

Use of a Microsatellite Assay to Differentiate Strains of the Entomopathogenic Fungus *Beauveria bassiana*

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Abstract

Beauveria bassiana is an entomopathogenic fungus that is being studied for its potential as a biocontrol agent for several pest arthropods. Because this fungus is naturally present in soils the world over, highly specific molecular markers are needed to differentiate between naturally present soil strains and the strains that are applied in biocontrol programs. Eight polymorphic microsatellite loci have been identified for *B. bassiana*. I report here on the variation at four of these microsatellite loci between two isolates of the fungus, one commercial biocontrol product and one soil sample. Size fractionation of PCR products indicates that at least two of the microsatellite loci tested exhibit polymorphisms that may be utilized to differentiate natural and applied strains of the fungus.

Introduction

Entomopathogenic fungi are naturally-occurring pathogens of arthropods which have gained recognition as useful biological control agents for a variety of arthropod pests. Species of entomopathogenic fungi have already been utilized successfully in the control of the gypsy moth (*Lymantria dispar*), the spotted alfalfa aphid (*Therioaphis trifolii* f. *maculata*) and other aphid species, the desert locust (*Schistocerca gregaria*), and the European cockchafer beetle (*Melolontha melolontha*) (reviewed in Shah and Pell, 2003). Research on the potential of entomopathogenic fungi to control other arthropod pests is ongoing, as are studies of potential non-target effects of these fungi. Highly specific genetic markers would greatly facilitate such

studies by allowing researchers to distinguish between naturally present strains of fungus and strains that are artificially applied.

Beauveria bassiana is one species of entomopathogenic fungus that has been pursued for its biocontrol potential. Early molecular analyses of *B. bassiana* and other *Beauveria* species indicated that there was very little genetic variation within this genus (e.g., Cravanzola et al., 1997; Berretta et al., 1998). It was initially proposed that the lack of genetic diversity was a result of clonal reproduction (Cravanzola et al., 1997), but it was later discovered that *Beauveria* species do not propagate clonally (Wang et al., 2003). Recent work has uncovered both inter- and intraspecific variation within *Beauveria*. Specifically, Coates et al. (2002) identified one polymorphic minisatellite locus, and Rehner and Buckley (2003) characterized eight highly polymorphic microsatellite loci for *B. bassiana*.

Microsatellite loci, first discovered in 1982 (Hamada et al., 1982), are tandemly repeated di- or trinucleotide sequences known to exhibit high rates of evolution and therefore a relatively high degree of intraspecific variability. For each of the eight such loci described by Rehner and Buckley (2003) for *B. bassiana*, four to ten alleles were found across 24 representative strains of *B. bassiana*. For this study, the four microsatellite loci exhibiting the most alleles (Ba02, Ba06, Ba08, and Ba12; see Table 1) were utilized.

The objective of this study was to assess the capacity of these four microsatellite markers to differentiate between strains of *B. bassiana* that are relevant to ongoing research on the biocontrol potential of this fungus. I report here on the variation between two isolates of *B. bassiana* at these four loci. The first isolate is the strain of *B. bassiana* present in Botanigard ES, a commercially available suspension of fungal spores that is marketed for use against whiteflies, aphids, thrips, psyllids, mealybugs, scarab beetles, plant bugs, and weevils and is presently being

investigated for its efficacy in controlling black-legged ticks. The third isolate is an unknown strain that most likely contains a mixture of Botanigard and strain of *B. bassiana* naturally present in the soil at the Louis Calder Biological Field Station in Armonk, NY

Materials and Methods

DNA Extraction

Mycelia of each fungal isolate described above were grown on plates of potato dextrose agar containing antibiotics and stored at 20°C until the start of this experiment. A scrape of mycelia was taken from each plate for DNA extraction using the DNeasy Plant Mini Kit (Qiagen). The manufacturer's Mini Protocol was followed with the following modifications. In lieu of the TissueRuptor or TissueLyser system, scrapes of mycelia were placed in individual 1.5 mL microcentrifuge tubes, dipped in liquid nitrogen to flash-freeze, and crushed with a sterile glass pestle. In the final steps of the extraction, samples were eluted twice with 50 μ L nuclease-free water instead of 100 μ L buffer AE.

Initial PCR Amplification

Four microsatellite loci were PCR-amplified using the primers listed in Table 1 (from Rehner and Buckley, 2003). Reaction mixtures consisted of 12.5 μ L GoTaq polymerase master mix, 0.5 μ L of each primer (at a concentration of 10 μ M), and 11.5 μ L DNA template. PCR conditions were as follows: initial denaturation at 94°C for 5 min, 50 cycles of 94°C denaturation for 30 sec, 57°C annealing for 30 sec, and 72°C extension for 30 sec, and final extension at 72°C for 7 min. To ensure that the primers successfully amplified DNA from my samples, these PCR products were visualized on a 1.5% agarose gel.

Size Fractionation Analysis of Microsatellite Repeats

The products amplified by the 4 primer sets in Table 1 were also size-fractionated on a polyacrylamide (sequencing) gel. Separate PCR's were performed for this using 6.25 μ L GoTaq, 0.25 μ L of each primer (at 10 μ M), 3.45 μ L nuclease-free water, 0.3 μ L 33 P-dATP, and 2 μ L DNA template. Cycling conditions were as described above but with no final extension time. Following PCR-amplification, 7 μ L of sequencing "stop solution" was added to each product, and products were denatured at 95°C for 2 min and then cooled on ice before loading in the sequencing gel. This procedure was only carried out for isolates 2 (Botanigard) and 3 (unknown).

Table 1. Four Microsatellite Loci for *Beauveria bassiana*
(adapted from Rehner and Buckley, 2003)

Locus	Repeat Motif	Primer Sequences	Approximate Size of Product (bp)
Ba02	(CA) ₂₀	F: AACGCTATGCCTTGACGAC R: GACGCCGAGCAATGTAACA	140
Ba06	(GTT) ₁₀	F: GCGATTGACGAAAAGCTAGA R: ACTTGCTTTGCTGTTGCACA	114
Ba08	(AAG) ₁₀	F: TGTTGCCGACACGAATTGT R: GGCTCAAGCGCAAAGAGAAA	210
Ba12	(CTT) ₇	F: GGGTCCATCATGTACGGC R: AGGCGTATACAGGTCGTG	231

Results

All four primer sets successfully amplified DNA from each of the two isolates, as shown in the agarose gel in Figure 1. All products were of the expected sizes.

Size-fractionating the PCR products on a sequencing gel allowed me to distinguish between distinct microsatellite haplotypes without knowing the precise sequence of each. The results, using this technique, for the Ba08 primers were inconclusive because there appeared to be a very large size difference between the two isolates, which is unlikely to be real. There does appear to be a size difference between the two isolates at loci Ba06 and Ba12 but no difference at locus Ba02 (Figure 2).

Discussion

The ability to differentiate strains of *B. bassiana* with highly specific molecular markers would facilitate several aspects of the ongoing research on this fungus. First, accurate assessments of the rate at which the fungus contacts organisms when applied in the field would be made possible. Currently, such assessments, when made, are very imprecise because it is impossible to distinguish between the fungus that is applied and background levels of the fungus that were present in the soil prior to application. Using molecular markers to make these assessments would improve evaluations of the efficacy of the fungus in controlling pest arthropods as well as increase our understanding of the effects of the fungus on non-target organisms. Further, researchers could use these markers to track the fate of the fungus when it is applied and, thus, examine the spatial and temporal impacts of applying the fungus in a given system.

While attempts to sequence the PCR products shown in Figure 1 failed due to errors in PCR, I was able to detect microsatellite polymorphisms by fractionating the products on a sequencing gel. This gel, seen in Figure 2, supports the conclusion that there are polymorphisms for at least two of these four loci among the isolates tested. There are clear size differences in the

alleles seen at loci Ba06 and Ba12 in isolates 2 and 3, indicating that these isolates have distinct haplotypes for each of these loci. Isolate 2 was the fungal strain present in Botanigard, and isolate 3 was an unknown isolate most likely containing strains from both the soil and Botanigard. Thus, the differences seen between these two isolates are likely indicative of differences between strains of *B. bassiana* present in Botanigard and in the soil in Armonk, NY. Therefore, I suggest that loci Ba06 and Ba12 are suitable candidates for molecular markers capable of differentiating between applied and natural strains of *B. bassiana*. Although further research is necessary to firmly establish the haplotype differences suggested herein, these results are very encouraging because such markers would be exceptionally useful in field studies of this entomopathogenic fungus.

References

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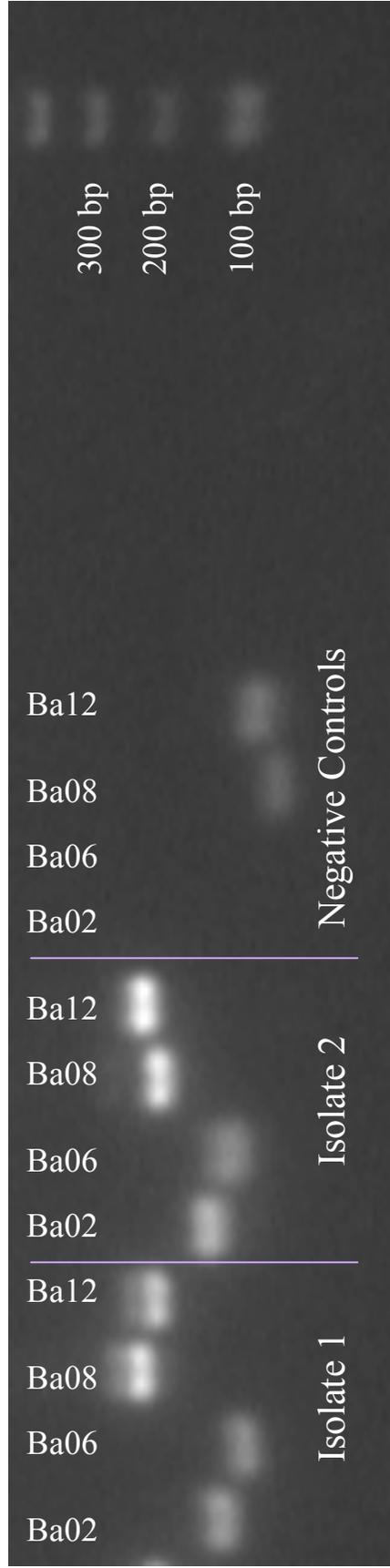


Figure 1. Size Fractionation of PCR Products on 1.5% Agarose Gel. DNA purified from mycelia of 2 fungal isolates (labeled 1 & 2 in the figure) was PCR-amplified for each of 4 microsatellite loci. Products were run on a 1.5% agarose gel stained with EtBr.

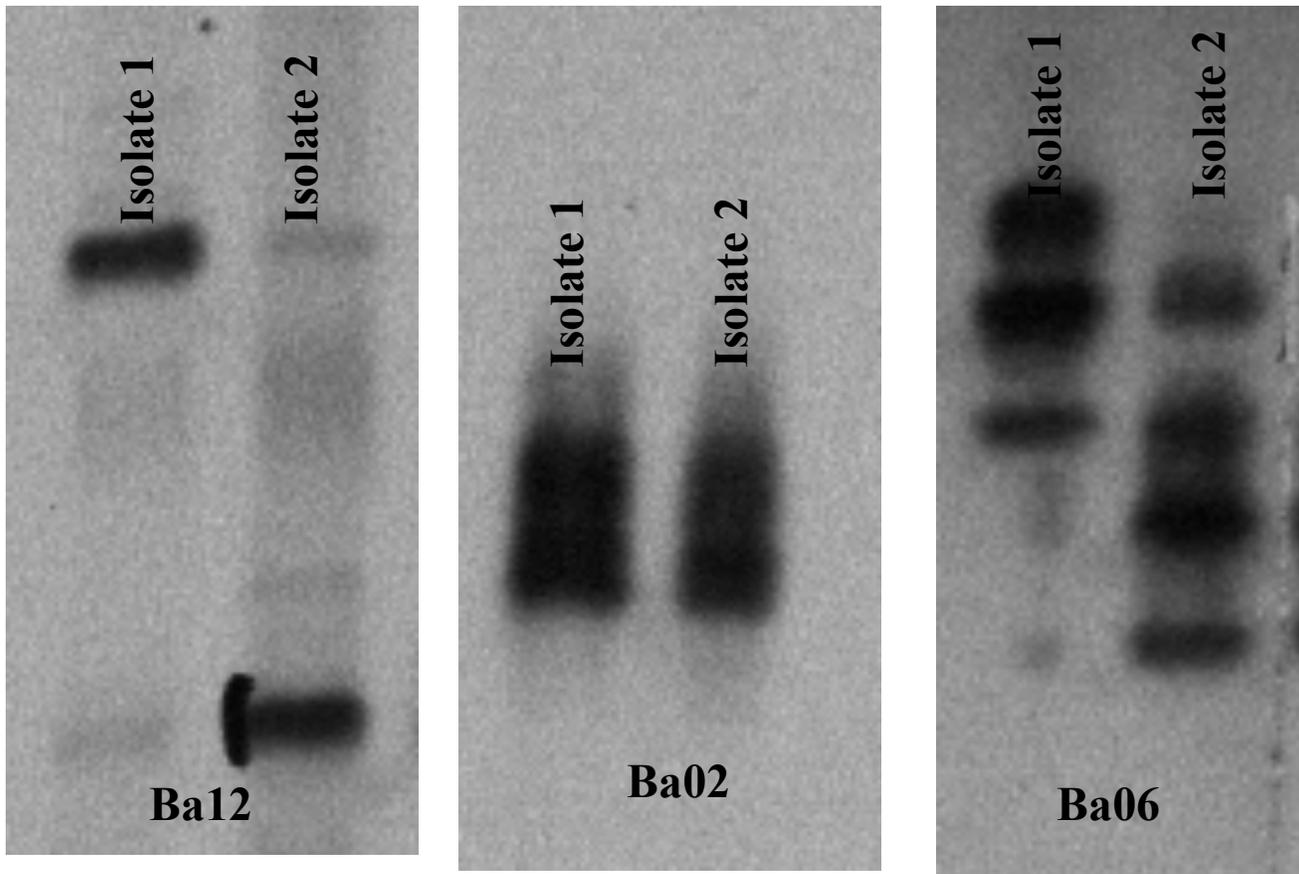


Figure 2. Size Fractionation of Loci Ba02, Ba06, & Ba12 on a Sequencing Gel. DNA purified from mycelia of 2 fungal isolates (labeled 1 & 2 in the figure) was PCR-amplified for each microsatellite locus in the presence of ^{33}P -dATP. Products were run in a polyacrylamide (sequencing) gel to assess small size differences between isolates. Size polymorphisms were detected at loci Ba06 & Ba12 but not at locus Ba02. Locus Ba08 is not shown because the fractionation of isolates 1 & 2 at this locus could not be clearly interpreted.