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Optimization of qPCR for detection and quantification of *Beauveria bassiana* in soil and insect cocoon samples

SYAIFUL AMRI SARAGIH♥, WIDIHASTUTY, AISAR NOVITA, RINI SULISTIANI
Department of Agrotechnology, Faculty of Agriculture, Universitas Muhammadiyah Sumatera Utara. Jl. Kapten Muchtar Basri No. 3, Medan 20238, North Sumatra, Indonesia. Tel.: +62-61-6622400, Fax.: +62-61-6625474, ♥email: syaifulamrisaragih@umsu.ac.id

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Abstract. *Saragih SA, Widihastuty, Novita A, Sulistiani R. 2026. Optimization of qPCR for detection and quantification of* Beauveria bassiana *in soil and insect cocoon samples. Biodiversitas 27 (2): d270231. https://doi.org/10.13057/biodiv/d270231.* The optimization of white muscardine fungus Beauveria bassiana-based biocontrol programs and comprehensive risk assessment remain limited due to the lack of standardized, sensitive, and rapid molecular detection methods capable of accurately measuring *B. bassiana* density in complex environmental matrices. Traditional culture-based detection methods have limitations, including an inability to detect viable but non-culturable fungal states and lengthy incubation periods. In this study, we developed and validated a quantitative PCR (qPCR) assay to detect and quantify *B. bassiana* in soil and insect cocoon samples. Soil samples were collected from a natural beech forest, while insect cocoon samples were collected from dead insects of the larch sawfly, *Pristiphora erichsonii*. The qPCR was performed with a specific primer pair obtained during the specificity test, and the standard curve was used to determine the density of *B. bassiana* in the mixture of DNA extract from the soil and insect cocoon samples. Using ten concentration levels of both genomic DNA and a standard soil DNA, two standard curves were produced. The results showed that the best primer pair among them was BB1962F (5'-GACGGCGTCGGTGTAACGT-3')/BB2156R (5'-GATGTTGTCTGGCGTCTCC-3'). This primer demonstrated specificity by producing a single melting curve peak. The qPCR revealed a standard curve generated with linear correlation with the log10 of *B. bassiana* genomic DNA (slope = -3.6193). The qPCR employing *B. bassiana* soil DNA revealed a linear relationship with log10 of *B. bassiana* dry weight, with a standard curve using *B. bassiana* soil DNA (ng/g soil) (slope = -3.3935). Although *B. bassiana* was not found in all soil samples, it was found in five out of 80 dead cocoon samples. In this study, a specific and quantitative qPCR method for the detection of *B. bassiana* was successfully developed.

Keywords: *Beauveria bassiana* monitoring, biocontrol agents, molecular detection, soil DNA, TEF1α

INTRODUCTION

The white muscardine fungus *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) is an entomopathogenic fungus that can be isolated from various organisms and environments, including insects, soils, and phylloplanes (leaf surfaces) (Zemek et al. 2021; Keçili et al. 2022; Islam et al. 2023). Over 700 arthropod species from a variety of taxonomic orders have been shown to be infected by *B. bassiana* (Goettel et al. 2021). This fungus can survive saprotrophically in soil environments for extended time periods (Sayed et al. 2021; de Oliveira Barbosa Bitencourt et al. 2023; Cappa et al. 2024). According to studies, *B. bassiana* is present in both natural and agricultural ecosystems, greatly assisting in the development of pest management strategies for a variety of crops and habitats (Bamisile et al. 2021; Geremew et al. 2024).

Over the last forty years, scientific research has produced a thorough understanding of the biology, ecology, and use of *B. bassiana* as a biocontrol agent (Dannon et al. 2020; Idrees et al. 2022; Feng et al. 2023). Numerous cryptic species with distinct virulence patterns, host preferences, and environmental adaptations have been identified through molecular phylogenetic analyses (Gasmi et al. 2021; Manfrino et al. 2024). *B. bassiana* has been effectively applied in Indonesia to control various plant pests (Fitriana et al. 2021; Liswandi et al. 2021; Lakshita et al. 2024; Faddilah et al. 2025). While in other countries, such as Japan, *B. bassiana* has been studied as a biological control of *Monochamus alternatus* (Coleoptera: Cerambycidae) (major vector of the pinewood nematode *Bursaphelechus xylophylus* that is the causative agent of pine wilt disease (Shimazu et al. 2002). *B. bassiana* is also considered a mortality agent of the beech caterpillar pupae, *Syntypistis punctatella*, in the soil (Kamata and Kamata 2002). *B. bassiana* was also found on dead larvae and cocoons of the larch sawfly, *Pristiphora erichsonii* (Pinkantayong et al. 2015).

Despite research advances, critical knowledge gaps persist, limiting the optimization of *B. bassiana*-based biocontrol programs and comprehensive risk assessment. Foremost among these is the lack of standardized, sensitive, and rapid molecular detection methods capable of accurately measuring the density of *B. bassiana* in complex environmental matrices, particularly in agricultural soils with high microbial diversity and in insect cocoons at varying stages of decomposition. It is also difficult to accurately measure *B. bassiana* biomass in agricultural soils due to interference from indigenous microorganisms (Ridwan et al. 2025). Some experiments have already been conducted to measure density of *B. bassiana* using traditional culture-based detection methods by counting

colony-forming unit from forest air and soil (Awan et al. 2021; Goettel et al. 2021; Liu et al. 2022; Zhang et al. 2022; Krutmuang et al. 2023; Petkova and Spasova-Apostolova 2024), and the use of the baiting method (insect bait) (Donga et al. 2021; Keçili et al. 2022). However, traditional culture-based detection methods suffer from limitations, such as the inability to detect viable but non-culturable fungal states and lengthy incubation periods of 7-14 days, which are time-consuming (Saragih et al. 2015, 2021a), and the need for large numbers of insects for fungal isolation (Saragih et al. 2021b).

To overcome the limitations of traditional culture-based methods, a culture-independent method was necessary. The quantitative PCR (qPCR) is a culture-independent method based on DNA that is widely used for an accurate and quantitative method of tracking DNA amplification reactions (Wang et al. 2020). qPCR is one of the most popular molecular techniques for its efficiency, accuracy, cost-effectiveness, and user-friendliness, enabling precise measurement of target DNA in various environmental samples (Prencipe et al. 2022). Several studies have reported the use of qPCR for specific detection of microorganisms from environmental samples (Borchardt et al. 2021; Langlois et al. 2021; Zhou et al. 2023). Using qPCR, it will be possible to detect viable but non-culturable fungal states, including indigenous microorganisms, in real time. Therefore, this study aimed to develop and validate a highly specific and sensitive qPCR assay for the absolute quantification of *B. bassiana* in complex environmental matrices, specifically soil and insect cocoons, using a novel primer set targeting the TEF1α gene.

MATERIALS AND METHODS

Research locatation

The entire research activities, including soil and insect cocoon sampling, DNA extraction, sequencing, and qPCR, were completed in October 2025 at The University of Tokyo Forests in Chichibu, Saitama, Japan, and at Universitas Muhammadiyah Sumatera Utara, Medan, Indonesia.

Procedures

Primers design specific to detect Beauveria bassiana

The Molecular Evolutionary Genetics Analysis (MEGA) was used to design eight primers (Tamura et al. 2021), using TEF 1 (Translation Elongation Factor 1) alpha gene region as molecular marker from 9 strains of *B. bassiana* (Table 1). TEF1α was chosen as an appropriate genetic marker due to its high interspecies and low intraspecies sequence divergence, indicating variation across species while being constant within the same species (Meyer et al. 2019). TEF1α was also chosen for its strong species selectivity across fungal taxa and capacity to construct universal primers, making it usable across multiple fungal groups rather than just specific genera (Hoang et al. 2019). The sequence alignment was conducted by first downloading the sequence data from NCBI and uploading it to MEGA. The Clustal W was used to align all data sequences. Then, the sequences that caused gaps or outliers were removed. During primer design, we also considered amplicon size, %GC, absence of secondary structures, and in silico specificity score.

The primer pairs were created using 25 strains of *B. bassiana* (Table 2). The melting temperature (Tm) was raised above 54°C in order to preserve efficiency and specificity (Saragih et al. 2025). The eight primer pairs in the GenBank (National Resource for Molecular Biology Information) nucleotide database were examined for specificity using the Basic Local Alignment Search Tool (BLAST) similarity search. The specificity was checked using *B. bassiana* (isolate F1042), a control, and four similar entomopathogen species, including *Cordyceps militaris* (isolate F1105-1), *Metarhizium anisopliae* (isolate F1035), *Isaria farinosa* (isolate F1075), and *Isaria fumosorosea* (isolate F2223). All isolates were collected from Forestry and Forest Products Research Institute, Tsukuba, Japan. The best primer pair was selected based on several criteria, including melting curve analysis, primer dimer formation, non-specific amplification products, the DNA amplification curve of *B. bassiana*, and BLAST similarity search.

PCR and sequencing

PCR was performed using genomic DNA as described by Saragih et al. (2023). The reaction was carried out in a 0.5-mL tube containing TaKaRa Ex Taq Polymerase 0.1 µL, TaKaRa Ex Taq buffer 2 µL, DNA template 2 µL, primer 0.4 µL for each, dNTP Mixture 1.6 µL, and DNAase-free water to complete a total of 20 µL. The cycling parameters were an initial denaturing step for 3 min at 95°C, followed by 30 cycles of denaturing for 15 sec at 95°C, annealing for 30 sec at 60°C, extension for 30 sec at 68°C, and a final extension for 5 min at 68°C. 10 mL of each reaction mixture were electrophoresed on a 1.5% agarose gel containing ethidium bromide following PCR. A UV transilluminator was used to visualize the bands, and images were captured. Sequencing was conducted to check the success of PCR amplification. PCR primers were used as sequencing primers for automated sequencing of both amplicon strands. After that, the sequence was examined in MEGA, aligned with ClustalW, and compared to the database sequence using BLAST.

Soils and insect cocoons sampling

Fifteen samples of soil were taken from two sites in a natural beech forest in Hachimantai and one site in a Japanese larch plantation in Furano, Japan. These two locations were chosen due to the presence of outbreaks of the beech caterpillar *S. punctatella* and the larch sawfly *P. erichsonii*. We assumed the presence of *B. bassiana* as it infected *P. erichsonii* and *S. punctatella* pupae. Three soil sub-samples were taken from the A0 layer and placed in a sterile plastic bag (Saragih et al. 2015).

Table 1. Eight *Beauveria bassiana* specific primer pairs were created in MEGA using TEF 1 (Translation Elongation Factor 1) alpha gene region. The melting temperature (Tm) was raised above 54°C (Saragih et al. 2025)

Primer ID	Primer sequences (5'-3')	T_m value	% GC	Length (bp)
BB820F	F : TTTCTGATAGATGGATCTCCTG	62	41	180
BB1005R	R : TAGAGGGCAAAGAGGAACATC	62	48	
BB958F	F : CCCGTCTCCCTCCTTATTC	60	58	162
BB1123R	R : TGGAACGACGGCGCGGCA	62	72	
BB1020F	F : TCGCCCTCCACAAGGGTC	60	67	176
BB1195R	R : GCAGAGCAGAGCCAGCGA	60	67	
BB1088F	F : CGACCGCTGCTGCATGAGT	62	63	142
BB1229R	R : GCGGCAGGAGCTGTCACTA	62	63	
BB1491F	F : GTCAACGCTCTCCGTGGGT	62	63	178
BB1668R	R : CAACTTCTCTCCGGACCCG	62	63	
BB1962F	F : GACGGCGTCGGTGTAACGT	62	63	195
BB2156R	R : GATGTTGTCTGGCGTCTCC	60	58	
BB2119F	F : GCGCTTTGAGCAGCCATTAG	62	55	271
BB2289R	R : AAATGGAGACAGTTCTCTTATTTT	62	29	
BB2217F	F : GCGTTCGGCAATCCAGTGG	60	63	204
BB2420R	R : GCAGTTGCTTCTGCTAAAGTC	62	48	

Table 2. Reference sequences from GenBank database to design a specific *Beauveria bassiana* primer using TEF 1 (Translation Elongation Factor 1) alpha gene region

Isolate name	Accession ID
Beauveria bassiana strain ARSEF 2686 EF1 alpha	DQ380135.1
Beauveria bassiana strain Bb9201 EF1 alpha	DQ380137.1
Beauveria bassiana strain IBL 03010 EF1 alpha	DQ380100.1
Beauveria bassiana strain ARSEF 2693 EF1 alpha	DQ380090.1
Beauveria bassiana strain EBCL 99043 EF1 alpha	DQ380129.1
Beauveria bassiana strain ARSEF 3018 EF1 alpha	DQ380102.1
Beauveria bassiana strain IBL 03022 EF1 alpha	DQ380134.1
Beauveria bassiana strain IBL 03034 EF1 alpha	DQ380106.1
Beauveria bassiana strain ARSEF 1831 EF1 alpha	DQ380095.1
Beauveria bassiana strain ARSEF 3440 EF1 alpha	DQ380096.1
Beauveria bassiana strain Bb9205 EF1 alpha	DQ380105.1
Beauveria bassiana strain ARSEF 5446 EF1 alpha	DQ380114.1
Beauveria bassiana strain IBL 03025 EF1 alpha	DQ380109.1
Beauveria bassiana strain ARSEF 1564 EF1 alpha	EF222318.1
Beauveria bassiana strain ARSEF 1966 EF1 alpha	DQ380098.1
Beauveria bassiana strain IBL 03023 EF1 alpha	DQ380127.1
Beauveria bassiana strain IBL 03027 EF1 alpha	DQ380111.1
Beauveria bassiana strain ARSEF 2691 EF1 alpha	DQ380117.1
Beauveria bassiana strain ARSEF 3445 EF1 alpha	DQ380120.1
Beauveria bassiana strain IBL 03028 EF1 alpha	DQ380133.1
Beauveria bassiana strain ARSEF 319 EF1 alpha	DQ380093.1
Beauveria bassiana strain ARSEF 3818 EF1 alpha	DQ380091.1
Beauveria bassiana strain ARSEF 1007 EF1 alpha	DQ380138.1
Beauveria bassiana strain ARSEF 3456 EF1 alpha	DQ380108.1
Beauveria bassiana strain IBL 03030 EF1 alpha	DQ380113.1

In the University of Tokyo Forest in Hokkaido, Japan, ten topsoil samples from each of eight separate Japanese larch plantations were used to collect dead insect cocoon samples of the larch sawfly (*P. erichsonii*). Cocoons of *P. erichsonii* were collected by hand sorting and sieving. Healthy-looking, unopened cocoons (without any shell damage) were placed in nonwoven nylon bags and allowed to overwinter in pots filled with sterile vermiculite (Pinkantayong et al. 2015). Following their overwintering period, these cocoons were removed from the planters and examined to determine whether mycelia were visible on their surface.

Genomic and soil standard curve for Beauveria bassiana

A standard curve was generated using 10-fold serial dilutions of *B. bassiana* genomic DNA, as described by Saragih et al. (2015). Using the method outlined by Saragih et al. (2025), another standard curve was created using soil DNA (standard soil DNA) and the dry weight of *B. bassiana*. Autoclaved soil was combined with a sterile fungal solution to produce standard soil DNA. Before extraction, the fungus was transferred to fresh Potato Dextrose Agar (PDA) plates and cultured for about a week at 25°C. The fungal mycelia and conidia were placed in a 2-mL microtube with 400 µL of free water, and homogenized for six minutes at 3200 rpm using a bead beater-type homogenizer (µT-12, TAITEC CORPORATION). Fungal suspension levels from 1 to 10 were created using ten-fold serial dilutions. Following the manufacturer's instructions, 100 µL of each level of suspension was added to 0.5 g of soil that had been autoclaved and extracted using a MO BIO Laboratories Soil DNA Isolation Kit. The PikoReal Real-Time PCR System software (Product ID # 14-955-184, Thermo Scientific) was used to determine the threshold line and cycle number using the default parameters in the early cycles of the exponentially increasing phase of the PCR amplification process to detect substantial fluorescence signals. The PikoReal Real-Time PCR System software will automatically generate a standard curve by plotting the starting quantity of the fungus and Ct values. The software will produce a regression line (standard curve) with a given slope and y-intercept.

DNA extraction from soil and insect cocoon samples

DNA extraction from soil and insect cocoon samples was performed at the University of Tokyo Forest in Chichibu, Saitama, Japan. For soil samples, 0.5 g of each soil sample was used to extract soil DNA using the Soil DNA Isolation KIT (MO BIO Laboratories).

For insect cocoon samples, direct DNA extraction was performed by placing the insect cocoon containing fungal mycelia in a 2-mL microtube with 500 µL of free water, then shaking for 6 minutes at 3200 rpm using a bead beater-type homogenizer. The DNA in the suspension was then extracted using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's protocol. The extracted DNA was then stored at -30°C before use.

Quantification of the Beauveria bassiana *density in the soil and insect cocoon samples by qPCR*

qPCR was used to detect and quantify *B. bassiana* in soil and insect cocoon samples using a pooled DNA extract from all samples. qPCR was performed with a specific primer pair obtained during the specificity test, along with a standard curve, to determine the density of *B. bassiana* in the samples. The qPCR was performed following Saragih

et al. (2015) in a 20 µL reaction volume using a 96-well plate and a PikoReal Real-Time PCR System (Thermo Scientific), containing SYBR Green PCR Master Mix (10 µL), template DNA (2 µL from each soil and insect cocoon sample), and each primer (4 µL). The cycling parameters were an initial denaturation step for 7 min at 95°C, followed by 50 cycles of denaturation for 10 sec at 95°C, annealing for 30 sec at 67°C, extension for 20 sec at 68°C, and a final extension for 30 sec at 60°C. The melting curve ranged from 60 to 95°C. The Ct value for each sample reaction will be measured by the qPCR machine's software as the cycle number at which the fluorescence signal crosses the background threshold. The quantity of *B. bassiana* in the samples was determined by converting Ct values using the slope and intercept of the standard curve.

RESULTS AND DISCUSSION

Beauveria bassiana primer specificity and sequencing analysis

The primer pair BB1962F (5'-GACGGCGTCGGTGTAACGT-3')/BB2156R (5'-GATGTTGTCTGGCGTCTCC-3') was selected to be the best primer pair out of all tested primers. This primer demonstrated specificity by producing a single melting curve peak at 67°C for an annealing temperature of 50 cycles (Figure 1.A). A thorough melting curve analysis showed the specificity and dependability of the proposed quantitative real-time PCR test for *B. bassiana* detection. After 50 cycles of amplification, the melting curve was analyzed using continuous fluorescence collection and a temperature ramp from 60°C to 95°C at 0.3°C per second. Accurate amplicon melting temperature measurement and high-resolution melting transition detection are made possible by this progressive temperature increase. *B. bassiana* amplicons generated with the BB1962F/BB2156R primer pair exhibit a single, distinct melting peak in the derivative melting curve (Figure 1). The peak maximum in the derivative figure indicates that the melting temperature (Tm) of the particular amplicon is about 86°C (Figure 1.A). A single dominant melting peak is a vital indicator of amplification specificity, suggesting that the primers amplify only the intended target sequence without generating multiple products of varying lengths or compositions (Xu et al. 2024). Non-specific amplification, on the other hand, is usually characterized by several peaks at different temperatures or a broad, irregular melting profile caused by a heterogeneous combination of amplification products (Ruiz-Villalba et al. 2017). The absence of non-specific amplification, including four similar entomopathogen species, *C. militaris, M. anisopliae, I. farinosa*, and *I. fumosorosea* in the observed melting curves clearly supports the specificity of the BB1962F/BB2156R primer pair for detecting *B. bassiana*. In addition, the melting curve showed consistency, indicating that the primers maintain specificity across the assay's dynamic range, from high to low template concentrations, which is necessary for producing standard curves and precise quantification of unknown samples.

Moreover, the existence of possible primer dimer formation and non-specific amplification products is a crucial part of the melting curve study for checking specificity (Tokamani et al. 2023; Yusuf et al. 2024; Fröder et al. 2025). At temperatures lower than the main amplicon peak, primer dimers, which normally melt at lower temperatures (usually 70-78°C depending on primer design), would show up as separate peaks in the derivative plot. The result showed that the BB1962F/BB2156R primer pair does not create primer dimers under the ideal reaction conditions (Figure 1). Similarly, based on their sequence composition and length, non-specific amplification products from non-target species including four similar entomopathogen species, *C. militaris, M. anisopliae, I. farinosa*, and *I. fumosorosea*, would normally produce extra melting peaks at temperatures distinct from the target amplicon. For field applications in which several fungal species may coexist in natural settings, this result is crucial. The solid peak in the clean melting profile indicates that non-target sequences are not amplified by the primers (Xu et al. 2024). For applications requiring complex matrices, such soil DNA extracts or insect tissue samples, where various microbial communities or host DNA may serve as templates for non-specific amplification, this specificity is essential.

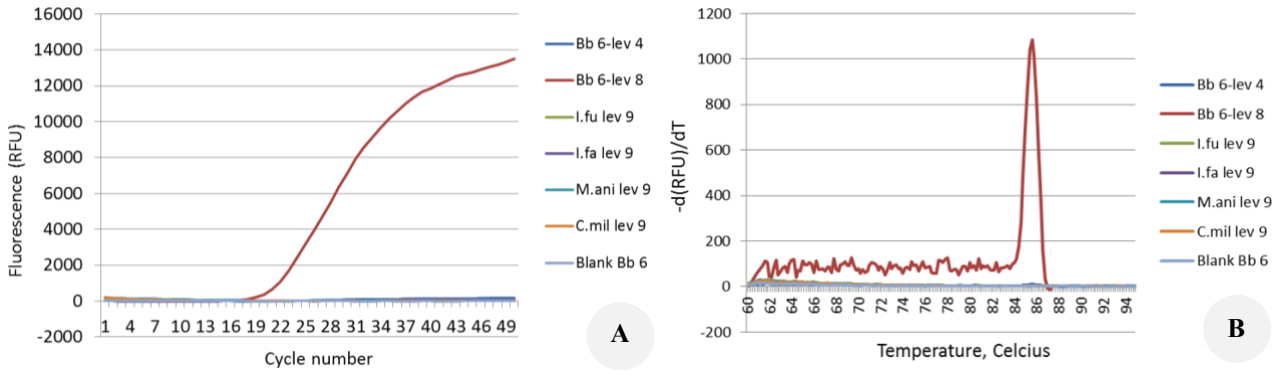

Figure 1. A. Melting curve of *Beauveria bassiana* using primer BB1962F / BB2156R showing a single and distinct melting temperature (Tm) peak of 86°C, B. qPCR amplification curve plotting cycle number and normalized fluorescence showing exponential DNA amplification of *Beauveria bassiana* without any DNA amplification of other entomopathogenic fungi as well as primer dimers

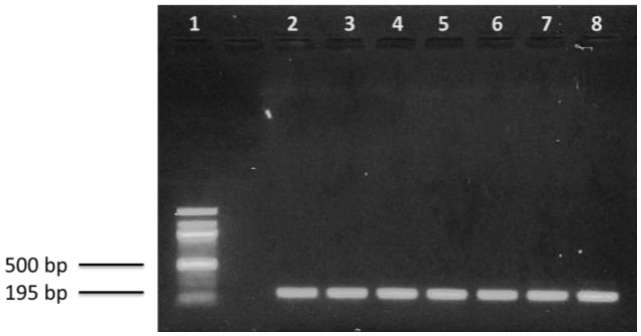

Figure 2. Eight TEF1 fragments of *Beauveria bassiana* samples on gel post electrophoresis showing the amplification of genomic DNA of *Beauveria bassiana* using 2 µL of PCR product with primer pair BB1962F and BB2156R that generated single PCR product of 195 bp. 1: Standard marker, 2-8: *Beauveria bassiana*

The specificity of the primer was also further validated by the exponential DNA amplification curve of *B. bassiana* (Figure 1.B). The area where PCR functions most efficiently is known as the exponential amplification phase across multiple cycles, which is represented by the sharp, almost vertical rise in fluorescence that starts at various cycle numbers. Assuming 100% efficiency, the amount of amplicon theoretically doubles with each cycle during this phase, leading to a logarithmic increase in product accumulation (McNair et al. 2025; Ruijter and van den Hoff 2025). Non-specific amplification products and primer dimers would usually produce curves with unusual morphologies, such as early-cycle amplification with gentle slopes, irregular exponential phases, or curves that do not reach standard plateau fluorescence levels (McNair et al. 2025). The clean and uniform appearance provide evidence that the BB1962F/BB2156R primer pair specifically amplifies the intended *B. bassiana* target sequence without producing off-target products (Figure 1.B). The specificity was also confirmed by the absence of contamination.

The quality of melting curve profiles directly affects the Limit of Detection (LOD) and test sensitivity (Ferreira et al. 2021). This study's distinct, sharp melting peaks show that amplicons with consistent melting properties can be produced from materials with low template concentrations. The preservation of melting peak integrity throughout the dynamic range of the assay indicates that the qPCR instrument's amplification efficiency and fluorescence detection sensitivity, rather than non-specific amplification or primer artifacts, are the main factors controlling the detection limit. This feature enables confident identification and quantification of *B. bassiana*, even in samples with extremely low fungal biomass, which is crucial for tracking the low-level persistence of applied biocontrol formulations in agricultural soils or for early detection of natural infections in field-collected insects. The stability of melting temperatures across repeated dilutions gives assurance that amplification efficiency stays constant throughout the quantification range for quantitative applications using standard curves. Any variation in Tm across samples or standards may indicate template degradation, PCR inhibition, or other factors that could compromise quantification precision (Huang et al. 2024). The result showed the distinctive melting profile of B. bassiana, which can be used to distinguish it from other closely related *Beauveria* species or entomopathogenic fungi.

The computational primer design approach used to create the BB1962F/BB2156R primer pair is validated by the successful melting curve profiles. To balance the conflicting demands of amplification efficiency, specificity, and resolution in melting curve analysis, the primers were designed to produce an amplicon of the ideal length (195 bp, based on primer designations) for qPCR applications (Figure 2). It appears that both primers bind precisely to their intended target sites without forming hairpins, self-dimers, or cross-dimers that could interfere with target amplification, as there are no secondary structures or alternative amplification products. This shows that primer sequences, concentrations, and annealing temperatures were successfully evaluated in silico and empirically optimized during assay development. The primer pair's high specificity, as evidenced by single-peak melting curves, stable performance across template concentrations, and the absence of artifacts, makes it ideal for environmental detection applications.

Furthermore, result of BLAST similarity searches on 195 bp sequences also showed match with strains of *B. bassiana,* with 100% match with three strains, 99% match with three strains, and 98% match with 36 strains. No non-target species was detected with 98% match or higher. This suggests that the BB1962F/BB2156R primer combination is specific to *B. bassiana*. The sequence also showed 95% match with one strain of *B. brongniartii* (Accession: DQ380139.1). It has been stated that *B. brongniartii* is an anamorph of *Cordyceps brongniartii* (Das et al. 2023; Tello-Salgado et al. 2025). Within the anamorphic genus *Beauveria*, *B. bassiana* and *B. brongniartii* are recognized as separate species and have been phylogenetically and developmentally connected to several Cordyceps species as their teleomorphs (Das et al. 2023). According to phylogenetic analysis, strains of *B. brongniartii* and *B. bassiana* form a sister clade, suggesting that they are not different species but rather evolutionary differences (Wang et al. 2022). However, according to the original description, the teleomorph-anamorph association of *C. brongniartii* was documented in Korea, validating the anamorphic nature of *B. bassiana* (Tello-Salgado et al. 2025). This suggests that there is a possibility that *B. brongniartii* (Accession: DQ380139.1) that showed 95% match to *B. bassiana* used in this study, was the same species.

Standard curve for quantification of *Beauveria bassiana*

The quantitative capability of the developed real-time PCR test for *B. bassiana* detection was demonstrated by the creation and validation of standard curves using both pure genomic DNA (Figure 3) and soil-spiked DNA samples (Figure 4). By establishing the mathematical relationship between cycle threshold (Ct) values and known quantities of target DNA, these calibration curves serve as the basis for accurately quantifying fungal biomass in the tested samples. The qPCR standard curve, generated from repeated dilutions of pure *B. bassiana* genomic DNA, is shown in

Figure 3. The qPCR revealed a standard curve with a linear correlation between the corresponding Ct values and the log10 of *B. bassiana* genomic DNA (slope = -3.6193). This standard curve covers a dynamic range of five orders of magnitude (from level 4 to 8): level 8, which represents the largest concentration of DNA, yields Ct values of roughly 15-16 cycles, while level 4, which represents the lowest concentration, yields Ct values close to 33-35 cycles. The Ct values for replications at levels 1, 2, and 3 (the lowest level) varied greatly. These were associated with a low fungal density. The Ct value among the three replications varied greatly at levels 9 and 10. It could result from a high DNA density. For genomic DNA, the amplification Efficiency (E) was 0.89. Using this standard genomic DNA, the Ct value limit for DNA quantification was 34.39. The angle of the regression line can be used to visually determine the slope of the standard curve in Figure 3, which seems to be close to the optimal value for qPCR tests. The theoretical slope is -3.322 for a completely efficient PCR reaction in which the template doubles with each cycle (100% efficiency) (McNair et al. 2025). Usually, acceptable slopes fall between -3.1 and -3.6 (McNair et al. 2025). This acceptable range appears to be covered by the regression line in Figure 3, indicating that the BB1962F/BB2156R primer pair amplifies *B. bassiana* genomic DNA with a high and reliable efficiency. Figure 3 also shows a near-ideal slope, confirming that the assay is very efficient across the whole concentration range evaluated. In the end, quantitative analysis should focus on samples having Ct values within the approved linear range (levels 4-8) to ensure accuracy.

The y-intercept of the standard curve (Figure 3), which represents the theoretical Ct value is around 33-35 cycles based on regression line extrapolation. This y-intercept indicates the assay's theoretical sensitivity and the link between Ct values and absolute copy counts. A higher y-intercept suggests that fewer template quantities are required to achieve a given Ct value, indicating more assay sensitivity (Lindrose et al. 2021). The standard curve in Figure 3 shows outstanding repeatability, with the Coefficient of Variation (CV%) for repeated Ct values at each concentration level appearing to be negligible due to tight clustering of data points. To ensure high-quality qPCR experiments, the CV% of repeat Ct values should be less than 1% at high template concentrations and 2-3% at concentrations near the limit of detection (Zhao et al. 2021). The visual representation indicates that these conditions are met, suggesting that the assay yields highly repeatable results when applied to pure genomic DNA templates free of matrix effects or inhibitors. The regular spacing between concentration levels, with each 10-fold dilution producing about 3.3 cycles of Ct difference, supports the logarithmic relationship and validates the assay's quantitative accuracy across the dynamic range. Deviations from this regular spacing imply concentration-dependent variations in amplification effectiveness or nonlinearity in the dose-response relationship, neither of which is visible in Figure 3.

Additionally, Figure 4 shows a standard curve employing *B. bassiana* soil DNA using qPCR with seven concentration levels (levels 3-9), which represents a greater dynamic range than the pure genomic DNA standards in Figure 3. The assay's expanded range of roughly 10^3 to 10^9 spore equivalents (or similar quantification units) demonstrates its ability to measure *B. bassiana* in soil samples accurately. The Ct values range from 12-14 cycles at the highest concentration (level 9) to 37-38 cycles at the lowest concentration (level 3). This standard curve revealed a linear relationship with log10 of *B. bassiana* dry weight with a standard curve using *B. bassiana* soil DNA (ng/g soil) (slope = -3.3935) (Figure 4). The standard soil DNA amplification efficiency was 0.97. The Ct value among replications varied between levels 1, 2, and 10.

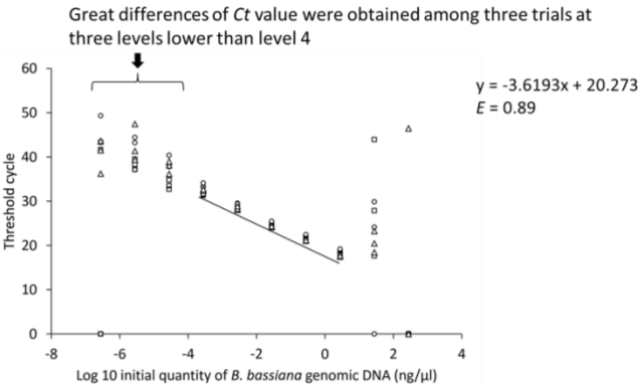

Figure 3. 10-fold serial dilutions of *Beauveria bassiana* genomic DNA that generated a standard curve by plotting log 10 of the quantity and the threshold cycle of Ct value using qPCR (ng/µL). The standard curve generated five orders of magnitude (from level 4 to 8) to create a regression line. The Ct values for replications at levels 1, 2, and 3 (the lowest level) and levels 9 and 10 (the highest level) varied greatly

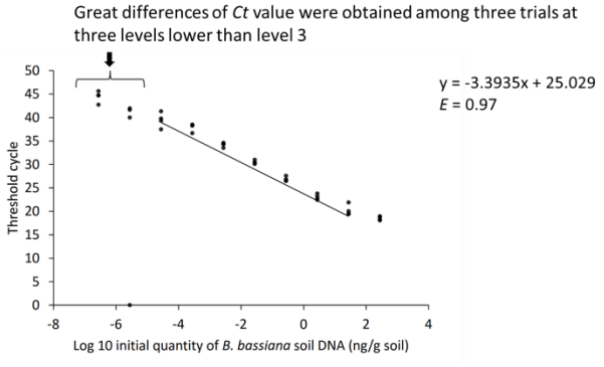

Figure 4. Standard curve of soil DNA showing the plotting of log 10 dry weight of *Beauveria bassiana* in soil and Ct value from level 3 to 9 (seven levels) (ng/g soil). The soil at Furano was autoclaved to extract the typical soil DNA, and then 10-fold dilutions (ranging from 10^1 to 10^{10}) of *Beauveria bassiana* solution were added. The Ct values for replications at levels 1 and 2 (the lowest level) and levels 10 (the highest level) varied greatly

Figure 4 shows a more environmentally relevant validation of the qPCR experiment using soil-spiked standards. The experiment involved autoclaving soil from Furano to eliminate indigenous microbial DNA while preserving the physical and chemical complexity of the soil matrix. The soil was then spiked with known quantities of *B. bassiana* solution across a 10-fold dilution series ranging from 10^1 to 10^{10} spores or cells. This method generates standards that include not only the target fungal DNA, but also soil components that are generally extracted during DNA isolation and may interfere with PCR amplification. Humic acids, fulvic acids, phenolic compounds, heavy metals, and other organic and inorganic substances can inhibit DNA polymerase activity, chelate essential magnesium ions, or interfere with primer-template annealing (Duff et al. 2025; Jeon and Yan 2025). Figure 4 validates the qPCR assay's quantitative performance even in the presence of soil-derived inhibitors, which is critical for accurate quantification of *B. bassiana* in real agricultural or environmental soil samples. Target not only fungal DNA, but also soil components that are often co-extracted during DNA isolation and may interfere with PCR amplification.

The ability to detect and quantify *B. bassiana* at level 9, producing Ct values near 37-38 cycles, is particularly noteworthy as it demonstrates that the assay maintains sensitivity even in soil matrix with low fungal loads. However, Ct values approaching 38-40 are generally considered near or at the limit of reliable quantification for most qPCR assays. Nevertheless, the inclusion of these low-concentration standards provides confidence that the assay can detect even minimal levels of *B. bassiana* in soil samples. This linearity shows that even when co-extracted soil components are present, the connection between log (DNA amount) and Ct values stays constant. The preservation of linearity indicates that either soil-derived compounds are successfully eliminated during DNA extraction and purification, or they are present at constant concentrations across dilutions, so that any inhibitory effects are proportional and do not affect samples with different concentrations of fungal DNA differently. The assay's ability to identify even low amounts of *B. bassiana* in soil samples is ensured by these low-concentration standards.

The slope of the standard curve in Figure 4 is comparable to that in Figure 3, indicating that the soil matrix does not affect amplification efficiency. This similarity indicates that the DNA extraction procedure effectively eliminates or dilutes PCR inhibitors to levels that do not affect amplification kinetics. The slope would be predicted to become increasingly negative (efficiency <90%) and possibly exhibit nonlinearity with increased inhibition at higher soil component concentrations if inhibition were present. Important information regarding matrix effects and assay robustness can be found by comparing Figures 3 and 4. When measuring pure genomic DNA or DNA taken from spiking soil, both standard curves show identical slopes and linearity, indicating that amplification efficiency is similar. Nonetheless, there are minor variations worth noting. The soil matrix standard curve (Figure 4) shows a slightly larger dynamic range at lower concentrations (level 9 vs. level 8). However, this is likely due to experimental design rather than underlying variations in assay performance inhibition at higher concentrations of soil constituents. Due to the different quantification scales used (pure genomic DNA quantity in Figure 3 vs. spore equivalents in spiked soil in Figure 4), the y-intercepts of the two curves may differ slightly, making it difficult to compare absolute sensitivity directly without knowing more about the relationship between spore number and extractable genomic DNA quantity. Regardless of sample origin, the assay yields accurate quantitative measures, as evidenced by consistent Ct values at similar template levels across both matrices.

The assay maintains good reproducibility even when applied to soil samples, as seen by the replication pattern in Figure 4, where many data points are clustered at each concentration level. Given the added variability brought forth by the soil matrix, DNA extraction procedure, and possible heterogeneity in fungal distribution within soil samples, the dispersion of replicate points seems larger than in Figure 3. The test is appropriate for quantitative applications in soil samples; nevertheless, the CV% for replicate Ct values seems to remain within reasonable bounds (<5% for the majority of concentration levels), allowing quantitative measurements independent of the sample source. With no obvious outliers or non-conforming data points, the results were consistent throughout the seven concentration levels, indicating that the soil spiking process produced homogenous samples with uniform fungal distribution. The uneven distribution of fungal spores or mycelium in soil could introduce sample error, which would show up as high variability in replicate data, making this validation crucial. The observed reproducibility indicates that representative samples are produced by the sample preparation procedure, including soil spiking, mixing, and subsampling for DNA extraction.

When measuring *B. bassiana* in unidentified soil samples taken from forest ecosystems, agricultural areas, or other environmental settings, the standard curve shown in Figure 4 is an essential tool. This standard curve's extended dynamic range of 10^3 to 10^9 spore equivalents allows for quantification in a variety of ecological contexts, from soil that has very low background levels of naturally occurring *B. bassiana* (potentially 10^3-10^9 spores/g) to soil that has been heavily amended with biocontrol formulations or is close to infected insect cadavers (potentially 10^3-10^0 spores/g). Because most environmental samples are expected to fall within this established range, this flexibility removes the requirement for substantial sample dilution optimization. The effectiveness and consistency of the DNA extraction procedure are indirectly validated by the successful creation of a linear standard curve utilizing soil-spiked samples (Figure 4). The standard curve would exhibit nonlinearity or a decreased slope if the effectiveness of DNA extraction varied with fungal load or if soil components impeded it. The extraction procedure recovers fungal DNA with consistent efficiency across the concentration range, enabling accurate quantification, as evidenced by the observed linearity and a suitable slope. The standard curves shown in Figures 3 and 4 show that *B. bassiana* can be reliably,

sensitively, and quantitatively detected using the proposed qPCR assay throughout a wide dynamic range of template concentrations. The assay is validated for quantitative environmental monitoring applications by its good linearity and suitable amplification efficiency (slope ~-3.3), and reliable performance in both pure DNA and complex soil matrices. Without requiring substantial dilution, the expanded dynamic range spanning 5-7 orders of magnitude allows quantification from low-level ambient levels to high-density biocontrol applications, as well as the specificity demonstrated by melting curve and amplification profile analysis.

The qPCR for detection of *Beauveria bassiana* from soil and insect cocoon samples

Three study locations in natural ecosystems, a Japanese larch plantation in Furano, Japan, and a beech forest in Hachimantai, were used to gather soil samples (Table 3). Each of the fifteen soil samples, five from each site, represented a different spatial location within the research areas. Interestingly, none of the soil samples taken from these three locations contained *B. bassiana* (Table 3). Fungal DNA was either absent or below the detection threshold in all samples, as indicated by Ct values exceeding the assay's quantification limit. Understanding the natural distribution and ecology of *B. bassiana* in temperate forest habitats is impacted by this unfavorable outcome from soil surveys. The lack of detectable *B. bassiana* in soil samples from the larch plantation and beech forest indicates that either: (i) the fungus is not naturally present at these locations; (ii) the fungal biomass is very low and below the assay's sensitivity threshold which prevented qPCR detection; or (iii) the fungal spores or mycelium may not persist in soil at levels detectable by qPCR. Given that *B. bassiana* is predominantly an insect pathogen. Since its presence in soil is frequently fleeting, residing mostly as latent spores or mycelium associated with infected insect cadavers rather than as a free-living soil dweller, the latter hypothesis is especially pertinent. On the other hand, the qPCR sensitivity was possibly reduced by the soil at each dilution stage. The assay's sensitivity appears to be influenced by soil type and extraction procedure (Sidstedt et al. 2020; Lee et al. 2025).

However, in order to find *B. bassiana*, a more targeted approach was taken by studying dead insect cocoons from the larch sawfly *P. erichsonii*, a known host of the fungus. The qPCR detected *B. bassiana* on five samples out of 80 insect cocoon samples from three of the eight sites (Table 4). This result is in line with a previous study that reported *B. bassiana* was isolated from dead larvae and cocoons of the larch sawfly, *P. erichsonii*, in Furano (Pinkantayong et al. 2015). This result implied that *B. bassiana* may be detected in soil samples from Furano, suggesting a patchy but present spread of the fungus within the larch plantation ecosystem. The distribution pattern showed several findings. There was no evidence of *B. bassiana* in cocoons at the remaining five locations (1, 4, 5, 6, and 8), showing 0% prevalence at these five sites (Table 4).

On the other hand, Site 2 produced two positive cocoons from ten samples (20% prevalence), and Site 7 also produced two positive cocoons from ten samples (20% prevalence) (Table 4). With one positive cocoon out of ten samples, Site 3 demonstrated intermediate detection (10% prevalence). These findings show that the prevalence of *B. bassiana* is not evenly distributed throughout the Furano study region. Of the 80 cocoons analyzed, 5 were positive at all 8 sites (6.25% overall infection rate). However, when compared to baseline predictions for naturally occurring entomopathogenic fungi in temperate forest habitats, the 6.25% overall detection rate indicates a modest level of fungal presence. This prevalence is high enough to imply ecological interactions between populations of *P. erichsonii* and *B. bassiana*, suggesting that the fungus uses natural infection pathways to exert mortality pressure on sawfly hosts. Beauveria bassiana's emergence as an endemic disease rather than a random or unintentional colonizer of larch sawfly populations is further supported by its constant identification across multiple sites.

Instead of random diffusion or uniform distribution throughout all surveyed sites, *B. bassiana* detection shows a clear geographic grouping. Sites 1, 4, 5, 6, and 8 comprise the majority of the study sites; sites 2, 3, and 7, which produced positive detections, are scattered around the study territory. *B. bassiana* populations appear to be localized in particular geographic zones based on this clustering pattern, which may indicate local environmental circumstances or ecological traits that support fungal establishment and survival. The uneven distribution suggests that some isolated habitats or microclimatic zones within Furano offer better conditions for *B. bassiana* colonization of sawfly cocoons than others. *B. bassiana*'s varied geographic distribution throughout the eight study locations has an impact on comprehending the potential for natural biocontrol and creating management plans for Furano's larch sawfly infestations. The quantity of fungal cocoons seen at sites 2 and 7—both of which are hotspots of natural infection—indicates that *B. bassiana* may naturally reside in or originate from these regions. Knowing what makes these high-prevalence places unique could help with ecological restoration initiatives or focused conservation biocontrol tactics that encourage the emergence of natural enemies in currently unfavorable areas. On the other hand, the absence of *B. bassiana* at half of the investigated sites may be due to sampling constraints, temporal factors, or recent events affecting local fungal populations, rather than necessarily indicating adverse conditions for fungal colonization.

Table 3. Detection of *Beauveria bassiana* from soil samples taken from a natural beech forest in Hachimantai and a Japanese larch plantation in Furano, Japan

Site number	Location	Number of soil samples	Total samples contained *Beauveria bassiana*
1	Hachimantai	5	0
2	Hachimantai	5	0
3	Furano	5	0

Table 4. Detection of *Beauveria bassiana* from dead insect cocoons of the larch sawfly *Pristiphora erichsonii* at eight research sites in Furano

Site number	Number of tested cocoons	Total cocoons contained *Beauveria bassiana*	Percentage of infection (%)
1	10	0	0
2	10	2	20
3	10	1	10
4	10	0	0
5	10	0	0
6	10	0	0
7	10	2	20
8	10	0	0

Out of the 50 cocoons that were sampled from all eight research sites, five of them produced no detectable *B. bassiana*. This lack could be the result of multiple non-exclusive mechanisms: (i) actual lack of *B. bassiana* in these geographic zones; (ii) unfavorable environmental conditions for fungal colonization and survival; (iii) temporal factors where sampling took place during times of low fungal transmission or prevalence; or (iv) limitations in detection methodology, such as inadequate sample size or unfavorable storage and processing conditions affecting fungal viability. In contrast to the positive detections at three sites, the total absence at five sites indicates that there is actual regional heterogeneity in fungal presence throughout the Furano region rather than random fluctuation caused by sampling artifacts.

Detection of *B. bassiana* from *P. erichsonii* cocoons showed that the entomopathogenic fungus actively colonizes Furano's larch sawfly populations, although with relatively limited prevalence. *B. bassiana* has successfully penetrated host defenses and established mycelial development within protected microhabitats, as evidenced by the fungus's presence in cocoons, constructs intended to shield developing pupae during overwintering and pre-eclosion phases. Since successful colonization of cocoon interiors involves efficient spore adherence, cuticle penetration, and nutrition intake within the host resource, our study supports active host-pathogen interaction rather than merely incidental ambient contamination.

Beauveria bassiana naturally occurs in larch sawfly cocoons, as evidenced by the discovery of five infected cocoons. This suggests that the pathogen plays a role in *P. erichsonii* population regulation and natural mortality. When paired with the effects of other natural enemies (parasitoids, predators), fungal-induced mortality may contribute to density-dependent population reduction, even at the reported prevalence of 6.25%. *B. bassiana*-mediated mortality may be sufficiently prominent in these geographic zones to affect local population dynamics and epidemic potential, according to the clustering of infections at particular places (2, 3, 7). Sites 1, 4, 5, 6, and 8, on the other hand, may lack this aspect of natural enemy-mediated mortality, potentially making local sawfly populations more vulnerable to rapid growth.

The successful detection of *B. bassiana* from cocoons demonstrates sufficient preservation of fungal vitality throughout sampling, transport, and processing and validates the effectiveness of qPCR. However, these methodological limitations imply that the 6.25% overall detection rate is not a conclusive assessment of *B. bassiana* prevalence, but rather a minimal estimate.

In contrast to its presence in insect cocoons, *B. bassiana* was not detected in soil samples, indicating that the fungus's ecological niche is closely linked to insect hosts rather than being a generalist soil dweller. The ecology of B. bassiana, which specializes in infecting arthropods and depends on insect hosts for reproduction and dissemination (Kovač et al. 2020; Yerukala et al. 2022; Gálvez et al. 2023), is consistent with this trend. The fungus appears to exist in soil mainly as temporary propagules connected to infect insect remnants, and soil is an unfavorable habitat for long-term persistence. Compared with clean DNA samples, soil samples contain a variety of organic and inorganic compounds that may impede PCR amplification, leading to lower efficiency and higher Ct values. It is crucial to remember that DNA extraction efficiency and qPCR performance both affect absolute quantification accuracy. The real fungal biomass in soil samples will be underestimated if the extraction efficiency is less than 100%, which is usually the case, unless the extraction efficiency is specifically calculated and taken into account. To ascertain extraction efficiency and correction factors, future research should involve recovery experiments using soil spiked with known amounts of *B. bassiana* spores, followed by improvements to DNA extraction processes or the incorporation of sample concentration techniques and qPCR quantification to increase detection sensitivity in soil. Samples with Ct values between 35 and 38 should be handled cautiously for environmental monitoring applications and may require verification by repeat analysis or other detection techniques (e.g., conventional PCR with sequencing). Although samples with Ct > 38 are typically considered below the reliable quantification limit, if melting curve analysis verifies amplification specificity, they can still indicate the presence of the target organism. The significant fluctuations in Ct values at extreme dilutions suggest that measurements should be limited to the validated linear range (levels 4-8). This precaution prevents incorrect results from samples with extremely low or very high levels of fungal DNA. Furthermore, the establishment of soil-specific standards was required for accurate detection in soil samples, emphasizing the value of matrix-matched controls in environmental qPCR research.

In conclusion, the BB1962F/BB2156R primer pair produces reliable, specific, and efficient amplification of *B. bassiana* genomic DNA throughout a wide dynamic range of template concentrations. The typical sigmoidal curve morphology, steady exponential-phase kinetics, and systematic Ct distribution across the dilution series all verify this qPCR assay's performance for quantitative applications. These findings, along with the melting curve analysis, demonstrate amplification specificity. A specific and quantitative qPCR methodology for *B. bassiana*

detection was also successfully developed, and its application to natural environmental samples was proven. The fungus was found in *P. erichsonii* cocoons at a 6.25% infection rate, indicating its presence and ecological activity in larch plantation ecosystems. The lack of discovery in soil samples underscores the host-dependent interactions of this entomopathogenic fungus. This study provides a solid foundation for using this molecular detection system in biocontrol efficacy studies, environmental fate assessments, and epidemiological investigations of *B. bassiana* in natural and managed ecosystems. Future studies on the ecological distribution, population dynamics, and the survival of imported fungal strains, as well as the ecological impact on target and non-target insects and the biocontrol potential of *B. bassiana* in wild and managed forest habitats, can build on the qPCR approach developed here. Future studies can also determine the factors influencing geographic variability in fungal occurrence, the environmental variables influencing infection dynamics, and the effectiveness of *B. bassiana* in integrated pest control programs for larch sawfly and other forest pests.

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