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A Novel Molecular Diagnostic Tool for Detection of *Ilyonectria radicicola*, Causal agent of Root Rot Disease of Ginseng

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ABSTRACT

The fungal genus *Ilyonectria* consists of several pathogenic species that can infect plant roots. An invasive and devastating pathogenic fungi, *Ilyonectria radicicola* (synonym *Cylindrocarpon destructans*), is responsible for root rot in ginseng. A culture-based method has traditionally been used for detecting of *I. radicicola* in field soil, but the process is time-consuming and labor-intensive. While molecular-based diagnostic tools for detection of *I. radicicola* have been developed, improvement in the detection level and correlation with disease severity is necessary. The current study developed new PCR primers based on the internal transcribed spacer (ITS) region of the rRNA operon from known *I. radicicola* strains. These new primers produced fast and precise detection of *I. radicicola*, *I. mors-panacis*, and *I. robusta* in ginseng soils, distinguishing between soil samples from non-infected and infected ginseng fields in both New Jersey and Korea. The newly-developed PCR primers were detected the presence of *I. radicicola* in soil samples, including two soils that had previously tested negative when earlier primers (Dest1 and Dest4) were used. Phylogenetic analysis based on DNA sequence homology indicated that the newly-developed primers could positively detect and identify the *Ilyonectria* strains *I. radicicola* and *I. mors-panacis* that cause severe root rot disease of ginseng. This new detection method will improve detection of root rot disease in ginseng fields before planting.

INTRODUCTION

Ginseng roots, traditionally used as a medical herbs against a wide range of diseases (Chu and Zhang, 2009), are primarily Asian ginseng (*Panax ginseng* C.A. Meyer) and American ginseng (*Panax quinquefolium* L.), cultivated, respectively, in Asia (mainly in Korea and China) and in the U.S. and Canada. The genus *Panax*, exclusively, contains ginsenosides (saponin glycosides), natural biologically active plant products largely responsible for the medicinal activity of both the Asian and American ginsengs (Christensen, 2009; Thompson, 1987).

As a light-sensitive species, populations of wild ginseng naturally grow in shaded woodlands. In contrast to wild ginseng, cultivated ginseng requires intense management being grown in raised beds in fields under artificial shade for four years so that the roots reach merchantable quality (Proctor, 1996).

In contrast to the more natural and simulated woodland grown and wild crafted populations (Carroll *et al.,* 2007; Persons and Davis, 2008; Proctor and Bailey, 1987).Under the intensive horticultural production systems for cultivated ginseng in growing regions, the planta are very vulnerable to infection by a number of fungi,. Because of the need for shade structures and high production costs of their construction, many commercial ginseng farmers use these same raised beds for successive cropping of ginseng, thus furthering the disease pressure as these root rot diseases can survive in the same soil for 10 years or more once in the soil (Cho *et al.,* 1995b; Kang *et al.,* 2007). Various pathogenic fungi, including

Ilyonectria radicicola (Synonym *Cylindrocarpon destructans*), *Fusarium solani, Rhizoctonia solani, Botrytis cinerea, Phytophthora cactorum, Pythium* sp. and *Alternaria panax*, have been isolated from infected ginseng roots (Kim *et al.,* 1997; Punja, 1997). Of these, *I. radicicola*, one of the most invasive pathogenic fungal species, is responsible for initial infection of ginseng root (Lee, 2004; Matuo and Miyazawa, 1984; Park *et al.,* 1997; Rahman and Punja, 2005; Reeleder *et al.,* 2002).

Because of considerable variation among taxa, *Cylindrocarpon*-like anamorphs (Jang, 2005) and questionable anamorphs (*Cylindrocarpon*) – teleomorph (*Nectria*) (Halleen et al, 2004, 2006), clearly defining the phylogenetic status of *I. radicicola* responsible for root rot disease in ginseng has been difficult. Recently, five genera of *Neonectria*- and *Cylindrocarpon*-like morphology were proposed, based on their morphological, molecular, and other characteristics (Chaverri, 2011) to belong to the *Ilyonectria radiciola* classification, which belongs *Ilyonectria* gen. nov. (anamorph *Cylindrocarpon*-like). Another recent study showed that at least two or more species could be distinguished within *I. radicicola* (Cabral, 2011).

Ilyonectria radicicola is a soil-borne fungus that grows at relatively low temperature, 5 to 25 °C, with an optimal temperature of approximately 20 °C (Cho *et al.,* 1995a; Cho and Yu, 2001). Under unfavorable conditions, such as low nutrient levels or temperature, *I. radicicola* forms a chlamydospore and enters into a dormant stage (Cho *et al.,* 2003). Chlamydospores of *I. radicicola* can survive in the soil for 10 years without a host (Kang *et al.,* 2007). When ginseng is planted in chlamydospore-contaminated fields, ginsenosides exudated from ginseng root induce germination of *I. radicicola* (Nicol, 2003) that subsequently causes root rot disease. Therefore, a sensitive method to detect the presence or absence of chlamydospores of *I. radicicola* is necessary in order to predict the presence of the pathogen in a field.

A culture-based method, in which soil samples are serially diluted and spread on selective media, has been used for detection of *I. radicicola* (Davet and Rouxel, 2000). This method, however, is time-consuming and labor-intensive with low-specificity as identification based on fungal morphology is. problematic and germination rates are influenced by physical and chemical factors, such as temperature and pH (Cho and Yu, 2001; Yoo *et al.,* 1996). In 1996, a culture-independent PCR-based detection method was developed targeting internal transcribed spacer (ITS) region of ribosomal RNA gene cluster in *I. radicicola* (Hamelin *et al.,* 1996). Because fungal species are phylogenetically close to each other, using the ribosomal RNA gene for classification, as used in bacteria, is impossible. Instead, highly variable ITS regions are widely used to further classify fungal species.

A primer set, Dest1 and Dest4, has been used for detection of *I. radicicola* (Jang *et al.,* 2005, 2010; Song *et al.,* 2014). In 2007, a quantitative PCR-based detection method was developed and studied for correlation of the density of *I. radicicola* with disease severity (Kernaghan *et al.,* 2007). The correlation value (*r*) with disease severity was 0.494 in steam-pasteurized soils mixed with chlamydospores formed from pathogenic *C. destructans* f. sp. *panacis* DAOM 234582.

The objective of the current research was to develop and optimize a nested PCR-based molecular method with newly developed primer sets for improved detection of pathogenic *I. radicicola* by targeting the ITS region of the ribosomal RNA gene cluster. In addition, DNA sequencing of PCR products enabled exploration of the phylogenetic status of related pathogenic *I. radicicola* species. The findings indicate the current procedure can be used as an effective tool to detect to potential disease pressure in ginseng fields before planting.

MATERIALS AND METHODS

Soil samples. Soil samples manually collected with a shovel to a depth of approximately 5 to 15 cm from the surface were used in this study. The soils were dried in the shade, sealed in plastic bags, and stored at 4 °C prior to analysis. The occurrence of a root rot disease of ginseng was determined by the presence of dark sunken lesions on planted ginseng roots. *I. radicicola* infected soil (S08) was pasteurized by exposure to 55 °C water for 3 h. *Isolates.* A total of five strains of *I. radicicola* isolates, generously provided by Dr. Hong-Gi Kim (Department of Agricultural. Biology.at Chungnam National University, Daejeon, Korea) were used in this study (Jang *et al.,* 2005). The strains were isolated from ginseng roots where root rot disease of ginseng was observed. The pathogenicity of the *I. radicicola* strains was confirmed by inoculating isolates into sterilized soil (120 °C for 30 min) to which disease-free young ginseng plants were transplanted. The root rot disease strains on the transplanted ginseng were subsequently

confirmed as the same species as the isolates added to the soil (Jang *et al.,* 2005).

Primer design. Six primers were manually designed based on the ITS regions of the rRNA operon of known *I. radicicola* species (Fig. 1). ITS regions of other major plant root pathogens (*Alternaria panax*, *Botrytis cinerea*, *Fusarium solani*, *Rhizoctonia solani*, *Fusarium oxysporum f. sp. fragariae*, *Phytophthora cactorum*, *Pythium ultimum*, *and Colletotrichum gloeosporioides*) were compared with excluded homologous regions for better *I. radicicola* specificity. The primer specificities were confirmed by a sequence homology search using BLAST (Altschul *et al.,* 1997). Oligo-Analyzer 3.1 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/), an online service of IDT Biotools (Coralville, IA), was used to ensure minimal self-complementarity and to prevent the presence of secondary structures.

DNA extraction, PCR, and nested PCR. DNA was extracted from 0.3 g of soil using the PowerSoil™ DNA Isolation Kit, according to the manufacturer's instructions (MoBio Laboratories Inc., Carlsbad, CA). The DNA extracts were stored at −20 °C prior to analysis. General fungal primers, ITS1 and ITS4 (White *et al.,* 1990), were used to selectively amply the fungal ITS region of the rRNA operon, including the ITS1 region, 5.8S rRNA gene, and ITS2 region (Fig. 1). The PCR reaction conditions with the ITS1 and ITS4 primers were the same as described previously (Hamelin *et al.,* 1996).

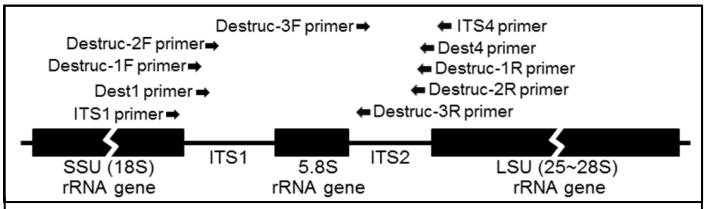

Figure 1. Schematic diagram of the primer design for *Ilyonectria radicicola*-specific primer sets from ITS region of fungal ribosomal RNA gene repeat unit. ITS = internal spacer region, SSU = small subunit, LSU = large subunit.

PCR products were purified using a PCR purification kit (MoBio Laboratories Inc., Carlsbad, CA) and then used as a template for nested PCR. The eight sets of "Destruc-series" primers were used for nested PCR: Destruc-1F/1R, Destruc-1F/2R, Destruc-1F/3R, Destruc-2F/1R, Destruc-2F/2R, Destruc- 2F/3R, Destruc-3F/1R, and Destruc-3F/2R (Table 1).

Table 1. Primers for detection of *Ilyonectria radicicola*.

Primer	Sequence (5′ to 3′)	Reference
ITS1	TCCGTAGGTGAACCTGCGG	White *et al.* (1990)
ITS4	TCCTCCGCTTATTGATATGC	White *et al.* (1990)
Dest1	TTGTTGCCTCGGCGGTGCCTG	Hamelin *et al.* (1996)
Dest4	GGTTTAACGGCGTGGCCGCGCTGTT	Hamelin *et al.* (1996)
Destruc-1F	CCGAGTTTACAACTCCCAAAC	this study
Destruc-2F	GTGCCTGYTTCGGCAGC	this study
Destruc-3F	GCGCCGKCTCCCAAATATAG	this study
Destruc-1R	GGTTTWACGGCGTGGCCACGCTGTT	this study
Destruc-2R	CTGTTTYCCAGTGCGAGGTGTGC	this study
Destruc-3R	CTATATTTGGGAGMCGGCGC	this study

Nested PCR reaction mixtures with Destruc-series primers contained approximately 1 µg of template DNA, 10 mM Tris–HCl (pH 8.3 at 25 °C), 2.5 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphates, 10 picomoles of each primer, and 1 U of Taq polymerase. The temperature profile was 94 °C for 3 min followed by 35 cycles at 94 °C for 20 sec, annealing temperature for 30 sec, and 72 °C for 30 sec. A final extension step was done for 7 min at 72 °C, after which the DNA was stored at 4 °C. The annealing temperature was tested from 50 °C to 65 °C for optimization.

The final optimized annealing temperature for PCR with Destruc-2F/2R was 65 °C. Nested PCR reactions with Dest1/Dest4 (Hamelin *et al.,* 1996) or with CDU1/CDL1b and CDU3/CDL1b (Kernaghan *et al.,* 2007) were done as previously described. The PCR primer pair ITS-Fu-f (5`-CAA CTC CCA AAC CCC TGT GA-3`) and ITS-Fu-r (5`-GCG ACG ATT ACC AGT AAC GA-3`) was used to detect *Fusarium* species in soil samples as described previously (Abd-Elsalam *et al.,* 2003).

The PCR reactions were the same, except that 1.75 mM of $MgCl_2$ and 5 picomoles of each primer were used. The PCR temperature profile was 95 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 30 sec, and 72 °C for 1 min. The expected size of the PCR product was 389 bp.

Cloning, sequencing, and phylogenetic analysis. Nested PCR products were purified using a PCR-purification kit (MoBio Laboratories Inc., Carlsbad, CA), for use as a template for DNA sequencing. In instances where direct sequencing of the PCR fragments were unsuccessful, the sequences were cloned into a pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Corp. Carlsbad, CA) according to manufacturer instructions. DNA sequencing was done by Genewiz, Inc. (North Brunswick, NJ). Similarity searches using BLAST

version 1.64b (Thompson *et al.,* 1997) and GeneDoc version. 2.0.004 (Nicholas et al., 1997) were used to align sequences and determine similarities. Phylogenetic and molecular evolutionary analyses were done using MEGA version 4 (Tamura *et al.,* 2007). The partial ITS regions of the rRNA operon sequences are deposited in GenBank under accession numbers HM208274 to HM208281.

RESULTS

Primer optimization and specificity to I. radicicola species. A total of eight pairs of Destruc-series primers (Table 1) were tested under various PCR con-ditions to choose the most reliable primer set based on the observed pathogenicity of root rot disease of ginseng (Table 2). The Destruc-2F/Destruc-2R pair was chosen and the PCR results were compared to those with three sets of previously developed primers specific to *I. radicicola* - Dest1/Dest4, CDU1/CDL1b, and CDU3/CDL1b. No PCR product was amplified with CDU1/CDL1b or with CDU3/CDL1b where any of the environmental DNA samples were used as a template (data not shown). The PCR detection pattern with Dest1/Dest4 corresponds to that with Destruc-2F/Destruc-2R, except for two samples that could be amplified with Destruc-2F/Destruc-2R (Figure 2).

Phylogenetic analysis. PCR products of soils S01, S04, S05, S08, S12, S14, S15 and S16 amplified with Destruc-2F/Destruc-2R were sequenced to deter-mine any phylogenetic relationships with *I. radicicola* strains. DNA (296 bp) sequences were used for analysis, excluding primer regions. All eight PCR products belong to Clade III (Mantiri *et al.,* 2001) or genus *Ilyonectria* (Cabral *et al.*, 2011).

The rRNA operon ITS region of six PCR products from soils S01, S04, S05, S12, S14, and S16 belong to Clade III-a or species *I. mors-panacis*, while two other PCR products from soils S08 and S15 belong to Clade III-b or species *I. robusta* (Cabral *et al.,* 2011; Mantiri *et al.,* 2001). Clade III-a includes pathogenic strains of *I. radicicola* (Jang *et al.,* 2005) with most strains isolated from *Panax ginseng* (Halleen *et al.,* 2006; Jang *et al.,* 2005).

The hosts of *I. mors-panacis* are *P. ginseng* and *P. quinquefolium* (Chaverri *et al.* 2011). In contrast, strains belonging to Clade III-b were isolated from various hosts, including *P. ginseng* (Halleen *et al.,* 2006; Jang *et al.,* 2005), but their pathogenicity to ginseng root has not been tested. The species *I. robusta* has been isolated from different plant roots including *P. quinquefolium*, but not *P. ginseng* (Cabral *et al.,* 2011).

Table 2. Soil samples, pathogenicity, detection, and clade based on their ITS sequences

Abbreviation	Sampling site and description	Infection[a]	Location	PCR product[b]	Clade[c]
S01	Ginseng field #1	Detected	Suwon, Korea	+	III-a
S02	Grass field	N/D	New Brunswick, NJ	-	
S03	Wet ground (similar to paddy field)	N/D	Piscataway, NJ	-	
S04	Ginseng field #2	Detected	Suwon, Korea	+	III-a
S05	3-yr-old ginseng field	Detected	Suwon, Korea	+	III-a
S06	Paddy field #1 (never planted ginseng before)	N/D	Eumsung, Korea	-	
S07	Underground soil (1m below the surface)	N/D	Eumsung, Korea	-	
S08	Ginseng field	Detected	Eumsung, Korea	+	III-b
S09	S08 soil pasteurized with 55 °C water for 3 hrs	N/D	Eumsung, Korea	-	
S10	Soybean field	N/D	Suwon, Korea	-	
S11	Corn field	N/D	Suwon, Korea	-	
S12	Barley field	N/D	Suwon, Korea	+	III-a
S13	1-yr-old ginseng field (no disease found)	Not Detected	Suwon, Korea	-	
S14	2-yr-old ginseng field (no disease found)	Not Detected	Suwon, Korea	+	III-a
S15	Ginseng field #3	Detected	Suwon, Korea	+	III-b
S16	Ginseng field #4	Detected	Suwon, Korea	+	III-a
S17	Paddy field #2 (never planted ginseng before)	N/D	Suwon, Korea	-	

Key: N/D = not determined.
[a] Root rot disease of ginseng observed.
[b] Based on the result from nested PCR with Destruc-2F and Destruc-3R (Fig. 2); + = positive; - = negative.
[c] Based on ITS sequences determined by Mantiri, *et al.* (2001) and Sefert, *et al.* (2013).

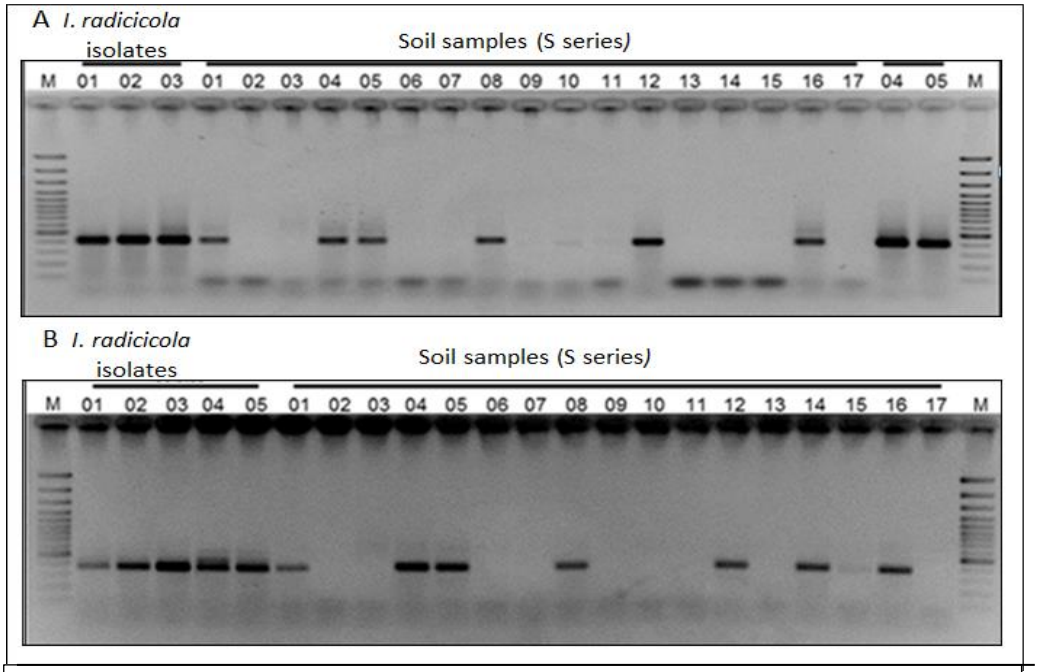

Figure 2. Detection of *lyonectria* species in various soils.
Nested PCR with universal fungal primers (ITS1 and ITS4) were done initially and then the resulting PCR products were re-amplified with Dest1 and Dest4 (A) or Destruc-2F and Destruc-2R (B). M = 100 bp size marker.

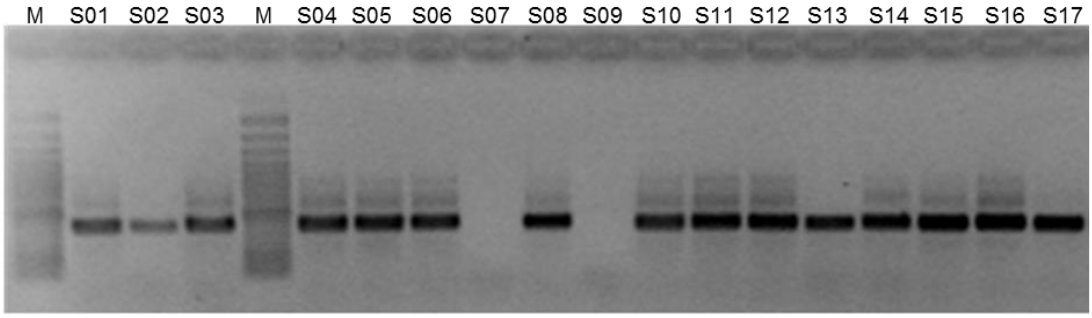

Figure 3. Detection of *Fusarium* species in various soil samples.
ITS-Fu-f and ITS-Fu-r primers were for PCR reaction. M = 100 bp size marker.

Detection of Fusarium species. Fusarium species are potential pathogenic fungi responsible for successive infection of ginseng root. PCR products of the expected size (389 bp) were amplified from all soils, except samples S07 and S09 using the PCR method previously described (Abd-Elsalam *et al.,* 2003). In addition, PCR tests on soil samples S07 and S09 with Dest1/Dest4 and with Destruc-2F/Destruc-2R did not indicate the presence of any *Ilyonectria* species (Fig. 3).

DISCUSSION

A nested PCR-based molecular method to improve the detection level and specificity of indigenous *I. radicicola* in agricultural soils was developed. The previous detection method used Dest1 and Dest4 applied in 15 cycles of nested PCR and 60 °C of annealing temperature (Hamelin *et al.,* 1996). Our newly developed method applies 35 cycles of nested PCR for improved detection and a 65 °C of annealing temperature for high stringency. Furthermore, the Dest4 primer contains one base pair mismatch at the 8th position when sequences are compared with the ITS regions of currently known *I. radicicola* strains, while all of the primer sequences match 100% or use ambiguous

codes for degenerate bases in target *I. radicicola* DNA sequences. For increased specificity to *I. radicicola*, the ITS regions of conventionally found major soil-borne plant root pathogens (*A. panax, B. cinerea, F. solani, R. solani, F. oxysporum f.* sp. *fragariae, P. cactorum, P. ulcimum,* and *C. gloeosporioides*) were aligned with those of *I. radicicola* and the homologous regions were exclud-ed from primer development.

DNA sequences of the ITS regions, Clade III-a could be further divided into two subgroups. Strains belonging to the first subgroup of Clade III-a were isolated from *Panax* species, including four isolates that cause severe root rot disease of ginseng (Jang *et al.,* 2005). These four, highly pathogenic isolates were strains CD1561 (AY295309), CY9801 (AY295310), CD1640 (AY295321), and CY9207 (AY295322). All of the PCR products, except S08 and S15, belonged to this subgroup with 100% sequence identity, suggesting virulence to ginseng root.

When the recent classification by Cabral *et al.* (2011) was used, all six PCR products within the first subgroup of Clade III-a belong to the species *I. mors-panacis* that infects two ginseng species, *P. ginseng* and *P. quinquefolium* (Cabral *et al.,* 2011). The second subgroup in Clade III-a included strains isolated from *Panax* species and other hosts, such as *Picea* (strain 94_1628) and *Pseudotsuga* (strain CR20). In this subgroup, three isolates exhibited pathogenicity to ginseng roots, but the disease severity was low (Jang *et al.,* 2005). These three low pathogenic strains were NSAC_SH_1 (AY295311), NSAC_SH_s2 (AY295313), and 94_1628 (AY295315). Based on Cabral's classification, these species belong to *I. panacis* (*N. radicicola* CDC-N-9a, AY295316), *I. rufa* (*N. radicicola* 94_1628, AY295315; *N. radicicola* CR26, AY295318), and *I. crassa* (*N. radicicola* NSAC_SH_1, AY295311; *N. radicicola* NSAC_SH_2, AY295313; *N. radicicola* NSAC_SH_2.5, AY295314) (Cabral *et al.,* 2011). The species *I. mors-panacis* is not a member of the second subgroup.

Of the PCR products, two derived from S08 and S15 belong to Clade III-b, which contains strains isolated from many other sources, including *Panax* species, such as *Panax* (strain CD1557 and CD1666), *Cornus* (strain JAT1378), *Prunus* (strain DAOM139398), and *Loroglossum* (strain CBS 321.34). The pathogenicity to ginseng roots, however, has not been tested. When the recent classification by Cabral *et al.* (2011) is applied, these two PCR products belong to *I. robusta* that infects various plant roots, such as *Loroglossum hircinum*, *P. quinquefolium*, *Quercus robur*, and *Quercus* sp. (Cabral *et al.,* 2011).

The nested PCR method with the Destruc-2F and Destruc-2R primer set, as compared with the Dest1 and Dest4 primers, was able to detect the presence of *Ilyonectria* species in two soil samples, S14 and S15. S14 was a soil sampled from a two-year-old ginseng field where no disease had yet occurred. Phylogenetic analysis based on the DNA sequence, indicated 100% identity with reported *I. radicicola* strains that cause severe root rot disease of ginseng (Jang *et al.,* 2005). The DNA sequence also indicated that *I. radicicola* strains could infect *I. mors-panacis*. These observations suggest the presence of potentially pathogenic *Ilyonectria* species in the soil of the two ginseng fields as detected by the Destruc-2F and Destruc-2R primer pair, but not by the previously developed Dest1 and Dest4 pair.

The nested PCR method was tested with four soil samples (S01, S04, S15, and S16) collected from different locations within the same ginseng field in which root rot disease of ginseng had been previously observed. The rRNA operon ITS region of the three strains detected in S01, S04, and S16 were identical and belong to clade III-a species *I. mors-panacis*. In S15, the rRNA operon ITS region belonged to clade III-b species *I. robusta*. These field samples strongly suggest the necessity of multiple sampling for a reliable assessment of *I. radicicola* contamination as the population density of the organism was not distributed uniformly within or between ginseng beds and soils, even within the same commercial region and farm.

Vernalized ginseng seed, planted in fall, geminates and begins to grow a young ginseng root the following spring. Generally, the root is allowed to continue to grow for four more years before being harvested (Proctor, 1996). Because the optimal temperature of *I. radicicola* is relatively low, approximately 20 °C (Cho *et al.,* 1995; Cho and Yu, 2001), *I. radicicola* tends to infect young ginseng roots during the spring, leaving a wound on the young ginseng root epidermis (Rahman and Punja, (2005). The tissue wound on the root enables *Fusarium* species, such as *F. solani* that has an optimum temperature of 25 to 30 °C (Saremi *et al.,* 1999), to successively breech and infect the wounded young ginseng roots the following summer, increasing the severity of root rot disease.

A *Fusarium* species-specific PCR developed by Abd-Elsalam *et al.* (2003) was able to detect *Fusarium* in all soil samples where *I. radicicola* was detected by Destruc-2F/2R or Dest1/Dest4 primer set. Neither *I.*

radicicola or *Fusarium* species were detected in soil samples S07 and S09. Except for these two samples, all other soil samples were infected with both *I. radicicola* and *Fusarium* species. Therefore the presence of *I. radicicola* may be a key factor to assess the risk of root rot disease of ginseng.

Treating a *I. radiciola* and *Fusarium* infected soil sample exposed to 55 °C water for 3 h pasteur-ized the soil, removing all traces of the disease organisms and preventing infection of ginseng roots. Development of a real-time PCR detection approach that indicates the need for field fumigation would undoubtedly enhance both ginseng root yield and quality.

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