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PRIMER NOTE

Isolation and characterization of microsatellite loci from the entomopathogenic fungus *Beauveria bassiana* (Ascomycota: Hypocreales)

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Abstract

***Beauveria bassiana*, an entomogenous fungus used for the biological control of pest insects, comprises a globally-distributed species complex of regionally endemic lineages. In order to study the population genetics of *B. bassiana*, detail species boundaries, conduct ecological studies of natural populations and track fates of experimentally-released strains, sensitive genetic markers are required. We describe the isolation and characterization of eight microsatellite loci that amplify successfully from strains representative of the phylogenetic diversity in the *B. bassiana* complex.**

Keywords: ascomycete, *Beauveria bassiana*, fungi, Hypocreales, microsatellite, population genetics

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Beauveria bassiana is a cosmopolitan, haploid, soil-borne entomopathogenic fungus used for the biological control of agricultural insect pests. Widely assumed to be clonal because it reproduces via mitotic spores, molecular phylogenetic analyses show that *B. bassiana* is a geographically-structured complex of recently-diverged lineages (S.A. Rehner, E.P. Buckley, unpublished) phylogenetically linked to the sexual species, *Cordyceps bassiana* (Ascomycota: Hypocreales) (Li *et al*. 2001; S.A. Rehner, E.P. Buckley, unpublished). In order to explore species boundaries in the *B. bassiana* complex more effectively, assess the relative importance of sexual vs. asexual reproduction on population genetic structure, and improve understanding of their ecology and pontential impacts when used as insect biological control agents, more sensitive genetic markers to *B. bassiana* are needed. A single polymorphic microsatellite locus to *B. bassiana* was described by Coates *et al*. (2002). However, many more polymorphic markers are required if precise strain genotyping and robust inference into population structure is to be achieved. Microsatellite markers developed for the related species *Beauveria brongniartii* demonstrate a low success rate when used on *B. bassiana* isolates (Enkerli *et al*. 2001). Here, we describe the isolation and characterization of eight polymorphic microsatellite markers and their polymerase chain reaction (PCR) amplification from 24 strains representative of the global diversity within the *B. bassiana* complex.

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Mycelium for DNA extraction was grown in SDY/4 medium (Goettel & Inglis 1997), harvested by filtration, washed in sterile distilled water and lyophilized. Lyophilized mycelium was pulverized in a FastPrep FP120 tissue grinder (ThermoSavant). DNA extractions were performed using the method of Cambereri & Kinsey (1994) with some modifications. The powdered mycelium was suspended in detergent solution (2 M NaCl, 0.4% w/v deoxycholic acid, 1.0% w/v polyoxyethylene 20 cetyl ether), incubated at 55 °C for 15 min, extracted with an equal volume of 24 : 1 chloroform : isoamyl alcohol (CIA) and the cellular debris pelleted by a 15 min centrifugation. The supernatant fluid was mixed with an equal volume of 6 M guanidinium thiocyanate; total nucleic acids were bound to glass powder (Vogelstein & Gillespie 1979), washed twice with ethanol buffer and the nucleic acids eluted into sterile distilled water. DNA for PCR amplifications was diluted to 1–2 ng/μL. DNA used for library construction was digested with RNase A for 30 min at 37 °C, extracted with CIA, ethanol precipitated and resuspended in sterile distilled water.

Microsatellite loci were isolated using an enrichment protocol based on modifications of methods by Hamilton *et al*. (1999), Edward *et al*. (1996) and Glenn *et al*. (2000). Genomic DNA of *B. bassiana* ARSEF strain 5436 (USDA-ARS

Collection of Entomopathogenic Fungal Cultures; origin: Ivory Coast; insect host: *Hypothenemus hampei*) was digested separately with *Rsa*I, *Hae*III and EcoRV. Each reaction contained 5 μg genomic DNA and 10 U restriction enzyme, and was digested overnight according to the manufacturer's instructions. Restriction fragment ends were dephosphorylated with 2 U shrimp alkaline phosphatase (Promega) at 37 °C for 20 min, after which the enzymes were inactivated by incubating at 80 °C for 20 min. Restriction fragment ends were capped with double-stranded SNX-linkers (Hamilton *et al*. 1999) by overnight ligation and simultaneous digestion with 10 U of *Xmn*I, which was included to eliminate linker–linker ligation products. The linker-capped fragments were size fractionated on Chromaspin + TE-400 columns (Clontech) and 5 μL of the eluate were amplified via PCR using SNX forward primer (Hamilton *et al*. 1999).

PCR reactions consisted of 25 cycles of 95 °C denaturation for 30 s, 60 °C annealing for 1 min, 72 °C for 2 min, followed by a 72 °C incubation for 30 min. Enrichment for microsatellites was achieved by mixing 45 μL PCR product with 140 μL 6 × SSC (saline sodium citrate)/0.1% SDS (sodium dodecyl sulfate) and 500 ng biotinylated oligonucleotides specifying either CA, GA, AAG or GTT microsatellite repeats, and brought to a final volume of 200 μL with sterile distilled water. These mixtures were denatured at 95 °C for 5 min and gradually cooled over 15 min to 50 °C; they were then incubated at 50 °C for 4–12 h to anneal microsatellite-containing fragments to complementary oligonucleotides. After annealing, the reactions were mixed with 300 μL TBT buffer (100 mM Tris, pH 7.5, 0.1% Tween 20) and 50 μL avidin beads (Vectrex), and then agitated for 30 min at 50 °C. The beads were pelleted by centrifugation, the supernatant fluid was discarded and the beads were washed three times with 0.2 × SSC/0.1% SDS to remove unbound DNA. The beads were then suspended in 100 μL TLE (10 mM Tris pH 8.0, 0.1 mM EDTA) and incubated at 95 °C for 5 min to elute the PCR fragments bound to the biotinylated probes. The eluted fragments were amplified by PCR as described previously, except that the total number of cycles was increased to 35. The PCR products were cloned using Topo-TA cloning kits (Invitrogen).

Recombinant colonies were randomly selected and cloned inserts were amplified by PCR with M13 forward and reverse primers. PCR products were gel-purified from 1.5% Nusieve agarose gels (BioWhittaker) and eluted from the gel by the freeze–squeeze method of Tautz & Renz (1983). PCR products were sequenced directly using BigDye Terminator v2.0 Cycle Sequencing kits (Applied Biosystems) and run on an Applied Biosystems 3100 DNA analyser. Clone sequences were edited using SEQUENCHER 4.1 (GeneCodes) software, and primers flanking microsatellite regions were designed with OLIGO v.6 (Molecular Biology Insights). PCR primers were designed with an estimated annealing temperature of 58 °C.

A 66–56 °C touchdown PCR cycle was used to amplify the microsatellite-containing loci from genomic DNA. The amplification cycle included a 2 min denaturation at 95 °C, followed by 10 cycles of denaturation at 94 °C for 30 s, 30 s primer annealing at 66 °C and decreasing the annealing temperature by 1 °C at each succeeding cycle, and 1 min elongation at 72 °C. Then, 36 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C were conducted, followed by a 30 min incubation at 72 °C to reduce artifactual stutter bands in dinucleotide repeats. The resulting microsatellite amplicons

Table 1 *Beauveria bassiana* microsatellite primers: locus identifier (GenBank accession number), repeat motif, forward (F) and reverse (R) primer sequences, sizes of cloned alleles (size range observed among different species lineages; number of alleles observed) in bp

Locus	Repeat motif	Primer sequences (5′–3′)	Size of cloned allele (no. of alleles; size range) in bp
Ba01 (AY212020)	$(CA)_{14}$	F: CCAACCCAATCAATCGTCAT R: GAGAGGCGGAGCTAAGCA	103 (4; 100–120)
Ba02 (AY212021)	$(CA)_{20}$	F: AACGCTATGCCTTGACGAC R: GACGCCGAGCAATGTAACA	140 (10; 110–160)
Ba03 (AY212022)	$(CA)_{26}$	F: GCATAGATATGTCTCGCACC R: ACTACCCTGTCCCGCTGA	146 (5; 120–160)
Ba05 (AY212023)	$(GAT)_{15}$	F: AGGCAATACCGAGGTTGGC R: ATCCATGGCGAGCCGTCC	160 (5; 110–175)
Ba06 (AY212024)	$(GTT)_{10}$	F: GCGATTGACGAAAAGCTAGA R: ACTTGCTTTGCTGTTGCACA	114 (7; 105–125)
Ba08 (AY212025)	$(AAG)_{10}$	F: TGTTGCCGACACGAATTGT R: GGCTCAAGCGCAAAGAGAAA	210 (8; 200–250)
Ba12 (AY212026)	$(CTT)_{7}$	F: GGGTCCATCATGTACGGC R: AGGCGTATACAGGTCGTG	231 (8; 200–240)
Ba13 (AY212027)	$(AAG)_{9}$	F: CAGGCAACAACACGATTTCA R: ATGCCATCTACGACTTTATGA	161 (5; 140–170)

were resolved on 4% Metaphor agarose gels (BioWhittaker), stained with ethidium bromide and visualized under UV light.

Primer sequences, allele size variation, and the number of alleles observed at eight microsatellite loci observed among 24 strains of *B. bassiana* representative of its global phylogenetic diversity (S.A. Rehner, E.P. Buckley, unpublished) are given in Table 1. These microsatellite loci were successfully amplified from the majority of strains examined, and the observed number of alleles per locus in this sample of strains ranged from four to eight alleles (Table 1). In summary, the polymorphic microsatellites presented here will benefit the investigation of population structure and species limits in *B. bassiana*. Additionally, these microsatellite markers provide powerful new tools for ecological and epidemiological studies, and for precise genotyping of proprietary and released strains of *B. bassiana*.

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