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Cultivation-Independent Analysis of Fungal Genotypes in Soil by Using Simple Sequence Repeat Markers▽

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Cultivation-independent analyses of fungi are used for community profiling as well as identification of specific strains in environmental samples. The objective of the present study was to adapt genotyping based on simple sequence repeat (SSR) marker detection for use in cultivation-independent monitoring of fungal species or strains in bulk soil DNA. As a model system, a fungal biocontrol agent (BCA) based on *Beauveria brongniartii*, for which six SSR markers have been developed, was used. Species specificity of SSR detection was verified with 15 fungal species. Real-time PCR was used to adjust for different detection sensitivities of the six SSR markers as well as for different template quantities. The limit for reliable detection per PCR assay was below 2 pg target DNA, corresponding to an estimated 45 genome copies of *B. brongniartii*. The cultivation-independent approach was compared to cultivation-dependent SSR analysis with soil samples from a *B. brongniartii* BCA-treated field plot. Results of the cultivation-independent method were consistent with cultivation-dependent genotyping and allowed for unambiguous identification and differentiation of the applied as well as indigenous strains in the samples. Due to the larger quantities of soil used for cultivation-dependent analysis, its sensitivity was higher, but cultivation-independent SSR genotyping was much faster. Therefore, cultivation-independent monitoring of *B. brongniartii* based on multiple SSR markers represents a rapid and strain-specific approach. This strategy may also be applicable to other fungal species or strains for which SSR markers have been developed.

Characterization and monitoring of fungi in the environment are important aspects for many research questions in fungal biology and ecology. These include, for example, characterization of fungal population structures (5, 44), investigations of fungal functions in ecosystems (34) or natural occurrence of specified fungal groups, e.g., entomopathogenic fungi (33), and studies of survival, spread, and persistence of fungal strains released to the environment (4, 16). Traditionally, identification and characterization of fungi has relied on cultivation, followed by morphological (26), biochemical (35, 40), or molecular (15) analyses. However, the cultivation step required makes these approaches both laborious and time-consuming.

Cultivation-independent molecular genetic detection of fungal populations directly in DNA extracted from complex environmental samples could reduce the time and cost for monitoring and analysis (8). Such analyses have successfully been applied to fungal community profiling and are highly valuable for analyzing population structures at various phylogenetic levels (1). Typically, specific PCR primers target conserved regions in phylogenetic markers, like the small as well as the large subunit of rRNA genes or their internal transcribed spacer regions. However, limited resolution often does not allow for identification of particular strains based on these markers (1). For identification of specific fungal groups in complex environmental samples, specific primers have been designed within variable regions of marker genes, such as the internal transcribed spacer region (2, 17) or sequence-characterized amplified regions (10, 13, 14). Because specificity of a single marker may be limited to particular ecosystems or a range of tested strains, the use of multiple markers would improve reliability and resolution of such analyses (23, 46).

Multilocus simple sequence repeat (SSR) genotyping is a commonly used technique for characterization of cultivated fungi based on PCR amplification of multiple markers (loci). The polymorphic character of SSRs produces highly discriminating fingerprints that often allow characterization of fungi at a strain level (3, 12, 16). Several fungal SSR markers have been reported to be species specific (3, 39, 46), and therefore multilocus SSR genotyping may be a promising option for cultivation-independent detection of fungal strains in soil samples (8). However, it is important to notice that detection sensitivities of individual SSR markers can be different (7, 20). Thus, in cultivation-independent analyses of environmental templates, SSR-specific detection sensitivities would have to be adjusted for reliable multilocus genotyping.

The filamentous ascomycete *Beauveria brongniartii* is a naturally occurring soil fungus and pathogen of the European cockchafer (*Melolontha melolontha*), a pest in permanent grasslands and orchards (28). Since 1991, *B. brongniartii* has been available as a commercial biocontrol agent (BCA) to control soil-dwelling larvae of *M. melolontha* (29). A cultivation-dependent monitoring approach based on six polymorphic SSR markers has been developed (15) and has successfully been used to characterize natural soil populations of *B. brongniartii* and to monitor applied BCA strains (16).

The objective of the present study was to develop a cultivation-independent approach for SSR genotyping of *B. brongniartii* strains in soil. For this purpose, the six *B. brongniartii* SSR primer pairs were tested for species specificity and performance in bulk soil DNA extracts. Sensitivities for detecting

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TABLE 1. Detection specificity of six *B. brongniartii* SSR markers tested on a collection of 15 fungal reference strains, including ubiquitous soil fungal species, close relatives of *B. brongniartii*, and other entomopathogenic fungi

Species	Order[a]	Family[a]	Strain collection accession no.[b]	Presence or absence of SSR locus[c]:					
				Bb1F4	Bb2A3	Bb2F8	Bb4H9	Bb5F4	Bb8D6
Beauveria brongniartii	Hypocreales	Clavicipitaceae	DSMZ 15205	+	+	+	+	+	+
Beauveria bassiana	Hypocreales	Clavicipitaceae	ARSEF 5066	−	−	−	−	−	+[d]
Hirsutella thompsonii	Hypocreales	Clavicipitaceae	ARSEF 2800	−	−	−	−	−	−
Trichoderma harzianum	Hypocreales	Hypocreaceae	DSMZ 63059	−	−	−	−	−	−
Tolypocladium cylindrosporum	Hypocreales	Incerta sedis	ARSEF 2777	−	−	−	−	−	−
Verticillium lecanii	Hypocreales	Incerta sedis	ARSEF 1102	−	−	−	−	−	−
Metarhizium anisopliae	Hypocreales	Nectriaceae	ARSEF 1066	−	−	−	−	−	−
Aspergillus parasiticus	Eurotiales	Trichocomaceae	ARSEF 5470	−	−	−	−	−	−
Paecilomyces fumosoroseus	Eurotiales	Trichocomaceae	ARSEF 1645	−	−	−	−	−	−
Penicillium chrysogenum	Eurotiales	Trichocomaceae	ACW 931	−	−	−	−	−	−
Conidiobolus coronatus	Entomophthorales	Ancylistaceae	IESR 04:427	−	−	−	−	−	−
Entomophthora culicis	Entomophthorales	Entomorphtoraceae	ARSEF 387	−	−	−	−	−	−
Erynia rhizospora	Entomophthorales	Entomorphtoraceae	ARSEF 1440	−	−	−	−	−	−
Zoophthora radicans	Entomophthorales	Entomorphtoraceae	ARSEF 388	−	−	−	−	−	−
Mucor hiemalis	Mucorales	Mucoraceae	ACW 922	−	−	−	−	−	−

[a] According to the CABI Bioscience database.
[b] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ARSEF, Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, NY; ACW, Agroscope Changins-Wädenswil, Changins, Switzerland; IESR, Collection of Microorganisms, Institute of Environment and Sustainability Research, Staffordshire University, United Kingdom.
[c] Presence (+) or absence (−) of characteristic SSR marker signal.
[d] Amplicon length was 163 bp and different from that of *B. brongniartii*, which was 172 bp.

the different SSR loci were determined, and differences were adjusted by adapted PCR conditions. A grassland plot treated with a commercially available *B. brongniartii* BCA strain was used as a model system to compare efficiencies and sensitivities of the established cultivation-dependent and the new cultivation-independent SSR genotyping approaches.

MATERIALS AND METHODS

Fungal reference strains. Fifteen reference strains, including typical soil fungal species, close relatives of *B. brongniartii*, and other entomopathogenic fungi, were obtained from several culture collections (Table 1). Reference strains were grown at 22°C for 3 weeks on Difco modified Sabouraud agar supplemented with Difco yeast extract (Becton Dickinson, Sparks, MD) (37). Egg yolk (17%) was added to the medium for growing the Entomophthorales strains (37).

Field application of the *B. brongniartii* BCA strain and soil sampling. Field experiments were carried out in an *M. melolontha*-infested hay meadow with a humus-rich eutric cambisol. Two adjacent plots of 400 m^2 (20 by 20 m) each were either treated with the commercially available *B. brongniartii* BCA product Beauveria-Schweizer (E. Schweizer Seeds Ltd., Thun, Switzerland) or left as an untreated control. The BCA product consisting of barley kernels overgrown with *B. brongniartii* strain DSMZ 15205 (BCA strain) was applied once in spring 2002 in quantities of 40 to 50 kg ha^{-1} (30). In September 2004, five soil samples from the treated plot (T1 to T5) and from the untreated control (C1 to C5) were collected. At each of the evenly distributed sampling points, two adjacent soil cores were taken using a stainless-steel corer with an internal diameter of 5.5 cm. The 5- to 15-cm-depth fractions of adjacent cores were pooled (30) and stored at 4°C until use (see below).

***B. brongniartii* density and field isolates.** Within 2 weeks after sampling, *B. brongniartii* density in each soil sample was determined. Twenty grams of soil was mixed with 100 ml of 4 mM tetra-sodiumpyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$) and suspended at room temperature for 2 h at 110 rpm (29). After sedimentation for 15 s, 100-μl aliquots of the supernatant were plated in triplicate on solid selective medium (SM) (43). After incubation for 14 days at 22°C, densities of *B. brongniartii* were determined as numbers of CFU per gram soil (dry weight). *B. brongniartii* isolates were obtained from single colonies randomly picked from SM plates, transferred to solid complete medium (41), and maintained at 22°C.

Extraction of genomic DNA. Mycelia for DNA extraction were produced by inoculation of 80 ml liquid complete medium with conidia collected from plates and growth for 2 to 6 days at 20°C at 120 rpm. Mycelia were harvested by filtration as described by Enkerli et al. (15). Genomic DNA was extracted from lyophilized mycelium by using a DNeasy plant mini kit (QIAGEN, Hilden, Germany) and quantified by gel electrophoresis using a GelDoc XRS (Bio-Rad Laboratories, Hercules, CA) gel imaging system with Quantity One analysis software (Bio-Rad Laboratories) and a high-mass DNA ladder (Promega, Madison, WI) as the standard. The suitability of DNA for PCR was tested by amplifying the 18S rRNA gene with universal primers SSU-uni-b-for (5′-TGCC AGCMGCCGCGGTA-3′) (modified from reference 19) and SSU-uni-b-rev (5′-GACGGGCGGTGTGTRCAA-3′) (6). PCR was performed on an iCycler (Bio-Rad Laboratories) in volumes of 25 μl containing 20 ng DNA, 1 U HotStart *Taq* polymerase (QIAGEN), 1× PCR buffer (QIAGEN), 2.5 mM $MgCl_2$, 0.2 μM of each primer, 0.4 mM deoxynucleoside triphosphate, and 0.6 mg ml^{-1} bovine serum albumin (BSA). Cycling conditions consisted of a 15-min initial denaturation at 95°C and 30 PCR cycles of 25 s at 92°C, 40 s at 53°C, and 3 min at 72°C, followed by a final extension for 10 min at 72°C. The quality of amplification products was confirmed by gel electrophoresis in 1.5% agarose gels and ethidium bromide staining.

Analysis of SSR markers in fungal genomic DNA. SSR marker detection for the six SSR loci Bb1F4, Bb2A3, Bb2F8, Bb4H9, Bb5F4, and Bb8D6 from *B. brongniartii* was performed according to the method of Enkerli et al. (15). Reaction volumes of 25 μl contained 20 ng genomic template DNA, 12.5 μl iQ SYBR green supermix (Bio-Rad Laboratories), 0.2 μM of fluorescently labeled forward primer, 0.2 μM of unlabeled reverse primer, and 0.6 mg ml^{-1} BSA. Cycling conditions consisted of a 3-min initial denaturation at 95°C and 36 PCR cycles of 40 s at 92°C, 40 s at 58°C, and 30 s at 72°C, followed by a final extension of 10 min at 72°C.

Detection sensitivities for the six SSR markers were compared based on cycle threshold (C_T) values of each primer pair determined from 2 pg genomic DNA of the BCA strain running real-time PCR with the same conditions as described above, except that 45 PCR cycles were applied. Real-time PCR data were analyzed using an iCycler iQ real-time PCR detection system and software v3.1 (Bio-Rad Laboratories). For comparison of detection sensitivities of the six loci, differences in C_T values were expressed as relative C_T ($C_{T\text{-rel}}$) values, calculated as quotients between C_T values of the most efficiently amplified SSR locus, Bb4H9 ($C_{T\text{-}4H9}$), and C_T values of each of the other five SSR loci.

PCR products were analyzed for SSR sizes and the presence of SSR characteristic stutter peak patterns on an ABI Prism 3100 genetic analyzer with 36-cm capillaries and POP-4 polymer (Applied Biosystems, Foster City, CA). For that purpose, 20 μl PCR product was purified (Montage PCR cleanup kit; Millipore, Bedford, MA), resuspended in 50 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), and further diluted 1 to 10. Two microliters of the diluted product was used for analysis. GeneScan ROX400 (Applied Biosystems) was used as an internal size standard, and signals were analyzed using GeneScan v3.7 and Genotyper v3.7 NT analysis software (Applied Biosystems).

TABLE 2. SSR fingerprints of the three *B. brongniartii* strains

Genotype	Allele length (bp) of SSR locus:					
	Bb1F4	Bb2A3	Bb2F8	Bb4H9	Bb5F4	Bb8D6
I[a]	242	118	172	171	208	172
II[b]	245	118	205	202	171	172
III[b]	235	124	208	153	205	172

[a] Genotype of the applied BCA strain.
[b] Genotypes of two indigenous *B. brongniartii* isolates from the experimental field.

SSR analysis in bulk soil DNA. Nucleic acids were extracted within 48 h after collection of soil samples. Six hundred milligrams fresh soil was extracted three consecutive times by using a bead-beating procedure, and bulk soil DNA of each sample was pooled and suspended in TE buffer at 1 ml g^{-1} (dry weight equivalent) of extracted soil (21). Twenty-five microliters of each extract was purified using NucleoSpin Extract-II DNA purification columns (Macherey & Nagel, Easton, PA) and quantified fluorometrically with PicoGreen (Molecular Probes, Eugene, OR) according to the method of Hartmann et al. (21).

The potential to amplify the six *B. brongniartii* SSR markers from bulk soil DNA was assessed with 50 ng *B. brongniartii*-free bulk soil DNA from sample C1 spiked with 2 pg of genomic DNA of the BCA strain (spiked-soil DNA). Prior to PCR, soil DNA was incubated with 30 μg BSA in a volume of 12 μl for 45 min at 37°C in order to scavenge PCR inhibitory substances present in the soil DNA extract (32). C_T and $C_{T\text{-rel}}$ of spiked-soil DNA were determined for each SSR locus as described above for genomic DNA of the BCA strain.

The potential to detect multiple genotypes of *B. brongniartii* in bulk soil DNA was tested using 50 ng of *B. brongniartii*-free bulk soil DNA from sample C1 spiked with various combinations of 20, 2, or 0.2 pg genomic DNA from three different genotypes (I, II, and III) of *B. brongniartii* (Table 2).

Variable template quantities in soil samples as well as differences in locus-specific detection sensitivities were accounted for by the use of adapted amplification cycle (C_a) numbers. The C_a values were determined in a three-step process for each of the 10 field soil samples and each locus. First, $C_{T\text{-4H9}}$ was determined experimentally in duplicate from each of the 10 field soil samples. Second, C_T values of loci Bb1F4, Bb2A3, Bb2F8, Bb5F4, and Bb8D6 were calculated as products of averaged $C_{T\text{-4H9}}$ and corresponding $C_{T\text{-rel}}$ values derived from spiked-soil DNA. Third, obtained C_T values were grouped in classes of adapted cycle numbers according to the following rules: $C_{a\text{-}28}$ for C_T of <30, $C_{a\text{-}32}$ for $30 \leq C_T < 34$; $C_{a\text{-}36}$ for $34 \leq C_T < 38$; and $C_{a\text{-}40}$ for C_T of ≥38. Therefore, PCR was run at 28, 32, 36, or 40 cycles according to C_a and amplification products were analyzed for SSR sizes and characteristics as described for genomic DNA of the BCA strain.

RESULTS

Specificity of *B. brongniartii* SSR marker detection. SSR analysis of the 15 reference strains revealed specific PCR amplifications from *B. brongniartii* with typical SSRs for all six loci Bb1F4, Bb2A3, Bb2F8, Bb4H9, Bb5F4, and Bb8D6 (Fig. 1a for locus Bb2F8 and Table 1). From *Beauveria bassiana*, one typical SSR product (163 bp) was obtained for locus Bb8D6 (Table 1). For the other loci tested, PCR products with lengths of 191 bp (locus Bb1F4), 203 bp (locus Bb4H9), and 133 bp (locus Bb5F4) were obtained from *B. bassiana*, and for locus Bb1F4 a product of 191 bp was obtained from *Trichoderma harzianum*; however, analyses revealed no SSR characteristic stutter patterns for these PCR products and they were therefore considered unspecific (data not shown). DNA of all reference strains was suitable for PCR as revealed by positive-control PCR using universal primers for the small-subunit rRNA gene (Fig. 1b).

Sensitivity of cultivation-independent SSR detection. The PCR amplification efficiency for each of the six SSR loci was determined for 2 pg genomic DNA of the BCA strain and resulted in C_T values ranging from 28.83 ± 1.86 (locus Bb4H9) to 34.57 ± 0.65 (locus Bb2A3) (Table 3). PCR detection in 50 ng bulk soil DNA from *Beauveria*-free sample C1 that was spiked with 2 pg genomic DNA of the BCA strain (spiked-soil DNA) resulted in values not significantly different ($P < 0.05$, two-sided Student's *t* test) from the results with pure genomic DNA (Table 3). The C_T values for these analyses ranged from 28.53 ± 0.68 (locus Bb8D6) to 34.35 ± 1.09 (locus Bb2A3). $C_{T\text{-rel}}$ values (with reference to locus Bb4H9) ranged from 1.02 (locus Bb8D6) to 1.20 (locus Bb2A3) for genomic DNA from the BCA strain and from 0.99 (locus Bb8D6) to 1.20 (locus Bb2A3) for spiked-soil DNA (Table 3).

Simultaneous detection of multiple genotypes in bulk soil DNA. The sensitivity for detecting multiple genotypes of *B. brongniartii* in bulk soil DNA was tested again using 50 ng *Beauveria*-free bulk soil DNA extract of sample C1 spiked with different quantities of genomic DNA from three different genotypes of *B. brongniartii* (Tables 2 and 4). All three genotypes had a common allele at locus Bb8D6, and genotypes I and II were identical at locus Bb2A3 (Table 2).

Marker detection sensitivities for this experiment were adjusted according to the total quantities of spiked genomic DNA and according to the relative efficiencies of the six loci according to $C_{T\text{-rel}}$ (Table 3). For mixtures containing a minimum of 6 pg spiked genomic template DNA (Table 4, mixtures a to c), cycle numbers for the more efficiently amplifying loci Bb4H9, Bb5F4, and Bb8D6 were set to 28 cycles and for the less efficiently amplifying loci Bb1F4, Bb2A3, and Bb2F8 to 32 cycles. For mixtures with less than 6 pg spiked genomic DNA (Table 4, mixtures d and e), the more efficiently amplifying loci were processed at 36 PCR cycles while the less efficiently amplifying loci were processed at 40 PCR cycles.

The unambiguous assignment of fingerprints to individual genotypes was possible for spiked genomic DNA quantities of 2 pg or 20 pg (Table 4, mixtures a to d). Analysis of samples with 0.2 pg spiked genomic DNA of a specific genotype yielded incomplete corresponding fingerprints, and thus no genotype could be assigned. In mixture b, containing 0.2 pg DNA of genotype I and 20 pg DNA each of genotypes II and III, genotype I-specific alleles at loci Bb1F4 and Bb5F4 were de-

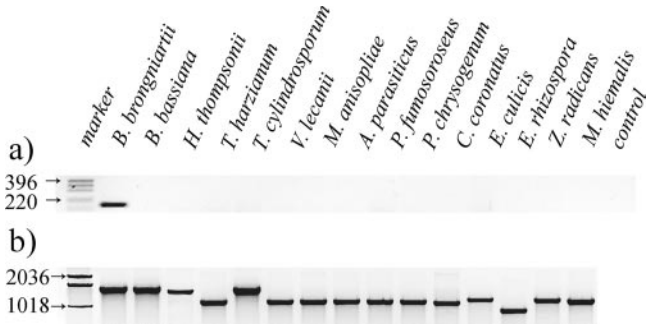

FIG. 1. Gel electrophoretic analyses of PCR products derived from the 15 fungal reference strains specified in Table 1. (a) The band at about 200 bp represents the PCR product for SSR locus Bb2F8 separated in 1.5% agarose. (b) The bands between 1,000 and 1,500 bp represent PCR products obtained with the universal primer pair targeting the small-subunit rRNA gene separated in 1.5% agarose. Marker, 1-kb DNA ladder (Promega).

TABLE 3. C_T values for PCR amplification of six *B. brongniartii* SSR loci and corresponding $C_{T\text{-rel}}$ values

Value measured	Template DNA	Value[a] for SSR locus:					
		Bb1F4	Bb2A3	Bb2F8	Bb4H9	Bb5F4	Bb8D6
C_T	Genomic DNA from BCA strain	31.93 ± 0.75	34.57 ± 0.65	31.07 ± 1.07	28.83 ± 1.86	30.97 ± 1.31	29.30 ± 1.74
	Spiked-soil DNA	32.05 ± 0.84	34.35 ± 1.09	31.10 ± 0.75	28.58 ± 0.22	29.75 ± 0.59	28.53 ± 0.68
$C_{T\text{-rel}}$	Genomic DNA from BCA strain	1.11 ± 0.04	1.20 ± 0.05	1.08 ± 0.06	1.00 ± 0.00	1.08 ± 0.07	1.02 ± 0.08
	Spiked-soil DNA	1.12 ± 0.02	1.20 ± 0.04	1.09 ± 0.02	1.00 ± 0.00	1.04 ± 0.02	0.99 ± 0.02

[a] $C_{T\text{-rel}}$ values were determined with reference to locus Bb4H9 derived either from 2 pg genomic DNA of the BCA strain (results are means of three replications) or from 50 ng bulk soil DNA spiked with 2 pg genomic DNA of the BCA strain (spiked-soil DNA) (results are means of four replications).

tected, but those of loci Bb2F8 and Bb4H9 were not detected. In mixture d, containing 0.2 pg spiked genomic DNA of genotypes II and III, genotype II alleles that are shared with genotype I (locus Bb2A3) or common to all three genotypes (locus Bb8D6) were detected. In mixture e, containing 0.6 pg DNA, i.e., 0.2 pg of each of the three genotypes, the shared allele at locus Bb8D6 and the genotype I-specific allele at locus Bb4H9 were detected (Table 4).

Cultivation-dependent analyses of *B. brongniartii* field populations. Plating on SM revealed the presence of *B. brongniartii* in all five soil samples (T1 to T5) from the field plot treated with the BCA strain (Table 5). The mean *B. brongniartii* density was 98,484 CFU g^{-1} (dry weight), with a minimum of 9,405 CFU g^{-1} (dry weight) (sample T3) and a maximum of 229,266 CFU g^{-1} (dry weight) (sample T2). From the control plot, two samples were free of *B. brongniartii*, while two samples contained *B. brongniartii* at low densities (261 and 816 CFU g^{-1} [dry weight]) and one sample contained *B. brongniartii* at the highest density observed, i.e., 724,441 CFU g^{-1} (dry weight). Genotypes of 24 isolates from the plating experiment were characterized by analysis of the six *B. brongniartii* SSR loci (Table 5). Three different genotypes were identified (Table 2). Genotype I of the applied BCA strain as well as indigenous genotype II was detected for 14 *B. brongniartii* isolates randomly selected from the treated plot (Table 5). A different indigenous genotype (genotype III) was found exclusively in 10 isolates from the control plot (Table 5). Genotypes I and II displayed the same allele at locus Bb2A3, while all three genotypes had a common allele at locus Bb8D6 (Table 2).

Cultivation-independent analysis of *B. brongniartii* field populations. DNA extraction from the 10 field soil samples yielded 261 to 299 μg DNA g^{-1} (dry weight) for the treated plot and 287 to 350 μg DNA g^{-1} (dry weight) for the control plot. Mean quantities of 276 μg DNA g^{-1} (dry weight) for the treated plot and 300 μg DNA g^{-1} (dry weight) for the untreated plot were not significantly different ($P < 0.05$, two-sided Student's *t* test).

Relative differences of SSR detection sensitivities ($C_{T\text{-rel}}$) derived from bulk soil DNA spiked with genomic DNA (spiked-soil DNA) (Table 3) were confirmed with field soil samples. Correlation coefficients between experimentally determined C_T values and C_T values calculated as products of

TABLE 4. Detection of genotype-specific alleles of six SSR loci in 50 ng *B. brongniartii*-free bulk soil DNA spiked with mixtures of different quantities of three different genotypes of *B. brongniartii*

Spiked genotype[a]	Template quantity[b] (pg)	Mixture	Presence (+) or absence (−) of alleles of locus:						Retrieved genotype[a]
			Bb1F4	Bb2A3[c]	Bb2F8	Bb4H9	Bb5F4	Bb8D6[d]	
I	2	a	+	+	+	+	+	+	I
II	20	a	+	+	+	+	+	+	II
III	20	a	+	+	+	+	+	+	III
I	0.2	b	+	+	−	−	+	+	UD[e]
II	20	b	+	+	+	+	+	+	II
III	20	b	+	+	+	+	+	+	III
I	2	c	+	+	+	+	+	+	I
II	2	c	+	+	+	+	+	+	II
III	2	c	+	+	+	+	+	+	III
I	2	d	+	+	+	+	+	+	I
II	0.2	d	−	+	−	−	−	+	UD
III	0.2	d	−	−	−	−	−	+	UD
I	0.2	e	−	−	−	+	−	+	UD
II	0.2	e	−	−	−	−	−	+	UD
III	0.2	e	−	−	−	−	−	+	UD

[a] According to Table 2.
[b] Based on genome size of *B. bassiana* (38), 2 pg of *B. brongniartii* genomic DNA corresponds to 45 genome copies.
[c] No discrimination of genotypes I and II possible (Table 2).
[d] No discrimination of the three genotypes possible (Table 2).
[e] UD, undefined.

TABLE 5. Monitoring of *B. brongniartii* in a BCA-treated plot (samples T1 to T5) and in a control plot (samples C1 to C5)

Soil sample	Cultivation-dependent analyses[i]			Cultivation-independent analyses[h]						
	CFU g^{-1} soil (dry wt)	No. of isolates genotyped	Genotype[a]	Detection of SSR locus:						Genotype[a]
				Bb1F4	Bb2A3	Bb2F8	Bb4H9	Bb5F4	Bb8D6	
T1	14,383	NA[b]	NA	+ (36)	+ (40)	+ (36)	+ (32)	+ (36)	+ (32)	I
T2	229,266	4	I	+ (36)	+ (36)	+ (32)	+ (32)	+ (32)	+ (32)	I
T3	9,405	2	I	+ (36)	+ (40)	+ (36)	+ (36)	+ (36)	+ (36)	I
T4	118,463	NA	NA	+ (32)	+ (36)	+ (32)	+ (28)	+ (28)	+ (28)	I
T5	120,904	8	II	+ (32)	+ (36)	+ (32)	+ (32)	+ (32)	+ (32)	II
C1	0	0	ND[c]	− (40)	− (40)	− (40)	− (40)[d]	− (40)[d]	− (40)[d]	ND
C2	724,441	8	III	+ (28)	+ (32)	+ (28)	+ (28)	+ (28)	+ (28)	III
C3	261	1	III	+ (40)	− (40)	− (40)	+ (40)[d]	+ (40)[d]	+ (40)[d]	UDC[e]
C4	0	0	ND	− (40)	− (40)	− (40)	+[f] (40)[d]	− (40)[d]	+ (40)[d]	UD[g]
C5	816	1	III	− (40)	− (40)	− (40)	− (40)[d]	− (40)[d]	− (40)[d]	ND

[a] According to Table 2.
[b] NA, not analyzed.
[c] ND, not detected.
[d] Analysis performed at C_a but repeated with 40 PCR cycles.
[e] UDC, undefined but consistent with genotype III.
[f] Allele of 215 bp, different from all three genotypes I, II, and III.
[g] UD, undefined.
[h] The presence (+) or absence (−) of SSR alleles and resulting genotypes was determined with cultivation-independent analyses of 50 ng bulk soil DNA of each sample. Numbers in parentheses are the numbers of cycles to which C_a values were set.
[i] Total CFU and genotypes of 24 selected isolates were determined with cultivation-dependent analyses.

$C_{T\text{-}4H9}$ and corresponding $C_{T\text{-}rel}$ from spiked-soil DNA were $r = 0.99$ for soil sample T2, with high *B. brongniartii* plate counts, and $r = 0.93$ for soil sample T3, with 24-times-lower plate counts.

Values for $C_{T\text{-}4H9}$ varied between 26.4 and 35.2 among 8 of the 10 soil samples, with a maximum difference of 1.1 cycles for duplicate analyses. Based on these results, C_a values were set to 28, 32, 36, or 40 cycles (Tables 3 and 5). If amplification at C_a did not allow for detection of a specific locus, PCR was repeated with 40 cycles (Table 5). All six loci were detected in the five samples from the treated plot. *B. brongniartii* genotype I, i.e., the applied BCA strain, was detected in four of the five soil samples, and genotype II was detected in sample T5 (Table 5). Among the samples from the control plot, only sample C2 yielded PCR products for each SSR locus. The resulting fingerprint corresponded to genotype III. Even though alleles corresponding to four loci of genotype III were detected in soil sample C3, the fingerprint remained incomplete and no genotype was assigned. In soil sample C4, only the allele at locus Bb8D6, which was common to all three genotypes, was detected, while for locus Bb4H9 an allele with the size of 215 bp, which was different from the corresponding alleles of any of the three genotypes isolated, was observed. None of the loci were detected in samples C1 and C5.

DISCUSSION

The goal of this study was to assess the feasibility of cultivation-independent multilocus SSR genotyping of fungal strains in bulk soil DNA extracts. The ascomycete fungus *B. brongniartii*, used in biological control of the European cockchafer, *M. melolontha*, served as a model system to develop a generally applicable strategy. A BCA strain applied in a field experiment was monitored by this strategy, and the results were validated by comparison with those of the established cultivation-dependent approach.

Specificity of SSR detection for reliable cultivation-independent identification of specific fungal isolates from complex samples was achieved by combined use of two criteria: species specificity of SSR PCR primers and multilocus SSR fingerprinting. Species specificity of the six *B. brongniartii* SSR primer pairs was demonstrated by the presence of characteristic SSR amplification products from the target species and their absence from nontargets, respectively (Table 1). Species specificity for SSR primers has also been reported for other fungi (3, 39, 46). However, there are also examples of either unspecific SSR primers, which detect various species (22, 25), or highly specific primers, limited to certain strains only (12). Therefore, detection specificity of SSR primers needs to be validated prior to cultivation-independent application to complex DNA samples.

Multilocus SSR fingerprinting of *B. brongniartii* has been reported to discriminate individuals in natural populations with high probabilities of 92 to 99% (16). Five of the six markers used have been reported to be highly polymorphic, while one marker (Bb8D6) was not (15). In the present study, amplification from locus Bb8D6 yielded a nontarget amplification product from genomic DNA of *B. bassiana*, which was in accordance with findings by Enkerli et al. (15). However, with a length of 163 bp, this allele was shorter than any allele recorded for this locus from *B. brongniartii* (172 to 190 bp) by others (15, 16) or in the present study. The absence of any unexpected allele or unspecific amplification products in the spiking experiments (Tables 3 and 4) further confirmed specificity of the six SSR primers if applied for detection in bulk soil DNA extracts. In addition, data from the experimental field plot indicated specificity, as they revealed the same genotypes for both analyses (Table 5). The allele of 215 bp derived from

locus Bb4H9 in soil sample C4 was the only one among 60 analyses that could not be attributed to the three genotypes detected (Table 5) and may indicate the presence of a fourth genotype at a very low abundance. All together, these data demonstrated that specificity of *B. brongniartii* SSR PCR primers is also retained in highly complex bulk soil DNA. Specificities of fungal SSR primers within plant DNA extracts have been reported previously (20, 34, 46), but to our knowledge the present study is the first report on SSR species specificity in bulk soil DNA.

Adjustment of detection sensitivities for multiple SSR loci needs to account both for template quantities in bulk soil DNA extracts and for locus-specific amplification efficiencies. Due to high sequence diversity related to the large numbers of different organisms present in a soil sample, typically, template DNA of individual genotypes is relatively nonabundant in bulk soil DNA (31). Sample-specific template quantities were accounted for by using C_a numbers, which also adjusted for primer-specific detection sensitivities. C_a numbers were set as low as possible in order to minimize the risk of PCR biases and artifact generation. For the 10 soil samples, four classes of C_a were sufficient to cover the range of all 60 C_T values. This allowed for efficient detection of all markers in all samples. Resulting quantities of all PCR products were similar; thus, no individual dilutions were necessary prior to fragment sizing. The observed reproducibility of $C_{T\text{-rel}}$ values both in genomic DNA and in the tested bulk soil DNA extracts indicated a general stability of SSR marker detection sensitivities for the *B. brongniartii* system.

Detection limits for reproducible cultivation-independent genotyping of *B. brongniartii* were estimated by analyzing either spiked-soil DNA or field soil samples. Unambiguous genotype identification from spiked-soil DNA was possible when 50 ng of bulk soil DNA was spiked with 2 pg genomic DNA of *B. brongniartii* but failed for assays with soil spiked with 0.2 pg genomic DNA (Table 4). Because in some reactions alleles were also amplified when present at quantities of 0.6 pg (locus Bb8D6 in mixture e) or 0.2 pg (loci Bb1F4 and Bb5F4 in mixture b and locus Bb4H9 in mixture e), the actual detection limit for *B. brongniartii* may be between 0.2 and 2 pg genomic DNA (Table 4). These quantities equal 4.5 or 45 genome copies, respectively, if assuming a genome size of 40 Mbp, as determined for the closest relative, *B. bassiana* (38). A similar detection limit of 26 copies per PCR has been reported for *Trichoderma atroviride* (11). For field soil samples, the detection limit of the cultivation-independent analysis of *B. brongniartii* genotypes depended on unambiguous identification of the least efficiently amplifying locus, Bb2A3, processed at a maximum of 40 PCR cycles (Table 5). The resulting detection limit corresponded to approximately 10^4 CFU g^{-1} (dry weight) soil (Table 5, sample T3). Similar cultivation-independent detection limits were reported for *Metarhizium anisopliae*, 4×10^4 CFU g^{-1} (dry weight) soil (17), *Fusarium solani*, 1×10^4 CFU g^{-1} (dry weight) soil (18), and *Paecilomyces lilacinus*, 3×10^3 CFU g^{-1} (dry weight) soil (2). With a mean of 275 μg DNA g^{-1} (dry weight) soil, the 50 ng DNA used for PCR corresponded to 0.18 mg soil. Thus, the 10^4 CFU g^{-1} (dry weight) would represent approximately 2 CFU per PCR. With such low template quantities, stochastic PCR amplification will provide unreliable results (45). However, 1 CFU may represent conidia, as well as mycelium carrying more than one nucleus (10). In addition, cultivation detects only viable material, whereas cultivation-independent analysis also detects target sequences from unculturable and possibly dead cells (31). For *B. brongniartii*, the relation between the detected CFU and the number of conidia added to soil was previously estimated to be 1:20; thus, 2 CFU may represent approximately 40 conidia (27). This almost perfectly supports the observed detection limit of 10^4 CFU g^{-1} (dry weight), corresponding to about 40 genome copies per PCR. These considerations are in agreement with the 2 pg detection limit for genomic DNA (Table 4). The use of a 100-times-higher quantity of soil, i.e., 20 mg per analysis, for plate counting resulted in a 100-times-higher sensitivity for *B. brongniartii*. This allowed detection of *B. brongniartii* at densities of 261 and 816 CFU g^{-1} (dry weight) in soil samples C3 and C5, respectively, which were not detectable with the cultivation-independent analysis (Table 5).

Cultivation-independent multilocus genotyping is an attractive alternative for *B. brongniartii* monitoring. The method has a detection limit of about 10^4 CFU g^{-1} (dry weight) of *B. brongniartii* as required to induce epizootics in *M. melolontha*-infested fields (27). In addition, it allows for substantial reduction of the time and cost for BCA monitoring. Analyses can be performed within 1 week, whereas the cultivation-dependent approach may require up to 2 weeks for density analysis and approximately 6 weeks for genotyping due to cultivation and subsequent DNA extraction (16). Furthermore, the method allowed monitoring of multiple cooccurring strains spiked into bulk soil DNA (Table 4). This can be important for fields where genotypic diversity may be high, e.g., 22 different *B. brongniartii* genotypes in a plot of 400 m^2 (J. Enkerli, unpublished data). As only one of the three occurring genotypes was detected per field soil sample (Table 5), spiking bulk soil DNA with three different genotypes per sample may cover the expected range of genotype diversity and may represent a realistic model (Table 4).

The three genotypes detected in the field experiment were unevenly distributed between the treated and the control plot (Table 5), which was in accordance with other studies revealing generally patchy distributions of soil fungi on small scales (8, 33, 36). The high plate counts caused by indigenous strains (Table 4, soil samples T5 and C2) emphasized the need for genotypic analyses when monitoring applied BCA strains in order to avoid false conclusions.

With a growing need to assess potential risks associated with the release of BCAs into the environment (24), effective and efficient monitoring becomes increasingly important (4). The cultivation-independent monitoring approach based on multiple SSRs combines species-specific detection of polymorphic markers with strain-level resolution. This allows detection of both BCA and indigenous strains of a target species. Furthermore, this approach will allow for genetic analyses of other organisms (2, 9, 11) as well as profiling of microbial community structures within the same DNA extract (21, 42). Cultivation-independent fingerprinting using SSR thus could ideally be used to study interactions between released fungal strains and indigenous soil microbial communities.

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