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Diagnosis of the major entomopathogenic fungus *Metarhizium anisopliae* using ultra-fast PCR

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Abstract
Because of the use of *Protaetia brevitarsis seulensis* (Coleoptera: Cetoniidae) larvae (for foods) and imago (for educational pets), the number of farmers breeding this insect has been increasing. Breeding requires a fast and accurate diagnostic method for early detection of insect diseases, to avoid losses. *Metarhizium* spp. frequently infect *P. b. seulensis* larvae; *Metarhizium anisopliae* (*Ma*) is the pathogenic species in more than 80% of cases. However, conventional PCR takes several days to identify the fungus. We utilized a portable ultra-fast PCR machine to identify entomopathogenic fungi from infected sawdust or tissues. We used a one-stop method that does not require genomic DNA to be isolated at the breeding farm. Pathogen samples were extracted at the farm, and detection of entomopathogenic fungi was possible within approximately 12 minutes. Relative quantification was also possible, which will enable estimation of disease progression.

Keywords: *Protaetia brevitarsis seulensis*, *Metarhizium anisopliae*, Ultra-fast PCR, One-stop method

1. Introduction

In Korea, there has been an increasing number of insect farmers due to a boom in insect industrialization [1]. *Protaetia brevitarsis seulensis* (*Pbs*) can be used as a functional food, and it has anti-cancer and anti-hepatofibrotic effects [2, 3]. A survey of insect farmers from 2012 to 2015 revealed that *Metarhizium* spp. were the primary fungal cause of death in *Pbs. Metarhizium* spp. were the causative pathogen for more than 80% of *Pbs* infections [4]. We developed a method of on-site diagnosis that analyzes sawdust or tissues, to detect infection before it spreads, and prevent economic loss. Specifically, this method uses relative quantification analysis with ultra-fast PCR to diagnose *Metarhizium* spp. early enough to prevent the spread of disease. Ultra-fast chip-type PCR has been used for early diagnosis of various targets that can affect humans, including A/H1N1 virus [5], and *Mycobacterium tuberculosis* [6]. Meanwhile, in terms of insects, ultra-fast PCR has been used for early diagnosis of *Paenibacillus larvae*, which causes American foulbrood in bees [7]. *Metarhizium anisopliae* (*Ma*) is a representative member of the *Metarhizium* genus. This genus is a member of the phylum Deuteromycota, and class Hyphomycetes. It infects more than 200 insect species, including *Pbs*, and other arthropods [8]. *Metarhizium anisopliae* permeates the insect's cuticle using the lytic action of enzymes like chitinase, and spreads conidia throughout the insect's body [9, 10], causing death by the toxin destruxin [11-13]. Dark-green conidiophores and conidia form, covering the body of the dead insect. The fungus can be verified using direct PCR to identify target DNA on the cuticle tissues or sawdust. Through early diagnosis, this study aims to prevent disease in insects, as well as the large losses incurred by insect farms when a rapid and precise diagnosis cannot be made. For on-site diagnosis with PCR, the portable lab-chip based micro PCR device GENECHECKER™ (Dongwoo Science Co., LTD., Hanam-si, Korea) is smaller and lighter than conventional PCR. This device is able to reduce the PCR run time to 16 min for 40 cycles, by using a 2–3-fold smaller sample volume than endpoint PCR, in wells on a chip. The reduced sample volumes mean that reaction temperatures can be changed more rapidly than in conventional PCR (Table 1). The PCR amplification process can be verified in real-time on a connected laptop with the included Gene reporter Program. Relative quantification is also possible, by comparison to a reference concentration.

2. Materials and Methods

2.1 Preparation of fungal genomic DNA for PCR

Metarhizium anisopliae was acquired from the Korean Agricultural Culture Collection (KACC), and cultured on Sabouraud Dextrose Agar (SDA) for 10~15days at 24 °C and 60% humidity. Genomic DNA was extracted using the Power Soil DNA preparation kit (MoBio, USA) for isolation and identification of the cultured fungi. The prepared template DNA was used for both conventional and ultra-fast PCR. Genomic DNA was used as the template, at concentrations of $1x10^4$, $1x10^3$, $1x10^2$, 10, and 0 copies/µL, to identify the optimal concentration for each PCR method.

2.2 Insect sample preparation and PCR using pathogen-infected insect tissues

Protaetia brevitarsis seulensis suspected of infection were collected from a farm located in Wanju, Korea that breeds large numbers of *Pbs*. For identification, fungi were cultured on SDA at 24 °C and 60% humidity, genomic DNA was extracted, and PCR was performed for the Transcribed Spacer 5 (ITS5) and Large Subunit RNA3 (LR3) regions. Fungi from the *Pbs* suspected of infection were cultured on SDA for 10 days at 24 °C and 60% humidity. Once the growth of mycelia had been verified, the fungus was confirmed to be *Ma*. For the ultra-Fast PCR, the larval tissue of *Pbs* with suspected infection was cut into 1×1 mm pieces, and the diced tissue was transferred into an Eppendorf tube containing 100 µL of 10x diluted buffer (Nanohelix Co. LTD., Daejeon, Korea). After 5–10 min of vortexing, the tube was centrifuged at 7000 rpm for 1 min, and the supernatant was retrieved. One-half microliter of the supernatant was mixed with 5 µL of Ssofast SYBR Green (Biorad, USA), 1 µL each of forward (5 pM) and reverse (5 pM) primer, and ddH_2O added to a total volume of 10 µL. This mixture was pipetted into the wells on a DNA lab chip for ultra-fast PCR. Ultra-fast PCR was run on the GENECHECKER™ machine. For conventional PCR, we used a Veriti Thermal Cycler (Applied Biosystems, USA), and the HelixAmp™ Direct PCR Buffer (Nanohelix Co. LTD., Daejeon, Korea). Insects with suspected infection were cut into 1×1 mm pieces, before placing in direct buffer and vortexing for approximately 5 minutes. One-half microliter of supernatant was mixed with 12.5 µL of 2x PCR Buffer, 1 µL each of forward (10 pM) and reverse (10 pM) primer. This solution was brought up to a total volume of 25 µL, for conventional PCR. The conditions for PCR were as follows: 50 °C for 5 min, 95 °C for 5 min, 95 °C for 20 s, 55 °C for 30 s, 72 °C for 20 s, and 72 °C for 5 min. The total time for PCR was 1 h 24 min.

2.3 Primers

Metarhizium anisopliae primers were based on 18SrRNA. The primer pair was designed based on the sequences with GenBank accession numbers AB712254, AY237118, FJ755243, and KC790106, producing the target gene, an 87 bp PCR product. Sequences and locations of the detection primers in the gene are as follows: 18SMF (5'-TTTCCAGCACAGCCGTCCCTTA-3') and 18SMR (5' GGCTCCTGTTGCGAGTGTTTT-3'). The specificity of detection primers was additionally tested by PCR reactions performed against gDNA from the bacterium *S. marcescens*. Primers for the detection of *S. marcescens* were luxF (5'-TACCATCACGGTATTTGATCTG-3') and luxR (5'-GAGATGTCGATAATCTCCACG -3').

3. Results and Discussion

The highly portable lab-chip system of the ultra-fast PCR machine was able to perform the reaction with a volume of only 10 µL, which was 2–3 times less volume than that used by conventional PCR (20–30 µL). The wider surface area of the lab-chip compared to standard plastic PCR tubes means that it has excellent thermal conductivity [5], which allows PCR results to be confirmed from 40 cycles, within approximately 16 minutes. The lab-chip system allows run time to be reduced by 40 min compared to conventional PCR, and provides real-time quantification results using SYBR green signal intensity [14]. Hence, our results show an increase in the SYBR green signal intensity as PCR amplification progresses. Improving this readout would make it possible to obtain the PCR results even more quickly. Moreover, the state of the reaction and the PCR results can be easily identified using SYBR green, without electrophoresis (Fig. 1). Eliminating electrophoresis reduces the time required by approximately 30 min. The run-time was also reduced by optimizing annealing temperatures, the number of cycles, and the template DNA concentration, which are the most important factors in the success of a PCR reaction that uses minimal template in minimal time.

Fig 1: Lab-chip based ultra-fast PCR and lab-chip fluorescence due to SYBR Green.
(A) The Lab-chip based ultra-fast PCR apparatus.
(B) In Lab-chip based PCR products, the amplification results using SYBR Green show different levels of fluorescence at six different template DNA concentrations (Lane 1: 1 ng; Lane 2: 100 pg; Lane 3: 10 pg; Lane 4: 1 pg; Lane 5: 100 fg; Lane 6: 0 ng)

Table 1: Ultra-Fast PCR vs. Conventional PCR

	Ultra-Fast PCR	**Conventional PCR**
Thermal Cycling Time (30 cycles)	11 min 35 s	39 min
Qualitative Analysis Result Confirmation	Possible immediately	Possible after electrophoresis
Quantitative Analysis Result Confirmation	Possible immediately	Possible after electrophoresis
Sample Carrier	Polymer chip (Rapi:chip™)	PCR tube
Sample Volume	10 µL	20–25 µL
On-site Analysis	Possible using the power jack of a car	Not recommended
Number of Wells	8	48–384
Volumetric Capacity of Well	10 µL	200–500 µL

The primer sequences and their specificity for *Ma* and *Ma* var. *anisopliae* genes were confirmed with Basic Local Alignment Search Tool (BLAST) analysis and ClustalW alignment. The annealing temperature was adjusted to 55 °C for conventional PCR and 57 °C for ultra-fast PCR, for the detection of *Ma.* The total run-time of the conventional PCR was 1 h 19 min, consisting of 5 min of predenaturation at 95 °C, 40 cycles of 20 s at 95 °C, 30 s at 55 °C, and 20 s at 72 °C, followed by 5 min of extension at 72 °C. When insect tissue samples were used in direct PCR, there was an additional 5 min at 50 °C before predenaturation, for a total run time of 1 h 24 min.

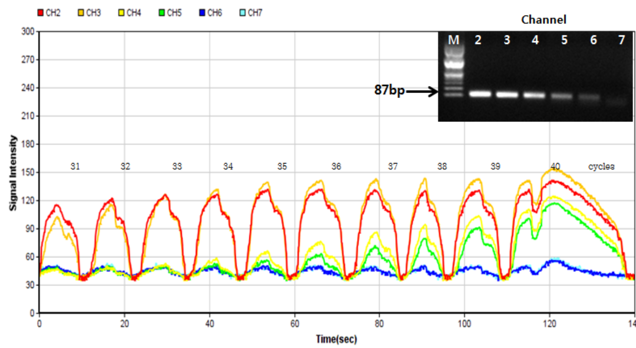

Fig 2: Measuring the detection sensitivity of ultra-fast PCR

The detection limit of ultra-fast PCR was measured at copy-numbers of $1x10^4$, $1x10^3$, $1x10^2$, 10, and 0, using the g DNA of *M. anisopliae.*

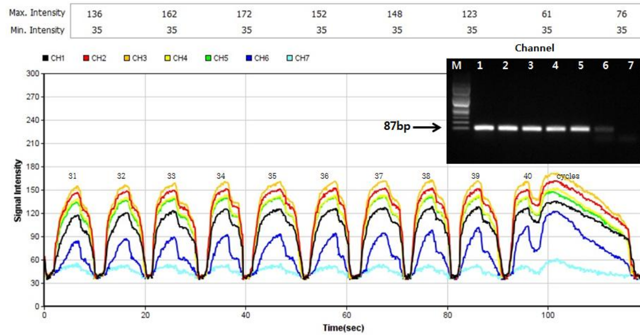

Fig 3: Direct Ultra-Fast PCR using insect tissue samples infected by *M. anisopliae.* As can be seen from the bands in the electropherogram, *M. anisopliae* genes were detected in the positive control (lane 1), and in lanes 2, 3, 4, 5, and 6. Channel 1: control $1x10^4$ copy/μL; CH 2: tissue 1; CH 3: tissue 2; CH 4: tissue 3; CH 5: tissue 4; CH 6: tissue 5; CH 7: negative control.

Meanwhile, the run-time of ultra-fast PCR was 16 min, with conditions of 40 s at 95 °C, followed by 40 cycles of 2 s at 95 °C, 10 s at 57 °C, and 4 s at 72 °C. Under these reaction conditions, it was possible to detect fungus using insect tissue samples (Fig. 3). The SYBR signal intensity in ultra-fast PCR was verified for the last 10 of the 40 total cycles, and was found to increase as the concentration of the template DNA increased (Fig. 2). For the $1x10^4$copies/μL template, ultra-fast PCR showed amplification of the target gene even after 30 cycles, and detection was possible even for the $1x10^2$ copies/μL template (Fig. 2). Ultra-fast PCR enables early diagnosis of insect diseases, through direct, on-site result confirmation. This method could be used in early detection of not only *Ma* in *Pbs,* but also in early detection of other fungi, bacteria, and viruses. *Metarhizium* spp., the main pathogenic fungi of *Pbs*, cannot be detected with the naked eye in early stages of infection. Because, Spores spread throughout the sawdust that *Pbs* eat, meaning that *Ma* can easily disperse to all breeding cages if it is not rapidly detected and contained. When a fungal disease occurs in one cage, it can spread rapidly if the conditions are right for spore proliferation [15]. This risk makes it essential to change the sawdust and quarantine the infected insects as soon as possible. In addition, *Metarhizium anisopliae* takes approximately 7 days to appear on the insects' surfaces, and until this time, detection with the naked eye is impossible [16]. Periodic diagnostic procedures must be performed to detect the fungus early, and the fastest and most efficient method of diagnosis is PCR. In conventional PCR, secondary infection can occur during transport from the farm to the laboratory, and the processes of pathogen isolation, PCR, and electrophoresis at the laboratory takes days. By comparison, ultra-fast PCR is a one-stop method that can be performed on-site to detect fungi, enabling prevention of disease spread and economic loss to the farm. On-site ultra-fast PCR using direct tissue templates vortexed with Direct PCR Buffer and diluted 1/10, without isolation of genomic DNA, produced the same results as conventional PCR, making it possible to diagnose the pathogenic fungi much sooner (Fig. 4).

However, based on a naked eye inspection of the SYBR green products, improvements to sensitivity are required (Figs. 1 and 3). Detection accuracy decreased for impure templates, and the Ct value could not be measured from the first cycle in ultra-fast PCR. In conventional PCR, infected insect tissues were vortexed with direct buffer and subjected to 40 cycles of PCR under the following conditions: 50 °C for 5 min, 95 °C for 5 min, 95 °C for 20 s, 55 °C for 30 s, 72 °C for 20 s, 72 °C for 5 min. The total time for PCR was 1 h 24 min. As with the results of ultra-fast PCR, *M. anisopliae* infection was confirmed for the positive control (lane 1), and lanes 2–6, but not for the negative control (lane 8). Lane 7 was found not to be infected by *M. anisopliae* (Fig. 4).

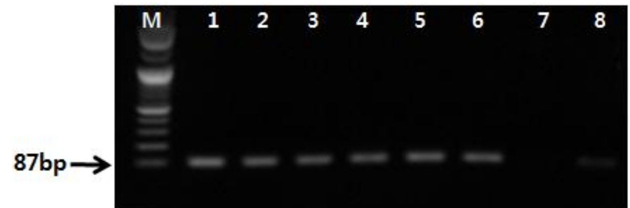

Fig 4: Conventional PCR using insect tissue samples infected by *M. anisopliae.*

Lane 1: control $1x10^4$ copy/μL; Lane 2: tissue 1; Lane 3: tissue 2; Lane 4: tissue 3; Lane 5: tissue 4; Lane 6: tissue 5; Lane 7: tissue 6; Lane 8: negative control.

Ultra-fast PCR should be highly useful in prevention of insect diseases at insect breeding farms, diagnosing diseases simply and rapidly, demonstrating amplification of target genes in real-time, identifying pathogens, and verifying the extent of disease progression.

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