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# PCR BASED DETECTION OF ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* IN HOST ORGANISMS

Do Van Hung[1], Katalin Posta[2] and Turóczi György[2]
[1]World Agroforestry Centre in Vietnam, Hanoi, Vietnam
[2]Plant Protection Institute, Szent Istvan University, Pater K. Street 1. H-2100 Godollo, Hungary
E-Mail: D.hung@cgiar.org

**ABSTRACT**

PCR based detection and identification of the entomopathogenic fungus *Metarhizium anisopliae* was conducted with specific primers F3 (5'-GGGTATATGAGAGGGAGGGC-3') and B3 (5'- GGTTCCTGGTCGGGACTT-3') which amplify a fragment of gene in the IGS (Intergenic spacer) region of rRNA (Ribosomal RNA) of *M. anisopliae*. The PCR amplification of IGS sequences yielded a unique fragment of 226-bp for all the four strains of *M. anisopliae* (M4, M16, M34 and M43). The results proved that the primers F3 and B3 were highly specific for *M. anisopliae*. PCR based detection *M. anisopliae* within host insects as Mealworm beetle (*Tenebrio molitor*) in the laboratory and cockchafer (*Melolontha* spp) in the field by using specific primers was applied. The PCR method could be a simple, rapid method to detect *M. anisopliae* within host insects just 8 days after infection. This study also showed that *M. anisopliae* exists in the soils in Felsőőrs-Köveskútpuszta region in Hungary. In fact, the results proved that DNA extracted from infected insects in laboratory and field could be used to identify the presence of the entomopathogen fungus *M. anisopliae* by using specific primers. Our study demonstrates an alternative approach for typing *M. anisopliae* strains within infected insects and reduces the need for time-consuming morphological and physiological tests.

**Keywords:** entomopathogen, *M. anisopliae*, host organisms, PCR, DNA, sequences, specific primers, F3, B3, amplification, detection.

## 1. INTRODUCTION

Since the middle of the nineteen century, microorganisms have been introduced to control pest and disease vector insects [1]. Until recently, they have widely applied in pest control and management [2]. Entomopathogenic fungi are significant factors to be used in biological control such as an integrated vector management [3] and microbial biologic control agent [4]. *Metarhizium* spp is considered as an important group of etomopathogenic fungi alternative to chemical pesticides in agriculture [5]. *Metarhizium anisopliae* var. *anisopliae* is a hyphomycetous fungus which infects more than 1000 insect species [6]. They have been used for the control of various pests such as *Manduca sexta* [7]; *Schistocera gregaria* [8] grasshopper (*Zonocerus variegates*) [9]; *Aedes aegypti* (L.) (Diptera: Culicidae) [10]; *Spodoptera frugiperda Sf9* [11]; rhinoceros beetle (*Oryctes rhinoceros* L.) [12] or cattle tick (*Boophilus microplus*) [13].

Traditional method for the identification of entomopathogenic fungi are based on spore morphology, biochemical characteristics and immunological properties. Furthermore, molecular techniques are also being intensively used as standard tools for the detection, identification and phylogenetic analysis of many fungal species [14]. Recently, different molecular techniques have been applied for the detection of *M. anisopliae*, such as lop-mediate isothermal amplification (LAMP) [15], bioassay probe [16], suppression-subtractive hybridization [17], microsatellite marker [18], using specific primers [19], restriction fragment length polymorphism (PCR-RFLP) [20]. The addition of PCR-based tools for detection of organisms has dramatically advanced our comprehending *M. anisopliae* [21].

The objective of this dissertation is: (i) to test specific primers F3 and B3 on *M. anisopliae*, (ii) to apply PCR (Polymerase Chain Reaction) technique using specific primers for the detection of *M. anisopliae* within host insects Mealworm beetle (*Tenebrio molitor* L) in laboratory, (iii) to evaluate the potential presence of *M. anisopliae* on cockchafer (*Melelontha* spp) in the nature.

## 2. MATERIALS AND METHODS

### 2.1 Entomopathogens and test organisms

Four strains of *M. anisopliae* (M4, M16, M34 and M43), *M. anisopliae* suspensions, *Beauveria bassiana, Lecanicillium lecanii*, two strain of *Fusarium* (*F. graminearum 1/3* and *F. culmorum 7603*) and Mealworm beetle (*Tenebrio molitor* L) larvae were provided by Institute of Plant Protection, Szent István University. All the entomopathogens were isolated in Hungary, either from infected insects (western corn rootworm, *Diabrotica virgifera virgifera*) or from soil samples. *Fusarium* strains were also isolated in Hungary and were used as negative control.

The cockchafer (*Melelontha* spp) larvae were collected from the location of field experiments (a stone-fruit orchard) of the Morello Ltd, Felsőőrs-Köveskútpuszta region in Hungary at four untreated plots (A, B, E and F).

### 2.2 Mycelium production of the fungal strains

*M. anisopliae* strains (M4, M16, M34, M43), *Beauveria bassiana*, *Lecanicillium lecanii* and two strains of *Fusarium* (*F. graminearum 1/3* and *F. culmorum 7603*) were cultured on tomato agar (75g tomato, 5g glucose, 10g agar, 500 ppm of Chloramphenical, 500 ml of water). Spores from these cultures were harvested, and spore suspension ($10^7$cfu/ml) of each strain was produced in physiological water. 1ml of these suspensions were used for inoculation of liquid cultures (100ml media: 1g

glucose, 15g tomato, 100 ppm of Chloramphenical, 100 ml of water). Flasks were shaken for 5 days at 20°C with 120 rpm to ensure necessary aeration in the liquid culture. Mycelium was separated with centrifugation for 10 minutes at 4000 rpm at 21°C. The supernatant was discarded and fungal biomass stored at – 20°C until DNA extraction.

**2.3 Infection of Mealworm beetle (*Tenebrio molitor* L.) larvae with *M. anisopliae***

*M. anisopliae* strain M43 and *M. anisopliae* conidial suspensions were used to infect *Tenebrio molitor* larvae. Four treatments were designed, each in 6 replicates. For treatments, 5 *Tenebrio molitor* larvae were placed in Petri dishes containing wheat meal, oat flakes and a slice of carrot to ensure necessary water for the larvae infected with *M. anisopliae* strain M43 and *M. anisopliae* conidial suspensions.

Treatment 1, 2, 3 and 4 included *Tenebrio molitor* larvae were exposed to infection with *M. anisopliae* for 24h (1 day), 120h (5days), 8 days and 21 days, respectively. After incubation period each larvae were collected and stored at – 20°C until DNA extraction. Control treatment means when no *M. anisopliae* was applied.

**2.4 DNA isolation**

Four strains of *M. anisopliae* (M4, M16, M34 and M43), *Beauveria bassiana*, *Lecanicillium lecanii* and two strain of *Fusarium* (*F. graminearum 1/3* and *F. culmorum 7603*) were used for DNA extraction to test specific primers for *M. anisopliae*. DNA was extracted from infected Mealworm beetle (*Tenebrio molitor*) in the laboratory and Cockchafers (*Melelontha spp*) collected from the field for detection the presence of *M. anisopliae* within insects, respectively. DNA isolations were followed by the FastDNA®SPIN Kit for Soil protocol. DNA concentration was checked by Nanophotometer™ P-Class (IMPLEN) equipment.

**2.5 PCR amplification**

PCR based method was used for the detection of *M. anisopliae* applying two primers F3 (forward) and B3 (reverse). The set primers consisted of F3 and B3 to amplify a fragment of gene in the IGS (Intergenic spacer) region of rRNA (Ribosomal RNA) of *M. anisopliae* [15]. PCR reactions were carried out in a total volume of 20 µl containing 2 µl of the fungal DNA or insect DNA, 2 µl of a pair of appropriate primers, 2 µl dNTP mixture (10 mM) and 0.4 µl of Dream Taq DNA polymerase (1U/µl) with the corresponding 2 µl polymerase buffer (10X Dream Taq Buffer) was mixed with 11.6 µl of sterile water. DNA thermal cycler TC-412 was used for the reactions.

**2.6 Gel electrophoresis**

The amplified products were analyzed in 1.5 % agarose gel by using Clever machine at 90 volts for 30 to 35 minutes. DNA marker (Ref. No 2500340, 5-Prime-pefect Size DNA Weight Ledders) with the amount of 4 µl were used as ladder on the gel. Mix containing 5 µl of PCR products and 1 µl of Loading dye (Loading buffer 6X) were used to fill into the wells. Then PCR products were run on the gel. Gels were analyzed by Gel Logic 200 Imaging System.

**3. RESULTS**

**3.1 Species-specific primers for *Metarhizium anisopliae***

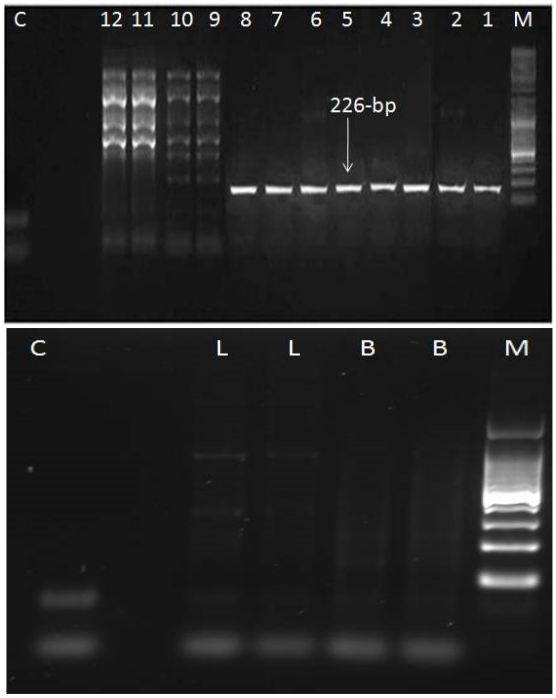


**Figure-1.** PCR based detection of *M. anisopliae* by using specific primers F3 and B3. M (Marker), C (negative control), 1: M4, 2: M4, 3: M16, 4: M16, 5: M34, 6: M34, 7: M43, 8: M43, 9: *F. graminearum 1/3*, 10: *F. graminearum 1/3*, 11: *F. culmorum 7603*, 12: *F. culmorum 7603*. B: *Beauveria bassiana*, L: *Lecanicillium lecanii.*

The specific primers (F3, B3) amplified a 226-bp product from one sequence of the IGS (the Intergenic Spacer in ribosomal DNA locus) of *M. anisopliae* (Figure 1). These primers were tested by using fungal DNA isolated from four strains of *M. anisopliae* (M4, M16, M34 and M43), *Beauveria bassiana*, *Lecanicillium lecanii* and two strain of *Fusarium* (*F. graminearum 1/3* and *F. culmorum 7603*).

All four strains of *M. anisopliae* (M4, M16, M34 and M43) gave strong PCR signals. Products of specific 226-bp length could be clearly observed in the gel. The specific primers also amplified some fragments in the genome of two strain of *Fusarium* (*F. graminearum 1/3* and *F. culmorum 7603*) and entomopathogenic *Lecanicillium lecanii*, but these were clearly different from the fragment characteristic for *M. anisopliae*. *Beauveria bassiana* did not show any PCR product with the specific

primers. Therefore, the primers tested were only specific for *M. anisopliae*.

### 3.2 Detection of *Metarhizium anisopliae* in *Telebrio molitor* L larvae

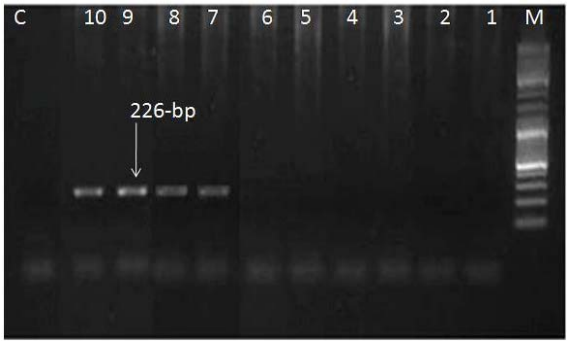


**Figure-2.** Detection of *M. anisopliae* in Mealworm beetle (*Tenebrio molitor*) larvae following laboratory infection. M (marker), C (negative control), 1: Insect control (8 days), 2: Insect control (21 days), 3: Insect infected with *M. anisopliae* strain M43 in 24 hours, 4: Insect infected with *M. anisopliae* conidial suspensions in 24 hours, 5: Insect infected with *M. anisopliae* strain M43 in 120 hours (5 days), 6: Insect infected with *M. anisopliae* conidial suspensions in 120 hours (5days), 7: Insect left in Petri dish with *M. anisopliae* strain M43 in 8 days, 8: Insect left in Petri dish *M. anisopliae* conidial suspensions in 8 days, 9: Insect left in Petri dish with *M. anisopliae* strain M43 in 21 days, 10: Insect left in Petri dish with *M. anisopliae* conidial suspensions in 21 days.

Sensitivity of specific primers was tested in laboratory on artificially infected larvae of Mealworm beetle (*Telebrio molitor*). Larvae were exposed to infection for 1 day, 5 days, 8 days and 21 days, respectively, with *M. anisopliae* strain 43 and *M. anisopliae* conidial suspensions. PCR was conducted with the specific primer F3 and B3, DNA extracted from the non-infected larvae were used as control.

The presence of *M. anisopliae* in the infected larvae was confirmed by the PCR which amplified 226-bp products (Figure-2). The results showed that non-infected as well as infected larvae from the samples of 24 hours and 120 hours infection did not show the PCR products. In contrast, larvae left in infected Petri dishes for 8 days and larvae left in infected Petri dish for 21 days presented the PCR products. These results indicated that in the laboratory, about 1 week of infection is necessary to detect the infection in Mealworm beetle larvae by PCR.

### 3.3. Detection the presence of *Metarhizium anisopliae* in untreated cockchafer larvae

The cockchafer (*Melelontha* spp) larvae were collected from the location of field experiments (a stone-fruit orchard) of the Morello Ltd, Felsőőrs-Köveskútpuszta in Hungary at four untreated plots (A, B, E and F). The presence of *M. anisopliae* was detected in only one of 9 larvae collected from untreated field. The presence of *M. anisopliae* was detected in larvae from plot B (Figure-3). These results indicated that entomopathogenic *M. anisopliae* exists in the soil of Felsőőrs-Köveskútpuszta region in Hungary.

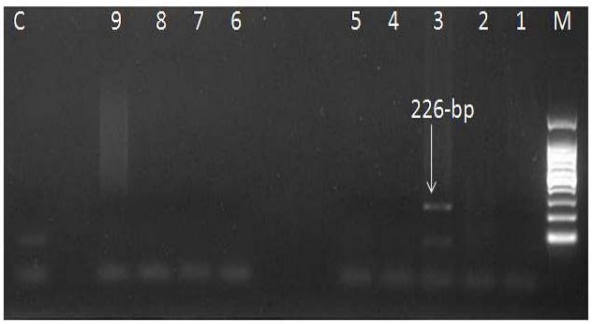


**Figure-3.** PCR to detect the presence of *M. anisopliae* on *Melolontha* spp in the field at different locations by using specific primer F3 and B3. M: (maker), 1: A, 2: A, 3: B, 4: E, 5: E, 6: E, 7 F, 8: F, 9: F, C: (negative control).

## 4. DISCUSSIONS

The experiments indicated that using the primers F3 and B3 to amplify the IGS sequences of *M. anisopliae* has advantages for molecular identification. These primers were tested on four *M. anisopliae* strains (M4, M16, M34 and M43), *Beauveria bassiana*, *Lecanicillium lecanii* and two strains of *Fusarium* (*F. graminearum 1/3* and *F. culmorum 7603*). The results agree with Li and Cai (2011) that F3 and B3 primers could amplify unique 226-bp PCR product form IGS sequences of *M. anisopliae*. The primers F3 and B3 were highly specific for the detection of *M. anisopliae* and suitable for species-level molecular identification.

PCR for the detection and identification of the entomopathogenic *M. anisopliae* in the hosts using species-specific primers have been applied on infected sugarcane borer (*Diatraea sccharalis*) by primers ITSMet and ITSMet14 [22]; infected immature stages of the fruit fly (*Anastrepha fraterculus*) by using set primer IST4 and ISMet [19]. In our study we used primers F3 and B3 for the detection of the entomopathogenic *M. anisopliae* in the laboratory were carried out with infected Mealworm beetle (*Tenebrio molitor*) larvae. We conducted PCR with primers F3, B3 and extracted DNA from infected Mealworm beetle larvae. The results showed that following 8 days of exposure to *M. anisopliae*, the pathogen can be detected within host by using specific primers F3 and B3. PCR based detection using specific primers F3 and B3 of *M. anisopliae* within host insects in the nature was also tested. In Felsőőrs-Köveskútpuszta region, cockchafer (*Melolontha* spp) larvae were collected from untreated plots (A, B, E and F) to check the presence of *M. anisopliae*. The presence of *M. anisopliae* was detected in larvae from plot B. These results indicated that entomopathogenic *M. anisopliae* exists in the soil of Felsőőrs-Köveskútpuszta region.

## 5. CONCLUSIONS

We concluded that PCR-based technique with specific primers F3 and B3 provide specific and rapid method for the detection of *M. anisopliae*. PCR technique also is an important tool for the detection of *M. anisopliae* within host insects. Our results proved that the species specific primers tested in our experiments are suitable for high sensitivity detection of entomopathogenic fungus species *M. anisopliae* either in artificially or naturally infected insects. These results make possible to follow and monitoring the *M. anisopliae* inoculums in soils where other methods (culturing on selective media) would be less sensitive and much more cumbersome. The sensitivity of the method (i.e. what amount of the fungus can be detected either in soil or in insects) is to be tested in following experiments.

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