African Journal of Biotechnology Vol. 9(27), pp. 4290-4294, 5 July, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Genotyping isolates of the entomopathogenic fungus *Beauveria bassiana sensu lato* by multi-locus polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis

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Accepted 27 April, 2010

Multi-locus denaturing gradient gel electrophoresis (DGGE) analysis was developed to investigate the genotypes of *Beauveria bassiana sensu lato*. Sensitive tests indicated all isolates with one or more nucleotide differences at EF-1α and Bloc could be distinguished by DGGE except for one pair of strains that differed at four nucleotide positions. Ten, twelve and five genotypes were identified at the EF-1α, Bloc and ITS locus, respectively, among seventeen isolates, which together differentiated 13 genotypes. These results demonstrated that multi-locus DGGE is a potentially useful molecular marker for genotyping, identifying and tracking the fates of experimentally released strains of *B. bassiana sensu lato*. Moreover, by multi-locus DGGE for scanning *B. bassiana sensu lato* isolates with different multi-locus sequences, genetic diversity of *B. bassiana sensu lato* was effectively investigated with substantially reduced time and cost in subsequent DNA sequencing.

Key words: Denaturing gradient gel electrophoresis, multi-locus sequences, genotyping, sensitive tests.

INTRODUCTION

Entomopathogenic fungi are natural enemies of numerous insects and are important regulators of host population in diverse ecosystems (Hajek, 1997). *Beauveria bassiana* is regarded as an important biological control agent for insect pests. However, *B. bassiana* is widely distributed as an anamorph in nature and has been reported as a heterogeneous ensemble of strains. Wang et al. (2003) used triplicate molecular methods to study strain genetic relatedness and population structure of *B. bassiana* and deemed gene flow was indirectly established to occur between *B. bassiana* populations. It is of great importance, therefore, to assess the genotypes and determine genetic diversity to investigate the ecology and evolutionary history of this fungus (Oulevey et al., 2009).

In recent years, many molecular genetic techniques have been developed for strain genotyping and to investigate genetic diversity and address the systematic and taxonomy of entomopathogenic fungi. Random amplified polymorphic DNA (RAPD) analysis has been used to differentiate entomopathogenic fungal isolates and genotyping *B. bassiana* isolates previously (Berretta et al., 1998; Wang et al., 2003; Wang et al., 2004). Moreover, inter simple sequence repeat (ISSR) markers revealed considerable intraspecific variability among the isolates of *Beauveria* spp. (Wang et al., 2005). However, the drawback of RAPD and ISSR methods is their low reproducibility. Meanwhile polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis was also used to discriminate isolates of *B. bassiana* (Hegedusge and Khachatourians, 1996), but optimal SSCP results generally require target fragments to be 100-250 bp in size and must include informative nucleotide variation detectable by this method.

Furthermore, Rehner and Buckley used simple sequence

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Abbreviations: DGGE, Denaturing gradient gel electrophoresis; RAPD, random amplified polymorphic DNA; **ISSR,** inter simple sequence repeat; **PCR-SSCP,** polymerase chain reaction single-strand conformation polymorphism; **SSR**, simple sequence repeat.

repeat (SSR) marker to study the population genetics of *B. bassiana* (Rehner and Buckley, 2003). However, development of microsatellite markers is time-consuming and costly compared to other DNA markers.

To understand the population genetic structure and evaluate their potential impact on ecological systems where *B. bassiana* is used as an insect biological control agent, more sensitive genetic markers to *B. bassiana* are needed for effective strain genotyping. PCR- denaturing gradient gel electrophoresis (DGGE) analysis can distinguish duplex DNA fragments that differ in a single base substitution (Myers et al., 1985). In a previous study, single-locus PCR-DGGE has been used to discriminate among isolates of yeasts (Manzano et al., 2004). However, precise genotyping and robust inference into population structure generally requires data from multiple loci. Prior sequencing studies in *B. bassiana* demonstrated that the nuclear loci ITS (not very variable), EF-1α (better) and Bloc regions (best) (Rehner et al., 2006) are polymorphic within *B. bassiana*. Therefore, variable regions with these three loci for PCR-DGGE analysis were targeted. In this study, a rapid and reliable method based on the multi-locus DGGE technique was developed to genotype 17 *B. bassiana sensu lato* isolates from different geographical origin and different host insects.

MATERIALS AND METHODS

Isolates used, DNA extraction and PCR amplification

Seventeen *B. bassiana sensu lato* isolates were obtained from the Research Center of Entomopathogenic Fungi, Anhui Agricultural University, Hefei, China (RCEF) collection. Genomic DNA was extracted from fresh mycelia by a modified method described by Rehner et al. (2006). 700 µL lysis buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 2% CTAB, 2% PVP) was used and extractions were processed in a FastPrep tissue homogenizer (MP Biomedicals, Irvine, CA) for 30 s at a speed of 4.5. The primers for PCR-DGGE analysis were designed based on variable regions from several related genomic loci. Primers for the amplification of ITS rDNA were ITS4 and ITS5 (White et al., 1990) and primers for Bloc sequence were Blocss1 (5`-TGCCGTCACCTACATTGCC-3`) and Blocss2 (5`-GCAATGAATCT CGCCTGAA-3`). PCR condition for ITS rDNA and Bloc sequence was: 5 min at 95℃; denaturation 1 min at 95℃, annealing 30 s at 52℃, extension 50 s at 72℃ (35 cycles) and 10 min at 72℃. The primers for the amplification of EF-1α are EF1 (5`-AAGGCTGTTGCTTTCGTCC-3`) and EF2 (5`-GCAAGCAATGTGGGCAGTG-3`) with these conditions: 5 min at 95℃; denaturation 1 min at 95℃, annealing 30 s at 60℃, extension 50 s at 72℃ (35 cycles) and 10 min at 72℃. In order to increase the sensitivity in the detection of one base difference by DGGE, a GC clamp (5`-CGCCCGCCGCGCGCGGCGGGCGGGG CGGGGGCCCGGGGG-3`) was added to the 5` end of the forward primer ITS5, EF1 and Blocss1.

DGGE analysis, DNA sequencing and sequence alignment

DGGE analyses for 17 isolates were performed with a Decode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA), with denaturant gradients of 35 to 60%, 30 to 70% and 30 to 70%, with running conditions of 60 V for 20 h, 120 V for 10 h and 120 V for 10 h for ITS, EF-1α and Bloc PCR products, respectively. 6 % polyacrylamide gels were run in 0.5×TAE buffer at a constant temperature of 60℃. The sequences of 17 isolates at these three loci regions were determined by clone sequencing. Sequence editing and alignment was performed using DNAMAN 6.0 software (Lynnon Biosoft USA). GenBank accession numbers for all sequence data generated in this study were listed in Table 1.

RESULTS AND DISCUSSION

DGGE-analysis of the 17 isolates for EF-1α discriminated 10 different DGGE-groups which are designated E1, E2, E3, E4, E5, E6, E7, E8, E9 and E10 (Figure 1A). In addition, 12 DGGE-groups were detected at the more variable Bloc locus and are designated B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11 and B12 (Figure 1B). By contrast, only 5 DGGE-groups were resolved at the ITS locus (Figure 1C). The results showed these were considered as low discriminative (ITS-DGGE analysis), moderately (EF-1α-DGGE analysis) and highly discriminative locus (Bloc-DGGE analysis).

The PCR products were sequenced to confirm the sensitivity of DGGE analysis. The cloned products of 17 *B. bassiana sensu lato* isolates for ITS, partial EF-1α and partial Bloc loci are 551, 517, 418 – 421 bp, respectively. For EF-1α locus, isolates in same DGGE group displayed identical sequence, while at least one base substitution was observed among different DGGE groups (Figure 1A). For Bloc locus, one or more base differences were observed among different DGGE groups. Sequence analysis however, showed that there were four base differences between isolates RCEF0813 and RCEF0829 in DGGE group B2 despite the fact that they had identical mobility in DGGE pattern (Figure 1B). However, for ITS locus, DGGE group I2 including 3 isolates with one base difference showed an indistinguishable DGGE pattern; similarly, no separation was observed among DGGE group I5 despite the nucleotide substitution among the isolates in this group (Figure 1C). ITS locus marker in further genotyping analysis was omitted because of its low genotypic diversity and sensitivity within 17 isolates.

Seventeen *B. bassiana sensu lato* isolates could be divided into 10 or 12 genotypes based on EF-1α or Bloc DGGE profile. Comparative analysis of two-loci DGGE pattern showed that 5 isolates in DGGE-group E5 were distributed into DGGE-groups B7 (4 isolates) and DGGE-groups B3 (1 isolates); and two, one and one isolates in DGGE-group E1 (4 isolates) were distributed into DGGE-groups B6, B10 and B12, respectively. Moreover, RCEF0 813 and RCEF0829 have identical mobility in Bloc DGGE, but they belong to two different DGGE-groups E9 and E10 in EF-1α DGGE profile. The results indicated that two-loci DGGE profiles possessed a certain complementtary characteristic in genotyping isolates and provided a higher differentiation power in genotyping isolates than analysis of single-locus DGGE. Seventeen *B. bassiana sensu lato* isolates were divided into 13 multi-locus genotypes by an analysis of the combined EF-1α and

Table 1. *B. bassiana* isolates used in this study and GenBank accession numbers and genetypes.

Isolates	Host	ITS rDNA	EF1-a	Bloc	Genetype
RCEF0025	Lepidoptera	GU233690	GU233707	GU233673	E5B7
RCEF0030	Malacosoma	GU233691	GU233708	GU233674	E5B3
RCEF0037	Lepidoptera	GU233692	GU233709	GU233675	E5B7
RCEF0333	Hemiptera	GU233693	GU233710	GU233676	E1B6
RCEF0334	Hymenoptera	GU233694	GU233711	GU233677	E1B6
RCEF0359	Lepidoptera	GU233695	GU233712	GU233678	E5B7
RCEF0393	Coleoptera	GU233696	GU233713	GU233679	E5B7
RCEF0493	Orthoptera	GU233697	GU233714	GU233680	E3B4
RCEF0496	Heteropter	GU233698	GU233715	GU233681	E1B12
RCEF0760	Coleoptera	GU233699	GU233716	GU233682	E8B8
RCEF0813	Coleoptera	GU233700	GU233717	GU233683	E10B2
RCEF0816	Homoptera	GU233701	GU233718	GU233684	E2B9
RCEF0823	Unknown	GU233702	GU233719	GU233685	E1B10
RCEF0829	Unknown	GU233703	GU233720	GU233686	E9B2
RCEF0830	Unknown	GU233704	GU233721	GU233687	E7B5
RCEF0835	Homoptera	GU233705	GU233722	GU233688	E4B1
RCEF0836	Coleoptera	GU233706	GU233723	GU233689	E6B11

Isolates RCEF0333, RCEF0334, RCEF0359, RCEF0393, RCEF0493 and RCEF0496 were originally obtained from Anhui Province in China. Isolates RCEF0025, RCEF0030, RCEF0037 and RCEF0823 were obtained from Guang xi, Bei jing, Guang dong and Jiang xi in China, respectively. Isolates RCEF0816 and RCEF0813 were obtained from Yun nan Province in China, isolates RCEF0760, RCEF0829 and RCEF0830 from USA, and isolates RCEF0835 and RCEF0836 from Bavi province in Vietnam.

Bloc DGGE profiles. Four isolates (RCEF0025, RCEF 0037, RCEF0359 and RCEF0393) have the same genotype E5B7 and isolates RCEF0333 and RCEF0334 have the genotype E1B6, the other 11 isolates all have different genotypes according to the combined EF-1α and Bloc DGGE patterns.

This study uniquely employed multi-locus DGGE analysis for genotyping *B. bassiana sensu lato* isolates. Multi-locus sequencing confirmed sensitivity of DGGE analysis in the detection of nucleotide differences, indicating that DGGE analysis is well suited for genotyping *B. bassiana* isolates. However, Meyling et al. (2009) recently demonstrated that SSR molecular markers identified 26 unique multi-locus genotypes for 17 or 18 loci sequences from 33 *B. bassiana* isolates from the same area. Obviously, SSR with multitudinous loci sequences, wide distributed in eukaryotic genomes and rich polymorphism, have a high ability to genotype *B. bassiana* isolates. Rehner et al. (2003) identified 10 alleles in 24 isolates by single microsatellite locus. Moreover, a single polymorphic microsatellite locus displaying the greatest genotypic variability resolved 12 alleles in 33 *B. bassiana* isolates (Meyling et al., 2009). In this study, 12 genotypes were identified by a single Bloc locus DGGE analysis in only 17 *B. bassiana sensu lato* isolates. Obviously, the resolving capacity of a single locus by DGGE analysis is similarly compared to a single SSR marker. Due to complementary characteristic of multi-locus DGGE analysis in genotyping isolates, if more hyper variable loci are developed, the resolving capacity of this method will increase dramatically. The results imply that multi-locus DGGE is a potentially useful molecular marker for genotyping, identification and tracking the fates of experimentally-released strains of *B. bassiana sensu lato*.

ACKNOWLEDGMENTS

We are grateful to Dr. Chunlong Ma and Dr. Rehner A Stephen that revised the manuscript. B. Huang is grateful for grants from the National Natural Science Foundation of China (30770008) and the Anhui Provincial Science

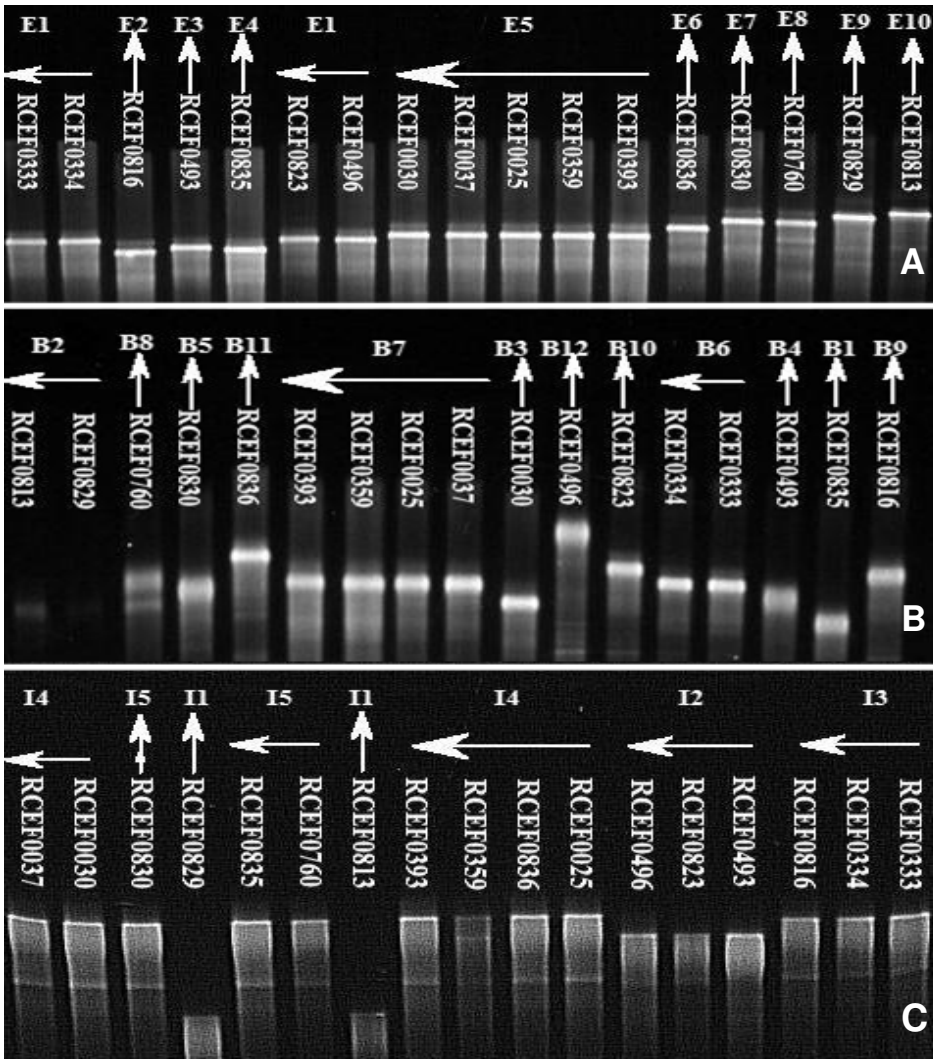

Fig. 1 (**A**): DGGE profiles of 17 *B. bassiana* isolates in the EF1-α partial sequence. Each lane denotes one isolates and relevant DGGE groups (E1-E10). (**B**): DGGE profiles of 17 *B. bassiana* isolates in the Bloc partial sequence. Each lane denotes one isolates and relevant DGGE groups (B1-B12). (**C**): DGGE profiles of 17 *B. bassiana* isolates in the ITS region. Each lane denotes one isolates and relevant DGGE groups (I1-I5).

Foundation for Excellent Youth (08040106902).

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