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Short Communication

A molecular tool for detection and tracking of a potential indigenous *Beauveria bassiana* strain for managing emerald ash borer populations in Canada

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ABSTRACT

Emerald ash borer is an invasive species from Asia. *Beauveria bassiana* strain L49–1AA is being tested for the control of emerald ash borer in Canada, using an autocontamination trapping system. We have developed a simplified allele discrimination polymerase chain reaction (PCR) assay to screen *B. bassiana* strain, L49–1AA from other *Beauveria* species by targeting the inter-strain genetic differences in 5′ end of EF1-α gene of the genus *Beauveria*. A single nucleotide polymorphism (SNP) site, T → C was identified only in L49–1AA and was used to develop a simplified allele discrimination polymerase chain reaction (PCR) assay based on a modified allelic inhibition of displacement activity (AIDA) approach for distinguishing *B. bassiana* L49–1AA from all background *Beauveria* isolates. The SNP site was employed to design inner primers but with a deliberate mismatch introduced at the 3′ antepenultimate from the mutation site in order to maximize specificity and detection efficiency. Amplification was specific to L49–1AA without cross-reaction with DNA from other *Beauveria* strains. In addition, the designed primers were also tested against environmental samples in L49–1AA released plots and observed to be highly efficient in detecting and discriminating the target strain, L49–1AA from both pure and crude DNA samples. This new method can potentially allow for more discriminatory tracking and monitoring of released L49–1AA in our autocontamination and dissemination projects for managing EAB populations. Additionally, the modified-AIDA format has potential as a tool for simultaneously identifying and differentiating closely related *Beauveria* species, strains/isolates as well as general classification of other pathogens or organisms.

1. Introduction

Emerald ash borer (EAB), *Agrilus planipennis* (Coleoptera: Buprestidae) is an invasive species from Asia that was discovered in North America within the Detroit, MI metropolitan area and Windsor, Ontario in 2002, and have since been detected in the Province of Quebec, Canada. To date, tens of millions of ash trees (*Fraxinus* spp.) have succumb to EAB infestations and still counting. Invasive species are prime candidates for biological control therefore there is an urgent need to accelerate the search for effective agents. Natural enemies that have become associated with and adapted to invasive species in their new environment will be prime candidates for used in their management. We conducted surveys to recover indigenous entomopathogenic fungi (EPF) infecting EAB in outbreak sites within the Province of Ontario, Canada for used in the management of EAB. Morphological and molecular characterization indicated that *Beauveria bassiana* isolates were the most predominant and virulent EPF from the collection and *B. bassiana* strain L49–1AA was the most virulent isolate against EAB adults (Johny et al., 2012). Currently this strain is being tested in autocontamination/dissemination trap to manage EAB under field condition (Lyons et al., 2012). Field release of biological control agents in new environment necessitates the development of methods that can reliably differentiate and monitor their presence/absence, establishment, persistence or recrudescence as well as population dynamics.

Molecular markers have been utilized to detect strains of *B. bassiana* and other entomopathogenic fungi, thereby providing means to identify strains of interest, determine origin of isolates, or study population structure. Few studies have monitored the fate of fungal strains released as biological control agents of insect pests. In the past, isoenzyme analysis have been used to study the fate of *Zoophthora radicans* for control of the spotted alfalfa aphid (Milner and Mahon, 1985) and *Erynia neoaphidis* for control of lettuce aphids (Silvie et al., 1990). Several PCR-based molecular tech-

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niques, e.g., PCR restriction fragment length polymorphism (RFLP) analysis, microsatellite analysis, and random amplified polymorphic DNA (RAPD) analysis, have also been used to monitor *Beauveria* strains released to manage various insect pests (Wang et al., 2004; Enkerli et al., 2004; Schwarzenbach et al., 2007). Additionally, RAPD, ISSR and AFLP can generate species- or strain-specific amplicons in filamentous fungi, thereby facilitating the development of species- or strain-specific sequence-characterized amplified region (SCAR) markers (Schilling et al., 1996; Hermosa et al., 2001; Castrillo et al., 2003; Lardner et al., 2005; Takatsuka, 2007). However, these methods have various advantages and limitations. They are laborious as well as time consuming in terms of initial screening with ideal isolates and different primers to identify unique amplicons from the target strain(s), then followed by cloning and sequencing of the target fragment before designing potential isolate(s)-specific primers. As a result, relatively easy, rapid, and more discriminatory methods are needed for detection and differentiation of specific fungal strains.

The allelic inhibition of displacement activity (AIDA) assay is a conceptually simple SNP genotyping strategy is based on the notion that an oligonucleotide which can be extended by Taq DNA polymerase is able to block the amplification of a PCR product when situated between two flanking PCR primers (Lewis et al., 1994; Galmozzi et al., 2013). The AIDA requires only two outer common primers and one inner primer with allele-specific 3′ terminus mismatch at the SNP site. The outer primers amplify the common fragments in inverse proportion to the primer-extension efficiency of the inner allele specific primer (Galmozzi et al., 2013). The method employed here is a modification of AIDA assay with a second deliberate mismatch at the 2nd nucleotide position from the 3′ terminals (SNP site) of the inner primer. The modified AIDA assay summarizes to a genotyping method that can elucidate and differentiate closely related *Beauveria* strains/isolates. Based on the modified protocol, we designed and evaluated strain-specific primers using EF1-α gene sequence region for the detection of *B. bassiana* strain, L49–1AA. The specificity and discriminatory power of our designed primers were validated *in silico* using various pure cultures of *Beauveria* spp. and isolates recovered from EAB populations in southern Ontario, Canada and Michigan, USA for the rapid, highly sensitive, reproducible and specific detection of *B. bassiana* strain, L49–1AA. These primers were also evaluated using the field collected samples to detect and distinguish L49–1AA from all background *Beauveria* isolates that are suspected to be present in the L49–1AA released plots.

2. Materials and methods

Eighteen *Beauveria* spp. isolates and cultures, previously characterized using nucleotide sequencing were used for validation of the designed *B. bassiana*, L49–1AA modified-AIDA assay. The isolates comprised seven *B. bassiana* and three *B. pseudobassiana* collected from EAB cadavers from southwestern Ontario, Canada (Johny et al., 2012), one *B. pseudobassiana* from Quebec, Canada (Lavallee R, unpublished) and three *B. bassiana*, one *B. pseudobassiana* and one *B. brongniartii* isolates from Michigan, USA (Castrillo et al., 2010; Johny et al., 2012) (see Table 1). In addition, the commercial strain *B. bassiana*, GHA (lot number 22WP100902) obtained from Lethbridge Research Centre (Lethbridge, Alberta, Canada) and *B. bassiana* isolate INRS-CFL-A (R. Lavallée, unpublished) were included as negative controls for the assay. These *Beauveria* spp. isolates were included purposely for testing potential assay cross-specificity.

All the fungal isolates used for the optimization of the modified AIDA assay were grown as mycelia in 5 ml of Sabouraud dextrose yeast (SDY), for 5–7 days at 150 rpm, 28 °C in darkness. Total genomic DNA was extracted from mycelia pellet using the Wizard genomic DNA purification kit (Promega Corporation, Madison, WI, USA) and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The extracted DNA was stored at −20 °C until use.

For the isolates, ARSEF 8130, ARSEF 8150, ARSEF 8153, ARSEF 8170, ARSEF 8187 and Car1, approximately 1200 bp segment spanning the 5′ end of EF1-α gene were amplified and sequenced with the primer pair, EF1T/1567R (Rehner and Buckley, 2005). The remaining *Beauveria* spp. EF1-α gene sequences (Table 1) were previously generated by Johny et al. (2012) and are available in the GenBank database. Newly generated sequences in this study have been submitted to the GenBank database (www.ncbi.nlm.nih.gov) and their accession numbers are provided in Table 1.

Selected *Beauveria* spp. EF1-α gene sequences were multiply aligned using the program ClustalW 1.82 (Chenna et al., 2003). A single nucleotide change (T → C) in the target DNA region discriminating L49–1AA from the next closest sequence(s) was visually selected from the sequence alignment (Table 2). A SNP detection technique based on ARMS PCR method described by Ye et al. (2001) was adopted in designing the allele specific primers using BatchPrimer3 v1.0 software (You et al., 2008). For specific amplification of *B. bassiana* strain, L49–1AA, the internal primer where the single nucleotide mutation occurs (Table 2; bold type) was designed to bind the specific SNP but with an additional deliberate mismatched nucleotide (lowercase letter) introduced at the 3′ antepenultimate of the internal primer. The primer sequences, major amplicon sizes, specificity and optimal PCR conditions of the targeted segment of the EF1-α gene are shown in Table 3.

To optimize the modified-AIDA assay, the primer pairs EFFO/EFRO and EFF1/EFRO theoretically specific for *B. bassiana* and L49–1AA respectively were applied in PCR to ensure non-specific amplicons. Then all 3 primers, EFFO/EFF1/EFRO were combined in a single-round PCR mix but in differential molar ratios to achieve a balanced and simultaneous amplification of all targets. Consequently, the amplicon yield was quantitatively normalized by adjusting the molar ratios of the primers until similar banding intensities were obtained. The final primer concentrations of the PCR mix resulting in similar amplicon intensities are shown in Table 3 and the optimized modified-AIDA assay conditions are described herein.

PCRs were performed with the modified-AIDA assay reaction among selected *Beauveria* spp. including L49–1AA using the primer set EFFO/EFF1/EFRO (Table 3). Additionally, the universal primer set ITS5F/ITS4R (ITS) (White et al., 1990) was used in a duplex PCR assay jointly with the primer set EFF1/EFRO. The combined ITS and EFF1/EFRO primers were used as control to check the quality of the DNAs as well as to detect and distinguished L49–1AA from tested *Beauveria* isolates. Control DNA included seventeen *Beauveria* spp. isolates (Table 1). The PCR reaction mixes (total volume 25 μl) contained template ~20 ng of DNA template, 1 × LA PCR buffer II (Mg^{2+} free), 1.5 mM $MgCl_2$ (TaKaRa); 4.0 μl of dNTPs (2.5 mM each) (TaKaRa), and 0.2 μl of TaKaRa LA DNA polymerase Taq (TaKaRa). For the semi-duplex PCR, concentrations of the three primers EFFO, EFRO, and EFF1 were 5 pM, 10 pM and 30 pM respectively (Table 3). However, for the duplex PCR protocol, we used a consistent 10 pM of each primer namely; ITS5F, ITS4R, EFF1 and EFRO. The PCR assays were performed in a Techne TC-512 thermal cycler (Techne Inc., Burlington, NJ, USA). PCR conditions for semi-duplex were as follows: 94 °C for 3 min initial denaturation, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 10 min. PCR conditions were similar in all assays with the exception of 58 °C annealing temperature for the duplex. The products were visualized by electrophoresis in 1.5% agarose gels stained with GelRed™ (Biotium, Hayward, CA, USA).