrucosum	CBS 223.71	*Penicillium verrucosum*	(100 %, 0 Mismatches)	*Penicillium verrucosum*
Rhizopus oryzae	CBS 607.68	*Rhizopus oryzae*	(100 %, 0 Mismatches)	*Rhizopus oryzae*
Stachybotrys chartarum	CBS 222.46	*Stachybotrys chartarum*	(99,81 %, 1 Mismatch)	*Stachybotrys chartarum*
Stemphylium solani		*Stemphylium solani*	(100 %, 0 Mismatches)	*Stephylium solani*
Trichothecium roseum	CBS 567.50	*Trichothecium roseum*	(100 %, 0 Mismatches)	*Trichothecium roseum*

ches, the best-matching reference sequences were aligned against the consensus, in order to assess the variability in single bases. If a matching species was represented by only one entry in the SmartGene database, the result's source was checked for an available, original publication and, in case of doubt, the result was not considered as definitive. Additionally, edited sequences were also analyzed using NCBI BLAST to compare these results with the results obtained from SmartGene ITS sequence database.

Primers and Probes

Primer and probe sequences specific for the genera *Alternaria, Penicillium* and *Aspergillus* used in this study (see Tab. 2) were published by the US Environmental Protection Agency; (http://www.epa.gov/nerlcwww/moldtech.htm). Primers were obtained from Thermo Fisher Scientific GmbH (Ulm, Germany). *Alternaria* spp., *Aspergillus* spp. and *Penicillium* spp. specific probes were obtained from TIB MOLBIOL GmbH (Berlin, Germany). The probes were labelled with 6-carboxy-fluorescein (FAM) and contained a black berry quencher (BBQ) conjugated to their 3'-terminal nucleotides. *Fusarium* spp. specific probes were obtained from Applied Biosystems Deutschland GmbH (Darmstadt, Germany) and were labelled with FAM and conjugated with TAMRA™ (6-carboxy-tetramethyl rhodamine) as quencher dye. Additionally they contained a Minor Groove Binder (MGB) ligand.

Real-time PCR

Real-time PCR was carried out according to the protocol provided by Roche Applied Science for the ERMI Mold Detection Plates Kit and was performed on LightCycler® 480 instrument running LightCycler™ software version 3.5 (Roche Diagnostics, Mannheim, Germany). The software was used to determine baseline and cycle of threshold (ct) values. Reactions were performed in 20 µL, using 10 µL LightCycler®480 Probes Master, 2x conc. (Roche Applied Science), 1 µM of each primer (5 µM for *Aspergillus versicolor* primer), 0.1 µM of each probe, 0.375 ng/µL DNA with the following program: initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 s.

Validation of the real-time PCR

The specificity of the primer/probe combinations was tested with genomic DNA of 43 reference strains. The reproducibility was analysed by running two PCR reactions in parallel and repeating the run in a second LightCycler® 480 experiment.

Results and Discussion

DNA sequencing of ITS region for species identification

The nuclear ribosomal ITS region has been recently proposed as the standard barcode for fungi because it combines the highest resolving power for discriminating closely related species with a high PCR and sequencing success rate. In this study we evaluated the applicability of ITS sequence analysis for rapid identification of food-associated fungi in routine analysis.

A total of 43 isolates from reference strains encompassing 38 species from 12 genera were analyzed. Out of important food spoiling fungi two isolates were included in the test collective.

The DNA isolated from 42 fungal isolates was successfully amplified by using universal ITS primers confirming a

high amplification success rate (ITS1–ITS4). Amplification resulted in a product of 500–600 bp. Amplification of DNA from *Penicillium solitum* (CBS 288.36) resulted in a mixture of sequences, which might be due to a bacterial or fungal contamination.

For microbial identification the sequences were analyzed applying the SmartGene Fungi Module. The embedded proofreading capability of this module allowed reducing sequencing errors significantly. The ITS reference database of the SmartGene Module contains quality controlled ITS sequences from the public domain: entries from clones or uncultured organisms are automatically filtered out in order to reduce the "noise" of potentially useless matches. Features such as an easy user interface, links to the original submission as well as corresponding publications. The possibility to easily perform multialignments and dendrograms on closely related reference sequences helped with the interpretation of the fungal sequences.

Using the sequence databases NCBI BLAST and SmartGene ITS database 42 moulds were identified on genus-level, 21 of these on species-level (Tab. 1). This result was expected because of high sequence similarities in that region among some fungal groups. ITS sequence similarities have been reported among species within genera *Cladosporium, Penicillium* and *Fusarium.* In *Aspergillus,* ITS sequences are identical in several complexes of important species. This has been confirmed by our results. From the genera *Aspergillus* only 3 out of 9, from *Fusarium* 4 out of 11 and from *Penicillium* 5 out of 13 species were identified on species-level.

In two cases species identification does not correspond with the tested reference strain. In this cases sequence analyses of additional marker genes such as the translation elongation factor 1-α, β-tubulin, or actin with more discriminatory power for species delineation are necessary.

Species identification by real-time PCR

In order to establish a TaqMan probe based real-time PCR method we tested 19 primer/probe combinations published by Haugland et al. and Waalwijk et al. The analysis tool published by Haugland et al. 2004 was designed for the detection and identification of mould species that are associated with indoor environments. Only those primer and probe sets, that were relevant for food spoilers were included into our analysis. To test the selectivity of the system a real-time PCR assay was performed with the DNA of 43 food isolates encompassing 38 food relevant fungi species (part of the described reference set).

The inclusivity of the tested primer/probe sets was 100 % for all tested species.

The exclusivity of the primer/probe sets number 3, 5, 6, 7, 9, 11, 12, 13, 14, 16, 17, 18 and 19 (Tab. 2) was 100 %. The exclusivity for the remaining sets was restricted. Primer/probe sets

TABLE 2: *Primers and probes used for real-time PCR analysis.*

Number	Specifity	Primer/ probe name	Sequence (5'–>3')
1	*Alternaria alternata*	*Aaltr*F1 *Altr*R1-1 *Aaltr*P1	GGCGGGCTGGAACCTC GCAATTACAAAAGGTTTATGTTTGTCGTA TTACAGCCTTGCTGAATTATTCACCCTTGTCTTT
2	*Aspergillus flavus/ oryzae*	*Aflav*F1 *Aflav*R1 *Aflav*P1	CGAGTGTAGGGTTCCTAGCGA CCGGCGGCCATGAAT TCCCACCCGTGTTTACTGTACCTTAGTTGCT
3	*Aspergillus fumigatus/Neosartorya fischeri*	*Afumi*F1 *Afumi*R1 *Afumi*P1	CGAGTGTAGGGTTCCTAGCGA CCGTTGTTGAAAGTTTTAACTGATTAC CCCGCCGAAGACCCCAACATG
4	*Aspergillus niger/ awamori/foetidus/ phoenicis*	*Anigr*F1 *Anigr*R1 *Anigr*P1	GCCGGAGACCCCAACAC TGTTGAAAGTTTTAACTGATTGCATT AATCAACTCAGACTGCACGCTTTCAGACAG
5	*Aspergillus ochraceus/ ostianus*	*Aochr*F1 *Aochr*R1 *Aochr*P1	AACCTCCCACCCGTGTATACC CCGGCGAGCGCTGTG ACCTTGTTGCTTCGGCGAGCCC
6	*Aspergillus parasiticus/sojae*	*Aflav*F1 *Apara*R3 *Aflav*P1	CGAGTGTAGGGTTCCTAGCGA GCCCGGGGCTGACG TCCCACCCGTGTTTACTGTACCTTAGTTGCT
7	*Aspergillus versicolor*	*Avers*F2 (5x) *Avers*R1-1 *vers*P1	CGGCGGGGAGCCCT CCATTGTTGAAAGTTTTGACTGATCTTA AGACTGCATCACTCTCAGGCATGAAGTTCAG
8	*Penicillium brevicompactum/ stoloniferum*	*Pbrev*F3 *Pen*R2 *Pbrev*P2	GGCGAGCCTGCCTTTTG GATCCGTTGTTGAAAGTTTTAAATAATTTATA CTCGCCGAAGACACCTTAGAACTCTGTCTGA
9	*Penicillium crustosum/camemberti/ commune/echinulatum/solitum*	*Pchry*F1: *Paura*R1-1 *Pen*P2	CGGGCCCGCCTTAAC GAAAGTTTTAAATAATTTATATTTTCACTCAGAGTT CGCGCCCGCCGAAGACA
10	*Penicillium chrysogenum*	*Pchry*F4-1 *Pchry*R8: *Pen*P6	GCCTGTCCGAGCGTCACTT CCCCCGGGATCGGAG CCAACACACAAGCCGTGCTTGAGG
11	*Penicillium expansum*	*Pexpa*F1-1 *Pexpa*R2-1 *Pen*P3	TTACCGAGTGAGGGCCGTT GCCCGCCGAAGCTACG TCCAACCTCCCACCCGTGTTTATTT
12	*Penicillium corylophilum*	*Pcory*F1 *Pcory*R3-1 *Pcory*P1	GTCCAACCTCCCACCCA GCTCAGACTGCAATCTTCAGACTGT CTGCCCTCTGGCCCGCG
13	*Penicillium roqueforti*	*Pchry*F1 *Proqu*R2 *Pen*P2	CGGGCCCGCCTTAAC TTAAATAATTTATATTTGTTCTCAGACTGCAT CGCGCCCGCCGAAGACA
14	*Penicillium verrucosum*	*Pverr*F2 *Paura*R1 *Pen*P2	CGGGCCCGCCTTTG GAAAGTTTTAAATAATTTATATTTTCACTCAGACTT CGCGCCCGCCGAAGACA
15	*Fusarium avenaceum*	*avenaceum*MGB-F *avenaceum*MGB-R *avenaceum*MGB-Probe	CCATCGCCGTGGCTTTC CAAGCCCACAGACACGTTGT ACGCAATTGACTATTGC
16	*Fusarium culmorum*	*culmorum* MGB-F *culmorum* MGB-R *culmorum* MGB-Probe	TCACCCAAGACGGGAATGA GAACGCTGCCCTCAAGCTT CACTTGGATATATTTCC
17	*Fusarium graminearum*	*graminearum* MGB-F *graminearum* MGB-R *graminearum* MGB-Probe	GGCGCTTCTCGTGAACACA TGGCTAAACAGCACGAATGC AGATATGTCTCTTCAAGTCT
18	*Fusarium poae*	*poae* 1-F: *poae* 1-R *poae* prob	AAATCGGCGTATAGGGTTGAGATA GCTCACACAGAGTAACCGAAACCT CAAAATCACCCAACCGACCCTTTC
19	*Fusarium solani f. sp. mori Mp III, F. solani f. sp. cucurbitae MP V, F. solani f. sp. solani MP VI, F. solani f. sp. robiniae MP VII*	*Fsola*F3 *Fsola*R3 *Fsola*P1mgb	CGCCGCAGCTTCCATC GATTCGAGGTCAACTTCAGAAGAG CGTAGTAGCTAACACCTC

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number 1 and 2 for the detection of *Alternaria alternata* and species group *Aspergillus flavus/A. oryzae* detected DNA extracted from *Mucor racemosus.* Primer/probe set number 4, specific for species group *Aspergillus niger, A. awamori, A. foetidus* and *A. phoenicis,* additionally detected DNA from *P. verrucosum.* The primer/probe set specific for species group *P. brevicompactum, P. stoloniferum* (set 8) detected DNA from *P. camemberti, P. commune, P. crustosum* and *P. echinulatum.* With the *P. chrysogenum* specific primer/probe set (set 10) DNA from *Fusarium verticilloides* was detected, too. *Fusarium avenaceum* specific primer/probe set (set 15) detected DNA from *Fusarium sambucinum.*

As these primer and probe sets are detecting the ITS region with its already described high similarity between species, several primer/probe sets detected more than one species. That is why selectivity depends on the tested collective. This work shows the applicability of the described primer/probe sets for the detection and identification of the described collection of food spoiling fungi.

Analysis of food samples with the real-time PCR method

To evaluate the application of the described real-time PCR assays for the identification of fungi in food we examined 20 food samples (24 isolates) from in-house routine diagnostics (Tab. 4). Initially food samples containing fungi of the genera *Aspergillus* and *Penicillium* were examined. These were chosen after morphological pre-identification. Our results were proofed by the fungal reference centre (Jena, Germany) or morphologically by our own routine laboratory. By the real-time PCR assays 14 isolates were identified to species-level or belonging to a species group. These results were confirmed by the fungal reference centre. Five Isolates (1a, 2a, b, 3 and 19) could not be identified because there was no appropriate primer/probe set available. In four food samples morphological and real-time PCR identification showed diverting results on species level while identification on genus level was identical.

Isolate 7 was identified as *Penicillium* spp. by the fungi reference centre. According to our results the isolate was identified as *P. brevicompactum* or *P. stoloniferum.* Isolate 13 was identified as *Neosartorya quadricincta* by the fungi reference centre, as *Aspergillus fumigatus* or *Neosartorya fischeri* by real-time PCR. Isolates 15 and 20 were identified as *Aspergillus tubingensis* by the fungal reference centre and as belonging to the species group *Aspergillus niger/awamori/foetidus/phoenicis* by real-time PCR. These fungi are belonging to the black *Aspergilli* (formerly *Aspergillus niger* group) and can be identified by a combination of ITS and beta-tubulin sequencing. An alignment of ITS sequences of these species (data not shown) showed no sequence differences. ITS sequences of *P. brevicompactum* and *P. bialowiezense* are highly conserved. This is the rea-

TABLE 3: *List of analyzed food samples. Results identified by real-time PCR compared with results from identification by the fungal reference centre (Jena, Germany).*

Number	Probe	Identification by the fungal reference centre	Identification by probe-specific real-time PCR
1a	cooked shrimp	*Eupenicillium terrenum* or *E. lapidosum*; 10 % Sectio *Eupenicillium citreonigrum* oder *E. ochrosalmoneum*	No result
1b	Cooked shrimp	*Penicillium chrysogenum*	*Penicillium chrysogenum*
2a	hungarian salami	*Penicillium nordicum*	No result
2b	hungarian salami	*Penicillium nordicum*	No result
3	Salad with strips of bologna-type sausage	*Penicillium nalgiovenese*	No result
4	pasta	*Penicillium roqueforti*	*Penicillium roqueforti*
5	salmon	*Penicillium commune*	*Penicillium echinulatum/crustosum/camemberti/commune/solitum*
6	Low fat Salad with strips of bologna-type	*Penicillium roqueforti*	*Penicillium roqueforti*
7	Bunny meat (raw)	*Penicillium* spp.	*Penicillium brevicompactum/stoloniferum*
8	Salad with strips of bologna-type	*Penicillium brevicompactum*	*Penicillium brevicompactum/stoloniferum*
9	Tomato jam	*Penicillium corylophilum*	*Penicillium corylophilum*
10	African vegetables (deep-frozen)	*Penicillium bialowiezense*	*Penicillium brevicompactum/stoloniferum*
11	Black grapes	*Penicillium expansum*	*Penicillium expansum*
12	Pu erh tea	*Aspergillus tubingensis/A. foetidus*	*Aspergillus niger/awamori/foetidus/phoenicis*
13	Cold-smoked sausage	*Neosartorya quadricincta*	*Aspergillus fumigatus/Neosartorye fischeri*
14	pork	*Aspergillus tubingensis/A. foetidus*	*Aspergillus niger/awamori/foetidus/phoenicis*
15	pasta	*Aspgergillus tubingensis*	*Aspergillus niger/awamori/foetidus/phoenicis*
16a	tuna	*Aspergillus flavus*	*Aspergillus falvus/oryzae*
16b	tuna	*Aspgergillus ochraceus*	*Aspergillus ochraceus/ostianus*
17a	lamb	*Aspergillus flavus*	*Aspergillus falvus/oryzae*
17b	lamb	*Aspergillus ochraceus*	*Aspergillus ochraceus/ostianus*
18	tuna	*Aspergillus fumigatus*	*Aspergillus fumigatus/Neosartorye fischeri*
19	Salad with strips of bologna-type	*Penicillium glabrum*	No result
20	pasta	*Aspgergillus tubingensis*	*Aspergillus niger/awamori/foetidus/phoenicis*

son why primer/probe set specific for *P. brevicompactum/stoloniferum* detected *P. bialowiezense,* too.

In summary our results demonstrate that ITS sequencing is a helpful tool to accelerate and supplement morphological identification of food-associated fungi even if not all can be distinguished. For more detailed species identification sequence analysis from additional markers might be necessary. Although the real-time PCR assays quickly identified 63 % of routine samples belonging to genera *Aspergillus* and *Penicillium* to species level or belonging to a species group, results must be considered critical and should be confirmed morphologically. To establish the real-time PCR assays in routine diagnostics more primer/probe sets, encompassing all food-relevant fungi are needed and more species should be included in the specificity tests.

We can conclude that the identification of food associated fungi on species level needs a combination of morphological and molecular methods.

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