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Specific Primer Design for Detection and Quantification of Entomopatogenic Fungi *Metarhizium anisopliae* using Quantitative PCR (qPCR) in Soil and Cocoon Samples

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ABSTRACT

The information on the abundance and dynamics of entomopathogenic fungus *Metarhizium anisopliae* on the soil is limited by the existence of insect hosts. In this study, to detect and quantify specifically for *M. anisopliae* from extract of soil DNA, a culture-independent approach based on DNA and employing quantitative Polymerase Chain Reaction (qPCR) was developed. Primer pairs were designed and tested for their specificity to get a specific primer pair. The best primer pair was determined to be MA6071F/MA6218R. Two standard curves were created using 10 concentration levels (10^1 to 10^{10}) by qPCR. Standard curves for genomic DNA showed a strong relationship and good fitting ($R^2 > 0.980$). Six levels were obtained to generate standard genomic DNA ($R^2 = 0.98$, E = 1.05). Eight levels ($R^2 = 0.9854$, E = 0.91) were created for standard soil DNA. By qPCR, *M. anisopliae* was not found in all soil samples, possibly due to the samples' low fungal density. However, 13 dead cocoon samples out of 80 showed positive for *M. anisopliae*. To successsfully detect and quantify *M. anisopliae* in soil, the method of DNA extraction and soil sampling should be enhanced.

INTRODUCTION

Metarhizium anisopliae (Hypocreales: Clavicipitaceae) is one of the most widely distributed entomopathogenic fungi used as biological control agents against a variety of insect pests, including locusts, thrips, whiteflies, and ticks (Kivett et al., 2016; Abdel-Raheem & Al-Keridis, 2017; Nardoni et al., 2018). Biological control has recently garnered interest as an alternative approach (Begg et al., 2017). *M. anisopliae* has widespread distribution around the world and has been isolated from many insect species such as Coleoptera (in the family of Curculionidae, Elateridae, and Scarabaeidae), Diptera, and Hymenoptera (Zimmermann, 2007).

This fungus has been found colonizing the rhizosphere and clinging to the surface of plant roots, and it may affect this ecological niche by repelling and killing soil insects and could survive for various years in the soil (Greenfield et al., 2016; Iwanicki et al., 2019; Razinger et al., 2020). *M. anisopliae* was also reported attacking the cocoon of *Syntypistis punctatella,* the caterpillar which attacks beech trees in Japan (Kamata et al., 1997). *M. anisopliae* even was considered the second-largest mortality factor for *S. punctatella* (Kamata et al., 1997). *M. anisopliae* was also found on the dead cocoon of a larch sawfly, *Pristiphora erichsonii* in *Larix kaempferi* plantation in Furano, Japan (Kamata et al., 1997).

However, information on the abundance, dynamics and population structures of *M. anisopliae* on the soil is limited to the existence of insect hosts, especially during latent periods. Fruit body and

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host insect pupae densities are low, which makes it difficult to evaluate the percentage of fungal infection in fields. Cultivation-based approaches are also laborious and time-consuming. Moreover, the result of cultivation-based approaches may also lack reliable. In the end, little is known about the abundance, dynamics, and population structures of *M. anisopliae* on the soil.

In this study, rather than the cultivation-based method (Saragih et al., 2021), quantitative PCR (qPCR) approach was developed. Quantitative real-time PCR (qPCR) is a quick, sensitive, accurate, and culture-independent technique that is frequently used to detect and quantify microorganisms in various environmental samples (Libert et al., 2016; Saragih et al., 2015; Saragih et al., 2023). Several primers were designed and standard curves were obtained. Using this method, we tried to detect and quantify *M. anisopliae* effectively using a specific primer from soil and dead cocoon samples.

MATERIALS AND METHODS

The research activities including soil and cocoon sampling, DNA extraction, PCR, qPCR, data analysis, and preparing the manuscript were finished in 2024 that were conducted at the University of Tokyo Chichibu Forest, Saitama, Japan and Universitas Muhammadiyah Sumatera Utara, Medan, Indonesia.

Fungal Isolates and DNA Extraction

The *M. anisopliae* isolate was obtained from the Forestry and Forest Product Research Institute (Tsukuba, Japan). For checking the specificity of the primers, *Cordyceps militaris*, *Beauveria bassiana, Isaria fumosorosea*, and *Isaria farinosa* were the four related fungal species used. All isolates were re-cultured on potato dextrose agar (PDA) media that contained 3 g of bacto yeast extract, 3 g of bacto malt extract, 5 g of bacto peptone, 10 g of bacto dextrose, 20 g of bacto agar, and mixed with 1 l of water after autoclaving for 20 minutes at 121 °C. The cultures were incubated for 7 days at 25 °C.

The five-fungus genomic DNA was recovered from liquid cultures after 48 hours of cultivation at 25°C and 180 rpm using a shaker SSR-2 machine. The mycelia were washed with 500 µl of 0.9% NaCl buffer after filtering the culture through filter paper (membrane filter with mixed cellulose ester), centrifuging, and harvesting. After extraction, the mycelial cells were frozen in liquid nitrogen and kept at -80°C (Liu et al., 2018). Then, by using the CTAB technique (Poon et al., 2019), DNA was extracted from the precipitated mycelia after crushing in liquid nitrogen.

Design of *M. anisopliae* specific primer

The specific primers were developed from the fungal ITS region, using MEGA 6 (Tamura et al., 2013). Thirteen primers to detect *M. anisopliae* were designed (Table 1). The melting temperature (T_m) for each primer pair was determined higher than 54°C (Saragih et al., 2015). The BLAST was used to check the specificity of the primers (Saragih et al., 2015).

PCR and Sequencing

The PCR was conducted using TaKaRa Ex Taq Polymerase (TaKaRa) according to Saragih et al. (2015) with the condition: initial denaturing at 95°C for 3 minutes, followed by 30 cycles of denaturing at 95°C for 15 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 30 seconds, and final extension at 68°C for 5 minutes. The QIAquick Gel Extraction Kit (Qiagen) was then used to cut and purify the DNA gel, yielding 48 µl of product. Sequencing was performed after the PCR and compared using BLAST (Ahmad et al., 2021).

Quantitative Real-Time PCR

The qPCR was conducted using SYBR Green PCR Master Mix (Applied Biosystems), according to Saragih et al. (2015). The condition of qPCR was the following condition using Schneider et al., (2011): initial denaturing at 95°C (15 minutes), 45 cycles of denaturing at 94°C (40 seconds), annealing at 66°C (40 seconds), extension at 72°C (2 minutes), and final extension at 72°C (10 minutes).

Soil and Dead Cocoon Samples

Fifteen soil samples were taken from a natural beech forest in Hachimantai and a Japanese larch plantation in Furano, Japan. From the A0 layer, three soil sub-samples were collected and put into a sterilized plastic bag (Thermo Fisher Scientific) (Saragih et al., 2015). Dead cocoon samples of larch sawfly (*P. erichsonii)* were collected from ten topsoil samples from each of eight disjunct plantations of the Japanese larch in The University of Tokyo Hokkaido Forest (UTHF), Hokkaido, Japan. *P. erichsonii* cocoons were gathered by hand sorting and sieving. Unopened, healthy-looking cocoons were put into non-woven nylon bags and

incubated in planters with sterilized vermiculite for overwintering (Pinkantayong et al., 2014). After overwintering, these cocoons were taken from the planters and diagnosed if mycelia appeared on their surface by the naked eye.

Standard Curve for *M. anisopliae* in Soil

After autoclaving, a certain dose of fungal suspension was added to autoclaved soil to create standard soil DNA. The fungus was transplanted to a new culture on potato dextrose agar medium (PDA) plates and incubated at 25°C for about a week before extraction. Mycelia and conidia of each entomopathogenic fungi were put into a 2-ml microtube with 400 μl of free water and homogenized using bead beater-type homogenizer (μT-12, TAITEC CORPORATION) at 3200 rpm for 6 minutes. After the homogenization, 200 μl of free water was added to the microtube and vortexed. From the original suspension, 10-fold serial dilutions (nine levels) were created. 100 μl of each suspension level was added to 0.5 g of autoclaved soil and then extracted. A standard curve was then generated from the ten leaves. After verifying specificity by qPCR, the best primer pair was used to quantify the fungus in the soil.

Soil DNA Extraction and Quantification

Soil DNA extraction was conducted at the University of Tokyo Chichibu Forest, Saitama, Japan. The Isolation KIT was used to extract soil DNA from 0.5 g of each soil sample, following the manufacturer's instructions (Saragih et al., 2015). The quantification of *M. anisopliae* in the soil was conducted by qPCR from the extracts using the standard curve (Saragih et al., 2015).

Table 1. Thirteen specific primer pairs designed from *M. anisopliae* ITS region using MEGA 6

No	Primer	Sequence (5' – 3')	T_m	% GC	Length (bp)
1	MA5821F	F : TGCTTCGGCGGGACTTC	62	65	134
	MA6009R	R : CGTTCTTCATCGATGCCAGAA	62	48	
2	MA6187F	F : CAGCCGTCCCTCAAATCA	59	56	138
	MA6334R	R : CCTACCTGATTCGAGGTCAACTA	58	48	
3	MA6071F	F : CACATTGCGCCCGTCAGT	62	61	141
	MA6218R	R : CGAGACCGCCAATTGATTTG	62	50	
4	MA6169F	F : GGCTGGTTTTCCAGCACAG	60	58	154
	MA6334R	R : CCTACCTGATTCGAGGTCAACTA	58	48	
5	MA5619F	F : TGGCTCAGTGAGGCGTCC	62	67	166
	MA5801R	R : ACAGGGGTTGGGAGTTGGAT	61	55	
6	MA6187F	F : CAGCCGTCCCTCAAATCA	59	56	252
	MA6452R	R : CCTGGGGACCAGATTTCAA	59	53	
7	MA5889F	F : GGGGACCCAAACCTTCTGA	60	58	310
	MA6218R	R : CGAGACCGCCAATTGATTTG	62	50	
8	MA6167F	F : GAGGCTGGTTTTCCAGCG	60	61	270
	MA6452R	R : CCTGGGGACCAGATTTCAA	59	53	
9	MA5975F	F : AACGGATCTCTTGGTTCTGGC	61	52	231
	MA6218R	R : CGAGACCGCCAATTGATTTG	62	50	
10	MA6191F	F : CCGTCCCTCAAATCAATTGG	61	50	161
	MA6452R	R : CCTGGGGACCAGATTTCAA	59	53	
11	MA5748F	F : GGAGGGATCATTACCGAGTTATCC	62	50	193
	MA6009R	R : CGTTCTTCATCGATGCCAGAA	62	48	
12	MA5762F	F : CGAGTTATCCAACTCCCAACCCC	65	57	331
	MA6165R	R : CCGATCCCCAACACCAAGT	62	58	
13	MA1763F	F : CCAACTCCCAACCCCTGTGAAT	65	55	336
	MA2097R	R : AAAACCAGCCTCGCCGAT	61	56	

Genomic DNA Extraction from Dead Cocoon Samples

Genomic DNA extraction was conducted at the University of Tokyo Chichibu Forest, Saitama, Japan. All fungi that grew on the surface of cocoon were collected using forceps and cultured on Sabouraud's dextrose agar (SDA) media in 9 cm petri dish, which contained 10 g of dextrose, 10 g of peptone, and 20 g of agar per 1000 ml of DNAse free water and was added with $N_{a2}CO_3$ (adjusting pH 10) and streptomycin (antibiotics) after autoclaving for 20 minutes at 120 °C. All cultures were incubated at 25°C for 5 days. After 5 days of incubation, all fungi that grew on the media were collected and extracted. The qPCR was conducted using a complex mixture of DNA extract from all samples for the detection of *M. anisopliae* inside the sample.

RESULTS AND DISCUSSION

Specificity of Primer Design of *M. anisopliae* and Sequence Analysis

Thirteen primers were created specifically for *M. anisopliae* (Table 1). Among thirteen primers, the primer pair MA6071F (5'CACATTGCGCCCGTCAGT 3') / MA6218R (5' CGAGACCGCCAATTGATTTG 3') with annealing temperature of 66°C and cycle number of 45 was the best combination of primer and conditions since only this primer showed specificity with a single peak of melting curve in qPCR (Fig. 1). This primer pair showed 137 hits with strain of *M. anisopliae* with 100% match. Additionally, four similar species were not found, such as *C. militaris, B. bassiana, I. farinosa,* and *I. fumosorosea*.

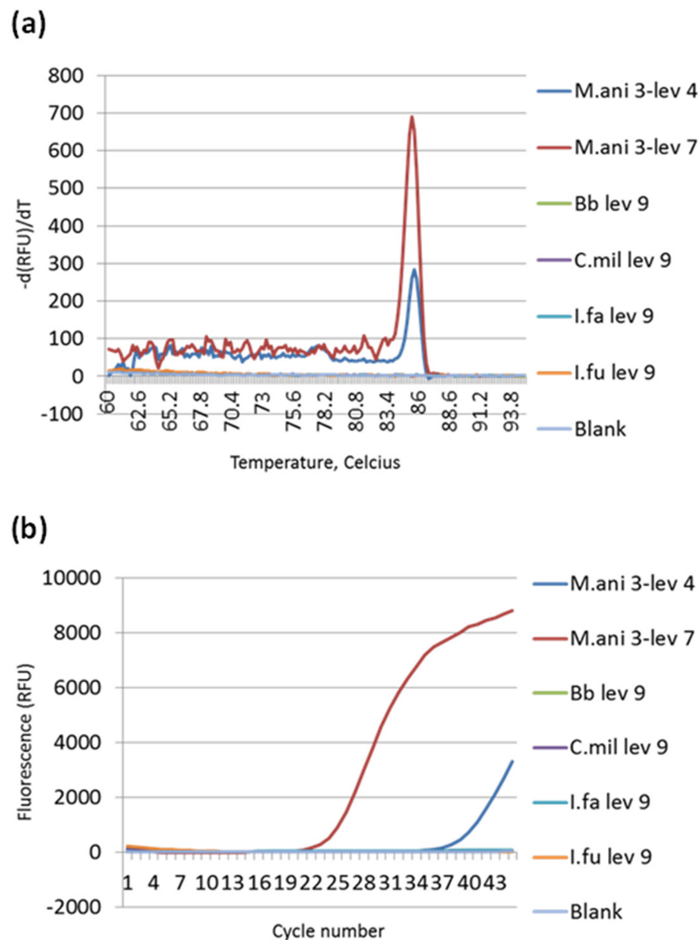

Fig. 1. (a) Melting curve; (b) Accumulation curve of *Metarhizium anisopliae* using primer pair MA6071F / MA6218R

From the DNA extract of *M. anisopliae*, a single amplicon (141 bp) was obtained by PCR amplification using primer pair MA6071F/MA6218R. BLAST similarity search with 141 bp of sequence data showed a 100% match with 87 strains of *M. anisopliae* and 3 strains of *Metarhizium brunneum* (GenBank: HM055438, FJ617325, FJ617318). The sequence data also showed a 99% match with 109 strains of *M. anisopliae*, and also with a complex group of *M. anisopliae* such as *Metacordyceps taii* (12 strains), *Metacordyceps indigotica* (5 strains), *Metarhizium robertsii* (9 strains), *M. brunneum* (3 strains), *Metarhizium quizhounse* (6 strains), *Metarhizium pingshaense* (2 strains), and *Metarhizium majus* (4 strains).

BLAST similarity searches for specificity test for primer pair MA6071F/MA6218R showed a 100% match with 137 strains of *M. anisopliae* and 8 strains of *M. brunneum*. The sequence of 141 bp also showed a 100% match with 90 strains of *M. anisopliae* and 2 strains of *M. brunneum*. These two strains that showed 100% match in sequence data are also included in 8 strains that match 100% with primer pair MA6071F/MA6218R. *M. brunneum* is a synonym of *M. anisopliae* (Bischoff et al., 2009). This indicates that the primer pair MA6071F/MA6218R is specific to *M. anisopliae.* The melting curve also showed a similar single peak for *M. anisopliae* and not for other non-target species amplified during the qPCR process. The annealing temperature of 66°C became the best annealing temperature. It was concluded that primer pair MA6071F/MA6218R could quantify *M. anisopliae* with an annealing temperature 66°C.

Standard Curve of qPCR

The standard genomic DNA of *M. anisopliae* was generated (slope = - 3.2073 and R^2 = 0.98) (Fig. 2a). The standard curve of genomic DNA showed a strong relationship with good fitting. Among 10 levels of dilutions of genomic DNA, only six levels could be used to obtain the regression line (from level 3 to level 8) with a range of *Ct* values around 14-31. Levels 1 and 2 showed great variation in *Ct* value among replications. It might be related to the low density of fungus. At levels 9 and 10, *Ct* value was not obtained for all replications. The high density of DNA might become a problem in qPCR process. Amplification efficiency (*E*) for genomic DNA was 1.05. The limit of *Ct* value for DNA quantification using this standard genomic DNA was 31.95.

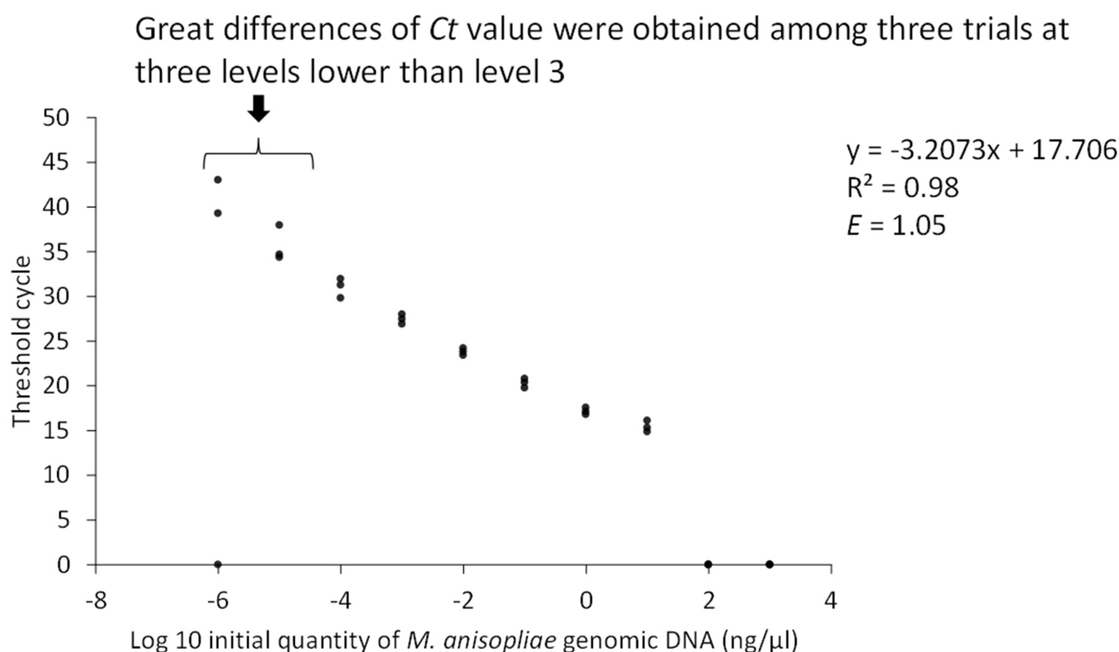

Fig. 2a. A standard genomic DNA of *Metarhizium anisopliae* that showed six levels from level 3 to level 8

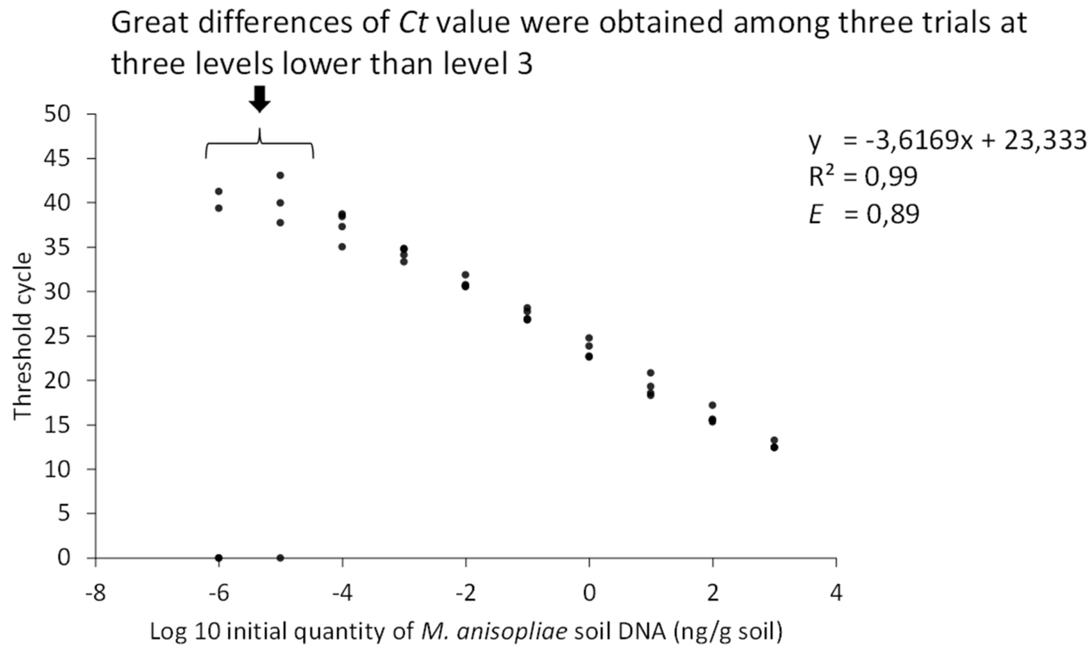

Fig. 2b. A standard soil DNA of *Metarhizium anisopliae* that showed eight levels from level 3 to level 10

Additionally, a linear correlation was shown between the *Ct* value and log10 of soil dry weight of *M. anisopliae* (ng/g soil) (ng/g soil) (slope = - 3.6169, R^2 = 0.9887 (Fig. 2b). Standard curve with log10 of dry weight of *M. anisopliae* (R^2 = 0.9887) also showed strong relationship with good fitting so that a mass of *M. anisopliae* in soil will be determined by qPCR with the primer pair MA6071F/MA6218R. Among 10 levels of dilutions, eight levels (3-10) were used for the standard curve with a range of *Ct* value around 12-38. Levels 1 and 2 showed great variation in *Ct* value among replications. The slope of standard soil DNA is greater than genomic DNA, but for the amplification efficiency (*E*), the genomic DNA was greater than soil DNA (*E*=0.91). The limit of *Ct* value was 38.67.

Detection of *M. anisopliae* from Soil Samples and Dead Cocoon Samples Using qPCR

M. anisopliae was not detected in all soil samples from Furano and Hachimantai by using qPCR. For dead cocoon samples, the result of qPCR showed positive for 13 samples of *M. anisopliae*. *M. anisopliae* were found on dead cocoons of the larch sawfly, *Pristiphora erichsonii* in *L. kaempferi* plantation in Furano. *M. anisopliae* was also found in the cocoon buried of *S. punctatella* in Hachimantai in 1994 (Kamata et al., 1997). *M. anisopliae* was considered the second-largest mortality factor for *S. punctatella* (Kamata et al., 1997). However, all soil samples showed negative qPCR, so the quantity of *M. anisopliae* in the samples could not be quantified. The low fungal density in soil samples that prevented qPCR detection possibly contributed to this result.

CONCLUSION

Although the detection of *M. anisopliae* from soil samples was unsuccessful, the qPCR approach was still effective and specific in detecting fungus. Other entomopathogenic fungi can be specifically detected using the specific pair. DNA extraction needs to be improved to effectively identify and quantify M. anisopliae in soil by modifying techniques for soil sampling and/or soil.

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