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Research article

Discovery of a new primer set for detection and quantification of *Ilyonectria mors-panacis* in soils for ginseng cultivation

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ABSTRACT

Background: Korean ginseng is an important cash crop in Asian countries. However, plant yield is reduced by pathogens. Among the *Ilyonectria radicicola*-species complex, *I. mors-panacis* is responsible for root-rot and replant failure of ginseng in Asia. The development of new methods to reveal the existence of the pathogen before cultivation is started is essential. Therefore, a quantitative real-time polymerase chain reaction method was developed to detect and quantify the pathogen in ginseng soils.
Methods: In this study, a species-specific histone H3 primer set was developed for the quantification of *I. mors-panacis*. The primer set was used on DNA from other microbes to evaluate its sensitivity and selectivity for *I. mors-panacis* DNA. Sterilized soil samples artificially infected with the pathogen at different concentrations were used to evaluate the ability of the primer set to detect the pathogen population in the soil DNA. Finally, the pathogen was quantified in many natural soil samples.
Results: The designed primer set was found to be sensitive and selective for *I. mors-panacis* DNA. In artificially infected sterilized soil samples, using quantitative real-time polymerase chain reaction the estimated amount of template was positively correlated with the pathogen concentration in soil samples ($R^2 = 0.95$), disease severity index ($R^2 = 0.99$), and colony-forming units ($R^2 = 0.87$). In natural soils, the pathogen was recorded in most fields producing bad yields at a range of 5.82 ± 2.35 pg/g to 892.34 ± 103.70 pg/g of soil.
Conclusion: According to these results, the proposed primer set is applicable for estimating soil quality before ginseng cultivation. This will contribute to disease management and crop protection in the future.
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1. Introduction

Korean ginseng (*Panax ginseng*), a member of the Araliaceae family, is a well-known medicinal plant in Asian countries. The roots are commonly used in traditional Asian medicine for many pharmaceutical purposes [1–3]. High-quality ginseng roots need to grow for 4–6 yrs. Because of this long cultivation period, the roots are exposed to soil-borne pathogen infections, which lead to a reduction in root quality and loss of the crop [4–7]. *Cylindrocarpon destructans* var. *destructans* is a soil-borne pathogenic fungus of the family *Nectriaceae* (Hypocreales) [7,8]. It causes severe root-rot in many hosts including ginseng plants [7,9–12]. As with several other pathogenic fungi, *C. destructans* var. *destructans* can be divided into weak or aggressive isolates on ginseng and other hosts [12–14]. Aggressive isolates are usually associated with severe root-rot disease [7,12,13] and were therefore named *C. destructans* f. sp. *panacis* [12]. Genetic diversity among the genus *Cylindrocarpon* is also a topic of interest in plant pathology. The genus *Cylindrocarpon* was recently re-classified into four different genera: *Ilyonectria*, *Neonectria*/*Cylindrocarpon s. str.*, *Rugonectria*, and *Thelonectria*.

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Cylindrocarpon destructans var. *destructans* was recently reclassified as *Ilyonectria radicicola* [7,15]. However, multigene molecular analysis, in particular histone H3 (HIS H3), revealed a polyphyletic relationship among *Ilyonectria macrodidyma* [16], *I. radicicola* [17,18], and *I. liriodendri* [19] isolates. Hence, each group of isolates is considered to be a different species. Species identity in the *I. liriodendri*-species complex did not explain the diverse pathogenicity profiles of the strains [19]. In contrast, isolates reported to be aggressive, namely *C. destructans* f. sp. *panacis* [12], were clustered in a distinct group and named *I. mors-panacis* [7,17], while isolates reported to be weakly aggressive [12] clustered into three different distinct groups and were designated *I. panacis*, *I. robusta* and *I. crassa* [7,17]. The genetic distinctiveness of *I. mors-panacis* isolates is of particular interest in the field of ginseng cultivation. Molecular detection and quantification of *I. mors-panacis* allows for better management of disease and early detection of pathogens prior to initiating cultivation. Seifert [12] developed intergenic spacer (IGS) region-specific primers to detect *I. mors-panacis* isolates. Kernaghan [20] later developed primers for detection and quantification of *I. mors-panacis* in soil based on quantitative real-time polymerase chain reaction (qRT-PCR). However, in the present study, our qRT-PCR analysis revealed that these IGS primers are not specific for *I. mors-panacis*. Therefore, an alternative molecular marker should be developed for better detection of this aggressive species. In earlier studies, specific HIS H3 primers were developed to detect *Fusarium subglutinans* f. sp. *pini* and *F. udum* [21,22]. In the present work, we report the first species-specific HIS H3 primer set for the specific detection of *I. mors-panacis* in soil.

2. Materials and methods

2.1. Microbial and plant materials

Strains of *I. mors-panacis* and other *Ilyonectria* species isolated from ginseng were obtained from the original author [23]. Strains of other genera were obtained from the Korean Agriculture Culture Collection (KACC) or isolated directly from ginseng fields, while bacterial strains were requested from their original isolator [24,25]. Fungal and bacterial strains were stored long-term in stock vials containing 10% and 30% glycerol, respectively, at −70°C. Fungal strains were cultured in potato dextrose broth (MB Cell, USA) at 25°C for 7 d with shaking (150 rpm). Then, the grown mycelia of each strain were filtered using sterilized filter paper, washed three time by sterilized distilled water, freeze dried, and kept at −70°C as stock mycelia for DNA isolation.

For estimation of the pathogenicity level of artificially infected soil samples, 2-yr-old ginseng seedlings were obtained from the Ginseng Bank (Kyung-Hee University, Yongin, Korea).

2.2. Genomic DNA extraction and isolation

The genomic DNA of fungal strains was extracted and purified using the DNeasy plant mini DNA extraction kit (Qiagen Inc., Germany). Genomic DNA from bacterial strains was isolated using GeneAll Exgene Clinic SV (GeneAll Biotechnology Co, Republic of Korea). The DNA of artificially infected and natural soil samples was extracted and quantified using a MoBio soil DNA isolation kit (MoBio Inc., USA).

2.3. Primer designation and PCR conditions

The HIS H3 sequence was used as the target genetic location for the design of species-specific primers that detect only the aggressive species, *I. mors-panacis*. HIS H3 sequences of all *Ilyonectria* strains used in this study were collected from the public GenBank database of NCBI according to the accession numbers mentioned in the previous study [23]. After collection, the HIS H3 sequences of the isolates were combined in a text file along with other species sequences available in NCBI, and aligned using the CLUSTAL X program [26]. Aligned sequences were carefully checked, and an *I. mors-panacis*-specific region was used to design a forward primer along with a universal reverse primer CYLH3R (5′-AGC TGG ATG TCC TTG GAC TG-3′) [17] for the specific detection of *I. mors-panacis*. PCR was performed on a MyCycler thermal cycler (BioRad, USA) using the following PCR conditions: 94°C for 10 min, followed by 30 cycles at 94°C for 15 s, 60°C for 2 s, and 72°C for 20 s, with a final elongation at 72°C for 5 min. qRT-PCR was carried out using the CFX Connect Real-Time System (BioRad, USA) in a Hard-Shell 96-well PCR plate (BioRad, USA). The 10 μl reaction comprised 5 μl of 2X iQ SYBR Green Supermix (BioRad, USA), 10 pmol of each primer, 1 μl of genomic DNA from a pure culture of the target species, and water to achieve the final volume. The cycling conditions were 94°C for 10 min, followed by 40 cycles at 94°C for 15 s, 60°C for 2 s, 72°C for 20 s, and 85°C for 2 s. The fluorescence was read at 72°C at the end of each cycle, followed by final melting curve analysis at 65−95°C at temperature increments of 0.1°C.

2.4. Validation of the primer set using standardized PCR and qRT-PCR conditions

The sensitivity of the primer set to DNA from *I. mors-panacis* was defined using the qRT-PCR conditions mentioned above with different concentrations of the target template. The analysis was started using the highest concentration of 6.4 ng/μL and ended using the lowest concentration of 0.10 ng/μL. The resultant cycle threshold (C_T) values were correlated with the corresponding concentrations and the regression coefficient (R^2) was determined. To check the primer set specificity for DNA from *I. mors-panacis*, the PCR conditions mentioned above were applied to DNA from *I. mors-panacis* in comparison with DNA from other *Ilyonectria* species as well as the other fungal and bacterial genera listed in Table 1. The specificity of the primer set for DNA from *I. mors-panacis* was further confirmed by detecting and quantifying the target template in complex DNA samples using the PCR and qRT-PCR reactions described above. These complex DNA samples were created using DNA from all tested microorganisms along with different defined concentrations of *I. mors-panacis* DNA (0.005−1 ng/μL). For comparison, conventional PCR and the qRT-PCR analyses described above were performed in parallel with the previously reported IGS primers, CDU1 and CDL1b [12,20].

2.5. Soil sample preparation

Artificially infected soil samples with different concentrations of pathogen were prepared as described previously [20,23] with some modifications; the strain HB11 was grown in V8 broth at 25°C for 2 wk with shaking (150 rpm), blended in water (50 mL/culture), and then mixed with sterilized artificial soil to a final concentration 10%. This prepared soil sample was considered to represent 100% infection; 50%, 10%, 5%, 0.1%, and 0.05% infections were prepared by diluting the originally prepared soil with sterilized artificial soil. Subsequently, soil samples were taken from each dilution for the estimation of *I. mors-panacis* DNA and colony-forming units (CFU). Non-infected soil and *I. robusta* HB3-infected soil were used in this experiment as mock and negative control samples, respectively. The remaining soil of each sample was used for ginseng infection. Each experiment was conducted using two replicates and three independent biological replicates were performed.

Table 1
Testing the specificity of *Ilyonectria mors-panacis*-specific HIS H3 primer set on the genomic DNA of other fungal and bacterial strains in comparison to IGS-specific primer set reported previously under conventional PCR condition

Name	Primer set	
	IGS-specific	HIS-specific
Ilyonectria mors-panacis HB9	+	+
I. mors-panacis HB10	+	+
I. mors-panacis HB11	+	+
I. mors-panacis HB12	+	+
I. mors-panacis HB13	+	+
I. mors-panacis HB14	+	+
Ilyoenctria robusta HB1	+	−
I. robusta HB2	+	−
I. robusta HB3	+	−
I. robusta HB4	+	−
I. robusta HB5	+	−
Ilyonectria leucospermi HB6	−	−
I. leucospermi HB7	−	−
I. leucospermi HB8	−	−
Pythium ultimum KACC 40705	+	−
Penicillifer spp. HR21	+	−
Rhizoctonia solani KACC 40123	−	−
Phialophora mustea KACC 43819	+	−
Fusarium solani KACC 44891	+	−
Fusarium oxysporum f. sp. *gladioli* KACC 44452	+	−
Fusarium equiseti KACC 42105	+	−
Fusarium culmorum KACC 42099	+	−
Alternaria panax KACC 42461	−	−
Penicillium spp. RR22	+	−
Paraconiothyrium spp. HR41	−	−
Volutella spp. HR42	−	−
Trichoderma harzianum KACC 44705	+	−
Burkholderia ginsengiterrae DCY85^T	+	−
Sphingomonas panacis DCY99^T	+	−

HIS, histone; IGS, intergenic spacer; PCR, polymerase chain reaction

2.6. Disease severity index

After soil sample preparation, each sample was transferred to a 15-cm pot. Then, 2-y-old ginseng seedlings were inserted in each soil sample pot and incubated in a greenhouse (22 ± 2°C, 12-h photoperiod). After 4 wk, seedlings were harvested for the estimation of the disease severity index (DSI), according to the scale described previously [23]. The symptoms were scored on a 6-point scale where 1 indicated symptomless roots and 6 indicated completely rotten roots. Seedlings were inserted in non-infected soil and *I. robusta* HB3 infected soil to represent the mock and negative control, respectively.

2.7. Detection and quantification of the pathogen in artificially infected soils

The pathogen was detected and quantified in artificially infected soils based on the standard curve constructed using different concentrations of highly purified DNA template from *I. mors-panacis*, as explained above using the conventional PCR and qRT-PCR conditions mentioned above except that the volume of DNA template used was 2 μL.

2.8. Estimation of CFU

The CFU of *I. mors-panacis* in each soil sample were estimated as follows: 1 g of soil was taken from each sample and mixed well with 9 mL of saline solution (0.85%). The resultant solution was then serially diluted in saline solution up to 10^{-3}. One hundred μL of each dilution was spread on semi-selective media (1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 50 mg rose bengal, 10 g dextrose, 5 g Bacto peptone, 15 g Bacto agar, 30 mg streptomycin sulfate, 250 mg ampicillin, 10 mg rifampicin, 500 mg pentachloronitrobenzene, 500 mg dicloran, and 1 L distilled water) [27]. Inoculated plates were incubated at 25°C in the dark for 2 wk. Then, the colonies grown in each soil sample were counted and the CFU/g soil was estimated using the following equation $\Sigma C/(n_1+0.1 \times n_2)d$, where ΣC is the number of the colonies counted on plates of two different dilutions; n_1 is the number of plates counted in the first dilution; n_2 is the number of the plates counted in the second dilution; and d is the initial dilution factor [28].

2.9. Pathogen detection and quantification in natural soil samples

Soil samples with either a bad or good yield history were collected in August from different fields for the detection and quantification of *I. mors-panacis*. Additionally, soil samples from fields being scheduled for ginseng cultivation were evaluated for pathogen presence. Information about the geographical locations where the samples were obtained is listed in Table 2. Soil DNA isolation and purification were performed as mentioned above. The pathogen concentration was quantified as for artificially infected soils. Experiments were conducted in triplicate.

3. Results

3.1. Amplification and sequencing of the HIS H3 gene

HIS H3 sequences were amplified and sequenced for all strains of the three *Ilyoenctria* species isolated from Korean ginseng root, *I. robusta*, *I. leucospermi*, and *I. mors-panacis* [23]. The partial sequence of the gene is approximately 500 bp and is composed of three exons separated by two introns (Fig. 1A). To identify regions specific to *I. mors-panacis*, HIS H3 sequences of all *Ilyonectria* species were obtained from the public GenBank database and aligned with the sequences obtained in this study. The alignment showed that the first 20 nucleotides of the second intron are highly conserved within the strains of each species while being variable between the different species (Fig. 1A). A nucleotide region specific to *I. mors-panacis* was identified and designated to be used as a forward primer for the specific detection of *I. mors-panacis*. The forward primer sequence was IMP 5′-CAC ACC CAA CGT GCC ACA T-3′. Unfortunately, it was not possible to identify another specific region to be used as a reverse primer, therefore the universal reverse primer was used in the detection and quantification analysis. The resultant product was expected to be approximately 230 bp.

3.2. Sensitivity of the primer set on pure DNA and standard curve analysis

qRT-PCR analysis of different concentrations of *I. mors-panacis* DNA mixed with the HIS H3 primer set showed a standardized exponential increase in PCR product. All the resultant products had the same single melting temperature (88°C). The lowest C_T value (which corresponded to the highest concentration of DNA) was 19.1 ± 0.03 while the highest C_T value (which corresponded to the lowest concentration of DNA) was 24.6 ± 0.20. Reactions with primers but without the *I. mors-panacis* template resulted in fluorescence after cycle 30; however, this amplicon had two melting curves with temperature points (79.5°C and 87.5°C) that were different from the target amplicon (Fig. 2A). The primer set detected the DNA template efficiently until the concentration of 1 pg/μL. Then, the efficiency was reduced until the concentration of 0.004 pg/μL (=4 fg/μL) and completely disabled using the lower concentrations (data not shown). The standard curve was observed

Table 2
Quantification of *Ilyonectria mors-panacis* template in soil samples collected from several locations for ginseng cultivation

History	Location	*I. mors-panacis* template quantity (pg/g)
Good yield	369-2, Sikhyeon-ri, Jeokseong-myeon, Paju	—
	San 161-8, Geumju-ri, Yeongjung-myeon, Pocheon	—
	San 161-8, Geumju-ri, Yeongjung-myeon, Pocheon	—
	665, Sajik-ri, Ildong-myeon, Pocheon	—
	575 Junggyo-ri Gasan-myeon Pocheon	—
	205-2, Hwangbang-ri, Nam-myeon, Yangju	—
	555-114, Hansan-ri, Nam-myeon, Yangju	—
	336-3, Ujeong-ri, Misan-myeon, Yeoncheon	—
Bad yield	338-54, Yulpo-ri, Jeokseong-myeon, Paju	280.41 ± 170.22
	351, Gaekhyeon-ri, Jeokseong-myeon, Paju	239.73 ± 20.71
	389-2, Dongmak-ri, Yeoncheon-eup, Yeoncheon-gun	51.18 ± 13.52
	256, Wangjing-myeon, Yeoncheon-gun	114.96 ± 58.39
	1000, Wangjing-myeon, Yeoncheon-gun	65.39 ± 16.87
	916, Geumju-ri, Yeongjung-myeon, Pocheon	119.47 ± 20.97
	575, Junggyo-ri Gasan-myeon Pocheon	892.34 ± 103.70
	205-2, Hwangbang-ri, Nam-myeon, Yangju	9.22 ± 1.81
	376, Sangbong-ri, Seolseong-myeon, Icheon	5.82 ± 2.35
	957, Gueup-ri, Jeokseong-myeon, Paju	12.40 ± 1.80
	74-4, Gueup-ri, Jeokseong-myeon, Paju	7.80 ± 0.38
	944, Gueup-ri, Jeokseong-myeon, Paju	41.68 ± 5.36
	851-1, Baekseok-ri, Misan-myeon, Yeoncheon-gun	—
	748-2, Daegwang-ri, Sinseo-myeon, Yeoncheon-gun	—
	861, Igapal-ri, Sohol-eup, Pocheon	—
	938-2, Jail-ri, Yeongbuk-myeon, Pocheon	—
Not yet cultured	259, Wangjing-myeon, Yeoncheon-gun	—
	706-3, Geumju-ri, Yeongjung-myeon, Pocheon	—
	Geumju-ri, Yeongjung-myeon, Pocheon	—
	1749, Sajik-ri, Ildong-myeon, Pocheon	—
	1749-1, Sajik-ri, Ildong-myeon, Pocheon	—
	517-5, Sajik-ri, Ildong-myeon, Pocheon	—
	190-1, Hwangbang-ri, Nam-myeon, Yangju	—
	423, Ibam-ri, Nam-myeon, Yangju	—
	423-1, Ibam-ri, Nam-myeon, Yangju	—
	938-2, Jail-ri, Yeongbuk-myeon, Pocheon	—
	222, Dapgok-ri,Sinseo-myeon, Yeoncheon	—
	1218, Juwol-ri, Jeokseong-myeon, Paju	18.00 ± 1.35
	260, Wangjing-myeon, Yeoncheon-gun	21.10 ± 8.15
	721, Junggyo-ri Gasan-myeon Pocheon	8.51 ± 2.78

to be precise as the linear correlation between the log values of C_T with the log values of different DNA concentrations was very high ($R^2 = 1$; Fig. 2B). Using the IGS-specific primer set, the amount of PCR product increased less exponentially. The amplicon melting point varied between 87°C, 87.5°C, and 88°C. The lowest C_T value (which referred to the highest concentration of template) was 11.6 ± 0.76, while the highest C_T value (which referred to the lowest concentration of template) was 17.52 ± 0.20 (Fig. S1A). The linear correlation between the log values of C_T with the log values of different DNA concentrations was lower than that of HIS primer set ($R^2 = 0.98$). Therefore, this standard curve was considered to be less precise (Fig. S1B).

3.3. *Specificity of a species-specific HIS H3 primer set using standardized PCR and qRT-PCR*

To determine the specificity of the primer set, pure DNA from *I. mors-panacis* was used. Using conventional PCR conditions, amplification of DNA from all strains resulted in a product size of 230 bp (Fig. 1B). The primer set was also used with pure DNA from other *Ilyonectria* species isolated from Korean ginseng as well as DNA from other fungal and bacterial genera. In these reactions, no PCR products were generated. When the primer set was mixed with DNA mixtures containing different defined concentrations of *I. mors-panacis* template, a band of the expected size (230 bp) was detected. A DNA mixture containing DNA from all the tested microbes except *I. mors-panacis* was used as a control and no PCR product was generated (Fig. 1B; Table 1). Using qRT-PCR conditions, DNA mixtures containing different defined concentrations of *I. mors-panacis* template resulted in an amplicon with a single melting point (88°C; Fig. S2A). The resulting quantification of *I. mors-panacis* template in the DNA mixtures was very similar to the real concentrations added to the mixtures (Table 3). The primer set for amplification of the IGS-specific sequence reported previously [12,20] for the detection and quantification of *I. mors-panacis* was used on the same strains' DNA using the optimized conditions mentioned in the previous report and was found to be not specific for *I. mors-panacis* detection. Using conventional PCR conditions, the primer set produced PCR bands when added to reactions containing genomic DNA from all tested microbes except *I. leucospermi*, *Rhizoctonia solani*, *Alternaria panax*, *Paraconiothyrium* spp., and *Volutella* spp. Also, when the primer set was mixed with a DNA mixture containing defined concentrations of *I. mors-panacis* template, a band of the predicted size (500 bp) was generated. However, two observations suggested the lack of specificity of the detection. Firstly, the brightness of the PCR products was not consistent with the known concentration of the target template in the DNA complex. Secondly, a band of the same size also appeared in reactions containing the control DNA mixture (Fig. 1B; Table 1). Using qRT-PCR, when the primer set was mixed with the complex DNA mixture, as with the HIS H3 primer set, fluorescence of the target PCR product was detected only in the sample containing the

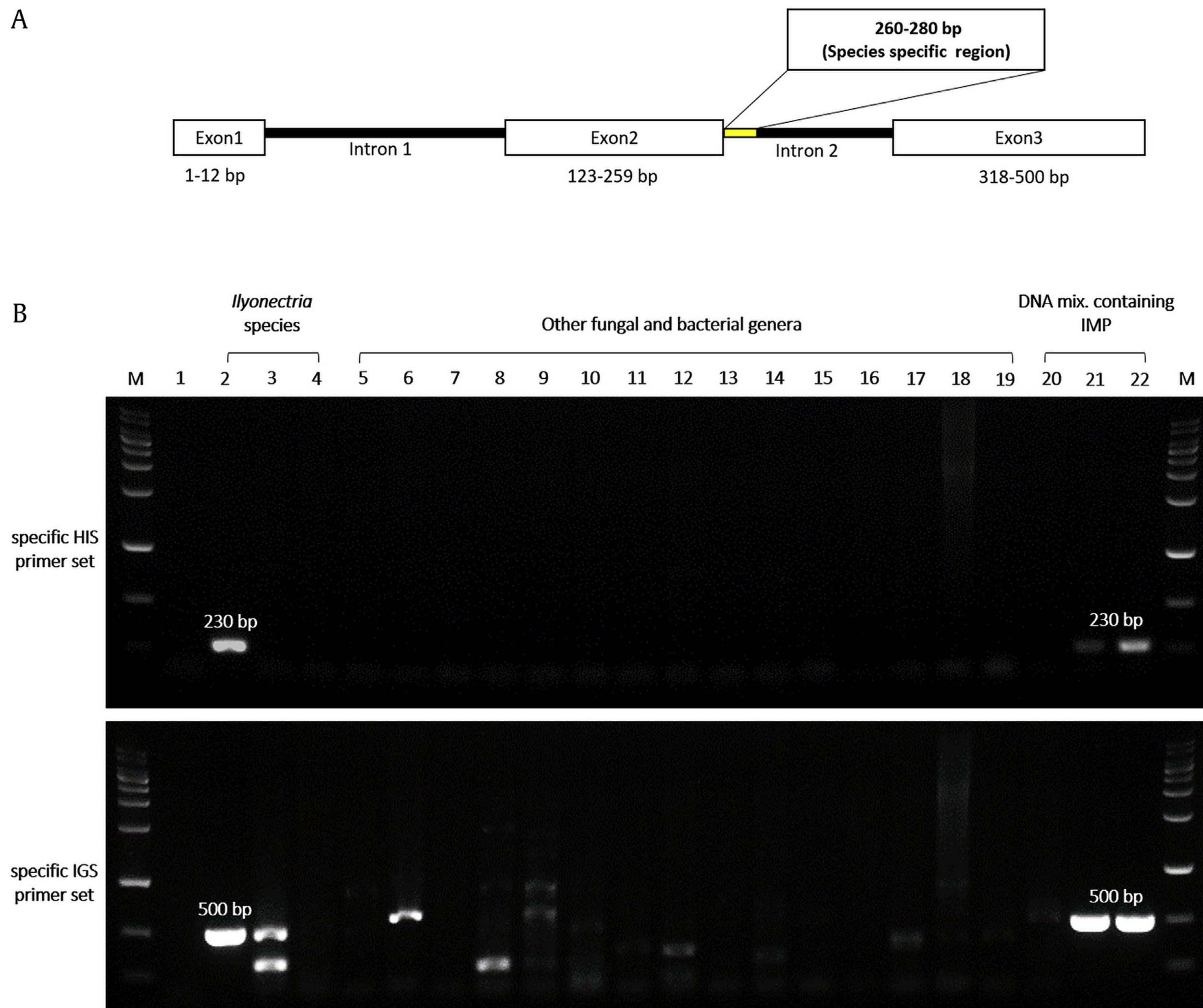

Fig. 1. Detection of *Ilyonectria mors-panacis*-specific HIS H3 sequence. (A) Schematic structure of the HIS H3 partial sequence showing the species-specific sequence in the second intron region. (B) Conventional PCR analysis demonstrating the specificity of the HIS H3 primer set using an *I. mors-panacis* template in comparison to the previously published IGS-specific primer set [12,20]. The samples used were: 1, water; and then DNA from 2, *I. mors-panacis*; 3, *Ilyonectria robusta*; 4, *Ilyonectria leucospermi*; 5, *Pythium ultimum*; 6, *Penicillifer* spp.; 7, *Rhizoctonia solani*; 8, *Phialophora mustea*; 9, *Fusarium solani*; 10, *Fusarium oxysporum*; 11, *Fusarium equiseti*; 12, *Fusarium culmorum*; 13, *Alternaria panax*; 14, *Penicillium* spp.; 15, *Paraconiothyrium* spp.; 16, *Volutella* spp.; 17, *Tichoderma harzianum*; 18, *Burkholderia ginsengiterrae*; and 19, *Sphingomonas panacis*. The specificity of the primer set on a complex DNA mixture of all tested microbes; 20, without *I. mors-panacis* template; 21, with 0.005 ng/μL of *I. mors-panacis*, 22, 1 ng/ μL of *I. mors-panacis*. bp, base pair; HIS H3, Histone H3; IGS, intergenic spacer; IMP, *I. mors-panacis*; M, 1 kb DNA ladder.

highest amount of *I. mors-panacis* template (1 ng/μL), while other DNA complex samples resulted in PCR products with a different melting point. This melting point was the same as that seen in the control DNA complex as well as the negative control reactions (Fig. S2B). Accordingly, we were unable to quantify the *I. mors-panacis* template accurately. Although the highest concentration of DNA was able to be amplified by the primer set, the detected concentration was vastly different from the adjusted concentration in the DNA complex (Table 3).

3.4. Sensitivity of the HIS H3 primer set on DNA isolated from artificially infected soil

DNA extracted from artificially infected soil resulted in amplified bands of the typical size obtained from the pure culture (230 bp). Soil DNA from non-infected soil or *I. robusta*-infected soil did not result in any amplification of a PCR product (Fig. 3C). To confirm the existence of *I. robusta* in the soil, soil DNA of non-infected and *I. robusta*-infected samples was mixed with the universal HIS H3 primer set. A band of the expected size for HIS H3 (500 bp) was obtained from the *I. robusta*-infected sample, while no bands were obtained from the noninfected soil sample (data not shown). Using qRT-PCR, the pathogen DNA was successfully quantified in each artificially infected soil sample. The concentrations of *I. mors-panacis* DNA ranged from 0.23 ± 0.0143 ng/μL of soil DNA, 91.20 ± 5.75 ng/g of soil particles (in 100% infected soil sample) to 0.02 ± 0.0004 ng/μL of soil DNA, 4.21 ± 0.16 ng/g of the soil particles (in 0.05% infected soil sample). To confirm the accuracy of pathogen detection in soil, CFU and the DSI in each soil sample were calculated. The CFU values ranged from 3 × 10^2/g of soil in the most highly infected soil to no detectable colonies in soil with the lowest level of infection (Fig. 3B). To calculate the DSI, roots cultured in soil with the highest level of infection were observed to be weakly germinated and completely rotten. The symptoms of rotting and weak germination decreased gradually as the level of infection decreased and fully disappeared in soil samples with the lowest levels of infection, yielding results similarly to the mock and negative control soil samples (Fig. 3A). The DSI recorded from the

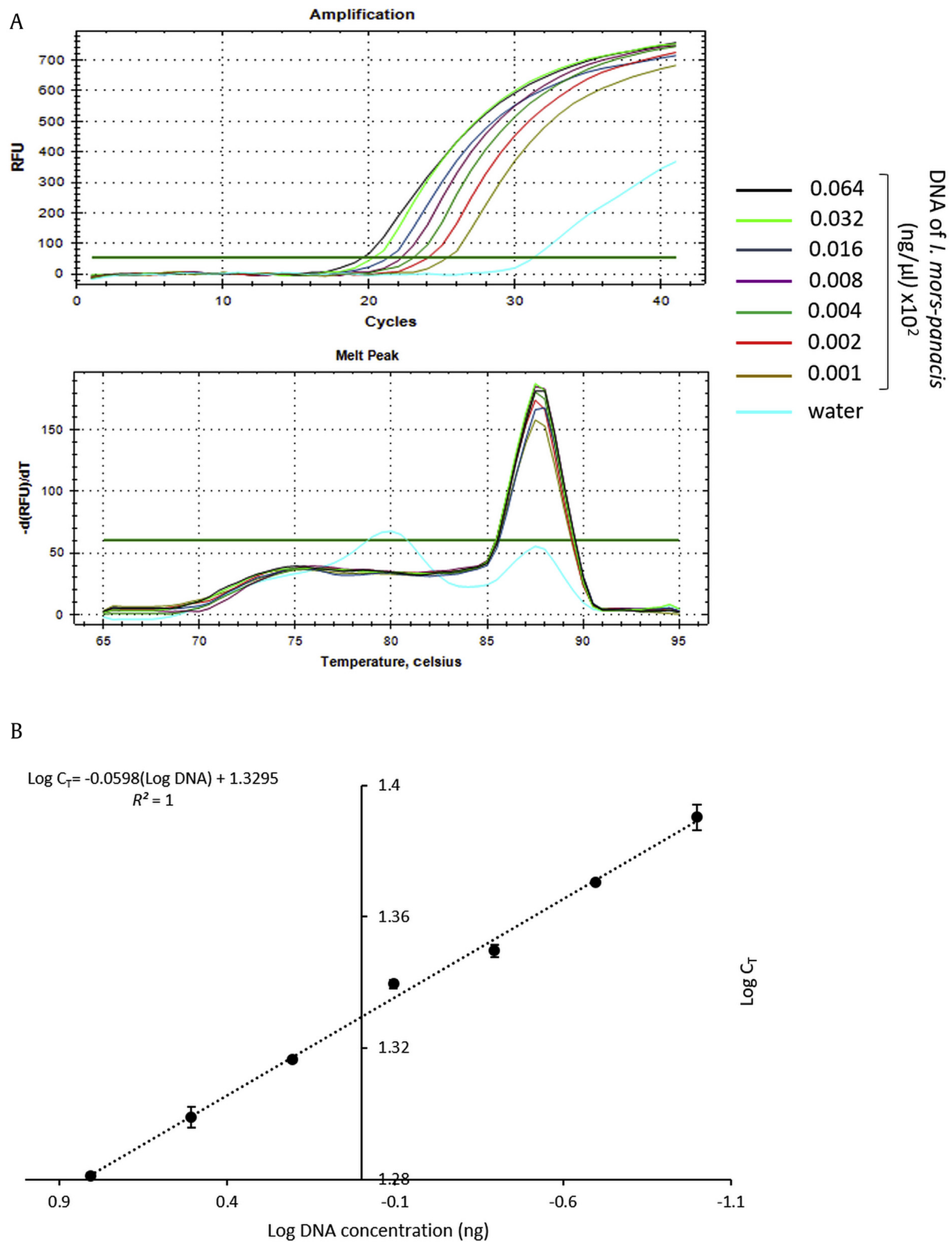

Fig. 2. Sensitivity of the specific HIS H3 primer set on pure DNA of *Ilyonectria mors-panacis*. (A) Fluorescence of the PCR products generated after mixing the specific HIS H3 primer set with different concentrations of *I. mors-panacis* DNA, with a characteristic melting temperature (88°C). Using the primer set without the target template resulted in dimerization with two melting temperatures (79.5°C and 87.5°C). (B) Standard curve analysis generated using the specific HIS H3 primer set demonstrating the logarithmic relationship between different concentrations of *I. mors-panacis* DNA and the corresponding C_T. C_T, cycle threshold; -d(RFU)/dT, negative rate of change of fluorescence versus temperature; HIS H3, Histone H3; PCR, polymerase chain reaction; RFU, relative fluorescence units.

Table 3
Quantification of *Ilyonectria mors-panacis* template in DNA complex samples containing adjusted concentrations of *I. mors-panaics* DNA using HIS H3-specific and IGS-specific primer set, respectively

Inserted DNA concentration (ng/μL)	Determined DNA concentration (ng/μL)	
	HIS primer set	IGS primer set
0.005	0.0028 ± 0.0013	—
0.1	0.1520 ± 0.0090	—
0.5	0.4305 ± 0.0580	—
1	0.8000 ± 0.0071	0.1038 ± 0.0595

HIS, histone; IGS, intergenic spacer

root cultures in each soil sample ranged from 5.5 ± 0.14 to 1.47 ± 0.22. The detection of pathogen DNA in the soil was found to be accurate since the DNA concentration was positively correlated with the range of soil infection ($R^2 = 0.95$; Fig. 4A), the range of DSI ($R^2 = 0.99$; Fig. 4B), and the range of CFU ($R^2 = 0.87$; Fig. 4C).

3.5. Detection and quantification of the pathogen in natural soil samples

In the natural soil samples, the soils were divided into three categories: soils used to be cultured by ginseng that resulted in good yields, soils used to be cultured by ginseng that resulted in bad yields, and soil samples scheduled for ginseng cultivation. In the first category of soil, the pathogen template did not be detected by qRT-PCR analysis. According to the qRT-PCR results, the pathogen template was detected in most of the soil in the second category, at levels ranging from 5.82 ± 2.35 pg/g to 892.34 ± 103.70 pg/g of the soil, while few soil samples did not have the pathogen template. Most of the soil of the last category of did not have the pathogen template except three soil samples, which were recorded to have a low quantity of the *I. mors-panacis* template (Table 2).

4. Discussion

Persistence of *I. mors-panacis* in soils for decades increases the possibility of ginseng crop loss [7,27]. Molecular detection is the best method for avoiding using infested fields for ginseng cultivation. HIS H3 is one of the genes used for the discrimination of the Ascomycetous fungi up to the species level. *Fusarium udum* can be differentiated from other *Fusarium* species using species-specific HIS H3 primers [22]. We designed *I. mors-panacis*-specific HIS H3 primers to successfully distinguish *I. mors-panacis* from other *Ilyonectria* species as well as other fungal genera. The sensitivity and specificity of the HIS H3 primers for *I. mors-panacis* were superior to that of the previously reported IGS primers. The sensitivities of both primers sets were evaluated according to the accuracy of the resultant standard curves as well as the stability of the melting point of the amplicon according to the template concentration. The HIS H3 primer set was highly accurate as the regression coefficient value of the standard curve reached the highest state ($R^2 = 1$) and the melting point of the resultant amplicon was found to be highly stable (88°C). By contrast, the IGS primer set was less accurate as the regression value of the standard curve was lower than that of the HIS primer set ($R^2 = 0.98$) and the

Fig. 3. Sensitivity of the HIS H3 primer set on DNA isolated from artificially infected soil by *Ilyonectria mors-panacis*. (A) Evaluation of the pathogenicity of *I. mors-panacis* inoculated into sterilized artificial soils at different concentrations in comparison with *Ilyonectria robusta* inoculated (-control) and mock soils. (B) CFU of *I. mors-panacis* in all inoculated sterilized artificial soil samples. (C) Conventional PCR analysis showing the specific detection of *I. mors-panacis* templates after using the specific HIS H3 primer set on artificially inoculated soils' DNA. bp, base pair; CFU, colony forming unit; HIS H3, Histone H3; M, 1 kb DNA ladder.

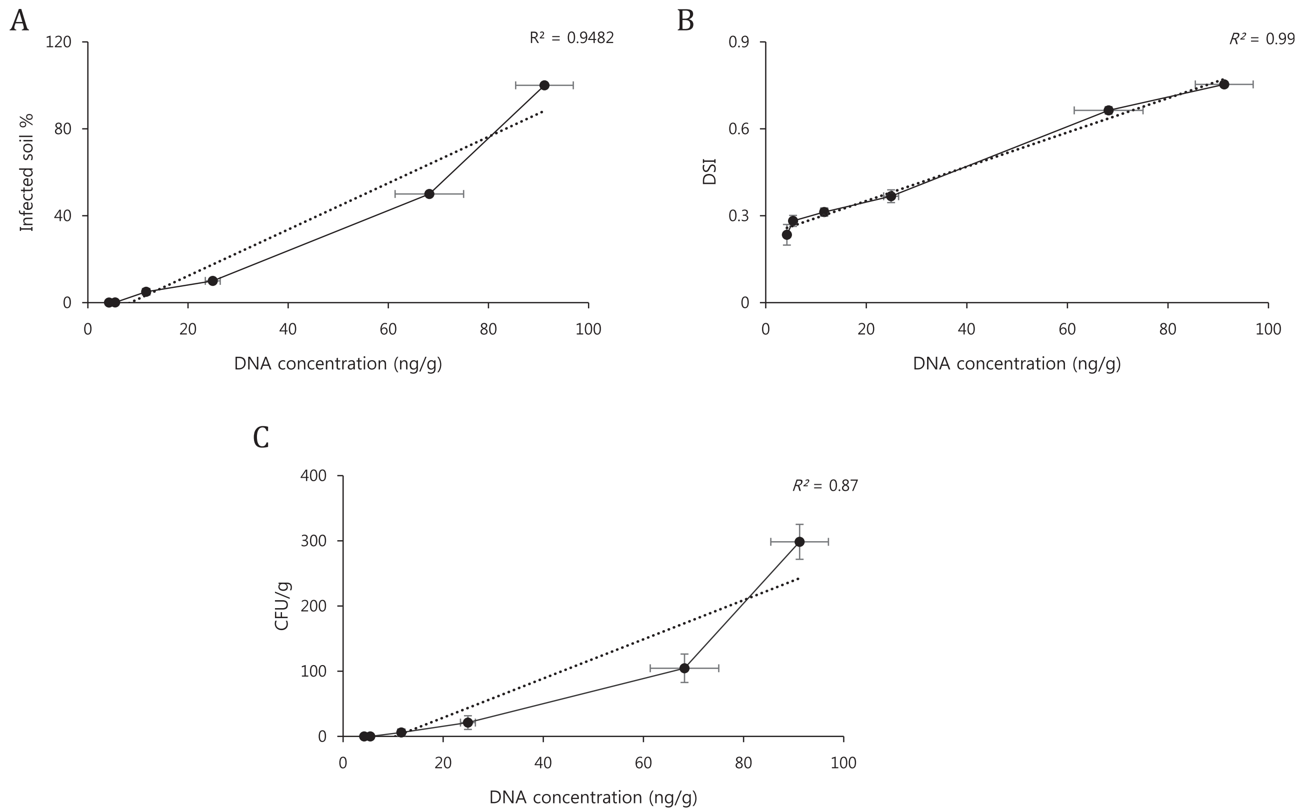

Fig. 4. The linear correlation between the estimated concentrations of *Ilyonectria mors-panacis* in artificially infected soil samples using the specific HIS H3 primer set and (A) The percentage of *I. mors-panacis* infection in the artificial soil samples. (B) DSI recorded on the roots cultured in the soil samples. (C) CFU of *I. mors-panacis* in the soil samples. CFU, colony forming units; DSI, disease severity index; HIS H3, Histone H3.

resultant amplicon melting point changed slightly according to the concentration of the template. The instability of the melting temperature of the amplicon was reported in the previous study [20]. The specificity of both primer sets was validated by conventional PCR and qRT-PCR on separate pure DNA samples from many fungal and bacterial strains and a complex DNA mixture of all microbes containing a defined concentration of *I. mors-panacis* complex. The IGS primer set resulted in a single PCR product of the expected size using DNA from *I. mors-panacis*, but it also amplified the template DNA from other microbes, resulting in PCR products. Furthermore, the low efficiency of this primer set became clear when it failed to amplify the target template in a complex DNA sample. This was due possibly to the length of the target template (500 bp), which is not ideal for use with the SYBR green detection method. However, the HIS H3 specific primers specifically generate a PCR product of the expected size only when used with DNA from *I. mors-panacis* and no product was amplified from DNA of other microbes. In addition, the primer set was used to successfully detect and quantify the target template in the complex DNA samples. The high efficiency of the primer set maybe due to the shortness of target sequence (200 bp). Using an annealing temperature of 60°C for only 2 s increased the specific detection of the HIS H3 primer set so that it detected only *I. mors-panacis.* Adding a 2 s at 85°C step after the elongation step was found to reduce the abundance of dimer formation. The accuracy of the primer set was further confirmed when a linear correlation was obtained between the template quantification of the pathogen in artificially infected soil samples and the pathogen concentration itself in the soil samples ($R^2 = 0.95$). Furthermore, the template quantification was linearly correlated with the DSI and CFU of infected soil samples ($R^2 = 0.99$ and 0.87, respectively). Selective detection in the soil system was confirmed when the soil sample infected with *I. robusta* (100% infection) did not result in any fluorescence. Based on these findings, this primer set was ultimately used to evaluate pathogen concentration in natural soil samples. Soil samples that had previously produced good yields were recorded by the farmer as being healthy soil. In addition, qRT-PCR analysis confirmed the absence of the pathogen template in these soil samples. These findings suggest that the soils could be used for ginseng cultivation in future seasons. Soil samples that had previously led to bad yields had the pathogen present at a range of different concentrations. This correlation suggests the high possibility that crop loss was caused by *I. mors-panacis*. However, a few soil samples had previously produced bad yields, but the pathogen template was not detected. In these cases, we propose that the root-rot symptoms might be induced by another pathogen in these areas. For example, it has been reported that *Phytophthora cactorum* causes root-rot in ginseng plants, but the symptoms are different from *Ilyonectria* root-rot. The root infection by *P. cactorum* occurs by transmitting the pathogen from infected leaves through the vascular system to the roots causing beige colored interior rotting, which develops at a late stage to become softy rot [29]. Therefore, it is thought that the recorded root-rot symptoms in these areas were due to *Phytophthora* not *Ilyonectria* root-rot. The last category of soil samples was evaluated for the predicted detection of the pathogen before starting ginseng cultivation. The pathogen template was not detected in most of the soil samples except three samples. Although the concentration of the pathogen template was low in these three areas, it is recommended that cultivation in these areas be avoided to prevent the possibility of crop infection.

5. Conclusion

In conclusion, a species-specific HIS H3 specific primer set was successfully designed for the specific detection of *I. mors-panacis*. The primer set specificity and sensitivity were superior to those of the previously reported IGS primer set. Also, the *I. mors-panacis* HIS H3 primer set successfully detected the pathogen in artificially infected and natural soils. Based on this, we propose this primer set for quantification of *I. mors-panacis* and the estimation of soil quality before starting ginseng cultivation.

Conflicts of interest

The authors have no conflicts of interest declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgr.2017.07.002.

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