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A species-specific multiplexed PCR amplicon assay for distinguishing between *Metarhizium anisopliae*, *M. brunneum*, *M. pingshaense* and *M. robertsii*

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A B S T R A C T

The fungal species *Metarhizium pingshaense*, *M. anisopliae*, *M. robertsii*, and *M. brunneum*, a monophyletic group informally referred to as the PARB species complex, are well known facultative entomopathogens, including many commercialized strains used for biological pest control. Accurate and expedient species identification of *Metarhizium* isolates represents an important first step when addressing ecological as well as application-related questions involving these fungi. To this end, a species-specific multiplexed polymerase chain reaction (PCR) assay was developed for identification and discrimination among *Metarhizium* PARB complex species, based on unique sequence signature differences within the nuclear ribosomal intergenic spacer (rIGS) and nuclear intergenic spacer regions MzFG546 and MzIGS2.

Species-specificities of the four primer pairs were assessed following a three-step approach including: (1) *in silico* verification of sequence signatures by BLASTN searches against publically available genome and amplicon sequence data, (2) corroboration of assay specificity and robustness by performing test PCR amplifications against a taxonomically curated reference strain collection of 68 *Metarhizium* strains representing 12 species, and (3) testing against a field collection of 19 unknown *Metarhizium* isolates from soil of a Swiss meadow. The specificity of these four primer pairs provide an efficient means to detect and discriminate PARB species in studies targeting ecological aspects of indigenous isolates, as well as efficacy, persistence and potential non-target effects of applied biocontrol strains.

1. Introduction

Entomopathogenic fungi are natural pathogens of diverse arthropods and used to control insect pest populations in biological pest management programs (Lovett and St. Leger, 2017). The entomopathogenic fungal genus *Metarhizium* Sorokin (Hypocreales: Clavicipitaceae) has a global distribution and comprises species that infect diverse insects and other arthropods and also colonize soil and plant rhizospheres (Barelli et al., 2016). *Metarhizium* represents one of the best characterized and widely studied entomopathogenic fungal genera with regard to ecology, evolution, pathogenicity, life history, and genome biology (Lovett and St. Leger, 2017). The insect pathogenic traits of several species have been exploited for biological control of various economically important arthropod pests, with multiple commercialized products available worldwide (Faria and Wraight, 2007).

Originally recognized on the basis of microscopical and cultural morphology (Sorokin, 1883), a recent taxonomic revision based on molecular phylogenetic criteria has revealed that *M. anisopliae* s.l. encompasses a clade of at least four cryptic phylogenetic species, the so-called PARB clade, that includes *M. pingshaense*, *M. anisopliae*, *M. robertsii* and *M. brunneum* (Bischoff et al., 2009; Rehner and Kepler, 2017). Among the steadily increasing array of nuclear loci used for

Abbreviations: PARB, *Metarhizium* clade consisting of *M. pingshaense*, *M. anisopliae*, *M. robertsii* and *M. brunneum*; rIGS, ribosomal intergenic spacer region; MzIGS2, nuclear intergenic spacer region between the pre-rRNA-processing protein ipi1 and the DEAD/DEAH box RNA helicase; MzFG546, nuclear intergenic spacer region between the vezatin and the proliferating cell nuclear antigen protein coding regions; PCR, polymerase chain reaction; BLASTN, nucleotide basic local alignment search tool; 5TEF, 5′-intron-rich portion of translation elongation factor one alpha; BCA, biological control agent; ARSEF, Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, U.S.A.; CBS, Centraalbureau voor Schimmelcultures, Westerdijk Fungal Biodiversity Institute Netherlands

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Table 1
Information for *M. anisopliae*, *M. brunneum*, *M. pingshaense* and *M. robertsii* specific primer pairs.

Primer*	Species	Locus	Sequence 5′ – 3′	Amplicon size for type species [bp]	Position of each primer based on type strain sequence	GenBank accession number of type strain sequence
Ma-rIGS-1648-F	*M. anisopliae*	rIGS	ACGGTCGCACACAAATC	328	1648–1664	MH604974
Ma-rIGS-1975-R			CAGCCTACCCGGTAC		1961–1975	
Mb-FG546-422-F	*M. brunneum*	MzFG546	TAGTCAGTCGTTGACGC	115	422–438	KC164538
Mb-FG546-536-R			TCCTGTGTCGACTGTGTCGA		517–536	
Mp-IGS2-240-F	*M. pingshaense*	MzIGS2	ACGGCATGGACATGCCC	535	240–256	KC164550
Mp-IGS2-774-R			GCCTCTCGTTACCTACGA		757–774	
Mr-rIGS-444-F	*M. robertsii*	rIGS	ATTACCAAGTCCAAAATACTGG	638	444–465	MH605000
Mr-rIGS-1081-R			CATATACCCACCAACTACCC		1062–1081	

* Primer name consists of abbreviation of species (i.e., Ma), locus, position of primer on type strain sequence and F or R for forward or reverse primer.

Table 2
Target and non-target hits with 100% identity at all positions of each primer assessed with BLASTN and non-target hits obtained with both forward and reverse primer.

Primer	Number of total hits	Number of target hits	Number of non-target hits/number of different species representing the non-target hits	Number of non-target hits of primer pair
Ma-rIGS-1648-F	4	1	3/2	0
Ma-rIGS-1975-R	69	1	68/23	
Mb-FG546-422-F	32	24	8/1	0
Mb-FG546-536-R	28	27	1/1	
Mp-IGS2-240-F	71	55	16/11*	0
Mp-IGS2-774-R	56	56	0/0	
Mr-rIGS-444-F	12	2	10/2**	0***
Mr-rIGS-1081-R	17	7	10/2**	

* Non-target hits included three *M. anisopliae* sequences (KX342364.1, KX342365.1 and KX342366.1).

** Non-target hits included one *M. pingshaense* sequence (Genbank Acc FJ2287.1) and nine *M. anisopliae* sequences (Genbank Acc AF487272.1, AF363471.1, AF363469.1, AF363466.1, AF363464.1, AF363463.1, AF363459.1, AF218207.1 and AY847512.1).

*** Based on the curated species affiliation of the sequences.

phylogenetic systematic studies of *Metarhizium* (Bischoff et al., 2009; Driver et al., 2000; Kepler et al., 2014), the 5′-intron-rich portion of the translation elongation factor one alpha (5TEF) has proven to be among the most useful single DNA markers for routine PARB species identification (Bischoff et al., 2009; Rezende et al., 2015) and has been used in numerous studies (Fisher et al., 2011; Garrido-Jurado et al., 2015; Steinwender et al., 2015; Steinwender et al., 2014; Wyrebek et al., 2011). Kepler and Rehner (2013) introduced seven carefully selected nuclear intergenic sequence markers whose phylogenetic informativeness extends across the epoch during which the PARB clade diversification occurred. Although not yet in as wide use as 5TEF, the utility of one or more of these intergenic markers for *Metarhizium* species identification (Kepler et al., 2015; Keyser et al., 2015; Rezende et al., 2015) and also for investigation of species limits and intraspecific genetic diversity within the PARB complex (Rehner and Kepler, 2017) has been demonstrated in several recent studies.

In keeping with recent taxonomic changes, several prominent *Metarhizium* biocontrol agents (BCA) species assignments have required correction. For example, BCA strain BIPESCO5 (F52), previously assigned to *M. anisopliae*, is now recognized as *M. brunneum* (Mayerhofer et al., 2015; Rehner and Kepler, 2017). Accordingly, species identifications in studies investigating host specificity, habitat association, diversity and prevalence *of Metarhizium* spp. performed prior to the currently accepted taxonomy likely need to be re-evaluated. For future studies, efficient and simple molecular identification assays such as species-specific PCR would greatly facilitate the identification process (Enkerli and Widmer, 2010), enabling insight into community composition, estimates of species diversity and abundance as well as a means to quickly assign species for population genetic analysis or monitoring applied BCA strains without the expense and effort of identification by sequencing. Species-specific PCR have been developed for detection of *M. brunneum* (Kabaluk et al., 2017), the *Metarhizium* clade 1 (Schneider et al., 2011), two strains originally assigned to *M. anisopliae* (Destéfano et al., 2004) and *M. acridum* (Entz et al., 2005), however, a single PCR assay enabling identification and discrimination among the four PARB species has not been developed.

The purpose of this study was the design of species-specific multiplexed PCR primers to identify and distinguish between PARB clade species *M. pingshaense, M. anisopliae, M. robertsii,* and *M. brunneum*. The specificity of the PCR primers developed here for the four species was tested *in silico* using BLASTN similarity searches and *in vitro* by assessing 68 and 19 *Metarhizium* isolates, which were obtained from culture collections or isolated from a Swiss meadow soil, respectively.

2. Material and methods

2.1. Source of Metarhizium spp. strains

A taxonomically curated reference culture collection and a field collection of *Metarhizium* isolates were assembled for this study. The former comprised 68 strains representing 12 *Metarhizium* species from 29 different countries (Table S1) and was used to develop and evaluate species-specific primer pairs for *M. pingshaense, M. anisopliae, M. robertsii,* and *M. brunneum*. Strains of the reference-culture collection are exemplars included in prior molecular systematic revisions of *Metarhizium* (Bischoff et al., 2009; Kepler et al., 2016; Kepler et al., 2014). The field collection included 19 *M. anisopliae* isolates obtained from soil sampled across a permanent grassland field of 100 × 100 m in Mellingen, Switzerland (47°24′25″ N 8°16′10″ E) applying a sampling protocol described by Schneider et al. (2012).

2.2. Genomic DNA extraction

Genomic DNA was extracted from fungal mycelium grown in liquid medium (Oulevey et al., 2009) using the Nucleo Spin Plant II DNA extraction kit (Machery & Nagel, Germany). Genomic DNA

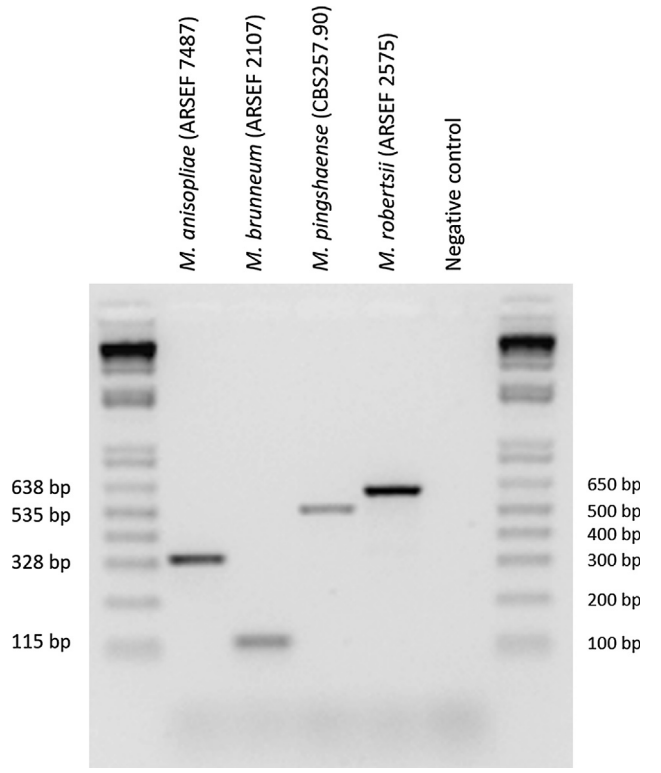

Fig. 1. Species-specific multiplex PCR with primer pairs specific for *M. anisopliae* (Ma-rIGS-1648-F and Ma-rIGS-1975-R), *M. brunneum* (Mb-FG546-422-F & Mb-FG546-536-R), *M. pingshaense* (Mp-IGS2-240-F and Mp-IGS2-774-R) and *M. robertsii* (Mr-rIGS-444-F & Mr-rIGS-1081-R) tested on the respective type strains. Species-specific amplicon sizes are indicate on the left and fragment sizes of the size standard are indicate on the right.

concentrations were assessed with PicoGreen® (Invitrogen, Carlsbad, CA) using a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, California, U.S.A.) and DNA extracts were diluted to 5 ng/µl in ddH_20.

2.3. Control PCR of small ribosomal subunit and 5TEF

The integrity of all sample DNA for PCR was first confirmed by amplification of the small ribosomal subunit with the primer pair uni-*b*-for (5′-TGCCAGCMGCCGCGGTA-3′; Pesaro and Widmer, 2006) and uni-*b*-r (5′-GACGGGCGGTGTGTRCAA-3′; modified from Amann et al., 1995). PCR reactions contained 15 ng DNA, 1× PCR Buffer with 1.5 mM $MgCl_2$ (Qiagen, Netherlands), additionally 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer and 0.5 U of HotStarTaq® Plus DNA Polymerase (Qiagen, Netherlands). The PCR cycling parameters included an initial denaturation for 5 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 2 min at 72 °C and concluded with a 10 min incubation at 72 °C. PCR results were assessed by electrophoresis in a 2% agarose gel and stained with GelRed™ (Biotium, California, U.S.A.).

Taxonomic assignment of individual isolates in both the reference and field collections were determined by phylogenetic analysis of their 5TEF sequences against corresponding reference *Metarhizium* spp. GenBank accessions from Bischoff et al. (2009), effectively providing a second positive control of DNA integrity for PCR amplification. 5TEF was amplified from different strains using primers EF1T (5′-ATGGGTAAGGARGACAAGAC-3′; Bischoff et al., 2006) and EFjmetaR (5′-TGCTCACGRGTCTGGCCATCCTT-3′; modified from EF-jR; Rehner and Buckley, 2005). All PCR amplifications were performed in volumes of 20 µl including 15 ng DNA, PCR Phusion HF buffer with 7.5 mM $MgCl_2$ (Qiagen, Netherlands), 0.2 mM dNTPs, 3% DMSO, 0.2 µm of each primer and 0.4 U Phusion polymerase HotStart II. 5TEF PCR cycling parameters consisted of an initial denaturation of 1 min at 98 °C, followed by 38 cycles of 10 s denaturation at 98 °C, 20 s annealing at 58 °C, 1 min elongation at 72 °C, and a final elongation of 5 min at 72 °C. PCR products were purified with a MultiScreen $PCR_{\mu 96}$ filter plate (Millipore, Billerica, Massachusetts, U.S.A.). Sequencing reactions were performed with a BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, California, U.S.A.) with primers used for amplification. Sequencing products were analyzed with an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, California, U.S.A.). Forward and reverse sequences were assembled and manually edited using DNABaser software version 3 (Heracle BioSoft, Romania). Sequences were aligned using CLUSTAL W (Thompson et al., 1994) implemented in Bioedit (Hall, 1999) followed by manual editing. Maximum likelihood phylogenetic analyses and model selection were performed in MEGA X with default settings under a best fitting DNA model of evolution selected by comparing BIC scores among competing models (Kumar et al., 2018). Bootstrap values were determined from 1000 bootstrap iterations. Sequence identities of rIGS sequences were calculated from pairwise distances assessed in MEGA X using default settings.

2.4. Design and application of species-discriminatory PCR primer pairs

The search for sequence signatures allowing design of species-specific primers discriminating PARB species focused on three nuclear intergenic loci, the rIGS, the MzFG546 and the MzIGS2 regions. The rIGS region, a highly variable DNA region between ribosomal operons, has been extensively used for taxonomic classification of Eukaryota (Hillis and Dixon, 1991) and has shown potential for species-specific signatures suitable for design of specific primers (Pantou et al., 2003). The MzFG546 is located between the genes encoding vezatin and the proliferating cell nuclear antigen. The MzIGS2 region is located between the pre-rRNA-processing protein ipi1 and the DEAD/DEAH box RNA helicase. Both loci have shown to be informative for phylogenetic discrimination among *Metarhizium* spp. by Kepler and Rehner (2013). The design of species-specific primer pairs was based on multiple sequence alignments of the rIGS and the MzFG546 and MzIGS2 nuclear intergenic spacers that included sequences from 31, 53 and 58 *Metarhizium* isolates from the reference culture collection, respectively (Table S1, File S1-S3). MzFG546 and MzIGS2 sequences were obtained from NCBI GenBank (Benson et al., 2015) except for five MzIGS2 sequences that were amplified and sequenced in the present study according to Kepler and Rehner (2013). The rIGS was amplified with primers LR12R (5′-GAACGCCTCTAAGTCAGAATCC-3′; Vilgalys et al., 1994) and CNS25 (5′-ATGTATTAGCTGTAGAATTACCAC-3′; Burt et al., 1996) (Table S1) using reaction components and concentrations identical to that described for amplification of 5TEF. Cycling parameters were also the same except for primer annealing at 60 °C and a 3 min final extension at 72 °C. Sequencing reactions were performed as described above using PCR primers and seven additional internal primers to provide complete bidirectional sequences (Table S2). Sequence assemblies, alignments and phylogenetic analyses were performed as described above.

Species-specific signatures for primer design were visually identified in the rIGS, MzFG546 and MzIGS2 sequence alignments (File S1-S3). *In silico* specificity of each primer was tested using the BLASTN 2.8.0 algorithm optimized for highly similar sequences (megaBLAST; Altschul et al., 1997). The tests were performed with the standard settings on the Nucleotide collection (nr/nt) listing 500 subjects and excluding uncultured/environmental samples on June 13th 2018.

2.5. Conditions of species-specific PCR

PCRs with individual species-specific primer pairs contained 15 ng genomic DNA, 1× PCR Buffer with 1.5 mM $MgCl_2$ (Qiagen, Netherlands), 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of the respective forward and reverse primer and 1 U of HotStarTaq® Plus DNA

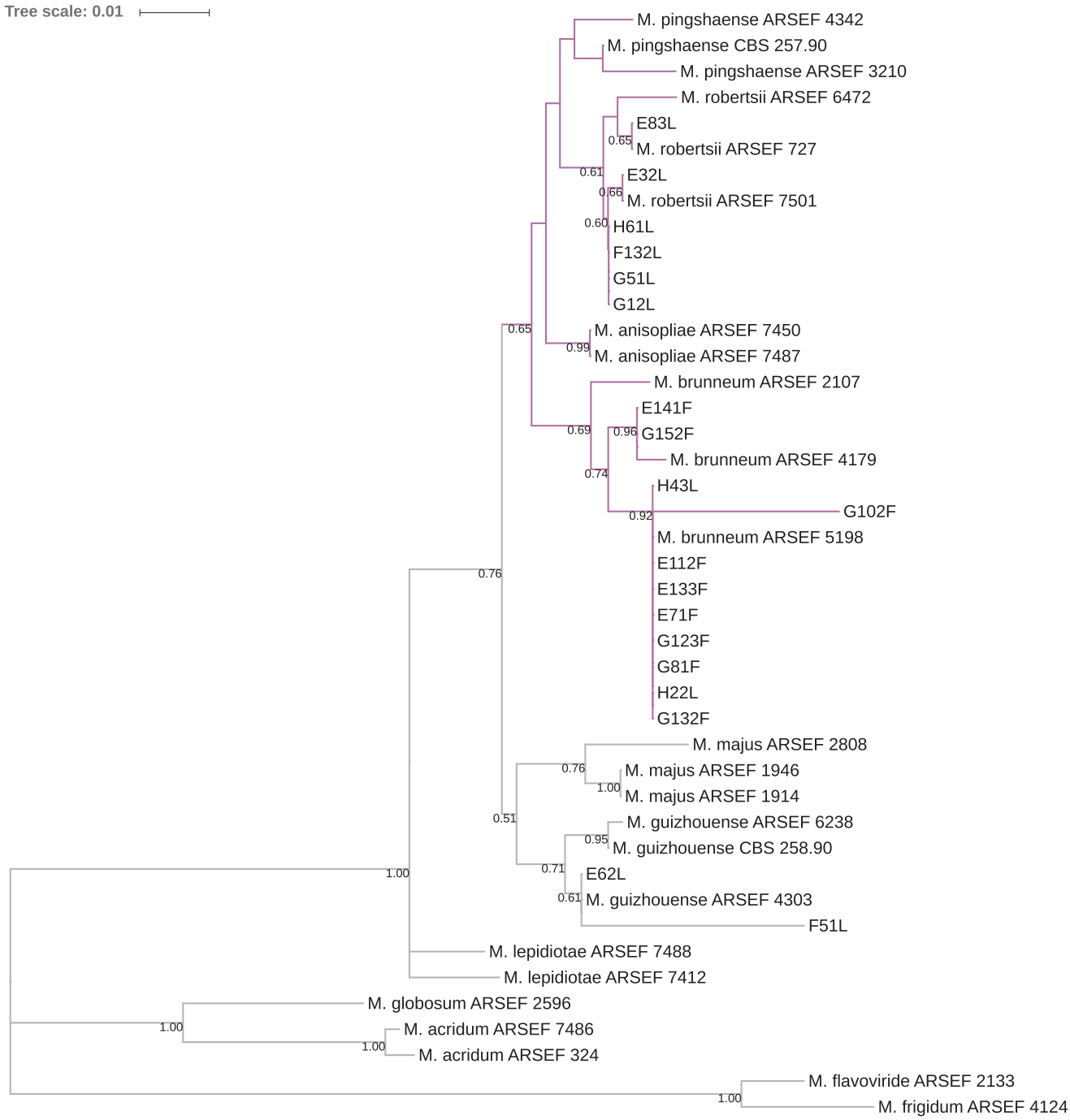

Fig. 2. Phylogenetic tree based on 5TEF (550 positions, 43 parsimonious informative sites) of 19 strains from the field-culture collection and 24 strains from the reference-culture collection inferred with maximum likelihood method, Kimura 2-parameter model, a Gamma distribution model for evolutionary rate differences among sites and 1000 bootstrap pseudoreplicates (0.5 bootstrap consensus). Branches within the PARB clade are colored in purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Polymerase (Qiagen, Netherlands) in a total reaction volume of 20 µl. Optimal annealing temperatures for the four species-specific primer pairs were determined empirically by performing gradient PCR using DNA from ex-type cultures for each species as template and primer lengths were adapted to allow multiplexed PCR at one annealing temperature. Cycling parameters included an initial step of denaturation at 95 °C for 5 min followed by 38 cycles of 94 °C for 30 s, 63 °C for 30 s and 72 °C for 30 s and a final elongation at 72 °C for 10 min. Multiplexed species-specific PCR was identical to PCRs with individual species-specific primer pairs. However, the concentration of each primer was 0.07 µM except for primers MbFG546_614_F and MbFG546_718_R with a concentration of 0.2 µM. PCR products were visualized by gel electrophoresis as described above.

3. Results

3.1. Primer design and optimization

rIGS, MzFG546 and MzIGS2 were selected as targets for primer design. A phylogenetic tree inferred from the rIGS alignment resolved monophyletic clades for *M. anisopliae*, *M. brunneum*, *M. pingshaense* and *M. robertsii* (File S1, Fig. S2). Species-specific signatures, were identified

for *M. anisopliae* and *M. robertsii* at rIGS (Table 1). No uniquely distinguishing sites for either *M. brunneum* or *M. pingshaense* were detected at rIGS. However, specific signatures for these two species were identified in the MzFG546 and MzIGS2 alignments, respectively (Table 1, File S2-S3). Species-specific PCR primers leveraging these unique signatures were designed for all four species yielding amplification products of 328 bp at rIGS for *M. anisopliae* ARSEF 7487, 115 bp at MzFG546 for *M. brunneum* ARSEF 2107, 535 bp at MzIGS2 for *M. pingshaense* CBS 257.90 and 638 bp at rIGS for *M. robertsii* ARSEF 2575 (Table 1).

3.2. Species-specificity tests

In silico specificity tests were performed using the BLASTN algorithm based on 100% sequence identity of each primer. Primers specific for *M. anisopliae* (Ma-rIGS-1648_F and Ma-rIGS-1975-R) and for *M. brunneum* (Mb-FG546-422-F and Mb-FG546-536-R) matched up to 68 non-target sequences representing up to 23 species, none of which were classified in GenBank as *Metarhizium* (Table 2). Searches with primers specific for *M. pingshaense* showed that the forward primer (Mp-IGS2-240-F) matched to sixteen non-target sequences of which three were classified as *M. anisopliae* and the remaining thirteen matched to species classified in genera other than *Metarhizium*. However, the reverse primer (Mp-IGS2-774-R) matched only sequences affiliated with the intended target species. None of the above mentioned non-target sequences matched to both the forward and the reverse primer of the primer pairs specific for *M. pingshaense*, *M. anisopliae* and *M. brunneum* (Table 2). Both primers specific for *M. robertsii* matched the same ten non-target sequences, which were identified in GenBank as either *M. anisopliae* or *M. pingshaense* (Table 2). Phylogenetic analysis of these ten sequences with those of the reference-culture collection placed them in *M. robertsii*. This was reflected in sequence identities, which were 98.4–100% to *M. robertsii* ARSEF 2575 and 94.3–96.2% to *M. anisopliae* ARSEF7487 and *M. pingshaense* CBS257.90.

Species-specificity tests with individual as well as multiplexed primer pairs on 68 *Metarhizium* strains including 12 *Metarhizium* spp. from 29 different countries (Table S1) revealed amplification products only from target species, and none from non-target species (Fig. 1 and Fig. S1). Fragment sizes of all products corresponded to the fragment sizes calculated for *M. pingshaense, M. anisopliae, M. robertsii,* and *M. brunneum.* Amplification results obtained from reference specimen are shown as examples in Fig. 1.

3.3. Testing and validating the PARB multiplexed PCR species identification assay

The identities of 19 *Metarhizium* isolates collected from soil of a Swiss meadow were diagnosed independently by PARB multiplexed PCR and by confirmatory sequencing of 5TEF (Table S3). Single amplification products were obtained for 17 isolates with 11 isolates producing an amplicon with the size predictive of *M. brunneum* (115 bp) and 6 isolates yielding a product with a size predictive of *M. robertsii* (638 bp). Species identities of all 19 isolates were confirmed by sequencing 5TEF and alignment to 24 reference strains. 5TEF sequence analyses confirmed the multiplexed PCR species assignments of 17 isolates, and showed that the two isolates for which no amplification products were produced clustered with *M. guizhouense*, which is not a target of the PARB multiplexed PCR (Fig. 2).

4. Discussion

Expedient identification of fungal isolates represents an important step when investigating ecological or applied aspects of entomopathogenic fungi. Here we developed species-specific PCR amplicon diagnostics for the four core species of the *M. anisopliae* species complex (PARB clade), i.e., *M. pingshaense, M. anisopliae, M. robertsii,* and *M. brunneum.* Species-specific signatures were identified within rIGS, for *M. anisopliae* and *M. robertsii,* within MzFG546 for *M. brunneum* and within MzIGS2 for *M. pingshaense*, enabling design of specific primer pairs for each target species.

A three-step approach was used to assess species-specificity of the *Metarhizium* PCR diagnostic tool described here including (1) *in silico* assessment of the specificity of individual primers by BLASTN analyses against the GenBank database, (2) testing assay specificity and robustness by performing PCR amplification with a taxonomically curated reference strain collection of 68 *Metarhizium* strains representing 12 species, and (3) assay validation with a field collection of 19 unknown *Metarhizium* isolates from soil of a Swiss meadow. *In silico* species-specificity tests using BLASTN also identified some non-target sequences for single primers, however, each primer pair included at least one primer that was uniquely specific to the species targeted. BLASTN searches of the GenBank database with the rIGS primers specific for *M. robertsii* identified ten presumptive non-target sequences that were assigned to *M. anisopliae* (9) and *M. pingshaense* (1). Phylogenetic analysis of the rIGS region including these sequences placed them within *M. robertsi*, illustrating our earlier point that PARB species assignments prior to Bischoff et al. (2009) should be carefully scrutinized. Nevertheless, a priori BLASTN searches constitute a time-efficient verification tool as to the potential specificity of a primer (or sequence) for taxa of interest. However, the accuracy of BLASTN-based identifications depend on the underlying accuracy of taxonomic assignments for sequence entries and sequence classifications, which poses a challenge in the light of the frequent taxonomic changes and the lack of taxonomic curation of the database as species circumscriptions and classifications are revised. Additionally, sequence databases include biases towards well-studied species and geographic regions and may therefore lack completeness. Accordingly, *in silico* tests permit meaningful conclusions only if the target locus is well represented in a database, e.g., the rIGS locus with currently 148,721 entries, including 17,619 fungal sequences (Nucleotide database, 09.07.2018).

In the soils sampled from a Swiss meadow only three *Metarhizium* species were detected, the PARB species *M. brunneum* and *M. robertsii,* and *M. guizhouense,* a species closely related to PARB (Bischoff et al., 2009). Several recent studies investigating *Metarhizium* species diversity in agricultural and non-agricultural soils and insects in Europe, North, Central and South America and Asia implementing current phylogenetic species concepts also reported the presence of two or more PARB species and either *M. brunneum*, *M. guizhouense*, *M. pingshaense* or *M. robertsii* predominating within the site sampled (Enkerli et al., 2016; Fisher et al., 2011; Garrido-Jurado et al., 2015; Hernández-Domínguez and Guzmán-Franco, 2017; Keyser et al., 2015; Nishi et al., 2011; Steinwender et al., 2015; Steinwender et al., 2014; Wyrebek et al., 2011). Although first insights into the occurrence of different *Metarhizium* spp. in soil have demonstrated the co-occurrence of two or more species within single habitats, ecological factors that may influence distribution of species, such as climate, have not yet been elucidated. Larger scale sampling efforts are required to further investigate these aspects. The assay described herein can greatly facilitate the characterization of *Metarhizium* communities by virtue of its speed and low cost.

The developed primers represent a useful identification tool for *M. anisopliae*, *M. brunneum*, *M. pingshaense* and *M. robertsii.* Validation of the method was performed with *Metarhizium* isolates from soil, however, this approach is applicable to strains from literally all possible sources. Compared to sequence-based methods commonly used for species identification (e.g. Keyser et al., 2015; Steinwender et al., 2014), the species-specific PCR method established in this study is simpler and more efficient as it includes only one multiplexed PCR and requires equipment that is widely available. Additionally, modification of PCR conditions to DNA extracts from different habitats, such as the insect host, may allow detection (standard PCR) or direct quantification (quantitative PCR) of the four *Metarhizium* spp. and hence complement

the molecular toolbox.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2019.01.002.

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