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Four Species of Montagnulaceae Unrecorded in Korea and Isolated from Plant Litter in Freshwater

Jaeduk Goh*, Hye Yeon Mun, Yoosun Oh and Namil Chung

Fungi Resources Research Division, Nakdonggang National Institute of Biological Resources, Sangju 37242, Korea

ABSTRACT : Freshwater is a diverse and complex environment for fungi and provides several types of habitat including plant litter, sediment, and carcasses. We collected plant litter from the main stream and branch streams of Nakdong River, Sohancheon in Samcheok, and Geumoreum in Jeju. From several samples of plant litter, we isolated 8 fungal strains belonging to 4 Montagnulaceae species unrecorded in Korea: *Paraconiothyrium archidendri*, *Paraphaeosphaeria sporulosa*, *Paraphaeosphaeria michotii*, and *Paraphaeosphaeria viridescens*. These fungi were identified by phylogenetic analysis using the internal transcribed spacer (ITS) region and examination of morphological characteristics. Montagnulaceae is known as one of the families in Pleosporales and includes coniothyrium-like fungi. In this study, we described phylogenetic analysis and mycological characteristics of these species, and this is the first report of these taxa in Korea.

KEYWORDS : Freshwater, Montagnulaceae, *Paraconiothyrium*, *Paraphaeosphaeria*, Plant litter

Introduction

Freshwater is a diverse and complex environment for microorganisms and provides several types of habitat for fungi, e.g., plant litter (for example, fallen leaves and decaying wood in freshwater), soil, aquatic insects, and aquatic plants. Especially, plant litter is an important habitat for many kinds of fungi because it provides surfaces for adhesion and nutrient sources. Freshwater fungi were identified in plant litter, and many of their characteristics are unclear.

Pleosporales Luttr. ex M.E. Barris is the largest order in the fungal class Dothideomycetes including 23 families, 332 genera, and 4,764 species [1]. The majority of species are saprobes on decaying plant litter in freshwater, marine, or terrestrial environments, but several species are also associated with living plants as parasites, epiphytes, or endophytes [2-4]. Montagnulaceae is the one of the families in Pleosporales and contains 7 genera and approximately 100 species [1, 5]. A tentative genus of Montagnulaceae is *Paraphaeosphaeria*, whose anamorph type is found to be *Paraconiothyrium*. Montagnulaceae fungi are known as saprobic or parasitic species in terrestrial or aquatic environments [6]. Nevertheless, only 2 species of Montagnulaceae have been reported in Korea until now [7].

In this study, we found and described 4 species of Montagnulaceae (*Paraconiothyrium archidendri*, *Paraphaeosphaeria sporulosa*, *Paraphaeosphaeria michotii*, and *Paraphaeosphaeria viridescens*), which were isolated from plant litter in freshwater for the first time in Korea. Herein, we described phylogenetic and morphological characteristics of these species.

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***Corresponding author**
E-mail: jdgoh@nnibr.re.kr

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Materials and Methods

Isolation of fungal strains and culture conditions

We collected deposits of plant litter in a freshwater stream and pond from October to December 2015. After washing with distilled water more than twice, we incubated plant litter in a pretreatment liquid medium (0.05% 3-morpholinopropane-1-sulfonic acid [w/v], 0.05% KNO_3 [w/v], 0.025% KH_2PO_4 [w/v], and 0.025% K_2HPO_4 [w/v]) at 20°C overnight. Next, 100 mL of the pretreatment medium was spread on a 1% water agar plate, and incubated

Table 1. Collection information of all strains in this study

Species	Strain name	Collection date	Location (GPS)	Stream or Pond
Paraconiothyrium archidendri	NNIBRFG29	2015.10.13.	Hamaengbang-ri, Geundeok-myeon, Samcheok-si, Gangwon-do (N 37°23'14.4", E 129°11'53.1")	Sohancheon
	NNIBRFG99	2015.10.13.	"	"
	NNIBRFG116	2015.10.13.	"	"
Paraphaeosphaeria sporulosa	NNIBRFG403	2015.10.29.	Togye-ri, Dosan-myeon, Andong-si, Gyeongsangbuk-do (N 36°43'21.0", E 128°51'49.6")	Nakdong river
	NNIBRFG404	2015.10.29.	"	"
	NNIBRFG436	2015.10.29.	Daeeun-ri, Yonggung-myeon, Yecheon-gun, Gyeongsangbuk-do (N 36°35'13.0", E 128°19'38.5")	Naesungcheon
Paraphaeosphaeria michotii	NNIBRFG243	2015.10.22.	Noeun-ri, Dongno-myeon, Mungyeong-si, Gyeongsangbuk-do (N 36°46'23.3", E 128°18'43.7")	Geuncheon
Paraphaeosphaeria viridescens	NNIBRFG469	2015.12.5.	Geumak-ri, Hallim-eup, Jeju-si,Jeju-do (N 33°21'21.9". E 126°18'21.4")	Geumoreum

at 20°C for 2 days. Hyphal tip and germinated conidia were isolated under a microscope and transferred onto 24-well plate of V8 agar (V8A; 8% V8 juice [v/v] and 1.5% agar [w/v] adjusted to pH 6.0 using 10N NaOH). All strains used in this study were grown on malt extract agar (MEA; 2% malt extract [w/v] and 1.5% agar [w/v]), oatmeal agar (OA; 5% oat meal [w/v] and 2% agar [w/v]), yeast peptone dextrose agar (YPDA; 0.3% yeast extract [w/v], 1% peptone [w/v], 1% dextrose [w/v], and 2% agar), and V8A at 25°C. Collection information of all strains in this study is listed in Table 1.

DNA extraction and phylogenetic analysis

Fungal genomic DNA was isolated by means of the NucleoSpin Plant II DNA extraction Kit (Macherey-Nagel, Düren, Germany). For identification of the fungi, amplification of an internal transcribed spacer (ITS) region using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') was performed [8]. Homology searches of DNA were carried out by BLAST algorithms available at the National Center for Biotechnology Information (NCBI). For the phylogenetic analysis, MEGA6 software was used [9]. A phylogenetic tree was constructed by the neighbor-joining (NJ) method, maximum-likelihood (ML) method with 1,000 bootstrap replications, and by the minimum evolution (ME) method. Reference sequences of other fungi were obtained from GenBank at NCBI (Table 2).

Results and Discussion

Phylogenetic analysis

We obtained sequences of reference species from NCBI (https://www.ncbi.nlm.nih.gov) for the phylogenetic analysis, which are listed in Table 2. A total of 23 taxa were used to analyze phylogenetic relations among the 4 unrecorded species and other Montagnulaceae species. Phylogenetic analysis was conducted by the NJ method after ClustalW alignment of ITS sequences for identification because our target species have distinct ITS sequence variance with other closely related species. The phylogenetic tree constructed by other statistical methods – ML method and ME method – showed a clustering pattern similar to that of NJ methods. Fig. 1 shows a phylogenetic relation between the newly recorded species and reference strains in Montagnulaceae. Three strains – NNIBRFG29, NNIBRFG99, and NNIBRFG116 (isolated in Samcheok) – were found to be in a cluster with *P. archidendri* CBS 168.77 showing 99.38% similarity. Although we isolated 3 strains of *P. archidendri* from plant litter in freshwater, the holotype of *P. archidendri* CBS 168.77 was isolated from leaf spots of a leguminouse tree in Burma [10]. Three other strains – NNIBRFG403, NNIBRFG404, and NNIBRFG436 – are clustered with *P. sporulosa* strains CBS 105.76 and CBS 109.72 showing 100% similarity. Although our strains of *P. sporulosa* were isolated from plant litter in freshwater, CBS 105.76 was isolated

Table 2. Information of sequences as reference in this study

Serial no.	Species	Strain name	Accesion no.
1	*Dothiorella iberica*	CBS 115041	NR111165.1
2	*Dothiorella longicollis*	CBS 122068	KF766162.1
3	*Leptosphaeria maculans*	CBS 260.94	JF740235.1
4	*Leptosphaerulina trifolii*	CBS 235.58	GU237806.1
5	*Neosetophoma samarorum*	CBS 138.96	FJ427061.1
6	*Paracamarosporium fagi*	CPC_24890	KR611886.1
7	*Paraconiothyrium africanum*	CBS 121166	JX496029.1
8	*Paraconiothyrium archidendri*	CBS 168.77	JX496049.1
9	*Paraconiothyrium brasiliense*	CBS 395.87	JX496083.1
10	*Paraconiothyrium cyclothyrioides*	CBS 432.75	JX496088.1
11	*Paraconiothyrium fuckelii*	CBS 508.94	JX496096.1
12	*Paraconiothyrium fungicola*	CBS 113269	JX496020.1
13	*Paraconiothyrium hawaiiensis*	CBS 120025	DQ885897.1
14	*Paraconiothyrium thysanolaenae*	MFLUCC_10_0550	KP744453.1
15	*Paraconiothyrium variabile*	CBS 413.84	JX496086.1
16	*Paraphaeosphaeria arecacearum*	CBS 614.75	JX496100.1
17	*Paraphaeosphaeria michotii*	CBS 340.86	JX496079.1
18	*Paraphaeosphaeria neglecta*	CBS 119637	JX496025.1
19	*Paraphaeosphaeria sporulosa*	CBS 105.76	JX496014.1
20	*Paraphaeosphaeria sporulosa*	CBS 109.72	JX496015.1
21	*Paraphaeosphaeria verruculosa*	CBS 263.85	JX496059.1
22	*Paraphaeosphaeria viridescens*	CBS 854.73	JX496085.1
23	*Phoma herbarum*	CBS 276.37	JF810524.1

from roots of Norway spruce (*Picea abies*), and another specimen (CBS 218.68) was isolated from wheat field soil [10]. NNIBRFG243 is in a cluster with *P. michotii* strain CBS 340.86 showing 100% similarity. Some of *P. michotii* strains were reported as pathogens of water plants, oak, sugarcane, and endophytic fungi of timothy [11, 12]. NN IBRFG469 isolated from Jeju was found to be in a cluster with *P. viridescens* strain CBS 854.73 showing 99.8% similarity. *P. viridescens* living Ex-type culture CBS 854.73 was isolated from freshwater, just as our strain NNIBR FG469 was [10]. This phylogenetic tree showed trends similar to those of other studies on phylogenetics of *Montagnulaceae* [13]. Above all, these results indicated that all four species (for the first time reported in Korea in this study) were isolated from a new habitat (except for *P. viridescens)*: plant litter in freshwater. These species are worthy of further research focused on diversity of freshwater fungi.

Taxonomic description

***Paraconiothyrium archidendri* Verkley, Göker & Stielow, Persoonia 32: 37 (2014) (Fig. 2)**

Shape of conidiomata was pycnidial globose, and diameter was 250~350 (~400) μm. Color of conidiomata became black because conidia were produced inside. Conidia shapes were variable, most of them ellipsoid, often subglobose, round-ended. Color of conidia was brownish. Conidia had no septa, and their size was 3.60~6.29 × 1.98~4.87 μm (average length 5.10 ± 0.65 μm, average width 3.10 ± 0.59 μm; n = 50). Average L/W (length/width) ratio was 1.69 ± 0.37 (n = 50). Colonies on MEA in 10 days showed growth of mycelia (50~55 mm in diameter) and glabrous-colorless margin; an aerial mycelium was absent. Immersed mycelium was reddish brown to cinnamon. Conidiomata developed after 20~25 days. Colonies on OA in 10 days showed growth of mycelia (55~60 mm in diameter), a colorless to buff margin, and fluffy white to gray aerial mycelia. Reverse side of colonies on

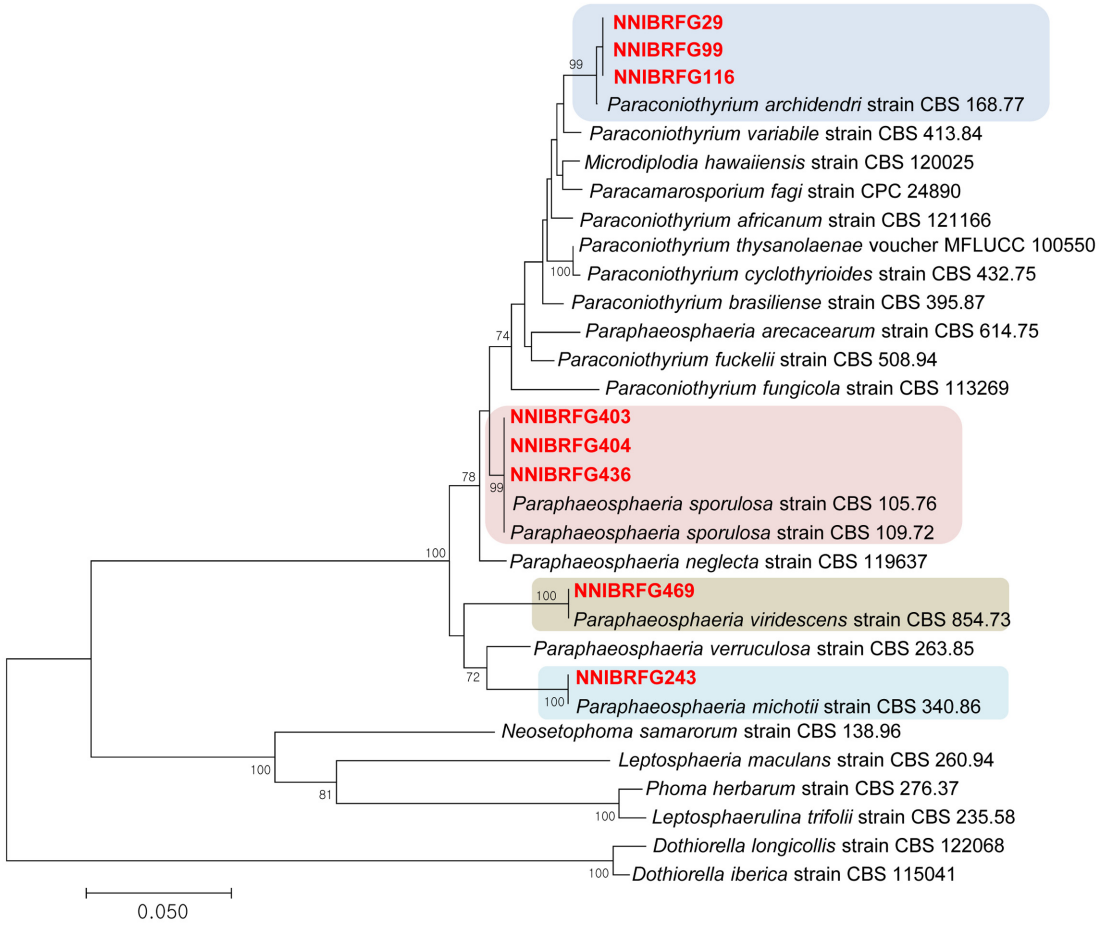

Fig. 1. Phylogenetic analysis using neighbor-joining method based on internal transcribed spacer region (ITS). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

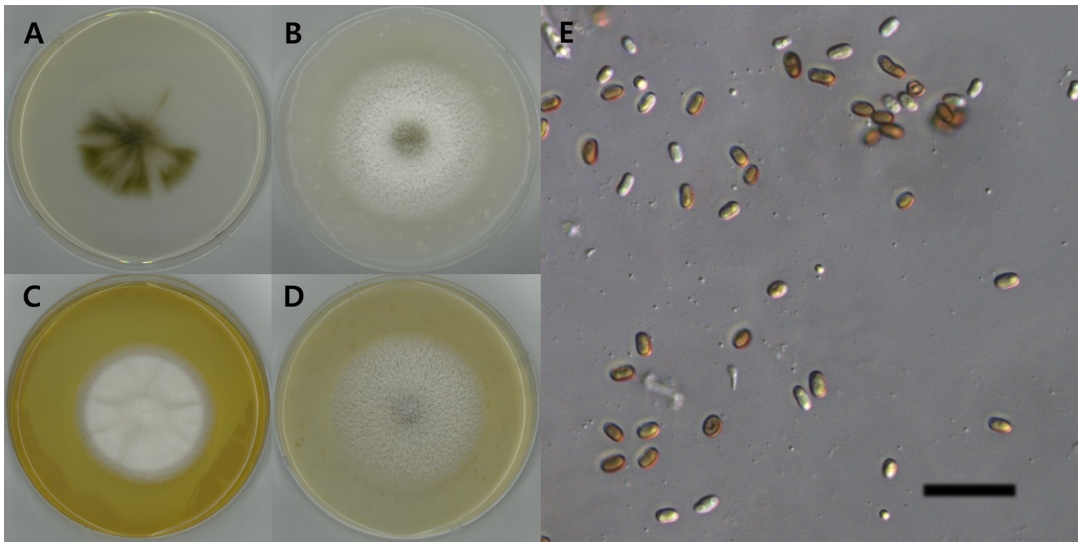

Fig. 2. *Paraconiothyrium archidendri* (NNIBRFG99). A, colony on malt extract agar at 10 days; B, colony on oatmeal agar at 10 days; C, colony on yeast peptone dextrose agar at 10 days; D, colony on V8A at 10 days; E, conidia morphology (scale bar = 20 μm).

OA was predominantly white to tawny, in center: olivaceous black. Conidiomata developed after 20~25 days. Colonies on YPDA in 10 days showed growth of mycelia 48~53 mm in diameter, colorless to buff margin, and highly dense light-yellowish aerial mycelia. Reverse of colonies on YPDA was predominantly lemon-colored to yellow, in center dark yellow with wrinkles. Colonies on V8A in 10 days showed growth of mycelia, 55~60 mm in diameter, colorless margin, highly fluffy aerial mycelium, and light gray aerial mycelia in center.

Specimens examined

Korea, Gangwon-do, Samcheok-si, Geundeok-myeon, Hamaengbang-ri, 37°23'14.4" N, 129°11'53.1" E, isolated from plant litter deposited in the stream Sohancheon, 13 October 2015, J. Goh, NNIBRFG29 (GenBank accession no. KY327411), NNIBRFG99 (GenBank accession no. KY 327412), and NNIBRFG116 (GenBank accession no. KY 327413).

***Paraphaeosphaeria michotii* (Westend.) O.E. Erikss., Arkiv før Botanik 6 (4–5): 405 (1967) (Fig. 3)**

Shape of conidiomata was pycnidial globose, and diameter was 250~350 (~400) μm. Color of conidiomata later turned black because of conidia produced inside. Conidia shapes were broadly fusiform to ellipsoid, ends rounded. Color of conidia was brownish. Conidia had no septa, and the size was 4.38~9.00 × 2.82~6.25 μm (average length 6.73 ± 1.05 μm, average width 4.70 ± 0.69 μm; n = 60). Average L/W ratio was 1.45 ± 0.25 (n = 60). Colonies on MEA in 10 days showed growth of mycelia 50~55 mm in diameter, glabrous and colorless margin, and aerial mycelia were absent. Immersed mycelium was reddish brown to cinnamon. Conidiomata developed after 20~25 days. Colonies on OA in 10 days showed growth of mycelia 55~60 mm in diameter, wide colorless to buff margin, and white to light brown fluffy aerial mycelia. Reverse side of colonies on OA was predominantly white to tawny. Conidiomata developed after 20~25 days. Colonies on YPDA in 10 days showed growth of mycelia 43 ~48 mm in diameter, narrow colorless to white margin, and highly dense goldenrod aerial mycelia. Center of colony on YPDA was weakly pinkish lemon-colored with aerial mycelium. Colonies on V8A in 10 days showed growth of mycelia 50~55 mm in diameter, wide colorless margin, and smooth yellowish brown aerial mycelium.

Specimens examined

Korea, Gyeongsangbuk-do, Mungyeong-si, Dongnomyeon, Noeun-ri, 36°46'23.3" N, 128°18'43.7" E, isolated from plant litter deposited in the stream Geumcheon, 12 October 2015, J. Goh, NNIBRFG243 (GenBank accession no. KY327414).

***Paraphaeosphaeria sporulosa* (W. Gams & Domsch) Verkley, Göker & Stielow, Persoonia 32: 47 (2014) (Fig. 4)**

Shape of conidiomata was pycnidial globose, and diameter was 120~250 (~350) μm. Color of conidiomata

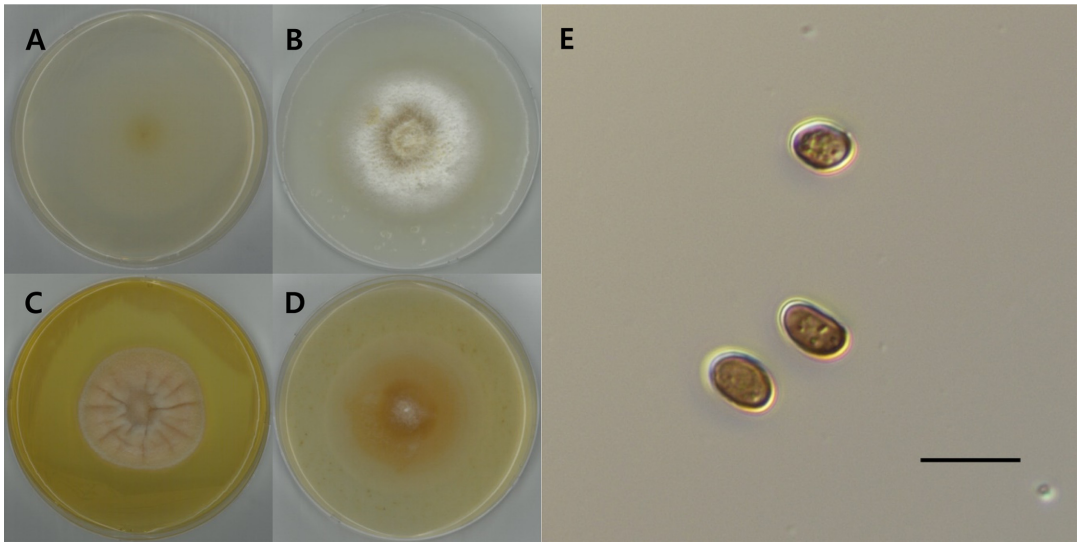

Fig. 3. *Paraphaeosphaeria michotii* (NNIBRFG243). A, colony on malt extract agar at 10 days; B, colony on oatmeal agar at 10 days; C, colony on yeast peptone dextrose agar at 10 days; D, colony on V8A at 10 days; E, conidia morphology (scale bar = 10 μm).

was initially pale to dark brown and finally black due to mature conidia inside. Conidia shape was ellipsoid, obovoid-pyriform, or subglobose. Color of conidia was initially hyaline to yellowish brown. Conidia had no septa and had one large and often 1~2 additional smaller oil droplets. Size of conidia was 2.58~6.20 × 2.30~4.92 μm (average length 4.94 ± 0.76 μm, average width 3.48 ± 0.53 μm; n = 60). Average L/W ratio was 1.45 ± 0.33 (n = 60). Colonies on MEA after 10 days showed growth of mycelia 46~50 mm in diameter, glabrous and with colorless margin, and hyaline reddish brown immersed mycelium; aerial mycelium was absent. Conidiomata developed after 20~25 days. Colonies on OA in 10 days showed growth of mycelia 55~60 mm in diameter, wide colorless margin, and fluffy light gray aerial mycelia. Reverse of colonies on OA appeared yellow in center where numerous pycnidia formed. Conidiomata developed after 7~10 days. Colonies on YPDA in 10 days showed growth of mycelia 48~53 mm in diameter, narrow colorless margin, and highly dense dove gray aerial mycelia. Center of colonies on YPDA in 10 days showed weakly pinkish lemon-colored aerial mycelium. Reverse was predominantly lemon-colored to yellow, in center: dark yellow with wrinkles.

Specimens examined

Korea, Gyeongsangbuk-do, Andong-si, Dosan-myeon, Togye-ri, 36°43'21.0" N, 128°51'49.6" E, isolated from plant litter deposited in Nakdong river, 29 October 2015, J. Goh, NNIBRFG403 (GenBank accession no. KY327415), NNIBRFG404 (GenBank accession no. KY327416); Korea, Gyeongsangbuk-do, Yecheon-gun, Yonggung-myeon, Daeeun-ri, 36°35'13.0" N, 128°19'38.5" E, isolated from plant litter deposited in stream Naeseongcheon, 29 October 2015, J. Goh, NNIBRFG436 (GenBank accession no. KY 327417).

***Paraphaeosphaeria viridescens* Verkley, Göker & Stielow, Persoonia 32: 49 (2014) (Fig. 5)**

Shape of conidiomata was pycnidial globose, and diameter was 250~450 μm. Color of conidiomata was initially light sea green, and turned black because of mature conidia inside. Shape of conidia was consistently colorless ellipsoid with thin and smooth wall. Conidia had no septa and had 1~2 large oil droplets. Size of conidia was 3.27~5.00 × 1.76~3.16 μm (average length 4.25 ± 0.36 μm, average width 2.40 ± 0.25 μm; n = 73). Average L/W ratio was 1.80 ± 0.26 (n = 70). Sexual morph is unknown. Colonies on MEA in 10 days showed growth of mycelia 55~60 mm in diameter and colorless to dark green mycelium with smooth surface. Reverse of colonies on MEA in center was dark brown to grayish-green. Colonies on OA in 10 days showed growth of mycelia 57~63 mm in diameter, slightly ruffled margin, and teal aerial mycelia. Immersed mycelium was initially colorless to very light green, later with dark green pigment. Reverse of colonies on OA in center was dark bluish green. Colonies on YPDA in 10 days showed growth of mycelia 55~60 mm in diameter, colorless glabrous margin, and rin-

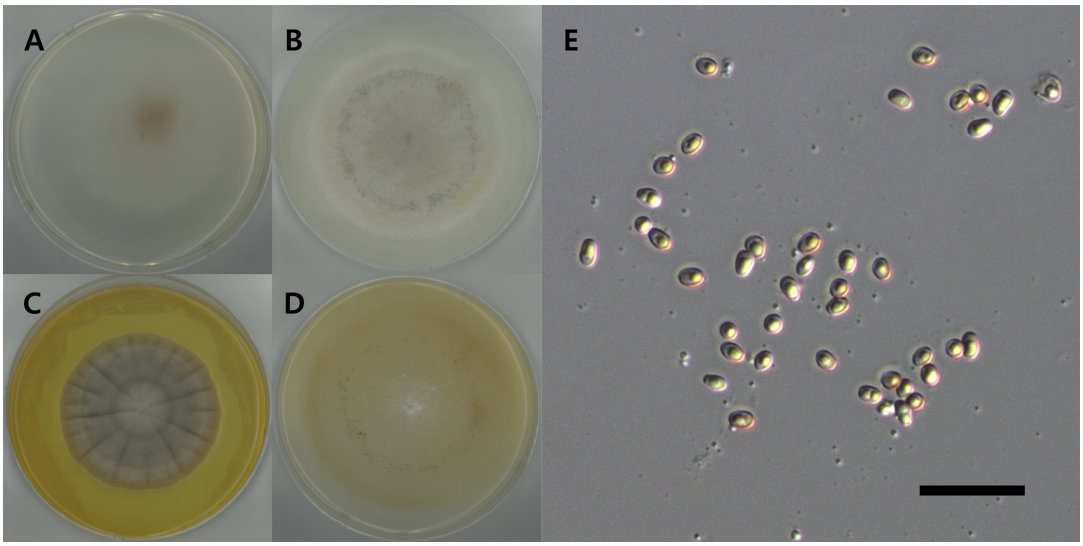

Fig. 4. *Paraphaeosphaeria sporulosa* (NNIBRFG436). A, colony on malt extract agar at 13 days; B, colony on oatmeal agar at 13 days; C, colony on yeast peptone dextrose agar at 13 days; D, colony on V8A at 13 days; E, conidia morphology (scale bar = 20 μm).

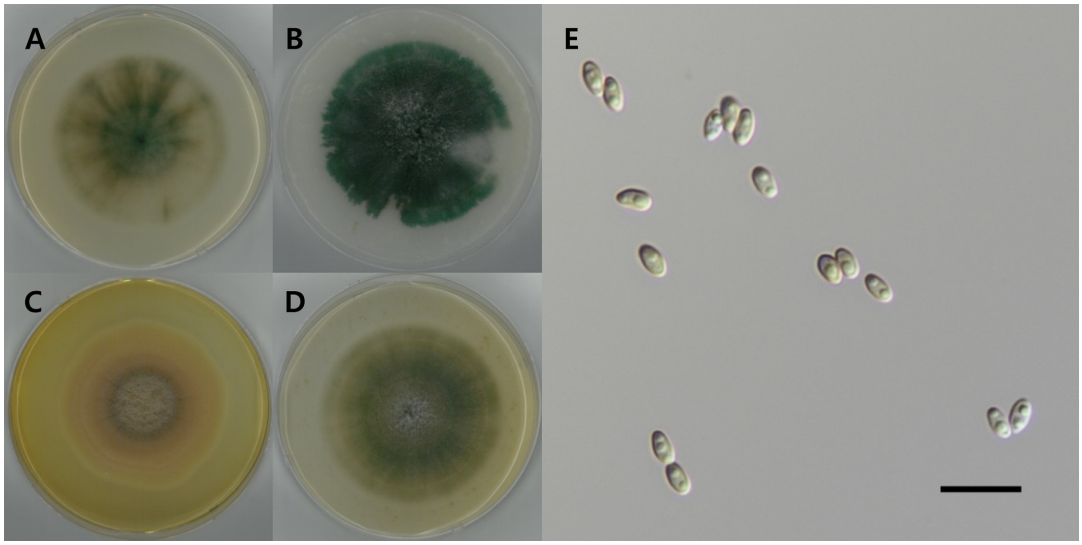

Fig. 5. *Paraphaeosphaeria viridescens* (NNIBRFG469). A, colony on malt extract agar at 10 days; B, colony on oatmeal agar at 10 days; C, colony on yeast peptone dextrose agar at 10 days; D, colony on V8A at 10 days; E, conidia morphology (scale bar = 10 μm).

ged yellow to gray aerial mycelia. Colonies on V8A in 10 days showed growth of mycelia 60~65 mm in diameter, colorless glabrous margin, and ringed olivaceous to gray aerial mycelia. This species is notable for producing green pigment diffusing in agar and for conidia with consistently ellipsoid shape and relatively high L/W ratio (1.80 ± 0.26), and relatively light green yellowish wall at maturity.

Specimens examined

Korea, Jeju-do, Jeju-si, Hallim-eup, Geumak-ri, 33°21' 21.9" N, 126°18'21.4" E, isolated from plant litter deposited in Geumorem, 5 December 2015, J. Goh, NNIBRFG469 (GenBank accession no. KY327418).

In this study, we for the first time reported 4 Montagnulaceae species in Korea. In particular, these fungi were isolated from plant litter deposited in freshwater. This finding indicated that a freshwater environment may be a good habitat for coniothyrium-like fungi. Further studies on a new substrate or habitat for unknown fungi may enhance domestic fungal biodiversity in Korea.

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