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Microsatellite markers to monitor a commercialized isolate of the entomopathogenic fungus *Beauveria bassiana* in different environments: Technical validation and first applications

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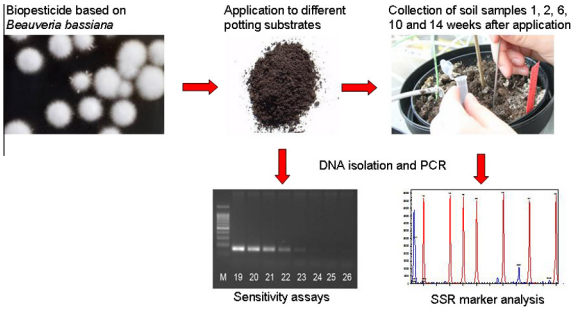
HIGHLIGHTS

- Three SSR markers confidentially discriminated between a world-wide collection of *Beauveria bassiana* isolates.
- Detection thresholds differed depending on the environment of the PCR assay.
- SSR markers detected a commercialized *B. bassiana* strain in different substrates up to 14 weeks after inoculation.

GRAPHICAL ABSTRACT



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ABSTRACT

Here, we report on the application of five previously developed microsatellite markers (simple sequence repeats, SSRs) to monitor an isolate of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. in different environments. Discriminatory power of these SSR markers was assessed in two commercialized *B. bassiana* isolates as well as in 16 *B. bassiana* isolates from a world-wide collection, and three of the five SSR markers were estimated to allow a confident discrimination among the given isolates. Sensitivity thresholds of 0.1 pg DNA were subsequently determined for all SSR markers in case pure genomic fungal *B. bassiana* DNA was used as a template for PCR assays, but threshold levels varied depending on the environment (soil, plant) of the PCR assay. Furthermore, presence of a commercialized *B. bassiana* isolate was monitored via these SSR markers in three different types of potting substrates over a period of 14 weeks. With two SSR markers, strain-specific products were detected up to 14 weeks after application of *B. bassiana* to the substrate. Infectivity of *B. bassiana* conidia in the respective soil samples was confirmed by the *Galleria* baiting technique. Together these results indicate that molecular markers like SSRs specific for commercialized strains of entomopathogenic fungi are important tools to monitor a particular fungal strain in complex environmental samples such as bulk soil or plant DNA.

1. Introduction

Entomopathogenic fungi are a very diverse and ubiquitous group of natural enemies of arthropods. As such they have attracted increased attention in recent times as potential microbial biocontrol agents to be used in integrated pest management programs in an inoculative or inundative manner (Hajek and Delalibera, 2010; Jaronski, 2010). More than 130 commercial products based on entomopathogenic fungi have been developed in the past, with around two-thirds of them consisting of conidial preparations of the two most widely studied entomopathogens, *Beauveria*

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bassiana (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sorokin. (both Ascomycota: Hypocreales) (Jackson et al., 2010; Jaronski, 2010). However, despite these numbers, fungus-based mycoinsecticides do not account for a substantial part of the US or European biopesticide market (Jaronski, 2010). Aspects on stability of achieved control levels, costs, product quality and shelf-life, as well as persistence of the fungal propagules in the environment are among the chief reasons for this limited use. The latter is mainly influenced by an array of biotic and abiotic factors such as temperature, solar radiation, moisture or heavy rainfall (Meyling and Eilenberg, 2007). Accordingly, a couple of recent studies have stressed the importance to not only focus on the interaction between an entomopathogenic fungus and its host insect, but also to identify critical environmental constraints and to understand fungal ecology and multi-trophic relationships in semi-natural or anthropogenic habitats (Bruck, 2010; Hesketh et al., 2010; Jackson et al., 2010; Meyling and Hajek, 2010; Meyling and Eilenberg, 2007; St. Leger, 2008). In the case of *M. anisopliae*, studies by Bruck and Donahue (2007) have shown that fungal propagules can be incorporated in different types of potting media at the time of planting and persist in the potting medium for one or more growing seasons, allowing control of soil-borne insects like larvae of the black vine weevil *Otiorhynchus sulcatus*. In addition, some *M. anisopliae* isolates are known to be rhizosphere competent, and insects like *O. sulcatus* larvae feeding on roots which were colonized with an entomopathogenic fungus showed high levels of fungal infection (Bruck, 2005). Furthermore, recent studies have proved that *B. bassiana* can endophytically colonize a wide array of plant species, while still maintaining its entomopathogenic habit (Akello et al., 2008; Gurulingappa et al., 2010; Ownley et al., 2010; Quesada-Moraga et al., 2009; Tefera and Vidal, 2009). Together these aspects again stress the importance of understanding the ecology and persistence of fungal entomopathogens in various environments such as the soil or plant (Bruck, 2010; Vega et al., 2009).

As a prerequisite for studying the ecology and persistence of entomopathogenic fungi in the soil or other environments as well as their interactions with microbial communities present in the respective habitat, a method allowing strain-specific identification of the particular fungal isolate is required. When applying classical, elaborate and time consuming cultivation-based techniques, a strain-specific identification is often not possible due to a lack of consistent and definite morphological characteristics, which might as well be influenced by culture medium or other physiological conditions (Hussain et al., 2010; Oliveira et al., 2011). Hence, cultivation-independent molecular genetic techniques have been increasingly applied to monitor entomopathogens in the environment (Enkerli and Widmer, 2010). Those PCR-based techniques usually allow a highly specific and high-throughput detection and quantification of the targeted fungal species or strain directly in the respective environment. For the entomopathogenic fungus *Metarhizium* spp. clade 1, a diagnostic PCR-based method was recently developed, which also allows quantification in complex bulk soil DNA samples (Schneider et al., 2011). For an entomopathogen of aphids, *Pandora neoaphidis* (Entomophtoromycota: Entomophthorales), Fournier et al. (2008) developed specific PCR primer pairs and applied them to monitor the presence of this fungus in bulk soil DNA. However, this approach did not allow a strain-specific identification of different *P. neoaphidis* isolates. Castrillo et al. (2003) have developed a marker specific for *B. bassiana* strain GHA based on a random amplified polymorphic DNA (RAPD) fragment and quantified its abundance using qPCR in various environmental samples such as leaf, bark and soil samples (Castrillo et al., 2008). Schwarzenbach et al. (2007) applied markers based on microsatellites (simple sequence repeats, SSRs) to detect *Beauveria brongniartii* strains in bulk soil DNA extracts. SSR markers are often highly polymorphic thus allowing a precise genotyping of different fungal strains and can be multiplexed during the reaction increasing the accuracy of identification and reducing both time and costs of the assays (Selkoe and Toonen, 2006). These SSR markers have been isolated in a variety of entomopathogenic fungi, among them *B. bassiana* (Rehner and Buckley, 2003), *B. brongniartii* (Enkerli et al., 2001), *Paecilomyces fumosoroseus* (Dalleau-Clouet et al., 2005) and *M. anisopliae* (Enkerli et al., 2005).

The intention of the present study was to apply five of the *B. bassiana* specific SSR markers previously published by Rehner and Buckley (2003) to monitor the presence of a commercialized *B. bassiana* isolate (ATCC 74040, active ingredient in the product Naturalis®) in various environments. For this purpose, these SSR markers were evaluated regarding their discriminative power and sensitivity and were subsequently applied to monitor the presence of *B. bassiana* isolate ATCC 74040 in three commercially available potting substrates.

2. Materials and methods

2.1. Fungal material

Throughout the present study, the commercial product Naturalis® (Intrachem, Italy) was used in the experiments. It was formulated as an oily fluid and contained 69.1 g/L *B. bassiana* isolate ATCC 74040 as an active ingredient with a concentration of at least 2.3×10^7/ml viable *B. bassiana* conidia. In addition, for SSR analysis, 17 *B. bassiana* strains were obtained from several culture collections, including *B. bassiana* strain GHA, which is the active ingredient in several mycoinsecticides registered worldwide, e.g. product BotaniGard® (Table 1). All *B. bassiana* strains including a suspension of product Naturalis® were grown on *Beauveria* selective medium (BSM, (Strasser et al., 1996)) to obtain pure reference DNA for molecular analysis.

2.2. DNA extractions

For DNA extraction, mycelium was directly collected from fungal colonies grown on BSM plates and cells were mechanically disrupted using the Precellys tissue homogenizer (Peqlab Biotechnologies, Erlangen). Fungal DNA was extracted using Power Soil DNA Isolation Kit (Mobio Laboratories, USA) according to the manufacturer's instructions. DNA from 250 mg of soil samples was extracted in the same way using Precellys tissue homogenizer for soil particle lysis and Power Soil DNA Isolation Kit. Genomic DNA from 5 to 10 mg about 3 weeks old plant leaves (grapevine, *Vitis vinifera* L. cv. Riesling, plants were about 8 weeks old) was extracted using the MasterPure DNA Purification Kit (Biozym Scientific GmbH, Hessisch Oldendorf) according to the manufacturer's instructions. We used grapevine as a model plant in this study since we currently apply strain-specific molecular markers to monitor endophytic establishment of *B. bassiana* in grapevine plants (Rondot and Reineke, 2013).

2.3. Amplification of microsatellite markers

Five *B. bassiana* specific microsatellite primers were used, called Ba01, Ba02, Ba08, Ba12 and Ba13 (Rehner and Buckley, 2003). To allow a fluorescent labelling of the generated PCR products, three primers were incorporated in the PCR reactions according to the method described by Schuelke (2000): a SSR-specific forward primer with an universal M13(−21) tail at it's 5′-end, an unlabelled SSR-specific reverse primer, and a fluorescently labelled CY5 universal M13(−21) primer, which will incorporate the fluorescent dye into the PCR product (Schuelke, 2000). PCR amplifications were