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Short Communication

Multiplexed microsatellite markers for seven *Metarhizium* species

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ABSTRACT

Cross-species transferability of 41 previously published simple sequence repeat (SSR) markers was assessed for 11 species of the entomopathogenic fungus *Metarhizium*. A collection of 65 *Metarhizium* strains including all 54 used in a recent phylogenetic revision of the genus were characterized. Between 15 and 34 polymorphic SSR markers produced scorable PCR amplicons in seven species, including *M. anisopliae*, *M. brunneum*, *M. guizhouense*, *M. lepidiotae*, *M. majus*, *M. pingshaense*, and *M. robertsii*. To provide genotyping tools for concurrent analysis of these seven species fifteen markers grouped in five multiplex pools were selected based on high allelic diversity and easy scorability of SSR chromatograms.

1. Introduction

Insect pathogenic species of the fungal genus *Metarhizium* Sorokin (Hypocreales, Clavicipitaceae) are widely used in biological control of arthropod pests (Faria and Wraight, 2007). Recent multilocus phylogenetic analyses of the genus resulted in delineation of a complex of nine species within the species *Metarhizium anisopliae* (Bischoff et al., 2009). The complex comprises a well-defined inner core, the PARB clade, including *M. pingshaense*, *M. anisopliae*, *M. robertsii* and *M. brunneum* (Bischoff et al., 2009). Furthermore, it includes a clade consisting of *M. majus* and *M. guizhouense* and three additional species, i.e., *M. acridum*, *M. lepidiotae*, and *M. globosum*.

In order to further improve our understanding of genotypic diversity and population genetic structures within *Metarhizium* species, to track introduced strains in the environment, to assess their possible effects on indigenous *Metarhizium* populations, and/or to characterize cultivars, highly resolving genetic markers are required (Enkerli and Widmer, 2010). Microsatellites, also known as simple sequence repeat (SSR) markers, have proven to be an ideal tool for such purposes (Taylor and Fisher, 2003). Forty-one SSR markers have been isolated from three different *Metarhizium* strains originally identified as *M. anisopliae* but now recognized as *M. anisopliae* (strain ART 2062), *M. brunneum* (strain ARSEF 7524) and *M. robertsii* (strain ARSEF 7532) (Enkerli et al., 2005; Oulevey et al., 2009). Selected subsets of these SSR markers have been used to characterize genotypic diversity of *Metarhizium* isolates from different environments (Freed et al., 2011; Kepler et al., 2015; Steinwender et al., 2014; Velásquez et al., 2007).

The goal of this study was to test the applicability of the 41 SSR markers for multilocus genotyping in different *Metarhizium* species, taking into account the recent taxonomic refinements in this genus. This information was used to compile SSR marker sets applicable to several different *Metarhizium* species.

2. Material and methods

A collection of 65 *Metarhizium* strains representing all nine *Metarhizium* species of the *M. anisopliae* species complex and two species of the *M. flavoviride* species complex (outgroup) was genotyped using 41 SSR markers (Tables 1 and S1). BLAST searches with reference sequences for these SSR markers (Enkerli et al., 2005; Oulevey et al., 2009) demonstrated their presence and broad distribution in the genomes of *M. anisopliae*, *M. brunneum* and *M. robertsii* (Hu et al., 2014; Pattemore et al., 2014; Supplemental Table S2). Fifty-four of the strains used in this study were included in the most recent revision of *Metarhizium* (Bischoff et al., 2009) and were obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF, Ithaca, NY, USA) or the Centraalbureau voor Schimmelcultures collection (CBS, Utrecht, Netherlands). The remaining eleven strains, among them the frequently used biological control agent *M. brunneum* strain BIPESCO 5 (also known as F52, M43, ATCC 90448), were obtained

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Table 1
The number of strains examined per species, SSR markers revealing PCR products for ⩾75% of all strains of a species, polymorphic markers and percentage of cross-species transferable markers.

Species	# of strains	# of markers		% of cross-species transferability[a]		
		Amplified	Polymorphic	*M. anisopliae*	*M. robertsii*	*M. brunneum*
M. pingshaense	4	35	28	100	100	77
M. anisopliae	4	34	28	100[b]	100	74
M. robertsii	7	33	22	86	100[b]	74
M. brunneum	13	37	34	100	83	89[b]
M. majus	9	25	22	63	50	63
M. guizhouense	9	22	17	50	67	52
M. lepidiotae	4	21	15	50	33	56
M. acridum	9	9	1	25	17	22
M. globosum	1	12	–[c]	25	33	30
M. flavoviride	4	2	0	0	0	7
M. frigidum	1	3	–[c]	0	17	7

[a] Percentage of SSR markers isolated from *M. anisopliae* (8 markers), *M. robertsii* (6 markers) and *M. brunneum* (27 markers) that are transferable to a different species.
[b] Percentage of SSR markers isolated from*M. anisopliae* (8 markers), *M. robertsii* (6 markers) and *M. brunneum* (27 markers) revealing PCR products for ⩾75% of the strains of the respective species.
[c] Not applicable because only one strain was tested.

from other culture collections (Table S1). Species affiliation of eleven strains not included in the study by Bischoff et al. (2009) was verified by sequencing and comparing the 5′ end of elongation factor 1 alpha (EF1alpha) as described by Bischoff et al. (2009). GenBank accession numbers are provided in Table S1. Each species was represented by at least 4 strains except for *M. frigidum* and *M. globosum* with only one strain each. The strains derived from insects or soils and originated from 29 countries representing all continents except Africa and Antarctica (Bischoff et al., 2009; Table S1). Cultures were maintained and fungal mycelia were produced as previously described (Oulevey et al., 2009). Genomic DNA was extracted using Nucleo Spin Plant II (Machery & Nagel, Germany) DNA extraction kit. Forty-one SSR primer pairs (Enkerli et al., 2005; Oulevey et al., 2009) were combined in sets of two or three pairs to perform multiplex touchdown polymerase chain reactions (PCR, Tables 2 and S3). PCR was conducted in 20 µl reaction volumes containing 10 ng genomic DNA, 0.2 µM of each primer, 0.2 mM dNTPs, 1 × GoTaq® Flexi Reaction buffer, 0.25 U of GoTaq® Flexi DNA Polymerase (Promega, WI, USA) and 3 or 4 mM $MgCl_2$ (Tables 2 and S3). One primer of each pair was labeled with NED, HEX or FAM (Applied Biosystems, CA, USA), respectively.

Table 2
Multiplex touchdown PCR conditions (final annealing temperature (T_a, °C), concentration of $MgCl_2$ (mM) and number of cycles (n) for five SSR sets comprising the most powerful of the 41 SSR markers to discriminate genotypes of seven *Metarhizium* species (*M. anisopliae*, *M. brunneum*, *M. guizhouense*, *M. lepidiotae*, *M. majus*, *M. pingshaense*, and *M. robertsii*).

Set	Markers[a]	T_a	$MgCl_2$	n[b]
I	Ma2049	58	4	12/22
I	Ma2054	58	4	12/22
I	Ma2063	58	4	12/22
II	Ma2089	58	4	12/22
II	Ma2103	58	4	12/22
II	Ma2296	58	4	12/22
III	Ma142	56	3	12/22
III	Ma2097	56	3	12/22
III	Ma2108	56	3	12/22
IV	Ma164	56	3	12/22
IV	Ma307	56	3	12/22
IV	Ma2099	56	3	12/22
V	Ma195	50	3	12/30
V	Ma327	50	3	12/30
V	Ma2287	50	3	12/30

[a] Markers labeled with 3 or 4 digits were published by Enkerli et al. (2005) or Oulevey et al. (2009), respectively.
[b] Number of cycles used for touchdown PCR/number of cycles used for subsequent PCR at T_a.

Touchdown PCR conditions consisted of 2 min initial denaturation at 94 °C, followed by 12 cycles of 30 s denaturation at 94 °C, 30 s annealing at $T_a + 12$ °C, (with 1 °C decrease per cycle) and 40 s extension at 72 °C followed by n (Tables 2 and S3) cycles of 30 s denaturation at 94 °C, 30 s annealing at T_a and 40 s extension at 72 °C. PCR was terminated with a final elongation step of 15 min at 72 °C. PCR fragment sizes were analyzed using capillary electrophoresis as described previously (Oulevey et al., 2009).

For each species and SSR marker the percentages of strains that produced a scorable PCR amplicon were determined. Markers revealing PCR products for ⩾75% of the strains of a species from which the marker was not isolated (*M. anisopliae* (8 markers), *M. robertsii* (6 markers) or *M. brunneum* (27 markers)) were considered as transferable to the respective species (cross-species transferability). Nei's unbiased genetic diversity ($H_e = \frac{N}{N-1}(1 - \sum p_i^2)$; Nei and Roychoudhury, 1974), was calculated for each marker and species.

3. Results and discussion

In seven species (*M. anisopliae*, *M. brunneum*, *M. guizhouense*, *M. lepidiotae*, *M. majus*, *M. pingshaense*, and *M. robertsii*; Table 1) at least 21 (15 polymorphic) markers were amplified from ⩾75% of the strains of a species. For the remaining species only 2–12 markers were amplified of which only one marker was polymorphic for *M. acridum*. For *M. globosum* and *M. frigidum* only one strain per species was included, thus results for these two species are considered tentative. The highest percentages of cross-species transferability and the highest numbers of polymorphic markers were obtained for species in the PARB clade (Table 1). All SSR markers isolated from *M. anisopliae* were transferable to *M. brunneum* and *M. pingshaense*, and all markers obtained from *M. robertsii* were transferable to *M. anisopliae* and *M. pingshaense*. Cross-species transferability of SSR markers isolated from *M. anisopliae*, *M. brunneum* or *M. robertsii* was negatively correlated with phylogenetic distance based on sequence analysis of EF1alpha (Spearman $\rho = -0.902$ to -0.951, $N = 10$, $p < 0.001$). Decreasing cross-species transferability with increasing taxonomic distance has also been observed for other fungal genera such as *Lobaria* and *Phytophthora* (Devkota et al., 2014; Schoebel et al., 2013). Cluster analyses performed on SSR marker data did not correspond to the multilocus sequence phylogeny of *Metarhizium* (Bischoff et al., 2009) and no species-specific clustering was obtained (data not shown). The complexity of evolution of SSR and their flanking region, which may lead to convergence in allele sizes among

species, render SSR markers as inappropriate for reconstructing phylogenetic relationships (Barthe et al., 2012; Colson and Goldstein, 1999; Goldstein and Pollock, 1997; Orti et al., 1997). Therefore, the use of SSR markers for species identification is limited and species affiliation should be based on DNA sequences, i.e. EF1alpha sequence comparison (Steinwender et al., 2014).

Nei's unbiased genetic diversity (H_e) ranged from 0.21 to 1 and varied substantially among species and SSR loci tested (Table S4). A significant correlation was observed between H_e and other indices of diversity such as Shannon index (Spearman $\rho = 0.98$, $N = 328$, $P < 0.001$) and evenness (Spearman $\rho = 0.99$, $N = 328$, $P < 0.001$).

PCR amplifications with all 41 SSR primer sets revealed single alleles for all strains, except for all *M. majus* strains, among which two alleles were obtained at one to eleven SSR loci per isolate (Tables S3 and S4). Polymorphism in *M. majus* strains depended on the locus and the particular strain. These results suggest duplication of the corresponding regions or possibly a diploid genome. Similar observations have been reported in previous studies of *M. majus* isolates using isozymes (St. Leger et al., 1992) or genome sequence analyses (Hu et al., 2014).

To provide robust and generally useful SSR genotyping tools for *Metarhizium*, polymorphic SSR markers that amplify reliably from as many *Metarhizium* spp. as possible, multilocus multiplex PCR methods were identified. For this purpose fifteen SSR markers were selected and grouped into five sets including three markers each. Selection was based on four criteria: (1) applicable to *M. anisopliae*, *M. brunneum*, *M. guizhouense*, *M. lepidiotae*, *M. majus*, *M. pingshaense*, and *M. robertsii*, (2) high within-species diversity, (3) amplification success and (4) easily scorable SSR peak patterns (Table 2). The markers were grouped according to matching PCR conditions and different allele size ranges to simplify marker scorability. Additional markers with high-resolution power for individual species can be selected from Tables S3 and S4 (Supplementary information).

The currently most efficient approach to genotype *Metarhizium* isolate collections is to first perform SSR marker analyses using the five marker sets, then to determine species affiliation (based on EF1alpha sequence comparison) of individual multilocus microsatellite genotypes and finally, if further resolution is required, to use additional SSR markers appropriate for the identified species. This approach will be applicable and useful for strain characterization, tracking introduced BCA strains in the environment, and analyses of population genetic structures of *M. anisopliae*, *M. brunneum*, *M. guizhouense*, *M. lepidiotae*, *M. majus*, *M. pingshaense*, and *M. robertsii*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2015.09.006.

References

Barthe, S., Gugerli, F., Barkley, N.A., Maggia, L., Cardi, C., Scotti, I., 2012. Always look on both sides: phylogenetic information conveyed by simple sequence repeat allele sequences. PLoS ONE 7. http://dx.doi.org/10.1371/journal.pone.0040699.

Bischoff, J.F., Rehner, S.A., Humber, R.A., 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia 101, 512–530.

Colson, I., Goldstein, D.B., 1999. Evidence for complex mutations at microsatellite loci in *Drosophila*. Genetics 152, 617–627.

Devkota, S., Cornejo, C., Werth, S., Chaudhary, R.P., Scheidegger, C., 2014. Characterization of microsatellite loci in the Himalayan lichen fungus *Lobaria pindarensis* (Lobariaceae). Appl. Plant Sci. 2. http://dx.doi.org/10.3732/apps.1300101.

Enkerli, J., Kolliker, R., Keller, S., Widmer, F., 2005. Isolation and characterization of microsatellite markers from the entomopathogenic fungus *Metarhizium anisopliae*. Mol. Ecol. Notes 5, 384–386.

Enkerli, J., Widmer, F., 2010. Molecular ecology of fungal entomopathogens: molecular genetic tools and their applications in population and fate studies. Biocontrol 55, 17–37.

Faria, M.Rd., Wraight, S.P., 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control 43, 237–256.

Freed, S., Jin, F.L., Ren, S.X., 2011. Determination of genetic variability among the isolates of *Metarhizium anisopliae* var. *anisopliae* from different geographical origins. World J. Microbiol. Biotechnol. 27, 359–370.

Goldstein, D.B., Pollock, D.D., 1997. Launching microsatellites: a review of mutation processes and methods of phylogenetic inference. J. Hered. 88, 335–342.

Hu, X., Xiao, G., Zheng, P., Shang, Y., Su, Y., Zhang, X., Liu, X., Zhan, S., St. Leger, R.J., Wang, C., 2014. Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. Proc. Natl. Acad. Sci. 111, 16796–16801.

Kepler, R.M., Ugine, T.A., Maul, J.E., Cavigelli, M.A., Rehner, S.A., 2015. Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. Environ. Microbiol. http://dx.doi.org/10.1111/1462-2920.12778.

Nei, M., Roychoudhury, A.K., 1974. Sampling variances of heterozygosity and genetic distance. Genetics 76, 379–390.

Orti, G., Pearse, D.E., Avise, J.C., 1997. Phylogenetic assessment of length variation at a microsatellite locus. Proc. Natl. Acad. Sci. 94, 10745–10749.

Oulevey, C., Widmer, F., Kölliker, R., Enkerli, J., 2009. An optimized microsatellite marker set for detection of *Metarhizium anisopliae* genotype diversity on field and regional scales. Mycol. Res. 113, 1016–1024.

Pattemore, J.A., Hane, J.K., Williams, A.H., Wilson, B.A., Stodart, B.J., Ash, G.J., 2014. The genome sequence of the biocontrol fungus *Metarhizium anisopliae* and comparative genomics of *Metarhizium* species. BMC Genom. 15. http://dx.doi.org/10.1186/1471-2164-15-660.

Schoebel, C.N., Jung, E., Prospero, S., 2013. Development of new polymorphic microsatellite markers for three closely related plant-pathogenic *Phytophthora* species using 454-pyrosequencing and their potential applications. Phytopathology 103, 1020–1027.

St. Leger, R.J., May, B., Allee, L.L., Frank, D.C., Staples, R.C., Roberts, D.W., 1992. Genetic differences in allozymes and in formation of infection structures among isolates of the entomopathogenic fungus *Metarhizium anisopliae*. J. Invertebr. Pathol. 60, 89–101.

Steinwender, B.M., Enkerli, J., Widmer, F., Eilenberg, J., Thorup-Kristensen, K., Meyling, N.V., 2014. Molecular diversity of the entomopathogenic fungal *Metarhizium* community within an agroecosystem. J. Invertebr. Pathol. 123, 6–12.

Taylor, J.W., Fisher, M., 2003. Fungal multilocus sequence typing – it's not just for bacteria. Curr. Opin. Microbiol. 6, 351–356.

Velásquez, V.B., Carcamo, M.P., Merino, C.R., Iglesias, A.F., Duran, J.F., 2007. Intraspecific differentiation of Chilean isolates of the entomopathogenic fungi *Metarhizium anisopliae* var. *anisopliae* as revealed by RAPD, SSR and ITS markers. Genet. Mol. Biol. 30, 89–99.