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Rapid and reasonable molecular identification of bacteria and fungi in microbiological diagnostics using rapid real-time PCR and Sanger sequencing

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ABSTRACT

Analyses of short subunit gene sequences have been established for taxonomic classification and identification of bacteria and fungi. To produce partial bacterial ribosomal 16S rRNA and *rpoB* and fungal ribosomal ITS/LSU gene sequences for DNA sequencing, real-time PCR assays supplemented with the nucleic acid stain SYBR Green were created. Generation of PCR products was monitored based on amplification and melting curves. The PCR products were subsequently subjected to Sanger sequencing on demand for identification of bacteria and fungi in routine microbiological diagnostics within a period of two days.

From a total of 78 bacterial isolates 40 (51%) or 67 (86%) could be identified at species level using only partial 16S rRNA or additionally *rpoB* gene sequences based on BLASTN (NCBI) database queries, respectively. Using partial 16S rRNA and *rpoB* gene sequencing unambiguous assignment was not possible for the closely related species of the *Bacillus* (*B.*) *cereus* group, *Bordetella (B.) pertussis/ B. parapertussis/ B. bronchiseptica*, *Brucella* spp., *Enterobacter cloacae* complex, *Escherichia/ Shigella* spp., *Staphylococcus* (*S.*) *hyicus/ S. agnetis* and *Yersinia (Y.) pseudotuberculosis/ Y. pestis*. However, partial *rpoB* gene sequencing succeeded in identifying 27 bacterial isolates at species level in addition to 16S rRNA gene sequencing.

Regarding ITS/LSU gene sequencing, best results could be achieved by ITS gene sequencing followed by LSU gene sequencing, resulting in 32 (63%) and 21 (43%) of a total of 51 fungal isolates that could be identified at species level, respectively. Insufficient identification at species level was observed for the genera *Apiotrichum*, *Aspergillus, Cladosporium, Cryptococcus, Microsporum, Nannizziopsis*, *Penicillium, Trichosporon*, and *Tolypocladium* included in this study.

The concept of this procedure is suitable for rapid and reasonable molecular identification of bacteria and fungi within two days and is therefore applicable in routine microbiological diagnostic laboratories.

1. Introduction

Analyses of short, well classified gene sequences have been established for taxonomic classification and identification of organisms. This has been achieved due to high accuracy, high throughput, immense expansions of sequence databases, fast processing of large amounts of data and reasonable prices for DNA sequencing. These favourable developments have promoted establishment of organism identification in routine microbiological diagnostic laboratories based on a single universal marker (Janda and Abbott, 2007; Woo et al., 2009), also called DNA barcode sequence (Chaudhary and Dahal, 2017; Purty and Chatterjee, 2016).

Recognising bacterial and fungal genera and species based on morphological features or biochemical reactions alone is often difficult or impossible. In contrast, DNA sequencing of taxonomically relevant genes facilitates unambiguous diagnostic identification of these microorganisms (Begerow et al., 2010; Chakraborty et al., 2014; Hebert et al., 2003; Kwiatkowski et al., 2012). Furthermore, genetic characterisation of bacteria and fungi is an indispensable basis for classification and detection of yet unknown microorganisms and for the creation of reliable databases (Chakraborty et al., 2014; Rau et al., 2016). This is particularly important for MALDI-TOF spectroscopic analyses of ribosomal proteins which are widely used for rapid identification of bacteria and fungi in routine diagnostics (Florio et al., 2018).

Rapid identification of microorganisms includes sequencing of the bacterial 16S rRNA and *rpoB* genes and the fungal internal transcribed

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Table 1
Forward (F) and reverse (R) primers used for the 16S rRNA, *rpoB* and ITS/LSU gene real-time PCR assays.

Primer name	Target gene	Primer sequence (5′ – 3′)	Position	Product size (bp)
16S_337-F	16S rRNA	ACTCCTACGGGAGGCAGCAGT	337–357[b]	860
16S_1196-R	16S rRNA	TTGACGTCRTCCMCACCTTCCTC	1174–1196[b]	
rpoB_gr-pos_2287-F	*rpoB*	ATRACTTGGGAHGGTTAYAACT	2287–2308[c]	929
rpoB_gr-pos_3215-R	*rpoB*	CCACCRAAYTGHGCTTTACC	3196–3215[c]	
rpoB_gr-neg_2620F	*rpoB*	TGGAACGGYTAYAACTTYGAAGA	2620–2642[d]	797
rpoB_gr-neg_3416-R	*rpoB*	TTACCGTGACGRCCBGCCAT	3397–3416[d]	
C2700F[a]	*rpoB*	CGWATGAACATYGGBCAGGT	2713–2732[e]	446
C3130R[a]	*rpoB*	TCCATYTCRCCRAARCGCTG	3139–3158[e]	
ITS1_modified-F	ITS/LSU	TCCGTWGGTGAACCWGCGG	1761–1779[f]	1288
NL4_modified-R	ITS/LSU	GGTCCGTGTTTCAAGACGGG	3029–3048[f]	

B: C or G or T, H: A or C or T, M: A or C; R: A or G, W: A or T, Y: C or T.
Relative positions of the nucleotides according to GenBank (NCBI)
[a] Primers described by Khamis et al. (2004)
[b] J01859, *Escherichia coli*
[c] NC_007795.1:522160-525711, *Staphylococcus aureus* subsp. a*ureus*, NCTC 8325.
[d] CP024256.1:1159297-1163727, *Escherichia coli*, strain ATCC 43886
[e] AY492230.1, *Corynebacterium diphtheriae*, strain CIP 100721
[f] AM946630, *Serpula himantioides*, isolate P19

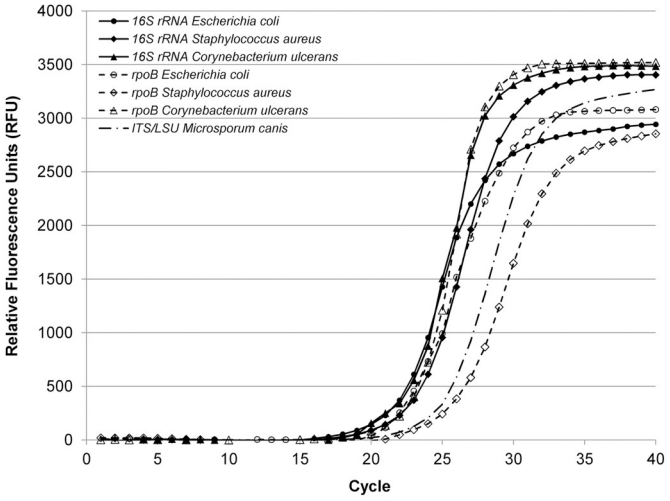

Fig. 1. Amplification curves for the real-time PCR assays targeting bacterial 16S rRNA, *rpoB* genes and the fungal ribosomal gene regions ITS/LSU.
Escherichia coli (MB10214 CVUAS, DSM 30083), *Staphylococcus aureus* (MB10223 CVUAS, ATCC 25923), *Corynebacterium ulcerans* (MB35160 CVUAS), *Microsporum canis* (11-7-D-00727 LHL-GI).

spacer (ITS) and 28S nuclear ribosomal large subunit rRNA (LSU) genes (Adékambi et al., 2009; Chakraborty et al., 2014; Purty and Chatterjee, 2016). Due to the lack of suitable DNA marker sequences for fungi (Chakraborty et al., 2014), both the ITS and the LSU are widely used either singly or in combination for identification and taxonomic classification of fungal species (Asemaninejad et al., 2016; Kwiatkowski et al., 2012; Romanelli et al., 2014; Schoch et al., 2012; Stockinger et al., 2010; Vu et al., 2019).

The fact that conserved gene segments alternate with variable gene regions in ribosomal genes enables generation of PCR products of different sizes. The size of the PCR product depends on the combination of universal primers which target specifically conserved segments and amplify variable gene regions. However, it is still a challenge to design universal PCR primers which are located within highly conserved gene segments and reliably produce amplicons bearing genetically variable taxon-specific information for the identification of a broad range of bacterial and fungal species (Barghouthi, 2011; Toju et al., 2012). This is especially true for the *rpoB* gene, for which no universal primer pair is available covering all bacterial species (Adékambi et al., 2009). Another issue is implementing an easy-to-perform, fast and economical procedure for generating amplicons for subsequent DNA sequencing. With this in mind, evaluation of real-time PCR on the basis of amplification and melting curves allows convenient assessment of the suitability of PCR products for DNA without additional characterisation of the amplicons by agarose gel electrophoresis (Chen et al., 2014).

The aim of the present study is to create real-time PCR assays targeting the bacterial 16S rRNA and *rpoB* and the combined ITS/LSU regions of fungal ribosomal gene sequences for easy-to-perform, rapid and robust generation and characterisation of amplicons in a single run procedure. Amplicons with a size of 800 to 1000 kb are particularly suitable for rapid and reasonable Sanger sequencing. Within a period of two days, this approach makes the receipt of DNA sequence data and easy evaluation of data possible, rendering this procedure suitable for routine microbiological diagnostic laboratories.

2. Material and methods

2.1. Bacterial and fungal isolates

The bacterial and fungal isolates used in this study originate from diagnostic samples, strain collections and from VETQAS proficiency testing (*Actinobaculum suis*, proficiency test 13,617/BA 2019, Animal and Plant Health Agency, United Kingdom) and are listed in Tables 2 and 3, respectively. The isolates included in this study were identified by MALDI-TOF using the Biotyper software (version 3.1.66) and by comparison with the commercial database version, containing 7311 entries (Bruker Biotyper, Bruker Daltonik, Bremen, Germany). Identification scores > 2.0 were considered as species identification. The isolates had been stored by cryopreservation in the collection of microorganisms at the CVUA Stuttgart and the Hessian State Laboratory Giessen using the Microbank™ system (Pro-Lab Diagnostics, Fisher Scientific, Schwerte, Germany) until use in this study.

2.2. Primer design

Primer design was based on multiple sequence alignments of 112 16S rRNA and 77 *rpoB* gene sequence entries deposited in GenBank (National Center for Biotechnology Information [NCBI], www.ncbi.nlm.nih.gov/; Pruitt et al., 2002). The bacteria used for DNA alignment were selected based on their relevance in veterinary medicine and as zoonoses. For the design of fungal primers 79 ITS and 89 LSU NCBI gene sequence entries were employed.

Multiple sequence alignments were carried out using the freely available internet program Clustal Omega provided by the European

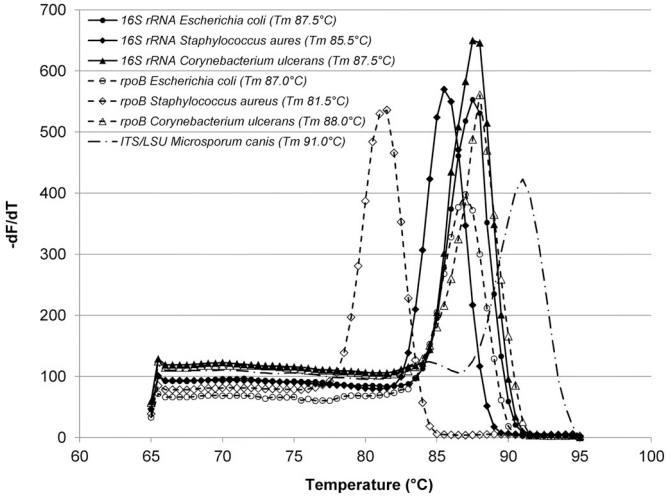

Fig. 2. Melting curves of the real-time PCR assays amplifying bacterial partial 16S rRNA and *rpoB* genes and the fungal ribosomal gene regions ITS/LSU. For bacterial and fungal strains see legend for Fig. 1.

Bioinformatics Institute (EMBL-EBI) (http://www.ebi.ac.uk/Tools/msa/clustalo/; McWilliam et al., 2013). The melting temperatures (T_m) of the designed primers were determined using the web-based T_m calculator OligoCalc (http://basic.northwestern.edu/biotools/OligoCalc.html; Kibbe, 2007).

For amplification of partial *rpoB* gene sequences from corynebacteria, the primers described by Khamis et al. (2004) were used. All primers used in this study are listed in Table 1.

2.3. DNA extraction

DNA was extracted from a loop full of bacterial culture material from a sheep blood agar plate suspended in 0.2 ml water (water for molecular biology, Merck Millipore, Darmstadt, Germany) by heating at 99 °C for 15 min. The cell-free supernatant obtained after centrifugation at 8000*g* for 1 min was directly used as a template for PCR. Fungal DNA was extracted from hyphae and/or spores that had been removed from the solid medium (Kimmig's agar) with a loop and transferred into 0.2 ml water. Fungal cells were mechanically disrupted by bead beating with ceramic beads (150 mg, 0.1 mm zirconia/silica beads, Roth, Karlsruhe, Germany) in a homogeniser (TeSeE Precess, Bio-Rad, Munich, Germany) for 3 min and subsequently heated for 15 min at 99 °C. After a final centrifugation step at 8000*g* for 1 min, DNA was extracted from the cell-free supernatant using the NuxEx Mag RNA/DNA kit (gerbion, Kornwestheim, Germany) processed on a King Fisher purification system (King Fisher Flex, Fisher Scientific, Schwerte, Germany) with an elution volume of 70 µl.

2.4. Real-time PCR assays and sanger sequencing

Real-time PCR assays were carried out with 12.5 µl master mix (VWR Taq DNA Polymerase Master Mix, VWR, Darmstadt, Germany) supplemented with 1 µl of a SYBR Green solution (SYBR Green I nucleic acid gel stain, 1:1000 diluted in water, Life Technologies, Darmstadt, Germany), 0.1 µM of each forward and reverse primer (Eurogentec, Seraing, Belgium) and 2.5 µl cell-free bacterial supernatant or 5 µl fungal DNA eluate. The reaction mixture was supplemented with water to a total volume of 25 µl.

The real-time PCR was performed with a universal thermal profile suitable for all PCR assays used (single run procedure). To achieve this, the optimal temperature for the annealing step of all assays was determined by running a temperature gradient ranging from 50 °C to 60 °C on the CFX Real-Time PCR System (Bio-Rad, Munich, Germany). Data were evaluated with the help of the CFX Manager Software V 2.0 program (Bio-Rad). The PCR run started with an initial step of DNA polymerase activation at 95 °C for 2 min, followed by 40 cycles including a denaturation step at 95 °C for 30 s, an annealing step at 55 °C for 30 s, and an elongation step at 72 °C for 30 s. The PCR runs were finalised by an extension step at 72 °C for 5 min. Data for melting curves were recorded for 5 s per temperature step during a temperature gradient from 65.0 °C to 95.0 °C with an increment of 0.5 °C. The CFX Real-Time PCR System (Bio-Rad) was applied for the PCR runs and data that had been recorded for creation of amplification and melting curves were evaluated using the CFX Manager Software V 2.0 program (Bio-Rad).

Selected PCR products were visualised by agarose gel electrophoresis (2% agarose, 100 V for 60 min) and finally stained with peqGREEN (VWR, Bruchsal, Germany) to verify the recorded real-time PCR data which characterise the PCR products (number of peaks, relative fluorescence units, and the heights of the melting curves).

PCR products obtained by the 16S rRNA, *rpoB* and combined ITS/LSU real-time PCR assays were purified using magnetic beads (MagBind TotalPure NGS Omega, Bio-Tek, VWR) and submitted for DNA sequencing on demand (Microsynth, Balgach, Switzerland). The bacterial forward primers were used for sequencing of the bacterial 16S rRNA and *rpoB* genes and the fungal reverse primer for decoding of the fungal ribosomal sequences in one direction, respectively. In addition, five bacterial and fungal isolates were sequenced in both directions. The sequence data used for bacterial identification encompassed at least 700 bp of the 16S rRNA and the *rpoB* genes of Gram-positive and Gram-negative bacteria and 350 bp of the *rpoB* genes of the corynebacteria, respectively. For the identification of fungi at least 400 bp of the ITS and the LSU genes were used.

The sequence data obtained were compared with sequence entries in the GenBank (http://www.ncbi.nlm.nih.gov/"; Pruitt et al., 2002) using the Basic Local Alignment Search Tool for nucleotides (BLASTN) on the NCBI website. Additionally, sequence data obtained from the fungal isolates were compared with entries in the Mycobank Database (MD; www.mycobank.org; Robert et al., 2013).

The sequences of the fungal regions ITS/LSU were separately employed for the database queries using the primer NL-1 (5′-GCATATCA ATAAGCGGAGGAAAAG-3′) as a separator between the ITS and LSU region (Kurtzman and Robnett, 1997).

The criteria used for assignment of a bacterial isolate to a species was set at a minimum of ≥ 99% homology combined with a minimum distance to the next best matching species of 0.8%. An isolate was assigned to a genus at a homology between 95% and 98.9% and also if it had ≥ 99% homology but with < 0.8% distance to the next best matching species (CLSI, 2008; Edgar, 2018; Jang et al., 2012; Koljalg et al., 2013).

The genus and species names were addressed according to the specifications provided by the free accessible web-based platform LPSN (list of prokaryotic names with standing in nomenclature for bacteria; http://www.bacterio.net, accessed 07.092018; Euzeby, 1997) and the Mycobank Database (http://www.mycobank.org, export date 03.07.2018; Robert et al., 2013).

3. Results and discussion

DNA sequencing has been increasingly used for taxonomy and identification of organisms (Hebert et al., 2003; Purty and Chatterjee, 2016). For bacteria the 16S rRNA and *rpoB* genes, and for fungi the ITS/LSU regions of ribosomal genes primarily serve as targets for DNA sequencing, ensuring high accuracy of microbial identification (Chakraborty et al., 2014; Petti, 2007; Purty and Chatterjee, 2016).

The first step in molecular investigations using PCR is the extraction of nucleic acids. This study shows that the procedure for DNA extraction has great influence on successful DNA amplification by PCR. Extraction of DNA from bacteria for the PCR assay was achieved by heating and using the cell-free supernatant after centrifugation (Chen

Table 2
Results retrieved from the Basic Local Alignment Search Tool (BLASTN, NCBI) based on partial bacterial DNA sequences obtained from the 16S rRNA and *rpoB* gene PCR assays and subsequent Sanger sequencing.

Isolate ID[a]	Intended bacterial species	Bacterial species identified by 16S rRNAgene sequences	Bacterial species identified by *rpoB* gene sequences
MB5898 CVUAS	*Acinetobacter lwoffii*	*Acinetobacter lwoffii* (sp.)	n.t.
MB11806 CVUAS	*Actinobacillus lignieresii*	*Actinobacillus lignieresii* (sp.)	n.t.
A19013088_3 CVUAS	*Actinobaculum suis*	*Actinobaculum suis* (sp.)	n.t.
MB9436 CVUAS	*Avibacterium gallinarium*	*Avibacterium gallinarum* (sp.)	n.t.
A18215078_2 CVUAS	*Basilea psittacipulmonis*	*Basilea psittacipulmonis* (sp.)	n.t.
MB3757.2 CVUAS	*Bibersteinia trehalosi*	*Bibersteinia trehalosi* (sp.)	n.t.
A16281684 CVUAS	*Clostridium perfringens*	*Clostridium perfringens* (sp.)	n.t.
A18214089-B CVUAS	*Clostridium tertium*	*Clostridium tertium* (sp.)	n.t.
MB1793.2 CVUAS	*Corynebacterium kutscheri*	*Corynebacterium kutscheri* (sp.)	n.t.
MB10231 CVUAS	*Corynebacterium renale*	*Corynebacterium renale* (sp.)	n.t.
MB10235 CVUAS (DSM 20478)	*Enterococcus faecalis*	*Enterococcus faecalis* (sp.)	n.t.
MB11480 CVUAS	*Erysipelothrix rhusiopathiae*	*Erysipelothrix rhusiopathiae* (sp.)	n.t.
A17053213_1 CVUAS	*Flavobacterium psychrophilum*	*Flavobacterium psychrophilum* (sp.)	n.t.
A18033627_17 CVUAS	*Flavobacterium succinicans*	*Flavobacterium succinicans* (sp.)	n.t.
MB5468.2 CVUAS	*Frederiksenia canicola*	*Frederiksenia canicola* (sp.)	n.t.
MB11809 CVUAS	*Haemophilus* sp.	*Haemophilus haemoglobinophilus* (sp.)	n.t.
MB2604.2 CVUAS	*Helcococcus kunzii*	*Helcococcus kunzii* (sp.)	n.t.
MB2603.2 CVUAS	*Helcococcus ovis*	*Helcococcus ovis* (sp.)	n.t.
VAR278368_1 CVUAS	*Histophilus somni*	*Histophilus somni* (sp.)	n.t.
MB4449.2 CVUAS	*Moraxella bovoculi*	*Moraxella bovoculi* (sp.)	n.t.
MB4844.3 CVUAS	*Necropsobacter rosorum*	*Necropsobacter rosorum* (sp.)	n.t.
MB2522.4 CVUAS	*Oligella urethralis*	*Oligella urethralis* (sp.)	n.t.
MB10238 CVUAS	*Pasteurella multocida*	*Pasteurella multocida* (sp.)	n.t.
MB6359 CVUAS	*Pasteurella testudinis*	*Pasteurella testudinis* (sp.)	n.t.
MB10239 CVUAS (DSM 50071)	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* (sp.)	n.t.
MB10220 CVUAS (ATCC 33701)	*Rhodococcus equi*	*Rhodococcus equi* (sp.)	n.t.
MB3141.4 CVUAS	*Rothia nasimurium*	*Rothia nasimurium* (sp.)	n.t.
MB766.3 CVUAS	*Salmonella enterica* serovar Abortusovis	*Salmonella enterica* (sp.)	n.t.
MB11188 CVUAS	*Salmonella enterica* serovar Enteritidis	*Salmonella enterica* (sp.)	n.t.
MB10221 CVUAS	*Salmonella enterica* serovar Typhimurium DT104L	*Salmonella enterica* (sp.)	n.t.
A16108380 CVUAS	*Shewanella putrefaciens*	*Shewanella putrefaciens* (sp.)	n.t.
MB10226 CVUAS (DSM 6784)	*Streptococcus agalactiae*	*Streptococcus agalactiae* (sp.)	n.t.
MB7649 CVUAS	*Streptococcus canis*	*Streptococcus canis* (sp.)	n.t.
MB11807 CVUAS	*Streptococcus dysgalactiae* subsp. *equisimilis*[b]	*Streptococcus dysgalactiae* (sp.)	n.t.
A18138302 CVUAS	*Streptococcus equi* subsp. *equi*[b]	*Streptococous equi* (sp.)	n.t.
A18103529 CVUAS	*Streptococcus equi* subsp. *zooepidemicus*[b]	*Streptococous equi* (sp.)	n.t.
MB9001 CVUAS	*Taylorella asinigenitalis*	*Taylorella asinigenitalis* (sp.)	n.t.
MB10242 CVUAS (DSM 10668)	*Taylorella equigenitalis*	*Taylorella equigenitalis* (sp.)	n.t.
MB10243 CVUAS	*Trueperella pyogenes*	*Trueperella pyogenes* (sp.)	n.t.
MB4339.2 CVUAS	*Yersinia ruckeri*	*Yersinia ruckeri* (sp.)	n.t.
MB4533.3 CVUAS	*Actinobacillus equuli* subsp. *equuli*[b]	*Actinobacillus equuli, A. suis* (gen.)	*Actinobacillus equuli* (sp.) [d]
MB4534.3 CVUAS	*Actinobacillus equuli* subsp. *haemolyticus*[b]	*Actinobacillus equuli, A. suis* (gen.)	*Actinobacillus equuli* (sp.) [d]
MB4219.2 CVUAS (Sterne strain 34F2)	*Bacillus anthracis*	*Bacillus anthracis, B. cereus, B. thuringiensis* (gen.)	*Bacillus anthracis, B. cereus, B. thuringiensis* (gen.) [c]
MB10201 CVUAS (DSM 4312)	*Bacillus cereus*	*Bacillus cereus, B. anthracis, B. thuringiensis* (gen.)	*Bacillus cereus, B. anthracis, B. thuringiensis* (gen.)[c]
MB11375 CVUAS	*Bacillus thuringiensis*	*Bacillus thuringiensis, B. cereus, B. anthracis* (gen.)	*Bacillus thuringiensis, B. cereus, B. anthracis* (gen.) [c]
MB10233 CVUAS	*Bordetella bronchiseptica*	*Bordetella bronchiseptica, B. pertussis, B. parapertussis and other species*(gen.)	*Bordetella bronchiseptica, B. pertussis, B. parapertussis* (gen.) [d]
MB2002.4 CVUAS	*Bordetella parapertussis*	*Bordetella parapertussis, B. pertussis, B. bronchiseptica,* and other species (gen.)	*Bordetella parapertussis, B. pertussis, B. bronchiseptica* (gen.)[d]
MB30917 CVUAS (CCM4915)	*Brucella microti*	*Brucella microti, B. abortus, B. canis, B. melitiensis, B. neotomae, B. suis*, and other species (gen.)	*Brucella microti, B. abortus, B. canis, B. melitiensis, B. neotomae, B. suis*, and other species (gen.)[d]
MB10204 CVUAS (ATCC 49941)	*Campylobacter coli*	*Campylobacter coli, C. jejuni* (gen.)	*Campylobacter coli* (sp.)[d]
MB395 CVUAS (ATCC 49943)	*Campylobacter jejuni*	*Campylobacter jejuni, C. coli* (gen.)	*Campylobacter jejuni* (sp.)[d]
MB31083 CVUAS (DSM 7528)	*Clostridium chavoei*	*Clostridium chavoei, C. septicum*	*Clostridium chavoei* (sp.)[c]
MB31084 CVUAS (DSM 7534)	*Clostridium septicum*	*Clostridium septicum, C. chauvoei* (gen.)	*Clostridium septicum* (sp.)[c]
MB3559.2 CVUAS	*Corynebacterium diphtheriae belfanti*	*Corynebacterium diphtheriae, C. vitaeruminis* (gen.)	*Corynebacterium diphtheria* (sp.) [e]
MB3365 CVUAS	*Corynebacterium pseudotuberculosis*	*Corynebacterium pseudotuberculosis, C. ulcerans* (gen.)	*Corynebacterium pseudotuberculosis* (sp.)[e]
MB5160 CVUAS	*Corynebacterium ulcerans*	*Corynebacterium ulcerans, C. pseudotuberculosis* (gen.)	*Corynebacterium ulcerans* (sp.) [e]
MB11691 CVUAS	*Edwardsiella tarda*	*Edwardsiella tarda, E. hoshinae* (gen.)	*Edwardiella tarda* (sp.) [d]

(*continued on next page*)

Table 2 (*continued*)

Isolate ID[a]	Intended bacterial species	Bacterial species identified by 16S rRNAgene sequences	Bacterial species identified by *rpoB* gene sequences
MB528 CVUAS	*Enterobacter cloacae*	*Enterobacter cloacae, E. asburiae, E. xiangfangensis* (*E. cloacae* complex) (gen.)	*E. hormaechei, E. asburiae* (*E. cloacae* complex) (gen.)[d]
MB10236 CVUAS	*Enterococcus faecium*	*Enterococcus faecium, E. durans* (gen.)	*Enterococcus faecium* (sp.)[c]
MB10214 CVUAS (DSM 30083)	*Escherichia coli*	*Escherichia coli, E. fergusonii, S. flexneri, S. boydii, S. dysenteriae, S. somnei* (gen.)	*Escherichia coli, Shigella flexneri, S. boydii, S. dysenteriae* (gen.) [d]
MB189.2 CVUAS	*Klebsiella (Enterobacter) aerogenes*	*Klebsiella (Enterobacter) aerogenes, Kluyvera cryocrescens* (gen.)	*Klebsiella (Enterobacter) aerogenes* (sp.) [d]
MB190.2 CVUAS	*Klebsiella pneumoniae*	*Klebsiella pneumoniae, K. variicola* (gen.)	*Klebsiella pneumoniae* (sp.) [d]
MB10522 CVUAS (DSM 20649)	*Listeria innocua*	*Listeria innocua, L. monocytogenes, L. seeligeri* (gen.)	*Listeria innocua* (sp.)[c]
MB7135.3 CVUAS	*Listeria ivanovii*	*Listeria ivanovii, L. monocytogenes, L. innocua* (gen.)	*Listeria ivanovii* (sp.)[c]
MB10219 CVUAS (ATCC 19115)	*Listeria monocytogenes*	*Listeria monocytogenes, L. innocua, L. seeligeri* (gen.)	*Listeria monocytogenes* (sp.)[c]
MB4862.3 CVUAS	*Listeria seeligeri*	*Listeria seeligeri, L. monocytogenes, L. innocua* (gen.)	*Listeria seeligeri* (sp.)[c]
MB30548 CVUAS	*Mannheimia haemolytica*	*Mannheimia haemolytica, M. glucosida* (gen.)	*Mannheimia haemolytica* (sp.) [d]
MB108.2 CVUAS	*Serratia marcescens*	*Serratia marcescens, S. nematodiphila* (gen.)	*Serratia marcescens* (sp.) [d]
A18262817 CVUAS	*Shewanella putrefaciens*	*Shewanella putrefaciens, S.oneidensis, S. xiamenensis* (gen.)	*Shewanella putrefaciens* (sp.) [d]
MB30980 CVUAS (ATCC 29903)	*Shigella flexneri*	*Shigella flexneri, S. boydii, S. dysenteriae, S. somnei, Escherichia coli, E. fergusonii* (gen.)	*Shigella flexneri, S. boydii, S. dysenteriae, Escherichia coli* (gen.) [d]
MB10223 CVUAS (ATCC 25923)	*Staphylococcus aureus*	*Staphylococcus aureus, S. haemolyticus* (gen.)	*Staphylococcus aureus* (sp.)[c]
MB10225 CVUAS	*Staphylococcus epidermidis*	*Staphylococcus epidermidis, S. capitis, S. caprae* (gen.)	*Staphylococcus epidermidis* (sp.)[c]
MB10240 CVUAS	*Staphylococcus hyicus*	*Staphylococcus hyicus, S. agnetis* (gen.)	*Staphylococcus hyicus, S. agnetis* (gen.)[c]
MB30303 CVUAS	*Streptococcus pneumoniae*	*Streptococcus pneumoniae, S. pseudopneumoniae, S. infantis, S. mitis, S. oralis* (gen.)	*Streptococcus pneumoniae* (sp.)[c]
MB7990 CVUAS	*Vibrio alginolyticus*	*Vibrio alginolyticus, V. parahaemolyticus* (gen.)	*Vibrio alginolyticus* (sp.) [d]
MB10227 CVUAS	*Vibrio cholera*	*Vibrio cholerae, V. alginolyticus* (gen.)	*Vibrio cholera* (sp.) [d]
MB10228 CVUAS (DSM 10027)	*Vibrio parahaemolyticus*	*Vibrio parahaemolyticus, V. alginolyticus* (gen.)	*Vibrio parahaemolyticus* (sp.) [d]
MB10229 CVUAS	*Yersinia enterocolitica*	*Yersinia enterocolitica, Y. rhodei* (gen.)	*Yersinia enterocolitica* (sp.) [d]
MB11690 CVUAS	*Yersinia pseudotuberculosis*	*Yersinia pseudotuberculosis, Y. pestis* (gen.)	*Yersinia pseudotuberculosis, Y. pestis* (gen.) [d]

n.t. = not tested because determination at species level was possible by 16S rRNA gene sequencing.
(sp.) = identification of bacterial isolate at species level
(gen.) = identification of bacterial isolate at genus level, the level of species complexes or closely related species.
[a] Isolates with the suffix CVUAS originate from the isolate collection of the Chemical and Veterinary Investigations Office Stuttgart (CVUAS).
[b] Differentiation between subspecies is not possible using 16S rRNA or rpoB gene sequencing
[c] *rpoB*-PCR for Gram-positive bacteria using the primer pair rpoB_gr-pos_2287-F/ rpoB_gr-pos_3215-R.
[d] *rpo*B-PCR for Gram-negative bacteria using the primer pair rpoB_gr-neg_2620F/ rpoB_gr-neg_3416-R.
[e] *rpo*B-PCR for corynebacteria using the primer pair C2700F/C3130R (Khamis et al., 2004)

et al., 2014). However, extraction of DNA from filamentous fungi proved to be more complex, because for successful DNA extraction a procedure including bead beating, heating and subsequent application of magnetic beads or silica based columns was favourable, as previously described by Romanelli et al. (2014). Furthermore, this technique allowed extraction of fungal DNA even in the presence of residual agar originating from the fungi cultivation media (Romanelli et al., 2014).

The products of the bacterial ribosomal RNA and *rpoB* and the fungal combined ITS/LSU ribosomal genes generated in this study showed a size of approximately 800–1000 bp and 1200–1300 bp, respectively, to be suitable for Sanger sequencing. Addition of DNA-binding dyes allowed the characterisation of PCR products based on amplification and melting curves during real-time PCR runs superseding detection of the amplicons in agarose gels (Chen et al., 2014). To achieve this, evaluation of data obtained from the PCR runs served as criteria for PCR products suitable for successful DNA sequencing. These criteria were melting curves with distinct peaks, RFU (relative fluorescence unit) values > 2000, −d(RFU)/dt (peak height) values of > 300 and T_m of > 80 °C (Figs. 1 and 2). PCR runs meeting these criteria showed distinct and single bands in agarose gel electrophoresis. Ultimately, the employment of a classical PCR master mix supplemented with SYBR Green facilitates the generation of PCR products and subsequent decoding using Sanger sequencing on demand within two days. Results of database queries obtained from PCR products of five isolates sequenced in one and in both directions revealed similar results, so that sequencing in the forward direction was considered sufficient (data not shown). This is in agreement with findings of Ellard et al. (2009) for semi-automated Sanger sequencing who found no evidence to support a requirement for bidirectional sequencing.

3.1. Bacteria

A crucial step in the identification of bacteria based on 16S rRNA and *rpoB* gene sequences is the design of appropriate primers. Therefore, primer design was based on multiple alignments of partial gene sequences originating from 112 (16S rRNA genes) and 71 (*rpoB* genes) bacterial species, respectively, which are deposited in the GenBank (NCBI) database. The primers were designed to target conserved segments amplifying the variable 16S rRNA gene regions V3 and V6 (Chakravorty et al., 2007; Yang et al., 2016). The designed 16S forward primer 16S_337-F shares common nucleotide sequences with the forward primer 357F described by Johnson (1994) and shows a complete agreement with the bacteria sequences included in the multiple sequence alignment. The designed reverse primer 16S_1196-R has partial sequences in common with the primer 1185aR provided by Hodkinson and Lutzoni (2009) and was supplemented with two degenerate nucleotides to compensate single nucleotide polymorphisms (SNPs). Regarding the *rpoB* gene, there is no universal primer pair which is able to produce amplicons for all bacterial species. Nevertheless, in this study it was possible to design broad-spectrum primers targeting the partial *rpoB* gene of Gram-positive and Gram-negative bacteria used in this study, respectively. These primers amplify a hypervariable region of the *rpoB* gene which is located between the

Table 3
Results retrieved from the Basic Local Alignment Search Tool (BLASTN, NCBI) and the MycoBank Database based on partial fungal DNA sequences obtained from the combined ITS/LSU PCR assay and subsequent Sanger sequencing.

Isolate ID[a]	Intended fungal genus or species	Fungal species identified by LSU gene sequences using BLASTN	Fungal species identified by ITS gene sequences using BLASTN	Fungal species identified by LSU/ITS gene sequences using MycoBank Database
A18200460-1 CVUAS	*Alternaria alternaria*	*Alternaria* sp. (gen.)	*Alternaria alternaria* (sp.)	*Alternaria alternata, A. burnsii* (gen.)
161014361 LHL-GI	*Alternaria infectoria*	*Alternaria* sp. (gen.)	*Alternaria infectoria* (sp.)	*Alternaria infectoria, A. graminicola* (gen.)
131007696 LHL-GI	*Aspergillus fumigatus*	*Aspergillus fumigatus, A. thermomutatus* (gen.)	*Aspergillus fumigatus* (sp.)	*Aspergillus fumigatus* (sp.)
MB30510 CVUAS	*Atelosaccharomyces pseudotropicalis (Candida kefyr)*	*Atelosaccharomyces pseudotropicalis (Candida kefyr), Kluyveromyces lactis* (gen.)	*Atelosaccharomyces pseudotropicalis (Candida kefyr)* (sp.)	*Atelosaccharomyces pseudotropicalis* (*Candida kefyr*) (sp.)
MB5206.2 CVUAS	*Botrytis cinerea*	*Botrytis cinerea* (sp.)	*Botrytis cinerea* (sp.)	*Botrytis cinerea, B. aclada* (gen.)
MB3957 CVUAS (DSM 1386)	*Candida albicans*	*Candida albicans* (sp.)	*Candida albicans* (sp.)	*Candida albicans* (sp.)
MB6900 CVUAS	*Candida glabrata*	*Candida glabrata* (sp.)	*Candida glabrata* (sp.)	*Candida glabrata* (sp.)
11-7-D-00568 LHL-GI	*Candida intermedia*	*Candida intermedia* (sp.)	*Candida intermedia* (sp.)	*Candida intermedia* (sp.)
MB3665 CVUAS	*Candida kruse*	*Candida krusei* (sp.)	*Candida krusei* (sp.)	*Candida krusei* (sp.)
09-7-D-05500/1 LHL-GI	*Candida palmioleophila*	*Candida palmioleophila, C. manassasensis* (gen.)	*Candida palmioleophila* (sp.)	*Candida palmioleophila* (sp.)
A18215193 CVUAS	*Candida rugosa*	*Candida rugosa* (sp.)	*Candida rugosa* (sp.)	*Candida rugosa* (sp.)
131013404 LHL-GI	*Candida tropicalis*	*Candida tropicalis* (sp.)	*Candida tropicalis* (sp.)	*Candida tropicalis* (sp.)
A17056283_5 CVUAS	*Cystofilobasidium infirmominiatum*	*Cystofilobasidium infirmominiatum* (sp.)	*Cystofilobasidium infirmominiatum* (sp.)	*Cystofilobasidium infirmominiatum* (sp.)
A17241291 CVUAS	*Diutina catenulata*	*Diutina catenulata* (sp.)	*Diutina catenulata* (sp.)	*Diutina catenulata* (sp.)
09-7-D-05172 LHL-GI	*Geotrichum candidum*	*Geotrichum candidum* (sp.)	*Geotrichum candidum* (sp.)	*Geotrichum candidum* (sp.)
MB5417 CVUAS	*Hanseniaspora uvarum*	*Hanseniaspora uvarum* (sp.)	*Hanseniaspora uvarum* (sp.)	*Hanseniaspora uvarum* (sp.)
A18162741 CVUAS	*Malassezia pachydermatis*	*Malassezia pachydermatis* (sp.)	*Malassezia pachydermatis* (sp.)	*Malassezia pachydermatis* (sp.)
Isolate collection Dr. Seidl, Munich LHL-GI	*Micorpsorum gypseum*	*Microsporum gypseum* (sp.)	*Microsporum gypseum* (sp.)	*Microsporum gypseum* (sp.)
11-7-D-02155 LHL-GI	*Mucor circinelloides*	*Mucor circinelloides* (sp.)	*Mucor circinelloides* (sp.)	*Mucor circinelloides* (sp.)
MB5210.2 CVUAS	*Penicillium digitatum*	*Penicillium digitatum, Eupenicillium molle* (gen.)	*Penicillium digitatum* (sp.)	*Penicillium digitatum* (sp.)
11-7-D-00082 LHL-GI	*Purpureocillium lilacinum*	*Purpureocillium lilacinum* (sp.)	*Purpureocillium lilacinum* (sp.)	*Purpureocillium lilacinum* (sp.)
10-7-D-00170 LHL-GI	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (sp.)	*Saccharomyces cerevisiae* (sp.)	*Saccharomyces cerevisiae* (sp.)
161009975 LHL-GI	*Sporothrix schenkii*	*Sporothrix schenckii, S. stenoceras* (gen.)	*Sporothrix schenckii* (sp.)	*Sporothrix sp.* (gen.)
Isolate collection Dr. Seidl, Munich LHL-GI	*Trichophyton (Microsporum) persicolor*	*Trichophyton (Microsporum) persicolor, M. gypseum* (gen.)	*Trichophyton*(*Microsporum*) *persicolor* (sp.)	*Trichophyton (Microsporum) persicolor* (sp.)
Isolate collection Dr. Seidl, Munich LHL-GI	*Paraphyton* (*Microsporum*) *mirabile*	*Trichophyton* (*Microsporum*) *persicolor, Paraphyton (M.) cookei* (gen.)	*Paraphyton (Microsporum) mirabile* (sp.)	*Nannizza* (*Microsporum*) *praecox, Microsporum gypseum* (gen.)
Isolate collection Dr. Seidl, Munich LHL-GI	*Trichophyton ajelloi*	*Trichophyton ajelloi* (sp.)	*Trichophyton ajelloi* (sp.)	*Trichophyton ajelloi* (sp.)
MB4706.3 CVUAS	*Trichophyton mentagrophytes* (hedgehog origin)	*Trichophyton mentagrophytes* (sp.)	*Trichophyton mentagrophytes* (sp.)	*Trichophyton mentagrophytes* (sp.)
161003164 LHL-GI	*Trichophyton mentagrophytes* (human origin)	*Trichophyton mentagrophytes* (sp.)	*Trichophyton mentagrophytes* (sp.)	*Trichophyton mentagrophytes* (sp.)
121016821 LHL-GI	*Trichophyton mentagrophytes* (reindeer origin)	*Trichophyton mentagrophytes* (sp.)	*Trichophyton mentagrophytes* (reindeer origin) (sp.)	*Trichophyton mentagrophytes* (animal origin) (sp.)
161009770 LHL-GI	*Nannizia (Microsporon) praecox*	*Trichophyton persicolor, Microsporum gypseum* (gen.)	*Nannizia* (*Microsporon*) *praecox* (sp.)	*Nannizia (Microsporon) praecox* (sp.)
11-7-D-01636/16 LHL-GI	*Trichophyton terrestre*	*Trichophyton terrestre* complex (gen.)	*Trichophyton terrestre* (sp.)	*Trichophyton terrestre* (sp.)
A18078681 CVUAS	*Trichophyton verrucosum*	*Trichophyton verrucosum* (sp.)	*Trichophyton verrucosum* (sp.)	*Trichophyton verrucosum* (sp.)
141001820 LHL-GI	*Apiotrichum loubieri*	*Apiotrichum loubieri, A. mycotoxinivorans, A. laibachii*, and other species (clade Gracile/Brassicae) (gen.)	*Apiotrichum loubieri, A. mycotoxinivorans, A. dulcitum*, and other species (clade Gracile/Brassicae) (gen.)	*Apiotrichum loubieri, A. mycotoxinivorans* (clade Gracile/Brassicae) (gen.)
131007518/2 LHL-GI	*Apiotrichum myotoxinvorans*	*Apiotrichum mycotoxivyrians, A. loubieri, A. dulcitum*, and other species (clade Gracile/Brassicae) (gen.)	*Apiotrichum mycotoxinivorans, A. loubieri, A. dulcitum* and other species (clade Gracile/Brassicae) (gen.)	*Apiotrichum loubieri, A. mycotoxinivorans, A. dulcitum* and other species (clade Gracile/Brassicae) (gen.)
MB30573 CVUAS	*Aspergillus brasiliensis*	*Aspergillus brasiliensis, A. niger (Aspergillus* section Nigri) (gen.)	*Aspergillus brasiliensis, A. niger (Aspergillus* section Nigri) (gen.)	*Aspergillus brasiliensis, A. niger (Aspergillus* section Nigri) (gen.)
MB5205.2 CVUAS	*Aspergillus niger*	*Aspergillus niger, A. tubingensis (Aspergillus* section Nigri) (gen.)	*Aspergillus niger, A. tubingensis (Aspergillus* section Nigri) *(gen.)*	*Aspergillus niger, A. tubingensis (Aspergillus* section Nigri) (gen.)
141002191 LHL-GI	*Aspergillus flavus*	*Aspergillus* section Flavi (gen.)	*Aspergillus* section Flavi (gen.)	*Aspergillus* section Flavi (gen.)
A18166548 CVUAS	*Aspergillus terreus*	*Aspergillus* section Terrei (gen.)	*Aspergillus* section Terrei (gen.)	*Aspergillus* section Terrei (gen.)
11-7-D-01435 LHL-GI	*Beauveria bassiana*	*Beauveria bassiana, B. brongniartii* (gen.)	*Beauveria bassiana, B. pseudobassiana* (gen.)	*Beauveria brongniartii* (sp.)
09-7-D-01436 LHL-GI	*Candida saitoana*	*Candida saitoana, C. glaebosa* (gen.)	*Candida saitoana, C. glaebosa* (gen.)	*Candida saitoana* (sp.)

(*continued on next page*)

Table 3 (*continued*)

Isolate ID[a]	Intended fungal genus or species	Fungal species identified by LSU gene sequences using BLASTN	Fungal species identified by ITS gene sequences using BLASTN	Fungal species identified by LSU/ITS gene sequences using MycoBank Database
A18200460-B CVUAS	*Cladosporium cladosporioides*	*Cladosporium cladosporioides* complex (gen.)	*Cladosporium cladosporioides* complex (gen.)	*Cladosporium cladosporioides* complex (gen.)
A17271552 CVUAS	*Cladosporium sphaerospermum*	*Cladosporium sphaerospermum* complex (gen.)	*Cladosporium sphaerospermum* complex (gen.)	*Cladosporium sphaerospermum* complex (gen.)
121002375 LHL-GI	*Cryptococcus neoformans*	*Cryptococcus neoformans, C. gattii* (gen.)	*Cryptococcus neoformans, C. gattii* (gen.)	*Cryptococcus neoformans, C. gattii* (gen.)
MB5212.2 CVUAS	*Phoma herbarum*	*Didymella* sp. (gen.)	*Didymella* sp. (gen.)	*Phoma herbarum* (sp.)
11-7-D-00727 LHL-GI	*Microsporum canis*	*Microsporum canis, M. audouinii* (*M. canis* complex) (gen.)	*Microsporum canis, M. ferrugineum* (*M. canis* complex) (gen.)	*Microsporum canis, M. ferrugineum* (*M. canis* complex) (gen.)
MB8051 CVUAS	*Nannizziopsis guarroi*	*Nannizziopsis draconii, N. guarroi, N. chlamydospora* (gen.)	*Nannizziopsis draconii, N. guarroi, N. chlamydospora* (gen.)	*Nannizziopsis draconii, N. guarroi, N. chlamydospora* (gen.)
MB5209.2 CVUAS	*Penicillium camemberti*	*Penicillium camemberti, P. commune* (sect. Camemberti) (gen.)	*Penicillium camemberti, P. crustosum* (sect. Camemberti) (gen.)	*Penicillium solitum, P. crustosum, P. expansum* (gen.)
MB5211.2 CVUAS	*Penicillium roqueforti*	*Penicillium roqueforti, P. carneum* (sect. Roqueforti) (gen.)	*Penicillium roqueforti, P. carneum* (sect. Roqueforti) (gen.)	*Penicillium roqueforti, P. carneum* (sect. Roqueforti) (gen.)
161013500 LHL-GI	*Tolypocladium* sp.	*Tolypocladium* sp. (gen.)	*Tolypocladium* sp. (only few species entries in NCBI) (gen.)	*Tolypocladium* sp. (only few species entries in NCBI) (gen.)
11-7-D-00669 LHL-GI	*Trichosporon asahii*	*Trichosporon asahii, T. asteroides, T. ovoides*, and other species (clade Porposum) (gen.)	*Trichosporon asahii, T. insectorum, T. asteroides*, and other species (clade Porposum) (gen.)	*Trichosporon asahii, T. ceremiiforme, T. ovoides*, and otherspecies (clade Porposum) (gen.)
171016086/1 LHL-GI	*Trichosporon coremiforme*	*Trichosporon coremiforme, T. insectorum, T. asahii*, and other species (clade Porosum) (gen.)	*Trichosporon coremiforme, T. faecale, T. asteroides*, and other species (clade Porosum) (gen.)	*Trichosporon coremiforme, T. insectorum* (clade Porosum) (gen.)

(sp.) = identification of fungal isolates at species level

(gen.) = identification of fungal isolates at genus level, the level of species complexes or sections or closely related species.

[a] Isolates with the suffix CVUAS originate from the isolate collection of the Chemical and Veterinary Investigations Office Stuttgart (CVUAS) Isolates with the suffix LHL-GI originate from the Hessian State Laboratory.

positions 2400 and 3300 and described as being suitable for identification and phylogenetic discrimination at species level (Adékambi et al., 2009; Khamis et al., 2004). In this regard, primers targeting *rpoB* gene sequences of Gram-positive and Gram-negative bacteria were created (Table 1). For amplification of partial *rpoB* gene sequences of corynebacteria, primers recommended by Khamis et al. (2004) were used successfully. The results of these PCR assays subjected to a temperature gradient showed that a one-run procedure works well and simplifies the process of identification of bacteria and fungi (data not shown).

Final evaluation of sequence data were performed with free accessible databases. Among common available databases, the RDP and SILVA databases are based on the genus level only and the EzBioCloud database is restricted to type strains (Yoon et al., 2017). By comparison, the GenBank (NCBI) and OTT databases also address species level (Balvočiūtė and Huson, 2017). All in all, the GenBank database (NCBI) was chosen for sequence analysis due to its comprehensive database, updates at short intervals and easy online accessibility.

Using only 16S rRNA gene sequencing resulted in identifications at genus level of only 40 (51%) of the 78 investigated bacterial isolates belonging to various genera (Table 2). These findings were confirmed by comparative BLASTN database queries including the partial nucleotide sequences obtained in this study compared to near-complete sequences retrieved from the GenBank database (NCBI) for the same bacterial species. Partial and near-complete 16S rRNA gene sequences returned comparable results. In this context, comparative studies on partial and near-complete 16S rRNA gene sequences and gene sequences obtained by unidirectional and bidirectional sequencing of the bacterial and fungal isolates used in this study returned comparable results from the GenBank database (NCBI), respectively.

In general, when using 16S rRNA gene sequencing, it must be kept in mind that identification at species level is not possible for various bacteria. Close relationships and near identity of 16S rRNA gene sequences require further exploration using additional methods such as *rpoB* gene sequencing (Adékambi et al., 2009). In this context, Janda and Abbott (2007) and Woo et al. (2009) reported identification of 65–100% and 24–40%, respectively, of the bacteria tested at species level using full or partial 16S rRNA gene sequencing. This is due to intraspecific variation in multiple 16S rRNA gene sequences that hamper differentiation of closely related species (Pei et al., 2010; Coenye and Vandamme, 2003). To alleviate these difficulties, alternative single copy housekeeping genes such as the *rpoB* gene have been used for species identification of bacteria (Adékambi et al., 2009; Case et al., 2007; Mollet et al., 1997; Petti, 2007). However, there is no universal *rpoB* primer pair that covers all bacterial species and as a general rule, *rpoB* primers must be targeted to amplify a range of bacterial groups (Adékambi et al., 2009; Case et al., 2007). Partial sequencing of *rpoB* genes has been taken up in this study using PCR assays for amplification of partial *rpoB* gene sequences of Gram-positive, Gram-negative bacteria and corynebacteria. With the help of sequencing of the hypervariable *rpoB* region located between position 2400 and 3300 (Adékambi et al., 2009; Khamis et al., 2004), an additional 27 bacterial isolates out of 38 could be identified at species level resulting in 67 (86%) species identifications of a total of 78 isolates. However, using either 16S rRNA or rpoB gene sequencing a clear species identification was not possible between the very closely related species *Bacillus* (*B.*) *anthracis/ B. cereus/ B. thuringiensis* (*Bacillus cereus* group), *Bordetella* (*B.*) *pertussis/ B. parapertussis/ B. bronchiseptica, Brucella* (*B.*) spp., *Enterobacter* (*E.*) *cloacae/ E. hormaechei/ E. asburiae* (*Enterobacter cloacae* complex), *Escherichia coli/ Shigella* spp., *Staphylococcus* (*S.*) *hyicus/ S. agnetis*, and *Yersinia* (*Y.*) *pseudotuberculosis/ Y. pestis*.

3.2. Fungi

In the present study, identification of fungi including dermatophytes, moulds and yeasts was based on DNA sequences of the internal transcribed spacer 1 and 2 (ITS1, ITS2) and the D1 and D2 regions of

the large-subunit rRNA gene (LSU). The primers used for the real-time PCR assay amplifying the combined ITS/LSU regions are listed in Table 1. The ITS and the LSU regions were chosen for DNA sequencing to provide sequence data of both, target genes that are relevant for taxonomy (Raja et al., 2017) and best identification rates have been achieved when using both targets (Kwiatkowski et al., 2012; Vu et al., 2019).

The primers used for generating PCR products for DNA sequencing amplify the ITS (ITS1, ITS2) and the LSU (D1, D2) regions in combination and were designed based on data provided by White et al. (1990) and O'Donnell (1993). The nucleotide sequences of the primers were modified based on multiple alignments of ITS and LSU gene sequences obtained from the GenBank database (NCBI) representing a total of 79 and 89 fungal species, respectively. The results retrieved from the GenBank database using separate queries for ITS and LSU sequences yielded higher coverage and identity values probably due to the higher proportion of partial ITS and LSU sequences deposited in GenBank database (Vu et al., 2019). The ITS and LSU sequences could be separated with the help of the primer described by Kurtzman and Robnett (1997) as a marker. This approach resulted in clearer species attributions. However, closely related filamentous fungal species included in this study, i.e. the genera *Apiotrichum*, *Aspergillus, Cladosporium, Cryptococcus, Microsporum, Nannizziopsis*, *Penicillium, Trichosporon,* and *Tolypocladium*, are difficult to differentiate based on ribosomal sequences (Table 3). Combining the results of ITS and LSU sequencing of the 51 fungal isolates examined, 32 (63%) could be identified at species level based on ITS sequences, but 19 (37%) were identified only at genus level or at the level of species complexes or closely related species according to BLASTN (Table 3). The comparison of the results of the queries retrieved from MD with those from GenBank database shows a complete correlation regarding the genus level. Concerning species identification, Vu et al. (2019) reported that for about 20% of the fungal isolates, identification at species level was not possible and the authors concluded that ITS and LSU sequencing is not sufficient for determination of all fungi. In general, ITS sequences have proved to be best suited for species identification, while LSU sequence data provide the basis for taxonomic classification (Das and Deb, 2015; Vu et al., 2019). However, other studies report a similar discrimination power of ITS and LSU sequences (Brown et al., 2014; Mueller et al., 2016; Porras-Alfaro et al., 2014). Nevertheless, Kwiatkowski et al. (2012) and Vu et al. (2019) point out that analyses of both the ITS and LSU regions are complementary and increase the degree of identification of filamentous fungi. Bearing these facts in mind, amplification of the ITS/LSU region in a single run procedure for sequencing was realised and makes database queries of both regions possible.

4. Conclusion

The combination of real-time PCR assay targeting partial bacterial 16S rRNA and *rpoB* gene sequences and combined ITS/LSU sequences in a single run procedure using SYBR Green real-time PCR and subsequent Sanger sequencing proved to be suitable tools for the identification of bacteria and fungi within a period of only two days.

For the robust amplification of partial gene sequences which are suitable for DNA sequencing extraction of DNA, the use of primers covering a broad range of taxa and the choice of a PCR master mix are crucial issues that must be considered.

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