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Research Article

Genetic Diversity and Pathogenicity of *Cylindrocarpon destructans* Isolates Obtained from Korean *Panax ginseng*

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Abstract We analyzed the genetic diversity of *Cylindrocarpon destructans* isolates obtained from Korean ginseng (i.e., *Panax ginseng*) roots by performing virulence tests and nuclear ribosomal gene internal transcribed spacer (ITS) and mitochondrial small subunit (mt SSU) rDNA sequence analysis. The phylogenetic relationship analysis performed using ITS DNA sequences and isolates from other hosts helped confirm that all the Korean *C. destructans* isolates belonged to *Nectria/Neonectria radicicola* complex. The results of *in vivo* and *ex vivo* virulence tests showed that the *C. destructans* isolates could be divided into two groups according to their distinctive difference in virulence and the genetic diversity. The highly virulent Korean isolates in pathogenicity group II (PG II), together with foreign isolates from *P. ginseng* and *P. quinquefolius*, formed a single group. The weakly virulent isolates in pathogenicity group I, together with the foreign isolates from other host plants, formed another group and exhibited a greater genetic diversity than the isolates of PG II, as confirmed by the mt SSU rDNA sequence analysis. In addition, as the weakly virulent Korean isolates were genetically very similar to the foreign isolates from other hosts, they were likely to originate from hosts other than the ginseng plants.

Keywords *Cylindrocarpon destructans*, Genetic diversity, Ginseng root rot, *Panax ginseng*, Pathogenicity

Cylindrocarpon destructans (teleomorph: *Nectria/Neonectria radicicola*), a soil-borne pathogenic fungus, can cause a primary root rot disease in ginseng (*Panax ginseng* and *P. quinquefolius*), reduce the yield of ginseng production, and result in great economic losses [1, 2]. In addition, *C. destructans* has been reported to lead to replant failure, due to its ability to survive in the soil for more than ten years after the harvest of ginseng [3]. *C. destructans* was originally described as *Ramuraria destructans* according to the root rot symptoms of American ginseng (*P. quinquefolius*) by Zinssmeister [4]. In South Korea, it had been identified only in ginseng, strawberries and peonies [5] since it was firstly discovered in ginseng roots by Chung [6], although it was shown to affect the roots of diverse woody plants and to cause one of the most severe fungal diseases worldwide [7-11].

A large number of pathogenic fungi in the genera of *Cylindrocarpon*, *Fusarium*, and *Cylindrocladium*, which belong to *Nectriaceae* (*Hypocreales*), show taxonomically close association with *C. destructans* [12]. Mantiri *et al.* [13] proposed the presence of approximately 125 species in the genus of *Cylindrocarpon*, and Booth [14] divided this genus into four groups, namely *C. magnusianum*, *C. cylindroides*, *Nectria mammoidea*, and *C. destructans*, according to the presence of microconidia and chlamydospores. Furthermore, Samuels and Brayford [15] reviewed the existing classification system and categorized *Nectria radicicola*, including the asexual generation of *C. destructans*, into three variations, known as var. *radicicola* (anamorph: *C. destructans* var. *destructans*), var. *coprosmae* (anamorph: *C. destructans* var. *coprosmae*), and var. *macroconidiales* (anamorph: *C. macroconidiales*), based on their morphological and culture characteristics.

Mantiri *et al.* [13] modified Booth's [14] classification and redefined the genus of *Nectria* into three clades (i.e.,

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clade I: *Nectria coccinea/galligena* group; clade II: *N. mammoidea/veuillotiana* group; and clade III: *N. radicicola* group) through the sequence analysis of mitochondrial rDNA, and included *C. destructans* into clade III. Seifert *et al.* [16] conducted DNA sequence analysis of nuclear ribosomal internal transcribed spacer (ITS) region and partial β-tubline gene using *C. destructans* isolates from diverse hosts and closely related species. They reported that *N. radicicola* complex isolates in the clade III established by Mantiri *et al.* [13] could be further divided into subclades a and b, and that all the isolates from Korean and Japanese ginseng (*P. ginseng*) and Canadian ginseng (*P. quinquefolius*) are genetically close to each other and belong to subclade b. Recently, several attempts have been made to establish a new type of classification system for the *Nectria/Neonectria* complex and to analyze the genetic diversity using various morphological, pathogenic, and genetic analytical approaches [9, 17-19]. Although the genetic variation of Korean *C. destructans* population that causes root rot in *P. ginseng* has been evaluated by random amplified polymorphic DNA analysis [20, 21], the pathogenic and taxonomical characteristics of *P. ginseng* in Korea have been rarely studied. As the genetically distinct populations may show difference in host range, aggressiveness, and susceptibility to disease control treatment, a better understanding of the genetic and pathogenic variations in *C. destructans* will be important for developing suitable root rot disease management strategies [16].

In this study, ITS region and mitochondrial small subunit (mt SSU) rDNA which were frequently used for analyzing the genetic diversity of phytopathogenic fungi [22, 23] were sequenced from Korean ginseng isolates, and compared with those from the foreign isolates to locate the taxonomic position of the *C. destructans* group. The genetic diversity of *C. destructans* isolates was analyzed by evaluating their virulence both *in vivo and ex vivo*. The data obtained from this study will be useful for constructing effective treatment strategies against ginseng root rot diseases.

MATERIALS AND METHODS

Isolation of pathogenic fungus. In this study, *C. destructans* was isolated from diseased ginseng roots that demonstrated typical symptoms of root rot; the ginseng roots were obtained from the main ginseng-cultivating regions of South Korea (Table 1). The diseased tissues on the collected ginseng roots showing brown discoloration were washed in running water. The tissues of both healthy and rotten parts were sliced into 5-mm pieces, treated with 2% NaOCl for 1 min for surface disinfection, washed two or three times in sterilized water, and placed on filter paper for dehydration. The affected tissues, whose surface had been previously disinfected, were placed on water agar or on pentachloronitrobenzene agar medium [24] and cultured at 15°C for seven days. Subsequently, the spores were isolated from the hypha grown on the tissues cultured in potato dextrose agar (PDA) medium at 15°C for ten days. Individual spores were then isolated, inoculated, and cultured in PDA medium at 20°C for 2 wk and re-inoculated into PDA medium for virulence tests. Furthermore, their morphological characteristics on PDA media were observed for identification of the isolates.

Isolation of genomic DNA from *C. destructans*. After the pure culture, the isolates were inoculated into a potato dextrose broth medium and subject to stationary culture at 20°C for ten days for isolating the genomic DNA. The mycelia were then harvested, split into 1.5-mL Effendorf

Table 1. A list of Korean ginseng (*Panax ginseng*) isolates of *Cylindrocarpon destructans* used in this study

Isolate	Geographic origin	Age of ginseng (year)	Accession No.		Pathogenicity group[a]
			ITS region	mt SSU rDNA	
CY001	Wanju, Jeonbuk	4	KF894988	KF895011	PG I
CY003	Yeoju, Gyeonggi	6	KF894989	KF895012	PG I
CY008	Icheon, Gyeonggi	4	KF894991	KF895014	PG II
CY010	Goesan, Chungbuk	4	KF894992	KF895015	PG II
CY030	Yeongju, Gyeongbuk	4	KF894993	KF895016	PG I
CY033	Icheon, Gyeonggi	6	KF894994	KF895017	PG II
CY036	Goesan, Chungbuk	4	KF894995	KF895018	PG II
CY037	Okcheon, Chungbuk	4	KF894996	KF895019	PG II
CY050	Boryeong, Chungnam	3	KF894997	KF895020	PG II
CY055	Boryeong, Chungnam	3	KF894999	KF895022	PG II
CY060	Goesan, Chungbuk	4	KF895000	KF895023	PG II
CY063	Hongcheon, Gangwon	6	KF895001	KF895024	PG I
CY066	Goesan, Chungbuk	4	KF895002	KF895025	PG II
CY075	Yangju, Gyeonggi	6	KF895003	KF895026	PG II
CY076	Goesan, Chungbuk	4	KF895004	KF895027	PG II

ITS region, internal transcribed spacer region; mt SSU rDNA, mitochondrial small subunit ribosomal DNA.
[a]Pathogenicity group: PG I (lesion size < 8.1 mm, diseased rate < 81%) and PG II (lesion size > 8.0 mm, diseased rate > 80%).

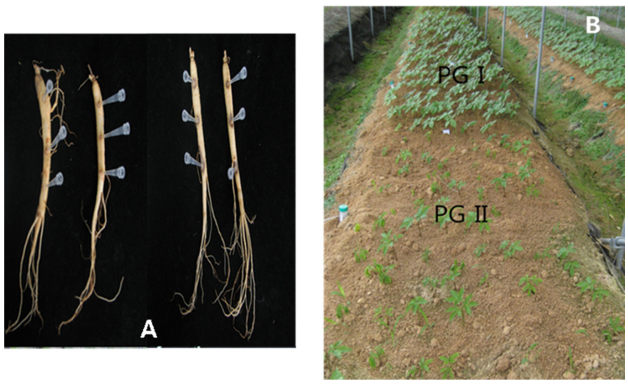

Fig. 1. Optical images showing symptoms of root rot due to *Cylindrocarpon destructans* on wounded 2-year-old ginseng plants *ex vivo* (A), and field-grown ginseng plants affected by weakly (PG I) and highly (PG II) aggressive *C. destructans* isolates *in vivo* (B).

tubes, lyophilized, and ground to extract the genomic DNA, as per the method of Doyle and Doyle [25].

Virulence test. *Ex vivo* and *in vivo* tests were performed to determine the virulence of *C. destructans* isolates obtained from the ginseng roots (Table 1, Fig. 1). In the *ex vivo* test, the surface of a 2-year-old ginseng plant was disinfected with 2% NaOCl. And the isolates cultured in PDA medium for 14 days were used as an inoculum. We placed a piece of paper towel wetted with sterilized water in a sealed container disinfected with 70% ethanol, fixed it onto the ginseng root pierced using plastic pipette tips (5 mm in diameter) containing the cultured mycelia, and stored it in an incubator for 4 wk, before measuring the size of the lesions. An average value of the lesion size was obtained to provide a mean value for the six replicate roots for each treatment. In the *in vivo* test, ginseng seedlings were dipped in the mycelia and spores suspension, which was prepared by suspending the ground isolates cultured at 20°C for 14 days after inoculation into a PDA medium in 40 mL of sterilized water, and transplanted into the pre-planting field for ginseng cultivation. Seven months later, an average value of diseased rate (%) was assessed to provide a mean value for the ten roots for each treatment. The ginseng isolates were divided into two groups according to the results of the virulence tests. The isolates showing an average lesion size less than 8.1 mm and diseased rate lower than 81% were categorized as pathogenicity group I (PG I); and those with an average lesion size more than 8.0 mm and diseased rate higher than 80% were classified as pathogenicity group II (PG II).

Genetic relatedness analysis. PCR amplification of approximately 600-bp region of ITS and mt SSU rDNA region was performed using ITS1 and ITS4 primers [26] and NMS1 and NMS2 primers [27], respectively. For each amplification, a 0.5 pmol primer, 2 ng of genomic DNA, 0.2 mM deoxynucleotide triphosphate (dNTP), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 units of Taq DNA polymerase were added to sterilized water to obtain a final volume of 50 μL. The PCR conditions used in analyzing the ITS region were as follows: an initial denaturation step at 95°C for 3 min; followed by 35 cycles of 95°C for 35 sec, 55°C for 1 min, and 72°C for 1 min; and a final elongation step at 72°C for 8 min. The PCR conditions for mt SSU rDNA region analysis were as follows: an initial denaturation step at 95°C for 3 min; followed by 34 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and a final elongation step at 72°C for 4 min. Each of the PCR products were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide for visualizing its amplification and size.

A Solgent PCR Purification Kit (Solgent Co., Ltd., Seoul, Korea) was used to purify the amplified PCR products, according to the manufacture's protocol and sequenced by Genotech Co., Ltd. (Daejeon, Korea). Sequences generated from the present study were deposited in Genbank (Table 1). The PHYDIT program ver. 3.2 [28] was used to align the sequences, and the unclearly aligned fragments were excluded from the analysis. Furthermore, PHYLIP 3.57c Package [29] was used to produce a neighbor-joining tree based on Kimura's two-parameter model [30], and an analysis of 1,000 bootstrap samples was carried out to assess its reliability.

RESULTS AND DISCUSSION

Virulence test. Virulence tests were performed on all the Korean ginseng isolates of *C. destructans* that formed white or brown colonies with microconidia, macroconidia, and chlamydospores on PDA media (data not shown). *C. destructans* isolates obtained from the diseased ginseng tissues can be divided into two groups based on their virulence (Table 1, Fig. 1) determined by the *in vivo* and *ex vivo* tests. In the *in vivo* test, an entire rotten root and weakened ginseng tissues were observed in the ginseng plants with withered leaves in the aerial part or in case of leaves failing to grow; and in such a case, normal ginseng growth was not observed, and the isolates were found to be highly virulent (PG II) after inoculation. Whereas, in the case where the root rot was found only in some parts of the plants, normal ginseng growth was observed, and weakly virulent isolates (PG I) were identified post-inoculation. Kim [31] observed that the symptoms of root rot in ginseng growth varied with the soil types, plant ages, and cultivating seasons, and he assumed that they also varied with the differences in the virulence among the *C. destructans* isolates rather than with the environmental factors. Matuo and Miyazawa [7] reported that the *C. destructans* isolates obtained from tea plants and cedar trees were weakly virulent against ginseng seedlings, although the isolates from ginseng plants were highly virulent. In this study, remarkable difference in virulence was identified

among the *C. destructans* isolates grown on *P. ginseng*, and the isolates could be clearly divided into two groups—PG I and PG II—on the basis of their virulence.

Analysis of genetic diversity. DNA sequence analysis of ITS region is often used to examine the genetic diversity of phytopathogenic fungi [22]. Seifert *et al.* [16] reported the presence of two groups, subclades a and b, in clade III, including the *Nectria/Neonectria radicicola* complex. In this study, the DNA sequences of ITS region from 15 *C. destructans* isolates of rotten ginseng roots collected from several different regions in Korea were used to analyze the genetic relatedness of *C. destructans* isolates with *Nectria/Neonectria radicicola* complex, as suggested by Seifert *et al.* [16] (Table 2, Fig. 2). All the Korean *P. ginseng* isolates of *C. destructans,* together with the isolates in clade III, formed a single group, with 98% bootstrap support. Among them, the highly virulent *C. destructans* (PG II) isolates from diverse ginseng-cultivation regions in Korea showed 100% similarity, and together with Japanese (IFO31881, IFO31882) and Canadian (NSAC SH 1, NSAC SH 2.5) ginseng isolates in clade III, formed the subclade a. Moreover, they showed 100% DNA sequence homology to the Japanese *P. ginseng* isolates, but not to the Canadian *P. quinquefolius* isolates. Though Seifert *et al.* [16] reported that all Korean, Japanese, and Canadian ginseng isolates belonged to subclade a in clade III, we observed 97.0~100% similarity among the weakly virulent *P. ginseng* isolates in PG I, which formed subclade b, along with the foreign isolates from diverse hosts. This finding indicates a great genetic diversity among the weakly virulent isolates, which is contrary to the results observed for the highly virulent isolates that form a single group with a high genetic homology.

Mantiri *et al.* [13] elucidated phylogenetic relationships among *Neonectria* species with *Cylindrocarpon* anamorphs using mt SSU rDNA region, and identified four well-supported clades or groups of the *Neonectria* complex. Based on this result, we analyzed the genetic relatedness between the DNA sequences of the PCR products obtained from Korean ginseng isolates of *C. destructans* and the DNA sequences of foreign *Neonectria*/*Cylindrocarpon* isolates and closely related species, which belonged to the *Neonectria* complex, as identified by GeneBank (Table 2, Fig. 3). In the analysis of the ITS region, both weakly (PG I) and highly (PG II) virulent isolates formed a monophyletic clade or group, and yet they formed different clusters in group III of *Neonectria*. Apart from the isolates in PG II, which showed 100% DNA sequence homology to each

Table 2. Identification of reference isolates used in the molecular analysis

Species	Strain No.	Host	Accession No.
C. cylindroides	CR6	*Pseudotsuga menziesii*	AY295301
	c2cun2ab2	*Pseudotsuga mensiezii*	AF315201
C. obtusisporum	94-1356	*Picea mariana*	AY295304
	JAT 1366	*Prunus armenica*	AY295306
C. radicicola	AR2553	Bark	AF220968
	RTDF14	*Pseudotsuga mensiezii*	AF315203
	FMd2.1	*Alnus rubra*	AF315204
Cylindrocarpon sp.	JAT 1591	*Pyrus* sp.	AY295302
	JAT1590	*Prunus armeniaca*	AY295308
Nectria cinnabarina	CBS 279.48	*Acer pseudoplatanus*	AF163025
N. radicicola	IMI 376404	*Malus* sp.	AJ007357
	IMI 061536	*Narcissus* sp	AJ007354
	CTR71-322	Wood	AF220969
	IMI 375719	*Malus* sp.	AJ007356
	IMI 375717	-	AJ007355
	IMI 376403	*Alnus* sp.	AJ007351
	IMI 376408	*Arbutus menziesii*	AJ007352
Neonectria galligena	JR0609B-2	*Malus pumila*	AF315206
Neon. liriodendri	USSO150	*Vitis* sp.	AY997585
	USST148	*Vitis* sp.	AY997584
Neon. radicicola	JAT1378	*Cornus floridae*	AY295328
	IFO31881	*Panax ginseng*, Japan	AY295323
	IFO31882	*Panax ginseng*, Japan	AY295324
	NSAC SH 2.5	*Panax quinquefolius,* Canada	AY295314
	CBS 129083; NSAC-SH-1	*Panax quinquefolius,* Canada	AY295311
	94-1628	*Picea glauca*	AY295315
	JAT 1551	*Prunus persica*	AF315202
	CR20	*Pseudotsuga*	AY295317
	CR26	*Pseudotsuga menziesii*	AY295318
Neon. ramulariae	CBS 730.87	-	AJ279446

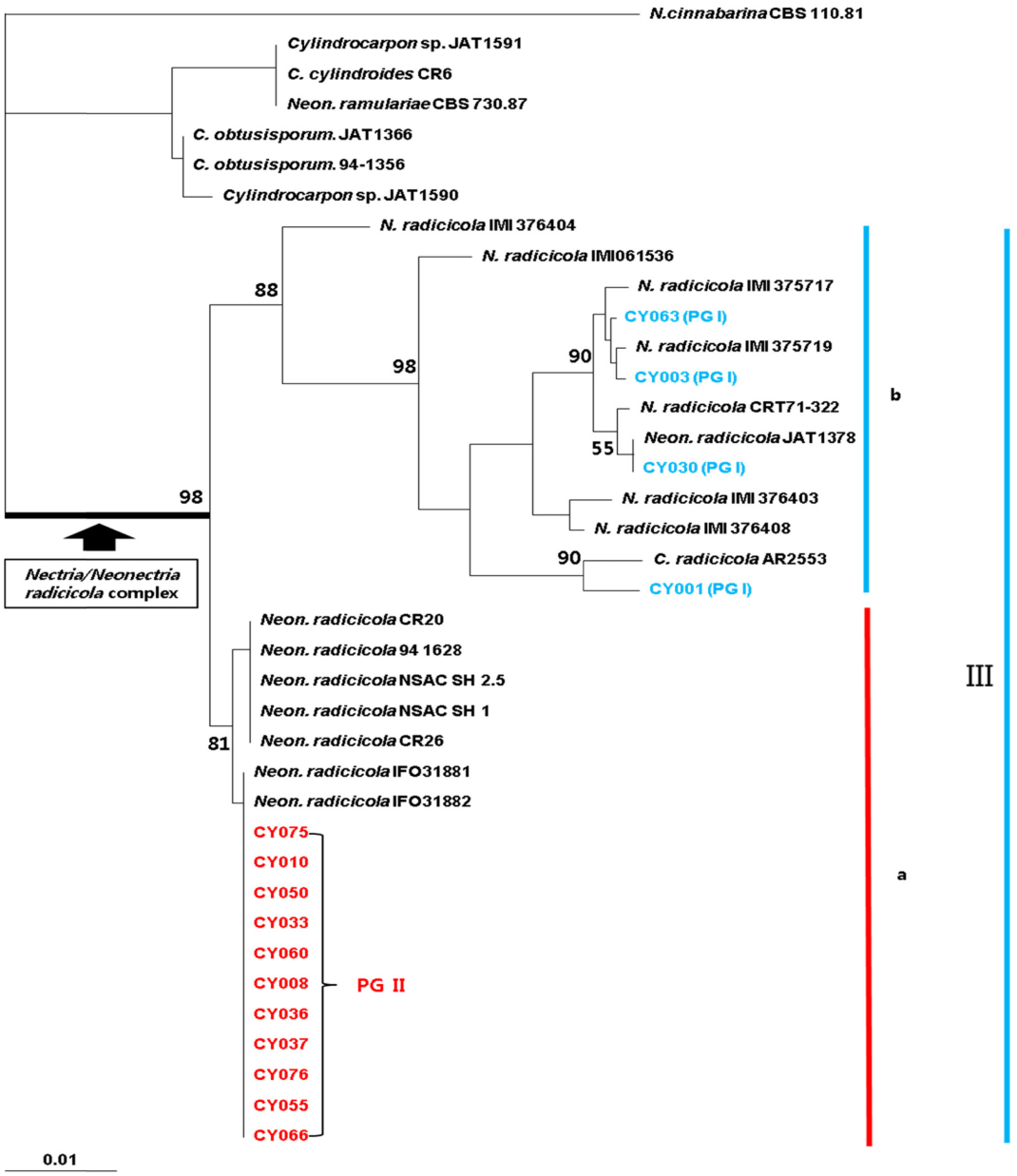

Fig. 2. Phylogenetic analysis of Korean ginseng *Cylindrocarpon destructans* isolates with respect to the *Nectria/Neonectria radicicola* complex of Seifert *et al.* [16] by using internal transcribed spacer sequence region. The bootstrap analysis was performed with 1,000 replications.

other and formed a specific group, the isolates in PG I showed 99.5~100% homology, and together with the foreign isolates from various hosts, formed an individual cluster.

Cabral *et al.* [17] recently renamed the *Nectria/Neonectria radicicola* complex as *Ilyonectria radicicola* complex after analyzing its morphological characteristics and multi-gene relatedness, and reclassified the fungi in this complex into 15 species under the genus of *Ilyonectria* based on their morphological characteristics and genetic diversity. They also reported the presence of genetically diverse fungi within the group of *P. quinquefolius* isolates, which were originally divided into four species: *I. mors-panacis*, *I. robusta*, *I. panacis*, and *I. crassa*. In particular, both Japanese *P. ginseng* isolates and those highly virulent to *P. quinquefolius* were included in *I. mors-panacis*. The Japanese isolates (IFO31881) in this study showed 100% hereditary homology to the highly virulent Korean isolates in PG II group. Thus, they were expected to belong to *I. mors-panacis*, as per the classification scheme of Cabral *et al.* [17].

In this study, we found that the *P. ginseng* isolates collected from several regions in Korea could be divided into two distinct groups on the basis of their virulence, although they all belonged to the same complex of *Nectria/Neonectria radicicola*. All the highly virulent isolates were

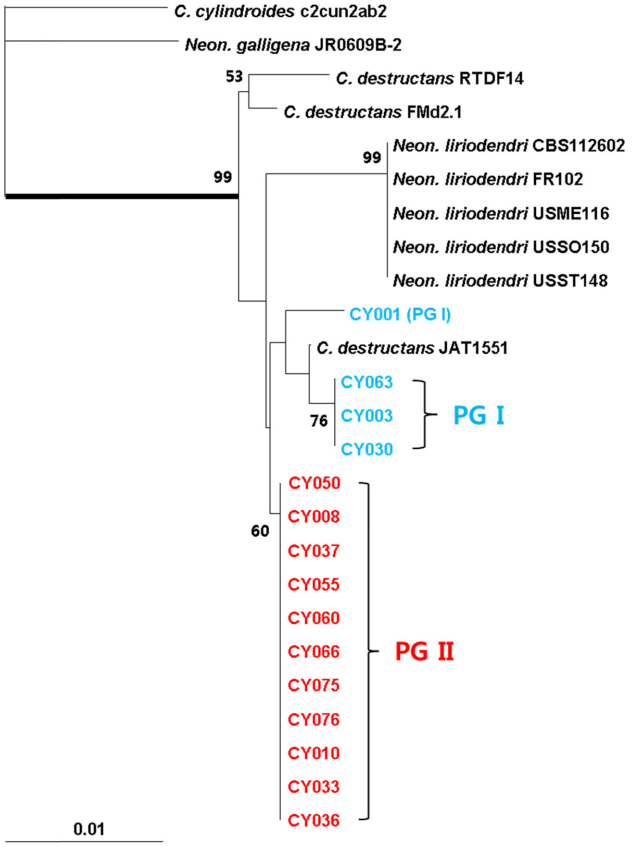

Fig. 3. Phylogenetic analysis of other host plants and closely related isolates with respect to Korean ginseng *Cylindrocarpon destructans* isolates by using mitochondrial small subunit rDNA. The bootstrap analysis was performed with 1,000 replications.

genetically homologous and formed a specific single group, whereas the weakly virulent isolates were genetically similar to the isolates from other hosts and showed a significant genetic diversity. These results suggest that the domestic group of weakly virulent isolates may possibly originate from hosts other than the ginseng plants.

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