Paraphaeosphaeria recurvifoliae, a new species causing leaf spots and necrosis on *Yucca recurvifolia*

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Brown leaf spot and necrosis was observed on pendulous yucca in house gardens of Korea. The causal fungus was isolated from the leaf lesion and identified as a new species of *Paraphaeosphaeria* based on pathogenicity tests, morphology of pycnidia and pycnidiospores, and ITS rDNA sequence data. This study reports the pathogenicity, morphological characteristics and phylogenetic status of a new species named *P. recurvifoliae*.

Key words: ITS rDNA sequence, new species, *Paraphaeosphaeria*, yucca.

Introduction

Yuccas are evergreen, perennial shrubs or trees that belong to the *Agavaceae* and produce tough, sword-shaped leaves and large clusters of white, rounded to bell-shaped flowers (Clemson Extension, 2002). *Yucca schidigera* is a plant native to South-Western United States, North Baja California and Mexican deserts and has been used as foodstuff and folk medicine by American Indians and early Californian settlers (Hristov *et al*., 1999; Marzocco *et al*., 2004). It is regarded as GRAS (generally recognized as safe) for human dietary use by the United States Food and Drug Administration. In Korea, yucca extract has also been used as an additive to enhance fermentation efficacy (In and Lee, 2004). Two exotic yucca species, pendulous yucca (*Y. recurvifolia*) and Adam's needle (*Y. filamentosa*) have been introduced to Korea as garden plants with tubular flowers.

Severe leaf spot and necrosis has frequently been observed on pendulous yucca in house gardens of Korea. In this study, the causal fungus was shown to be a species of *Paraphaeosphaeria*. *Paraphaeosphaeria* was originally described by Eriksson (1967) and has been revised by Hedjaroude (1968) and Shoemaker and Babcock (1985). The genus resembles *Phaeosphaeria* but is

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distinguished by the presence of a septum in the ascospores, the shape of ascospores and the *Coniothyrium*-type anamorph (Eriksson, 1967). Taxa differ in ascomatal structure, ascospore septation and size, and host-specificity (Wong *et al*., 2000; Camara *et al*., 2002). Species of *Paraphaeosphaeria* are found on a wide range of dicotyledonous and monocotyledonous plants (Checa *et al*., 2002). The genus is especially common on the *Agavaceae* from which *Paraphaeosphaeria agavensis*, *P. conglomerata*, *P. lamentosa*, *P. nolinae*, *P. obtusispora* and *P. quadriseptata* have been described (Checa *et al*., 2002). Recently, *P. barrii* was reported as a new species on *Y. schidigera* from Mexico (Checa *et al*., 2002).

Camara *et al*. (2002) described *Neophaeosphaeria* and *Phaeosphaeriopsis* as segregates of *Paraphaeosphaeria* based on small subunit nuclear ribosomal DNA (SSU nrDNA). Several *Paraphaeosphaeria* species including *P. agavensis*, *P. filamentosa*, *P. obtusispora* and *P. quadriseptata* commonly occur on living leaves of various yucca plants and possibly on other yuccas of the *Agavaceae* from Asia, Europe and North America (Morgan-Jones and White, 1987; Barr, 1992; Checa *et al*., 2002), but there have been no reports on the distribution of *Paraphaeosphaeria* taxa in Korea.

Some anamorphs of *Paraphaeosphaeria* are *Coniothyrium*-like, whereas others are more typical of *Microsphaeropsis* (Sutton, 1980; Morgan-Jones and White, 1987). A *Microsphaeropsis*-like coelomycete, *M. concentrica* Desm., has been reported only on *Y. aloifolia*, *Y. filamentosa* and *Y. recurvifolia* in the USA and Venezuela. In Korea, leaf spots and necrotic lesions have been frequently observed on yucca plants of house gardens, suggesting the possible occurrence of pathogenic fungal species on yuccas.

In this study, a new fungus was isolated from pendulous yucca, pathogenicity to yucca was tested, morphology of pycnidia and pycnidiospores was observed, and ITS regions including 5.8S rDNA were sequenced to compare with the related species of *Coniothyrium*, *Microsphaeropsis* and *Paraphaeosphaeria* in order to determine its phylogenetic position.

Materials and methods

Fungal strains

Brown to black leaf spots and necrosis lesions were collected from pendulous yuccas of house gardens located in Daejeon, Korea, in 2002 to 2004, and were maintained at room temperature. Collected samples were deposited at MRDB (HBL020815 to HBL020820; Microbial Resources Data Base, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea), at SFC

[L041010 (GenBank accession number AY957476); Seoul National University Fungus Collection, Seoul, Korea] and at CABI Bioscience (IMI387175; Centre for Agriculture and Biosciences International, Egham, UK) as dried materials. The causal fungus was isolated into pure culture on potato dextrose agar (PDA) or potato dextrose leaf extract agar (PDLA) amended with antibiotics, from conidial masses exuded from conidiomata of the leaf lesions. The isolated fungus was incubated on PDA at 25°C for 4-5 days. The new isolate and 27 strains of fungi used in this study are listed in Fig. 4.

Pathogenicity test

Pathogenicity tests were conducted twice by inoculating slightly wounded and non-wounded leaves (each five detached leaves) of young yucca plants with mycelial agar plugs (15 mm diam.) taken from a culture gown on PDLA medium, and placing those leaves in a moist chamber at 25°C. Control leaves were inoculated with pure agar plugs. Plugs were fixed in place with Parafilm and removed after one week.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from both natural leaf spot lesions and mycelia cultured on PDA plates using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). The ITS region (ITS1-5.8S-ITS2) of nuclear rDNA was amplified from the extracted genomic DNA with a primer pair, ITS5 and ITS4 (White *et al*., 1990), using Quick PCR Premix containing *Taq* DNA polymerase, dNTPs, reaction buffer and tracking dye (GENENMED, Seoul, Korea). The PCR reaction was conducted with an initial step of 10 minute at 95°C followed by 40 thermal cycles, each cycle consisting of 1 minute at 94°C for denaturation, 1 min at 52°C for annealing and 1 minute at 72°C for extension. A final cycle of 10 minutes at 72°C was performed for terminal extension. Amplified PCR products were detected on 0.75% agarose gel through electrophoresis. Checked amplicons were purified with AccuPrep® PCR Purification Kit (Bioneer, Daejeon, Korea). The purified PCR products were sequenced with the above primer pair using ABI3700 Automated DNA Sequencer (Applied Biosystems, Foster, CA, USA).

Phylogenetic analyses

Using CLUSTAL X ver. 1.83 (Thompson *et al*., 1997), sequences were aligned with those retrieved from GenBank with gap opening penalty 10.0 and

gap extension penalty 0.05. Using BioEdit ver. 5.0.9 (Hall, 1999), ambiguous and uninformative variable sites were excluded and an optimized sequence dataset was submitted to subsequent phylogenetic analyses. Based on parsimony analysis, phylogenetic analyses were conducted by the heuristic search with tree bisection reconnection (TBR) branch swapping using PAUP 4.0b10 (Swofford, 2002). All gaps were treated as missing data. *Leptosphaeria bicolor* was selected as an outgroup. To evaluate confidence levels for internal nodes of most parsimonious trees, 1000 nonparametric bootstrap replications (resampling size = 1000; TBR swapping; MAXTREES unrestricted) were used (Felsenstein, 1985). Sequence similarity between ITS sequences was calculated using BioEdit ver. 5.0.9.

Results

Description of the new species

Paraphaeosphaeria recurvifoliae H.B. Lee, K.M. Kim & H.S. Jung, **sp. nov.**
(Figs. 1-4)

Etymology: The Latin *recurvifoliae* refers to the specific name of the host, *Yucca recurvifolia*.

Coloniae in agaro PDA dicto primum albidae vel cremeae, deinde nonnumquam massa conidiorum nigrescens, ad 55.5 mm diam. post 12 dies 25°C. *Pycnidia* brunnea vel atra, ostiolata, superficialia vel partim immersa, 78-140 (in medio 128) µm diam. *Conidia* primum hyalina, deinde subhyalina vel fuscescentia, verruculosa, forma variabilia, rotundata vel ellipsoidea, 5 × 3(-4) µm vel breviora, in substrato naturali circum ostiolum aggregata. *In medio* seminaturali folia continente conidia copiosa. *Cellulae conidiogenae* phialides, discretae, subglobosae vel irregulariter ampulliformes, hyalinae, superficiem internam conidiomatum tegentes, 4-5 × 3-5 µm.

Colonies on PDA at first whitish to cream coloured, sometimes producing blackish conidial masses, with mycelial growth of 55.5 mm diam. over 12 days on PDA at 25°C. *Pycnidia* brown to black, ostiolate, superficial to partly immersed, ranging 78-140 (av. 128) µm in diam. Pycnidia observed around ostioles on natural lesions. *Conidial masses* abundantly produced on semi-natural leaf medium. *Conidia* hyaline, becoming subhyaline to dark, with uneven surface, variously shaped, round to ellipsoidal, 5 × 3(-4) µm. *Conidiogenous cells* phialidic, discrete, subglobose to irregularly ampulliform, hyaline at inner cells of conidiomatal wall, 4-5 × 3-5 µm in size.

Holotype here designated: from a pendulous yucca in a house garden of Daejeon, Korea, 15 August 2002, H.B. Lee (IMI387175, as a dried material).

Isotypes: Microbial Resources Data Base, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea (HBL020815 to HBL020820;); Seoul National University Fungus Culture Collection (SFCC), Seoul, Korea (SFCC L041010).

Comments: This new *Paraphaeosphaeria* species found on the *Agavaceae* is described from both leaf spot lesions and artificial medium. The

Fig. 1. Brown spots and necrotic lesions on leaves of *Yucca recurvifolia*. **A.** Healthy leaves. **B.** Old and new leaf spots and necrotic lesions. **C.** New leaf spots and necrotic lesions. **D.** Advanced leaf spots and discolouring necrotic lesions. **E.** Irregularly developed leaf spots and necrotic lesions of curling leaves (magnified).

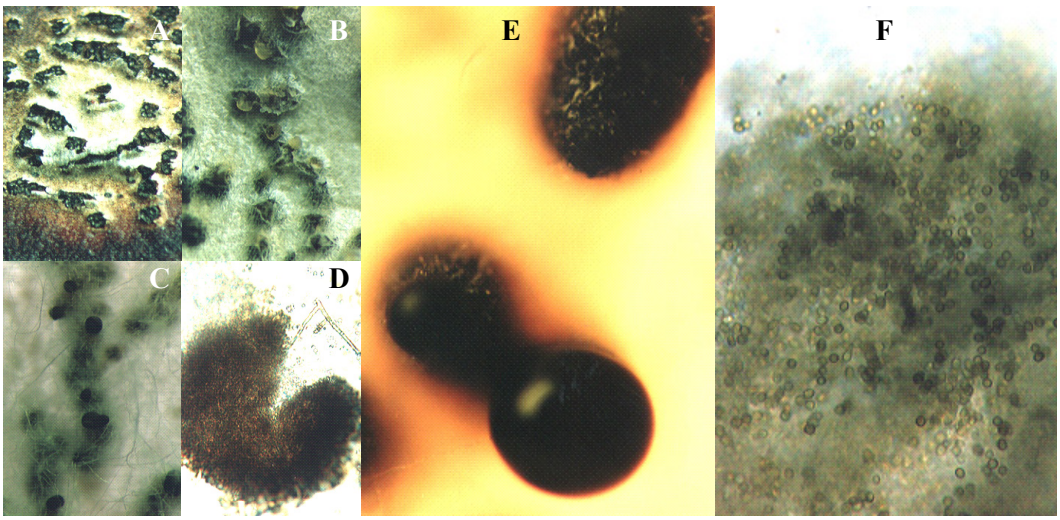

Fig. 2. Leaf spot lesions, pycnidia, conidiomata and conidia of *Paraphaeosphaeria recurvifoliae*. **A.** Lesions with pycnidia partly immersed below leaf surface on blotter under moist condition. **B.** Brownish conidial cirrhus on leaf lesions. **C.** Blackish conidial cirrhus on potato dextrose leaf medium. **D.** a small sized pycnidium. **E.** Blackish conidiomata from pycnidia formed on potato dextrose leaf medium. **F.** A number of conidia exuded from conidiomata.

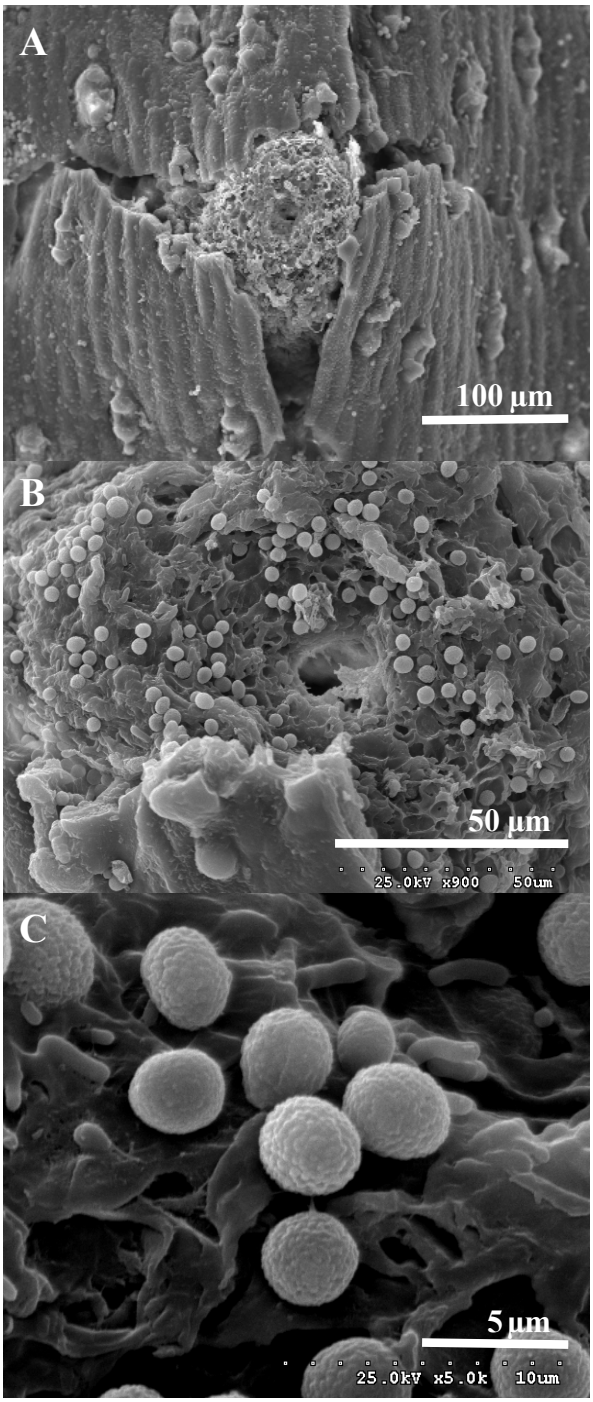

Fig. 3. Scanning electron microscopy of *Paraphaeosphaeria recurvifoliae* on a natural leaf lesion. **A.** A pycnidium (× 180). **B.** a conspicuous ostiole and pycnidiospores (× 900). **C.** pycnidiospores (× 5000).

initial symptoms were generally observed in early June and consisted of small brown leaf spots that expanded and coalesced, resulting in discolouration, blight, necrosis and defoliation (Fig. 1, B-E) compared to those of healthy yucca leaves (Fig. 1, A). Leaf lesions with conidiomata were amphigenous but chiefly epiphyllous, orbicular, irregular, distinct, bright or blackish-brown to black, and attained commonly 10 mm (sometimes up to 45 mm) in diam. Leaf spots were more severe on the leaves near the ground and progressed toward upper leaves. Leaf spots were observed in the following year, and the disease residues seemed to act as primary inoculum.

Colonies of the new species on PDA were first white but later changed and often formed a sector (data not shown). Pycnidia with a conspicuous ostiole were often observed on natural leaf lesions. Pycnidia and conidiomata were also observed from leaf lesions on both blotter and PDLA medium. A conidial cirrhus was frequently observed on leaf lesions and asceptically plated on PDA or on blotter under moist condition (Fig. 2, B and C). The surface of conidia were observed to be uneven and embossed under SEM (Fig. 3, A-C). A *Microsphaeropsis*-like coelomycete previously known as *M. concentrica* was observed on both natural yucca leaf lesions and artificial media. So far, the *Microsphaeropsis*-like anamorphs have been considered to consist of mostly saprobic forms on herbaceous stems and branches (Morgan-Jones and White, 1987). Figure 3 shows SEM pictures of a small pycnidium (partly immersed below the leaf lesion surface) and small pycnidiospores (5 µm or less in diam.) exuded from the pycnidium through the conspicuous ostiole. The new species is morphologically similar to *Microsphaeropsis*, but phylogenetically closely related to *Paraphaeosphaeria* species such as *P. obtusispora* and *P. glauco-punctata* based on ITS sequence data.

Pathogenicity

When the pendulous yucca plant was inoculated using cultured agar plugs, small, sunken spots and necrotic lesions appeared on 80% (av.) of the leaflets within 2-3 weeks. Results from pathogenicity tests were similar in a repeated test. The same fungus was successfully re-isolated from the leaves on which lesions developed after inoculation. However, any symptoms did not develop on the control leaves and *Paraphaeosphaeria* were not obtained from those leaves.

Phylogenetic analyses

In the analysis of phylogenetic relationships of the new species and 27 related taxa (11 *Paraphaeosphaeria* species, 3 *Phaeosphaeria* species, 1

Microsphaeropsis species, 5 *Coniothyrium* species, 7 related species, and 1 *Leptosphaeria* species as an outgroup using parsimony method), eight most parsimonious trees (tree length = 794 steps, CI = 0.519, RI = 0.709) were generated and one of them is presented in Fig. 4. Based on the most parsimonious tree, the new fungus (AY957476) clustered with *P. obtusispora* with a bootstrap value of 70%. *Paraphaeosphaeria glauco-punctata* was sister to the clade of the new fungus with 67% bootstrap value. ITS sequence analysis revealed that this fungus had sequence similarities of 94.4% and 94.1% to *P. obtusispora* (AF250822) and *P. glauco-punctata* (AF250819), respectively, showing that this new strain was phylogenetically separate at species level. As shown in Fig. 4, the sequence identity values obtained via NCBI BLASTN searching were essentially identical to the sequence similarities as above.

Discussion

Severe brown to blackish leaf spots and/with necrosis was repeatedly observed on pendulous yuccas in Daejeon, Korea, from 2002 to 2004. In this study, a *Microsphaeropsis*-like anamorph previously known as *M. concentrica* was observed on both natural leaf lesions and artificial media. As this causal fungus was identified as a *Paraphaeosphaeria* species in this study, it seems that the *Microsphaeropsis*-like species known as *M. concentrica* on yucca plants must have been wrongly identified.

A recent severe outbreak of leaf spot and necrosis on yucca occurred during the rainy summer of 2004 in Korea, which appeared to be due to humid weather conditions. It is expected that diseased leaf residues can act as a primary inoculum for the next year, causing the same symptoms on new or young yucca leaves. When the pathogenicity test was conducted on pendulous yucca, Koch's postulates were satisfied, with the same phytopathogenic fungus developing on host leaves. When the new species has been subcultured on artificial media repeatedly, the fungus tended to produce almost no spores or pycnidia on artificial media. However, the new fungus produced spores on semi-natural medium containing leaf slices or leaf extract such as PDLA (data not shown).

The genus *Paraphaeosphaeria* is classified in the family *Phaeosphaeriaceae*. Its anamorphs produce smooth-walled, pale brown conidia from inconspicuous phialides with some periclinal thickening, which is typical of *Microsphaeropsis*. So far, it has been reported that *M. concentrica* occurs on three yucca plants (Morgan-Jones and White, 1987; Checa *et al*., 2002). In this study, when our strain was compared to *M. concentrica*, it was found to be dissimilar to the species. The dark spores of *Microsphaeropsis* are borne within

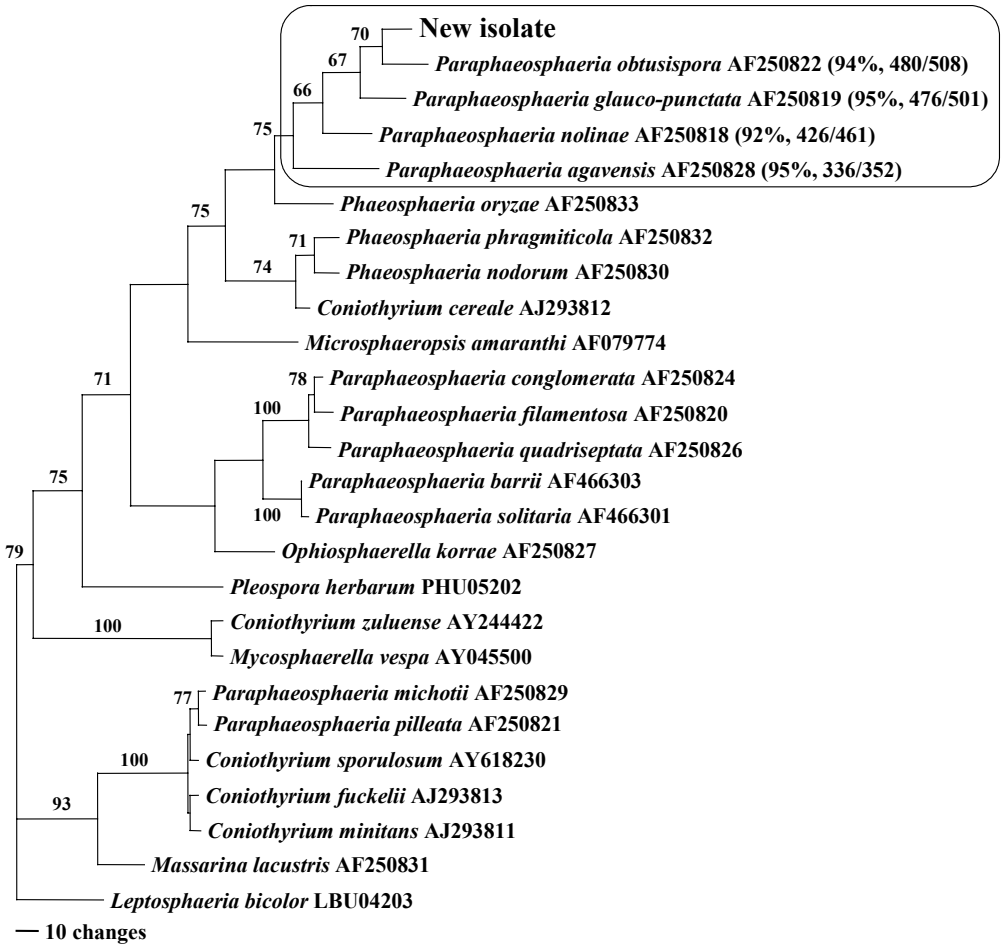

Fig. 4. Phylogenetic tree inferred from the analysis of nuclear ribosomal ITS1-5.8S-ITS2 sequences of the new species and 27 related taxa. This tree is one of 8 most parsimonious trees (tree length = 794 steps, CI = 0.519, RI = 0.709). The % sequence identity (the number of matches / the complete alignment length) values in the parentheses within a rectangular box were obtained via NCBI BLASTN searching of the new isolate (AF957476). Bootstrap values were shown at nodes supported by more than 50% from 1000 replications.

cellular, more or less round fruiting structures (pycnidia). *Paraphaeosphaeria* was previously described as a segregate of *Leptosphaeria* that produces ascospores with rounded ends, a submedian primary septum, an inflated cell above the primary septum, and a *Coniothyrium* anamorph. Described species of *Paraphaeosphaeria* also differ in anamorphic characteristics, such as the conidiomatal structure, type of conidiogenesis, and conidial morphology. Previous work demonstrated that *Paraphaeosphaeria* is polyphyletic (Camara *et al*., 2002; Checa *et al*., 2002), and ten species were grouped in at least three clades (Camara *et al*., 2002).

A number of species of *Coniothyrium* are known to be anamorphs of *Paraphaeosphaeria* (Morgan-Jones and White, 1987). A *Coniothyrium* anamorph was also reported for *P. glauco-punctata* (Grev.) Shoem. & Babcock by Muller and Tomasevic (1957). *Paraphaeosphaeria obtusispora* (Speg.) Erikss., a species described on *Yucca gloriosa* by Spegazzini, has also been reported to occur on the genus *Agave* (Hedjaroude, 1968; Morgan-Jones and White, 1987). In view of the result from this study, there is a possibility that *P. obtusispora* could be a teleomorph of *M. concentrica* (Morgan-Jones and White, 1987). Sivanesan (1984) cited *Coniothyrium henriquesii* Thüm. as an anamorph of *P. obtusispora*. However, there have been no phylogenetic analyses for these species.

So far, about four *Paraphaeosphaeria* species such as *P. filamentosa*, *P. obtusispora*, *P. quadriseptata* and *P. yuccae-gloriosae* have been isolated from different yucca hosts as possible pathogens (Systematic Botany and Mycology Laboratory, 2005). However, there have been no studies on their pathogenicity and host-specificity. Endophytic species from banana have been shown to be capable of becoming pathogens (Photita *et al*., 2005) and it would be interesting to establish if this event happens here. This might also explain how the pathogen entered Korea, i.e. as an endophyte in the host plant. This study on the taxonomy of anamorphs in relation to DNA sequence data in order to determine the relationships between species described in *Paraphaeosphaeria* is the first report on brown leaf spots and necrosis on pendulous yucca caused by a new *Paraphaeosphaeria* species in Korea. Further studies on the host-specificity, geographical distribution, and host ranges for the species of *Paraphaeosphaeria* from various yucca plants are in progress.

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