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Short Communication

Specific Primer Designing for Quantitative PCR (qPCR) of Entomopathogenic Fungi *Isaria fumosorosea* from Soil Samples

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ABSTRACT

Entomopathogenic fungus are important component in pests regulation in ecosystem. *Isaria fumosorosea*, as one of the entomopathogenic fungi, was reported to successfully control outbreaks of forest defoliators known to attack larch (*Larix kaempferi*) plantation in Furano, Japan and beech (*Fagus crenata*) forest in Hachimantai, Japan. Instead of semi-cultured methods, this paper developed and used a culture-independent method based on DNA using qPCR for specific *I. fumosorosea* detection and quantification directly from soil DNA extraction using specific primer. The primer IFU5821F/IFU6061R was designed and the best primer pair for *I. fumosorosea*. Strong relationship and good fitting with five levels (R^2 = 0.989, E = 0.58) were obtained standard soil DNA extraction. *I. fumosorosea* could not be detected from all soil samples possibly caused by low density of the fungi. The qPCR was likely a rapid and specific method to detect the fungus from soil.

Keywords: *Isaria fumosorosea*; qPCR; soil DNA; soil samples

INTRODUCTION

Entomopathogenic fungus are important components for pest regulation in ecosystem, mostly to control arthropod population (Meyling & Eilenberg, 2007). Some studies revealed the entomopathogenic fungi roles as insect population regulators and other arthropod pests including grasshoppers, locusts, and other hemipterans (Roberts & St. Leger, 2004; Zimmermann, 2007). More than 750 entomopathogenic fungal species have been isolated in the world, one of them is *Isaria fumosorosea* (Nielsen *et al.,* 2008).

I. fumosorosea (formerly known as *Paecilomyces fumosoroseus*) (Hypocreales: Cordycipitaceae) is an entomopathogenic fungus known as one of the most promising species with wide distribution and host range of more than 40 insect species mostly from order Lepidoptera (Zimmermann, 2008). *I. fumosorosea* has already been isolated from many arthropods that lives in the air, water, plants, and often soil (Zimmermann, 2008).

I. fumosorosea was found on buried beech caterpillar, *Syntypistis punctatella* cocoons (Lepidoptera: Notodontidae) in Japan (Kamata *et al.,* 1997). *I. fumosorosea* was reported to successfully control forest defoliators in larch (*Larix kaempferi*) plantation located at Furano, Japan and beech (*Fagus crenata*) forests in Hachimantai, Japan (Kamata, personal communication). *I. fumosorosea* has also been reported to become a biological control option for white fly, *Bemisia tabaci* in soybean (Lozano-Contreras *et al.,* 2013). *I. fumosorosea* has been isolated often from soil (Landa *et al.,* 2002; Meyling & Eilenberg, 2006;

Ayala-Zermeño *et al.,* 2011; Hu *et al.,* 2011). However, abundance information of *I. fumosorosea* in soil is limited especially during latent periods, and cocoon density is also low causing it to be difficult to observe epizootiology of this fungi in soil. Using lab-reared insects as bait to measure infection rate requires much time and great number of insects to acquire enough replicates for further statistical analysis.

Instead of semi-cultured method (Saragih *et al.,* 2021), we developed a culture-independent method based on DNA using qPCR in this paper. The qPCR is considered as an accurate, rapid, sensitive, and culture-independent method to detect and quantify microorganism directly from environmental samples (Chemidlin Prévost-Bouré *et al.,* 2011; Saragih *et al.,* 2015). We designed and tested several primers to detect and quantify *I. fumosorosea* specifically from soil samples using qPCR. The number of *I. fumosorosea* in soil was quantified using a standard curve that was created from fungus DNA extracted from the soil samples.

MATERIALS AND METHODS

Fungal Culture and Genomic DNA Extraction

Fungal culture of *I. fumosorosea* used in this study was collected from Forestry and Forest Product Research Institute (FFPRI) located in Tsukuba, Japan (Table 1). To confirm primer pairs specificity, four related fungal species were used, namely *Metarhizium anisopliae, Cordyceps militaris, Beauveria bassiana,* and *Isaria farinosa* (Table 1).

The five fungi were cultured on potato dextrose liquid and extracted after incubation at 25°C and centrifuge at 180 rpm for around 48 h. Then 0.9% NaCl buffer was used to wash the mycelium. Harvested mycelium was frozen in liquid nitrogen and stored at -80°C (Enkerli *et al.,* 2001). Intact DNA was extracted from the precipitated mycelia using CTAB (hexadecyltrimethylammoniumbromide) method (Zhou *et al.,* 1996) after being crushed using liquid nitrogen. A spectrophotometer was used to quantify genomic DNA of the extract.

Table 1. Four fungal isolates used to check the specificity of eleven primer pairs related to *I. fumosorosea* from the Forest Products Research Institute, Tsukuba, Japan

Fungal isolate	Species
F2223	*Isaria fumosorosea*
F1035	*Metarhizium anisopliae*
F1105-1	*Cordyceps militaris*
F1075	*Isaria farinosa*
F1042	*Beauveria bassiana*

Specific Primer Design and Primer Specificity Check

The specific primers were designed using MEGA 6 (Tamura *et al.,* 2013). Based on internal transcribed spacer (ITS) region, eleven primer pairs (Table 2) were designed from 17 strains of *I. fumosorosea* and compared to other entomopathogenic fungus (Table 3). For maintenance of efficiency and specificity, melting temperature (T_m) was determined higher than 54°C for each primer pair (Dieffenbach *et al.,* 1993). BLAST similarity search was used to check specificity of the eleven primer pairs in nucleotide database of GenBank (National Resource for Molecular Biology Information) using the default setting and the number of hits was set to 250. The qPCR was run using genomic DNA of each of five species of entomopathogenic fungi and a negative control.

PCR and Sequencing

PCR was carried out with a program consisting of initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension at 68°C for 30 s, and final extension at 68°C for 5 minutes (Saragih, *et al.,* 2015). Sequencing was performed using 20 µl of PCR as described by Saragih *et al.* (2015).

Quantitative Real-Time PCR

The qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) as described by Saragih *et al.*, (2015) with the condition of qPCR program consisting of initial denaturing at 95°C for 7 minutes, followed by 50 cycles of denaturation at 95°C for 10 s, annealing at 67°C for 30 s, extension at 68°C for 20 s, and final extension at 60°C for 30 s. Melting curve was set between 60–95°C. In preliminary experiments, best combination of primer pair and annealing temperature was done by comparing different melting curves annealing temperature between 61–67°C.

Table 2. Eleven primer pairs designed using MEGA 5 for checking *I. fumosorosea* specificity based on ITS region with melting temperature (T_m) higher than 54°C

Primer ID	Primer sequences (5' – 3')	T_m value	% GC	Estimated Length (bp)
IFU5819F	F : AGACCACGCAACCCTGCATC	64	60	181
IFU6019R	R : GCGCAATGTGCGTTCAAAG	58	53	
IFU5681F	F : CCAGAGTTTTCACAACTCCC	60	50	159
IFU5861R	R : GGATTCAGAGAGACTGATGG	60	50	
IFU609F	F : GTATCGACAACCAGGCCCTT	62	55	157
IFU768R	R : AGCTTGCGAAGGTCAGAGTTG	64	52	
IFU862F	F : CTCGGTTCCTGAGCTCACT	60	58	175
IFU1096R	R : GACGAAGTAGGTGGAGTTCTTG	66	50	
IFU1105F	F : TCCCAACAACATTCAGAATGCC	64	45	139
IFU1244R	R : TGCGACGGAACATGGCG	56	65	
IFU1147F	F : CGGCCTGAAGATGTCGTCTACT	68	55	168
IFU1315R	R : CATGTTGGACTCAGCCTCGG	64	60	
IFU919F	F : TGACTTCCGTAACGGTCGC	60	58	231
IFU1209R	R : CGCTTGAAGAGGTCCTGAATG	64	52	
IFU5821F	F : ACCACGCAACCCTGCATC	58	61	338
IFU6195R	R : TCCGGTGCGAGCTGTAGTACTT	68	55	
IFU5821F	F : ACCACGCAACCCTGCATC	58	61	221
IFU6061R	R : GAAATGACGCTCGAACAGGC	62	55	
IFU5699F	F : GTTTTCACAACTCCCAACCCTC	66	50	284
IFU6008R	R : GTTCAAAGATTCGATGATTCACG	64	39	
IFU860F	F : GTCTCGGTTCCTGAGCTCACT	66	57	214
IFU1133R	R : CGCAAAGGGCATTCTGA	52	53	

Table 3. Reference sequences from GenBank (National Resource for Molecular Biology Information) used for designing *I. fumosorosea* specific primer based on ITS regions

No.	Isolate name	Accession number
1	*Isaria fumosorosea* isolate SKCH-1	FJ765017.1
2	*Isaria fumosorosea* isolate NLWG	FJ765014.1
3	*Isaria fumosorosea* isolate KTU-13	FJ177461.1
4	*Isaria fumosorosea* isolate KTU-42	FJ177460.1
5	*Isaria fumosorosea* 18S ribosomal RNA gene	GQ244511.1
6	*Isaria fumosorosea* isolate NLHG-2	FJ765013.1
7	*Isaria fumosorosea* isolate NLHG-1	FJ765012.1
8	*Isaria fumosorosea* isolate CNXN	FJ765011.1
9	*Isaria fumosorosea* isolate CNBJ	FJ765010.1
10	*Isaria fumosorosea* isolate CNHG	FJ765009.1
11	*Isaria fumosorosea* isolate CNZH	FJ765008.1
12	*Isaria fumosorosea* isolate CNZG	FJ765007.1
13	*Isaria fumosorosea* isolate CNIM	FJ765006.1
14	*Isaria fumosorosea* strain NBAII-Pl-1	JF718689.1
15	*Isaria fumosorosea* strain ART2780	HQ380864.1
16	*Isaria fumosorosea* isolate KTU-16	FJ177464.1
17	*Isaria fumosorosea* strain KVL 07-37	GU354344.1

Soil Samples

Soil samples were collected from two sites in Hachimantai with beech trees and one site in Furano occupied by the Japanese larch in 2013 (Table 4). These sites were selected because reported outbreaks of forest defoliators (Table 4). Samples were collected from A0 layer and kept inside sterilized plastic bag (#14-955-184, Thermo Fisher Scientific)

Table 4. List of soil sample collected form three sites

Location	Latitude / Longitude	Elevation (ASL)	Forest type	Outbreak insect species	Number of samples
Furano	N43°12′58.49″ E142°30′34.94″	250 m	*Larix kaempferi* plantation	Larch sawfly (*Pristiphora erichsonii*)	5
Hachimantai A	N39°59′46.89″ E140°48′20.97″	890 m	Natural deciduous broadleaved forest predominantly occupied by *Fagus crenata*	Beech caterpillar (*Syntypistis punctatella*)	5
Hachimantai B	N39°58′17.50″ E140°48′10.88″	1050 m			5

Note: One from larch plantation with the larch sawfly (*Pristiphora erichsonii*) outbreaks (Furano), and the other two from natural beech forest with the beech caterpillar (*Syntypistis punctatella*) outbreaks (Hachimantai). Five soil samples, each contained three sub-samples (ϕ 7.5 cm × 7.5 cm), were collected from each site in August 2013.

and homogenized as described by Saragih *et al.* (2015). Fifteen soil samples from three sites were collected for detection and quantification of *I. fumosorosea* (Table 4).

Standard Curve for Entomopathogenic Fungi in Soil

Soil standard curve was obtained using methods as described by Saragih *et al.* (2015). To create standard soil DNA, sterile fungal suspensions were prepared and mixed with autoclaved soil. As a total 10 levels of suspension using 10-fold serial dilutions from level 1 (lowest) to level 10 (highest) of fungal suspension were prepared. As much as 100 µL of each suspension was placed into 0.5 g autoclaved soil from Furano and extracted using Soil DNA Isolation Kit (MO BIO Laboratories) according to manufacturer's protocol. Fungus quantification was carried out using qPCR with specific primer pairs obtained by specificity tests.

Extraction of Soil DNA and Quantification

Soil DNA was extracted from 0.5 g of soil using an Isolation Kit (MO BIO Laboratories). By using qPCR, the density of entomopathogenic fungi in soil was determined. Quantification was determined using a standard curve generated using 10-fold serial dilutions from 10^1 (lowest density = level 1) to 10^{10} (highest density = level 10) and plotted the *Ct* value against log10 of initial amount of genomic DNA (DNA quantity, ng/µl) (Smith & Osborn, 2009).

RESULTS AND DISCUSSION

Specificity of Primer Design and Sequence Analysis

Eleven specific primer pairs were designed for *I. fumosorosea*. Result showed that the primer IFU5821F (5' ACCACGCAACCCTGCATC 3')/IFU6061R (5' GAAATGACGCTCGAACAGGC 3') was the best primer pair that showed a single peak of melting curve confirming specificity (annealing temperature of 68°C and cycle number of 50) (Figure 1). This primer pair showed 38 hits with *I. fumosorosea* strains with 100% match. Primer dimer also did not occur since all lines showed identical melting peaks. While the other primers showed nonspecific amplification for both *I. fumosorosea* and other fungal species. PCR amplification using DNA extract of *I. fumosorosea* with designed primer pair IFU5821F/IFU6061R showed single amplicon of expected size (221 bp). Similarity searches using the 221 bp sequence with BLAST also showed 100% match with 37 strain of *I. fumosorosea* and 2 strain of *Cordyceps takaomontana* (GenBank: FJ765285, EU807996). No *I. fumosorosea* strains were found with matches less than 100%.

Primer pair IFU5821F/IFU6061R showed 100% match with 38 strain of *I. fumosorosea* after BLAST similarity searches. No non-target species with matches of 100% were detected. BLAST results on PCR sequence product of 221 bp showed 100% match with 36 strain of *I. fumosorosea* and 2 strain of *C. takaomontana* (GenBank: FJ765285, EU807996). *C. takaomontana* has conidial stages called *I. japonica* (Kobayasi, 1981) and *I. japonica* has been reported as a close relative to *I. fumosorosea* based on their clades (Luangsa *et al.* 2005). These two strains were not detected when checking specificity of primer pair IFU5821F/IFU6061R. These indicated that the primer pair IFU5821F/IFU6061R can inclusively detect *I. fumosorosea*. Melting curve with annealing temperature 68°C also showed specificity by creating a single peak for target species and no non-target

species were amplified during qPCR process. This concluded that the primer pair IFU5821F/IFU6061R could be used for specific quantification of *I. fumosorosea* using qPCR.

Standard Curve of qPCR

A linear correlation of *I. fumosorosea Ct* values (ng/g soil) was obtained (slope = -5.0478 and R^2 = 0.989) (Figure 2). However, only four levels (level 5–8) could be used to obtain the standard curve. At level 1 and 2, *Ct* values were not obtained from all replicates. Level 3 and 4 showed great variation of *Ct* values among replicates. Level 9 and 10 also showed great variation in *Ct* values. Amplification efficiency (*E*) was 0.58. The *Ct* value limit for quantification of DNA using this standard soil DNA was 36.93.

Standard soil DNA was created using four levels (levels 5-8) with range of *Ct* value around 20-45. *Ct* value showed great variation among three replications at level 1, 2, 3, and 4, as well as level 9 and in level 10 where *Ct* value was not obtained for all replicates.

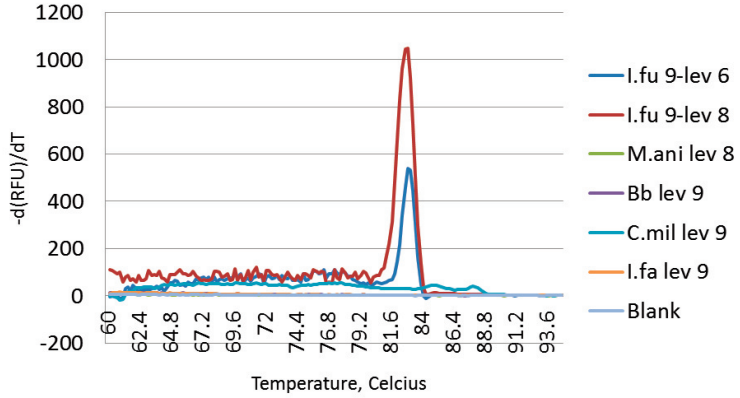

Figure 1. Melting curve of specific primer IFU5821F/IFU6061R for *Isaria fumosorosea* (I.fu) showed a single peak of melting curve that confirmed its specificity (annealing temperature of 68°C and cycle number of 50) and showed negative results for four related species namely *Cordyceps militaris,* (C.mil) *Metarhizium anisopliae* (M.ani), *Beauveria bassiana,* (Bb), and *Isaria farinosa* (I.fa) as well as a control without any non-specific amplification or primer dimer

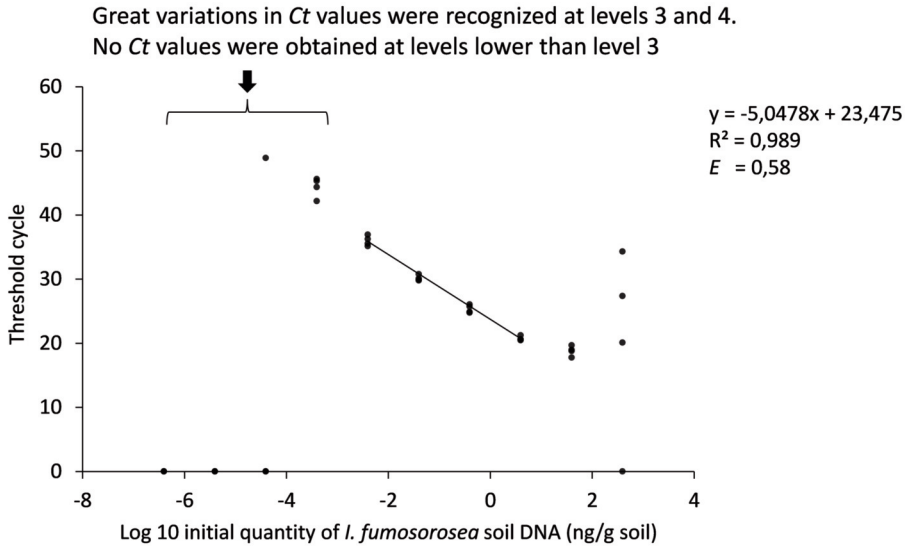

Figure 2. Soil standard curve of *Isaria fumosorosea* (level 5 to level 8) generated by plotting log10 of soil DNA initial quantity (ng/g soil) against *Ct* values; coefficient of determination (R^2) and amplification efficiency (*E*) are shown in the graph

Table 5. Cycle threshold (*Ct*) detection of *Isaria fumosorosea* in soil samples from Furano, Hachimantai A, and B in 2013

No.	Location	Soil Samples	Replications	*Ct*
1	Furano	FU – 01	1, 2 and 3	ND
2	Furano	FU – 02	1, 2 and 3	ND
3	Furano	FU – 03	1, 2 and 3	ND
4	Furano	FU – 04	1, 2 and 3	ND
5	Furano	FU – 05	1, 2 and 3	ND
6	Hachimantai A	HA – 01	1, 2 and 3	ND
7	Hachimantai A	HA – 02	1, 2 and 3	ND
8	Hachimantai A	HA – 03	1, 2 and 3	ND
9	Hachimantai A	HA – 04	1, 2 and 3	ND
10	Hachimantai A	HA – 05	1, 2 and 3	ND
11	Hachimantai B	HB – 01	1, 2 and 3	ND
12	Hachimantai B	HB – 02	1, 2 and 3	ND
13	Hachimantai B	HB – 03	1, 2 and 3	ND
14	Hachimantai B	HB – 04	1, 2 and 3	ND
15	Hachimantai B	HB – 05	1, 2 and 3	ND

Note: ND = Not detected.

Detection of Entomopathogenic Fungi from Soil Samples Using qPCR

Although high density of DNA was extracted from soil, *I. fumosorosea* was not detected from all Furano and Hachimantai soil samples by using qPCR. The *Ct* values of all samples were out of the detection limit for *I. fumosorosea* density in soil (Table 5). These results were possibly caused by low density of the fungi in soil samples that could not be detected using qPCR. There is also a possibility that *I. fumosorosea* could not survive in soil from Furano and Hachimantai. Therefore, soil DNA extraction methods should be improved to increase detection levels of *I. fumosorosea* from soil samples.

CONCLUSION

Results in this study indicated that qPCR method effectively and efficiently detected *I. fumosorosea* in soil by using specific primer pair IFU5821F/IFU6061R. The specific primer pair could be used for specific detection of other entomopathogenic fungi in soil or other ecosystems.

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LITERATURE CITED

Ayala-Zermeño, M.A., Reyes-Montes, M.R., Arroyo-Vázquez, E., Calderón-Ezquerro, M.C., Mier, T., Robledo-Retana, T., & Toriello, C. (2011). An *Isaria fumosorosea* SCAR Marker for Evaluation of Soil, Insect, and Airborne Samples. *Biocontrol Science and Technology*, *21*(9), 1091–1102. https://doi.org/10.1080/09583157.2011.60382

Chemidlin Prévost-Bouré, N., Christen, R., Dequiedt, S., Mougel, C., Lelièvre, M., & Jolivet, C. (2011). Validation and Application of a PCR Primer Set to Quantify Fungal Communities in the Soil Environment by Real-Time Quantitative PCR. *PLoS ONE*, *6*(9), e24166. https://doi.org/10.1371/journal.pone.002416

Dieffenbach, C.W., Lowe, T.M., & Dveksler, G.S. (1993). General Concepts for PCR Primer Design. *Genome Research*, *3*, S30-S37.

Enkerli, J., Widmer, F., Gessler, C., & Keller, S. (2001). Strain-specific Microsatellite Markers in the Entomopathogenic Fungus *Beauveria brongniartii*. *Mycological Research*, *105*(9), 1079–1087. https://doi.org/10.1016/S0953-7562(08)61970-X

Hu, Q., Liu, S., Yin, F., Cai, S., Zhong, G., & Ren, S. (2011). Diversity and Virulence of Soil-dwelling Fungi *Isaria* spp. and *Paecilomyces* spp. against

Solenopsis invicta (Hymenoptera: Formicidae). *Biocontrol Science and Technology*, *21*(2), 225–234. https://doi.org/10.1080/09583157.2010.515733

Kamata, N., Sato, H., & Shimazu, M. (1997). Seasonal Changes in the Infection of Pupae of the Beech Caterpillar, *Quadricalcarifera punctatella* (Motsch.) (Lep., Notodontidae), by *Cordyceps militaris* Link (Clavicipitales: Clavicipitaceae) in the Soil of the Siebold's Beech Forest. *Journal of Applied Entomology*, *121*, 17–21. https://doi.org/10.1111/j.1439-0418.1997.tb01364.x

Kobayasi, Y. (1981). Revision of the Genus *Cordyceps* and its Allies. 1. *Bulletin of the National Science Museum Tokyo, 7*, 1–13.

Landa, Z., Osborne, L., Lopez, F., & Eyal, J. (1994), A Bioassay for Determining Pathogenicity of Entomogenous Fungi on Whiteflies. *Biological Control, 4*(4), 341–350. https://doi.org/10.1006/bcon.1994.1043

Lozano-Contretas, M.G., Maldonado-Blanco, M.G., Elias-Santos, M., Gonzales-Hernandez, A., & Nava-Camberos, U. (2013). Application of *Isaria fumosorosea* Blastospores Produced in Liquid Culture for Control of *Bemisia argentifolii* on Cotton Plants. *Southwestern Entomologist, 38*(1), 57–66. https://doi.org/10.3958/059.038.0113

Luangsa, J.J., Nigel, L., Hywel-Jones, Leka, M., & Robert, A.S. (2005). On the Relationships of *Paecilomyces* sect. *Isarioidea* species. *Mycological Research*, *109*(5), 581–589. https://doi.org/10.1017/s0953756205002741

Meyling, N.V., & Eilenberg, J. (2006). Occurrence and Distribution of Soil Borne Entomopathogenic Fungi within a Single Organic Agroecosystem. *Agriculture, Ecosystems and Environment*, *113*, 336–341. https://doi.org/10.1016/j.agee.2005.10.011

Meyling, N.V., & Eilenberg, J. (2007). Ecology of the Entomopathogenic Fungi *Beauveria bassiana* and *Metarhizium anisopliae* in Temperate Agroecosystems: Potential for Conservation Biological Control. *Biological Control*, *43*, 145–155. https://doi.org/10.1016/j.biocontrol.2007.07.007

Nielsen, C., Jensen, A.B., & Eilenberg, J. (2008). Survival of Entomophthoralean Fungi Infecting Aphids and Higher Flies during Unfavorable Conditions and Implications for Conservation Biological Control. In Ekesi, S., Maniania, N.K. (Eds.), *Use of Entomopathogenic Fungi in Biological Pest Management* (pp. 13–38). Research Sign Post, Kerala, India.

Roberts, D.W., & St. Leger, R.J.S. (2004). *Metarhizium* spp., Cosmopolitan Insect Pathogenic Fungi: Mycological Aspects. *Advances in Applied Microbiology 54*, 1–70. https://doi.org/10.1016/S0065-2164(04)54001-7

Saragih, S.A., Takemoto, S., Hisamoto, Y., Fujii, M., Sato, H., & Kamata, N. (2015). Quantitative Real-Time PCR (qPCR) - Based Tool for Detection and Quantification of *Cordyceps militaris* in Soil. *Journal of Invertebrate Pathology 124*, 70–72. https://doi.org/10.1016/j.jip.2014.11.002

Saragih, S.A., Kusumoto, D., Takemoto, S., Torii, M., & Kamata, N. (2021). Virulence of Fungi Isolated from Ambrosia Beetles to *Acer amoenum* Branches. *Plant Disease*, *105,* 3087–3091. https://doi.org/10.1094/PDIS-11-20-2543-RE

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution 30*, 2725–2729. https://doi.org/ 10.1093/molbev/mst197

Zimmermann, G. (2007). Review on Safety of the Entomopathogenic Fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology*, *17*, 553–596. https://doi.org/10.1080/09583150701309006

Zimmermann, G. (2008). The Entomopathogenic Fungi *Isaria farinosa* (Formerly *Paecilomyces farinosus*) and the *Isaria fumosorosea* Species Complex (Formerly *Paecilomyces fumosoroseus*): Biology, Ecology and Use in Biological Control. *Biocontrol Science and Technology*, *18*(9), 865–901. https://doi.org/10.1080/09583150802471812

Zhou, J., Bruns, M.A., & Tiedje, J.M. (1996). DNA Recovery from Soils of Diverse Composition. *Applied and Environmental Microbiology 62*, 316. https://doi.org/10.1128/aem.62.2.316-322.1996