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Research Article

Molecular Phylogeny and Morphology of *Mycosphaerella nawae*, the Causal Agent of Circular Leaf Spot on Persimmon

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Abstract In this study, the phylogeny and morphology of *Mycosphaerella nawae* (Dothideomycetes, Ascomycota) were examined using Korean and Japanese isolates, to establish the phylogenetic relationship between *M. nawae* and its allied species. Korean and Japanese isolates of *M. nawae* were collected from circular leaf spot-diseased leaves and were confirmed based on internal transcribed spacer (ITS) sequence data. Phylogenetic analysis was conducted using multiple genes, including the ITS region, 28S rDNA, β-tubulin, translation elongation factor-1α, and actin genes. Our results revealed that *M. nawae* is closely related to members of the genus *Phaeophleospora* but are distant from the *Ramularia* spp. In addition, microscopic analysis revealed pseudothecia on the adaxial and abaxial surface of overwintered diseased leaves (ODL) and only on the abaxial surface of diseased leaves. Ascospores are oval to fusiform, one-septate, tapered at both ends, 1.7~3.1 × 8.1~14.1 μm, and were observed in ODL. Conidia are oval, guttulate, one-septate, 3.5~4.9 × 12.8~19.8 μm, and barely discernable on 30-day cultures. To our knowledge, this is the first report on the phylogeny of *M. nawae*, which is closely related to the genus *Phaeophleospora*, especially *P. scytalidii*.

Keywords Persimmon, *Phaeophleospora* spp., Phylogenetic analysis

Circular leaf spot (CLS) that is caused by *Mycosphaerella nawae* Hiura & Ikata, occurs only on persimmons (*Diospyros kaki* Thunb.) and has been reported in Japan, Korea, and Spain [1-3]. The typical symptoms of CLS include necrotic spots on leaves, chlorosis, red discoloration, and early defoliation [4]. This disease consequently leads to premature fruit maturation and abscission, ultimately resulting in economic losses [3, 5]. Previous studies have shown that *M. nawae* has a long latent period and that typical symptoms on leaves appear approximately 4 mon after infection [6, 7]. Similarly, it grows very slowly on cultured media [5]. Therefore, isolation of *M. nawae* from diseased leaves (DL) has proven difficult [4]. According to Kwon *et al.* [5-7], the anamorph of *M. nawae* is of *Ramularia*-type and can be observed in a 90-day-old growth on potato dextrose agar (PDA) media as well as in circular leaf spots. The authors identified the anamorphic type of *M. nawae* as *Ramularia* sp. based on their morphological characteristics; however, its classification was not supported by their phylogenetic analysis based on molecular marker genes [7].

The genus *Mycosphaerella* includes numerous fungal pathogens mainly associated with foliar diseases of various host plants [8, 9]. Classification of the genus *Mycosphaerella* has relied on host plant symptoms, morphological and cultural characteristics [10-14], and phylogenetic analyses using molecular markers [12-14], or molecular markers along with morphological characteristics [15]. Reassessment of taxonomic status has been performed for many fungal species in the genus *Mycosphaerella*, and most of these studies have used morphological characteristics and molecular methods [16-18]. Recently, new genera and combinations have been reported in *Mycosphaerellaceae* and *Teratosphaeriaceae* based on molecular marker genes and morphological characteristics, whereas several combinations only occurred based on phylogenetic analysis [19, 20].

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This study aimed to examine the morphological characteristics and compare the phylogenetic position of Korean and Japanese *M. nawae* isolates, based on the internal transcribed spacer (ITS) region, 28S rDNA, β-tubulin, and actin genes, in relation to fourteen allied species and *Ramularia* spp. which was reported as an anamorph of *M. nawae*. These comprehensive experiments were conducted to enhance our understanding of the phylogenetic position of *M. nawae*.

MATERIALS AND METHODS

Isolation of *Mycosphaerella nawae* from DL and their microscopic observation. CLS-diseased persimmon leaves were collected from seven different regions, including Sangju-si, Gumi-si, Gimhae-si, Miryang-si, and Changwon-si in Korea, and the Wakayama prefecture in Japan, from August to October 2014. To isolate *M. nawae* from the DL, dark green necrotic spots were sterilized in 70% ethanol for 30 sec and 1% sodium hypochlorite for 60 sec. The samples were then washed thrice in double distilled water (DDW). The sterilized samples were dried on filter paper at room temperature for 30 min, and DDW (50 μL) was then added on the back of the symptomatic spots, which were then spread on a PDA plate and then incubated at 25°C until colonies appeared. After 2~3 days, small black colonies were transferred onto a new PDA plate.

Genomic DNA preparation and PCR amplification of molecular markers. Total genomic DNA was extracted from the isolated *M. nawae* according to the cetyltrimethylammonium bromide method [21]. Using the genomic DNA of *M. nawae* isolates and their allied species, the ITS region, the partial region of 28S rDNA, *Tub*, and *Act* were amplified using the corresponding primer pairs [22-25]. A total reaction volume of 20 μL contained 1 μL of genomic DNA, 2 μL of 10× *Taq* buffer, 0.4 μL of 10 mM dNTP, 0.5 μL each of 10 pM forward and reverse primer, and 0.2 μL of *Taq* DNA polymerase (Solgent Co., Daejeon, Korea). PCR was performed in a Veriti 96-well Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA). The obtained PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide, and observed under a UV illuminator. All the amplified PCR products were purified using ExoSAP-IT (USB Co., Cleveland, OH, USA) and were directly sequenced (Solgent Co.).

Nucleotide sequences and phylogenetic analyses. All the obtained sequences of ITS, partial 28S rDNA, *Tub*, and *Act* were compared with the available sequence data, using BLAST search against the NCBI GenBank database to identify the sequences, and multiple sequence alignments were performed using CLUSTAL W [26]. Phylogenetic trees were constructed according to the maximum likelihood method with 1,000 bootstrap replications, using the MEGA 7 software ver. 7.0.14. Moreover, each of the homosynonyms and heterosynonyms of the allied species of *M. nawae* were surveyed through the MycoBank Database (http://www.mycobank.org).

Microscopic observation. Isolated colonies were observed under a light microscope (BX-50; Olympus, Tokyo, Japan) after 30 days of cultivation. To observe the conidia, aerial mycelia were collected from 30-day-old colonies, using DDW, and the suspension was spread onto a PDA plate. The PDA was observed under a light microscope to determine conidia before germination.

Observation of pseudothecia on diseased and overwintered DL To observe the pseudothecia on DL, DL and overwintered diseased leave (ODL) were collected from diseased trees and the leaf litter around the diseased trees in Sangju-si. The adaxial and abaxial sides of the DL and ODL were observed under a stereoscopic microscope (DIMIS-M; Siwon Optical Technology, Co., Ltd., Anyang, Korea) and a light microscope (BX-50; Olympus) after staining with 1% methylene blue. To prepare semi-thin sections, the diseased part was excised using a sterilized surgical blade. Samples were then treated with Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2) for 24 hr. The fixed samples were dehydrated in a graded ethanol series of 30%, 50%, 70%, 80%, 90%, and absolute ethanol for 20 min at each concentration and were then infiltrated with propylene oxide. Finally, the samples were embedded in Spurr's resin and polymerized at 70°C for 10 hr. The embedded samples were cut using an ultra-microtome (MT-7000; RMC Boeckeler, Tuscon, AZ, USA) and each section was observed using a light microscope after staining with 1% methylene blue.

RESULTS

Isolation of *Mycosphaerella nawae* from CLS-DL. Twenty isolates of *M. nawae* were obtained from collected leaves with CLS-disease from each region of collection. At

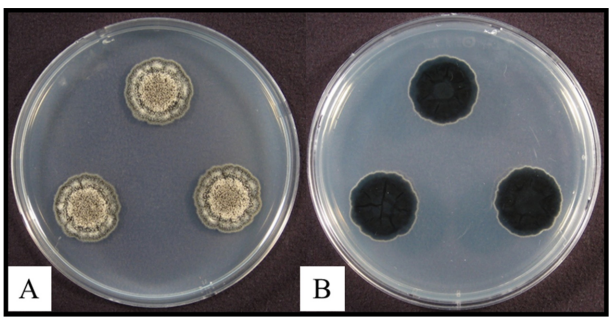

Fig. 1. Morphological characteristics of the isolated *Mycosphaerella nawae* on potato dextrose agar (PDA). A, Isolated *M. nawae* colonies after 4 wk of growth on PDA; B, Reverse side of the 4-week-old colony.

first, the colonies appeared white, dense, and round, and grew slowly on the PDA plates compared to other fungi. After 5 to 7 days, the colonies turned dark green toward the middle. After 4 wk, they transformed into grayish brown or dark brown colonies that were raised in the center, had a wave pattern with a wrinkled surface, and ranged from 19 to 21 mm in diameter at 25°C (Fig. 1A and 1B).

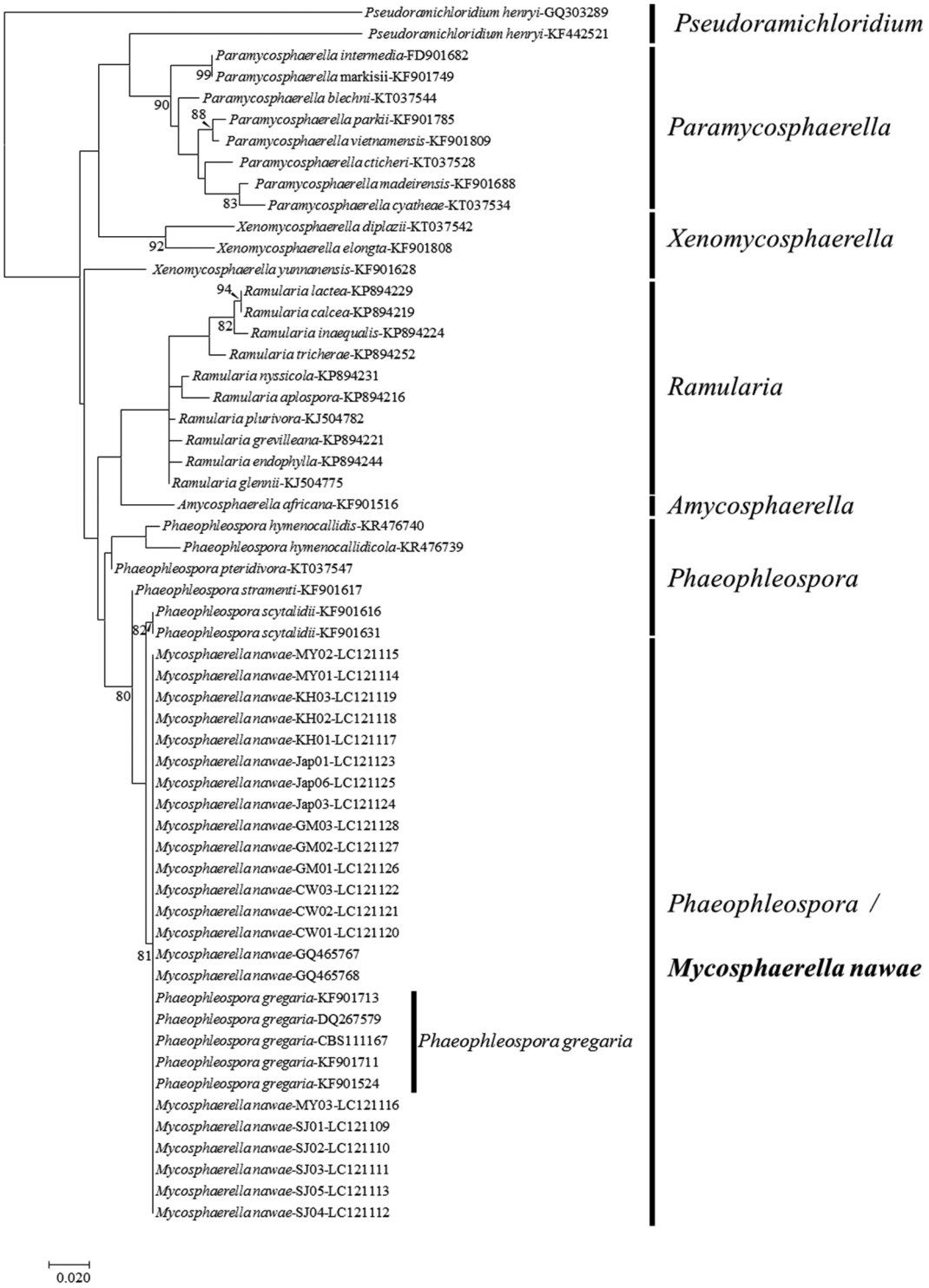

Fig. 2. Maximum likelihood tree of *Mycosphaerella nawae* inferred from the internal transcribed spacer sequences. *Pseudoramichloridium henryi* (GQ303289) was used as the outgroup. The numbers above the branches represent the bootstrap values obtained for 100 replicates (values smaller than 80 are not shown). The scale bar represents a phylogenetic distance of 0.02%.

Table 1. List of allied species of *Mycosphaerella nawae* for phylogenetic analysis

Species	Culture collection accession No.	Synonym[a] (= Heterosynonym/ ≡ Homosynonym)	Reference[b]	Isolated host	Genbank accession No.				
					ITS	28s rDNA	*Act*	*TEF-1α*	*Tub*
Amycosphaerella africana	CBS 110500	= *Mycosphaerella africana*	20	*Eucalyptus globulus*	LC121129	LC121200	LC121211	KF903115	LC121211
Lecanosticta acicola	CBS 133789	≡ *Dothistroma acicola*		*Pinus* sp.	LC121130	LC121201	LC121212	JX901648	LC121212
Mycosphaerella graminicola	CBS 398.52	-		*Triticum aestivum*	LC121131	LC121202	LC121213	JQ739795	LC121213
Mycosphaerella musae	CBS 121386	-		*Musa* sp.	LC121133	LC121204	LC121215	-	LC121215
Paramycosphaerella blechni	COAD 1183	-		*Blechnum serrulatum*	KT037544	KT037586	KT037611	KT037503	-
Paramycosphaerella sticheri	COAD 1422	-		*Sticherus penniger*	KT037528	KT037569	KT037615	KT037488	-
Paramycosphaerella cyatheae	CPC 24730	-		*Cyathea delgadii*	KT037534	-	-	-	-
Paramycosphaerella intermedia	CBS 114415	= *Mycosphaerella intermedia*	20	*Eucalyptus saligna*	KF901682	KF902027	KF903468	KF903143	-
Paramycosphaerella madeirensis	CBS 112301	= *Mycosphaerella madeirae*	21	*Eucalyptus globulus*	KF901688	KF902033	KF903453	KF903108	-
	CBS 112895			*Eucalyptus globulus*	LC121137	LC121203	LC121214	KF903109	LC121214
Paramycosphaerella marksii	CBS 110981	= *Mycosphaerella marksii*	20	*Eucalyptus* sp.	KF901749	KF902103	KF903417	KF903148	-
	CBS 110920			*Eucalyptus globulus*	LC121137	LC121209	LC121219	KF903145	LC121219
Paramycosphaerella parkii	CBS 387.92	≡ *Mycosphaerella parkii*	21	*Eucalyptus grandis*	KF901785	KF902143	KF903585	KF903392	-
					LC121139	LC121210	LC121221	KF903392	LC121221
Paramycosphaerella vietnamensis	CBS 119974	≡ *Mycosphaerella vietnamensis*	21	*Eucalyptus grandis hybrid*	KF901809	KF902171	KF903514	KF903114	-
Paramycosphaerellla intermedia	CBS 114356	= *Mycosphaerella intermedia*	20	*Eucalyptus saligna*	LC121136	LC121207	LC121218	KF903142	LC121218
Passalora fulva	CBS 119.46	≡ *Cladosporium fulvum*		*Lycopersicon esculentum*	LC121134	LC121205	LC121216	-	LC121216
Phaeophleospora concentrica	CPC 3615	-		*Protea caffra*	FJ493187	FJ493205	-	-	-
Phaeophleospora epicoccoides	CMW 22486	= *Kirramyces epicoccoides*		*Eucalyptus urophylla*	DQ632706	-	-	DQ632720	-
Phaeophleospora eucalypticola	CPC 26523	-		*Eucalyptus robusta*	KX228267	KX228318	-	KX228374	-
Phaeophleospora eugeniae	CMW 5351	-		*Eugenia uniflora*	DQ632710	-	-	EF011663	-
Phaeophleospora eugeniicola	CPC 2558	-		-	FJ493191	FJ493209	-	-	-
Phaeophleospora gregaria	CBS 114662	= *Mycosphaerella gregaria*	20	*Eucalyptus* sp.	KF901713	KF902060	KF903470	KF903165	
	CBS 111519			-	DQ267579	JX901861	JX902108	JX901655	-
	CBS 111167			*Eucalyptus cladocalyx*	KF901711	KF902058	KF903434	KF903163	-

Table 1. Continued

Species	Culture collection accession No.	Synonym[a] (= Heterosynonym/ ≡ Homosynonym)	Reference[b]	Isolated host	Genbank accession No.				
					ITS	28s rDNA	*Act*	*TEF-1α*	*Tub*
	CBS 110501			*Eucalyptus globulus*	LC121135	LC121206	LC121217	KF903161	LC121217
Phaeophleospora hymenocallidicola	CPC 25014	-		Fern	KR476739	KR476772	-	-	-
Phaeophleospora hymenocallidis	CPC 25018	-		Fern	KR476740	KR476773	-	-	-
Phaeophleospora parsoniae	CPC 22537	-			KJ869131	KJ869188	-	-	-
Phaeophleospora pteridivora	COAD 1182	-		*Serpocaulon triseriale*	KT037547	KT037582	KT037631	KT037499	-
Phaeophleospora scytalidii	CBS 516.93	= *Mycosphaerella scytalidii*	20	*Eucalyptus globulus*	KF901616	-	-	-	-
	CBS 118493			*Eucalyptus urophylla*	KF901631	KF901966	KF903493	KF903167	-
Phaeophleospora stonei	CBS 120830	-		*Eucalyptus* sp.	KF901525	KF901847	KF903645	KF903168	-
Phaeophleospora stramenti	CBS 118909	= *Mycosphaerella stramenti*	20	*Eucalyptus* sp.	KF901617	KF901942	KF903506	KF903169	-
Pseudoramichloridium henryi	CBS 124775	-		*Corymbia henryi*	GQ303289	KF442561	KF903559	KF903227	-
Ramularia aplospora	CBS 109013	≡ *Ramularia haplospora*		*Alchemilla xanthochlora*	KP894216	KP894107	KP894322	KP894432	-
Ramularia calcea	CBS 101612	= *Ramularia noneae*		*Symphytum* sp.	KP894219	KJ504744	KJ504449	KJ504700	-
Ramularia endophylla	CBS 117876	-		*Quercus robur*	KP894244	KP894137	KP894352	KP894462	-
Ramularia glennii	CPC 18468	-		-	KJ504775	KJ504734	KJ504439	KJ504690	-
Ramularia grevilleana	CBS 114732	= *Ramularia punctiformis*		*Fragaria ananassa*	KP894221	KP894438	KP894328	KP894113	-
Ramularia inaequalis	CBS 250.96	= *Ramularia inaequale*		*Taraxacum officinale*	KP894224	-	-	-	-
Ramularia lactea	CBS 114442	= *Ramularia violae*		*Viola hirta*	KP894229	KP894122	KP894337	KP894337	-
Ramularia nyssicola	CBS 127664	≡ *Mycosphaerella nyssicola*		*Nyssaogeche x sylvatica hybrid*	KP894231	KP894124	KP894339	KP894449	-
Ramularia plurivora	CPC 16123	-		*Knautia arvensis*	KJ504782	KJ504741	KJ504446	KJ504697	-
Ramularia tricherae	CBS 108994	= *Ramularia knautiae* var. *arvensis*		-	KP894252	KP894145	KP894360	KP894470	-
Xenomycosphaerella diplazii	CPC 24691	-		*Diplazium* sp.	KT037542	KT037584	KT037627	KT037501	-
Xenomycosphaerella elongata	CBS 120735	= *Mycosphaerella elongata*	20	*Triticum aestivum*	KF901808	KF902170	KF903528	-	-
Xenomycosphaerella yunnanensis	CBS 119975	= *Mycosphaerella yunnanensis*	20	*Musa cultiva*	KF901628	KF901962	KF903515	KF903375	-

[a]The synonym was searched in Mycobank.
[b]It indicates the reference that newly reclassified the species belonging to the genus *Mycosphaerella*.

Molecular identification based on ITS sequences. The obtained ITS region sequences from 20 Korean and Japanese *M. nawae* isolates were searched in the NCBI database, using the BLAST search. All the obtained sequences from the Korean and Japanese isolates were 665 bp long and were identical (data not shown). We observed that all the isolate sequences were identical to those of the Spanish *M. nawae* isolates (GQ465767 and GQ465768). The phylogenetic analysis showed that they were indistinguishable from the Spanish *M. nawae* isolates (GQ465767 and GQ465768) but were distinct from those of *Ramularia* spp. whereas the sequences of *Phaeophleospora gregaria* were not distinguished from *M. nawae* (Fig. 2).

Phylogenetic analysis based on molecular markers. To examine the phylogenetic relationship of *M. nawae* with its allied species, a maximum likelihood tree was constructed based on the combined dataset composed of concatenated sequences of ITS, 28S rDNA, *Tub*, and *Act*. The obtained sequences of all molecular markers were deposited in the NCBI database (LC121109~LC121232). The combined dataset was approximately 2,450 bp and included sequences from 20 *M. nawae* isolates and the derived allied species from the NCBI (Table 1). In the resulting tree topology, the Korean and Japanese *M. nawae* isolates were clustered together forming a single sister clade to the clade containing the genus *Phaeophleospora* (Fig. 3). In addition, we tested the phylogenetic relationship between the newly introduced species in the *Mycosphaerellaceae* and the Korean and Japanese isolates of *M. nawae* based on ITS, partial of 28S rDNA and translation elongation factor-1α (*TEF-1α*) genes (Table 1). The combined dataset was approximately 1,200 bp and a phylogenetic tree was constructed using the maximum likelihood method with 1,000 replicates. The

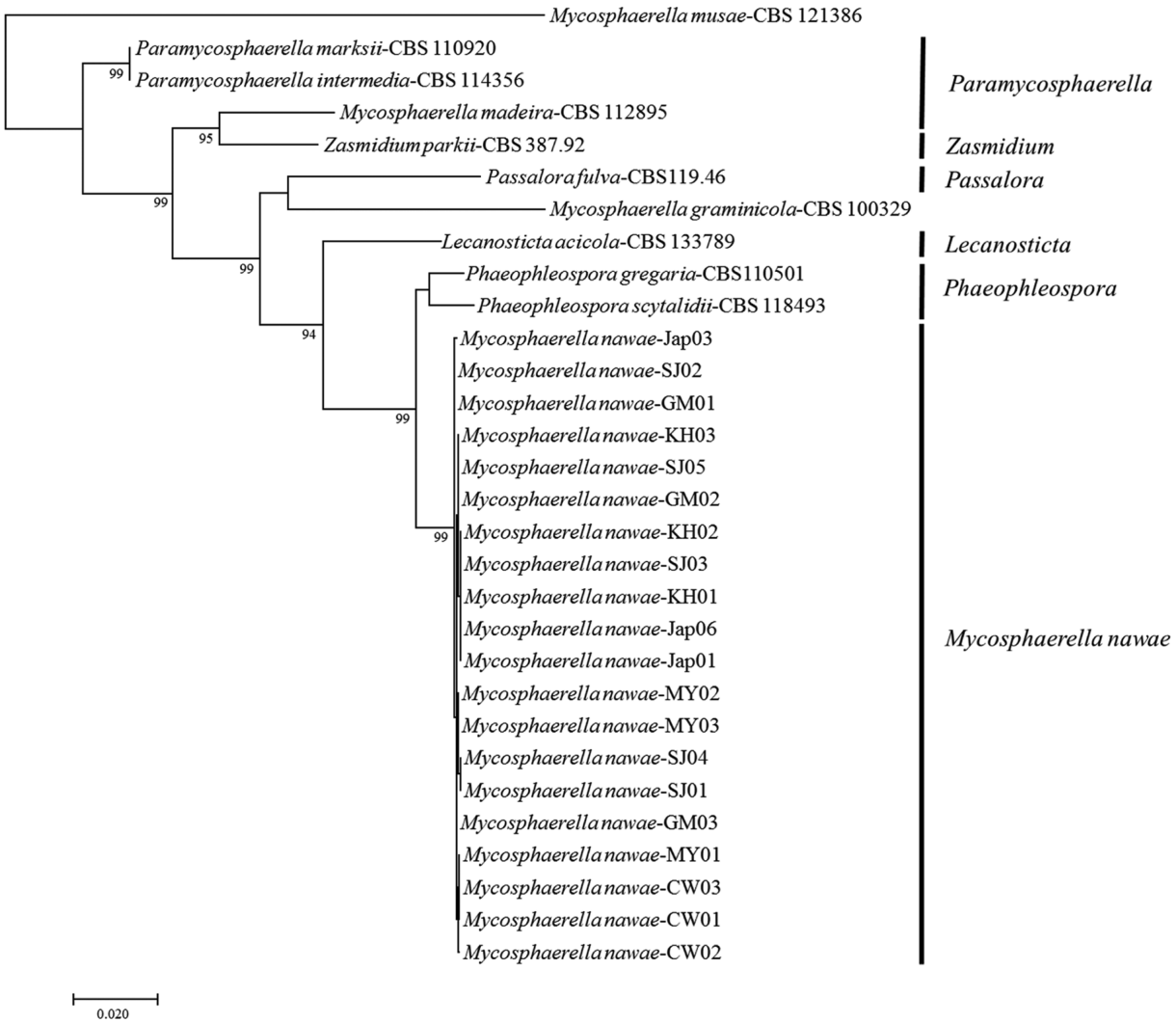

Fig. 3. Maximum likelihood tree of *Mycosphaerella nawae* and its allied species inferred from the combined internal transcribed spacer, partial 28S rDNA, β-tubulin, and actin gene sequences. *Mycosphaerella musae* was used as the outgroup. The numbers above the branches represent the bootstrap values obtained for 100 replicates (values smaller than 80 are not shown). The scale bar represents a phylogenetic distance of 0.02%.

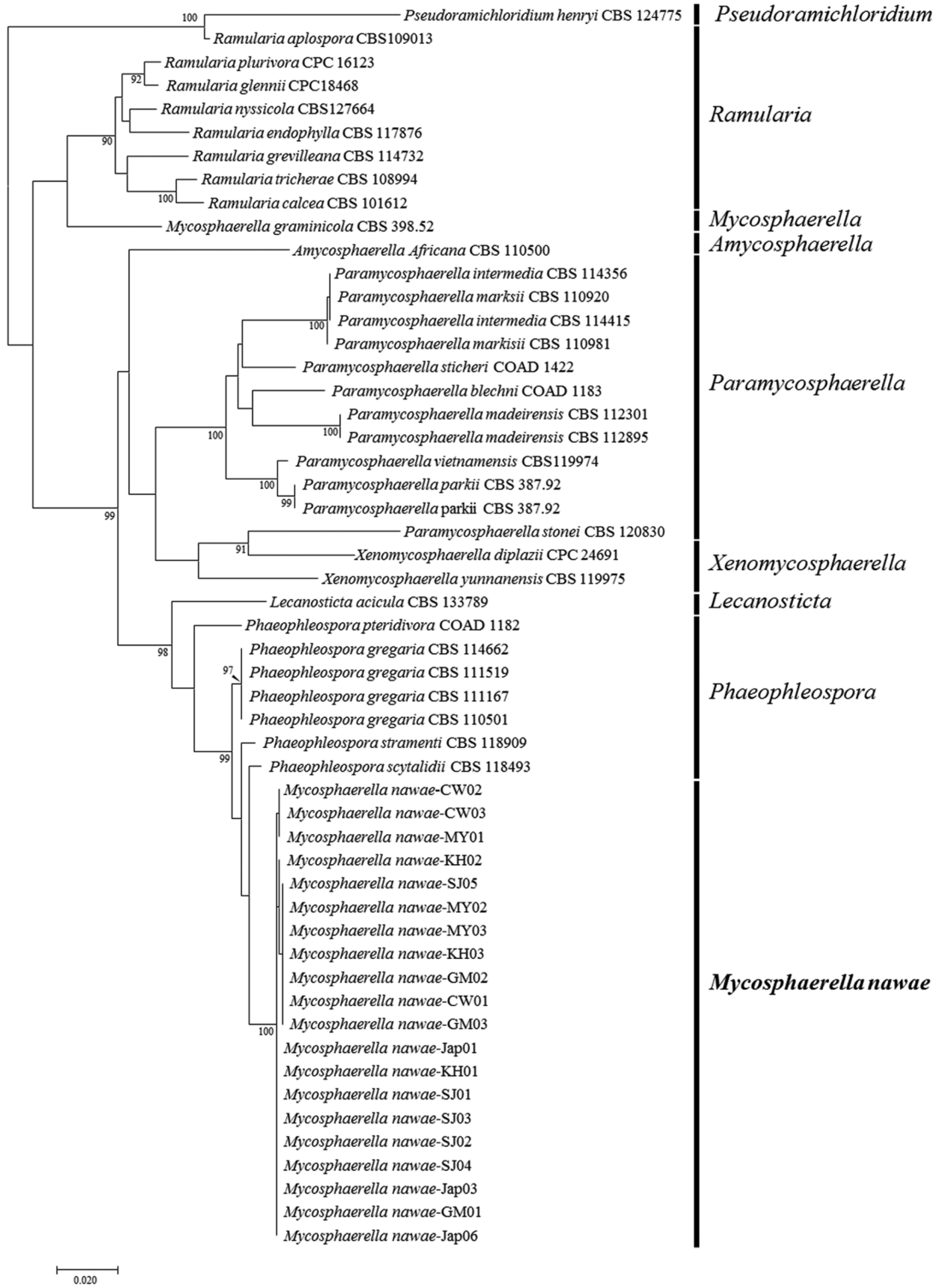

Fig. 4. Maximum likelihood tree of *Mycosphaerella nawae* and its allied species inferred from the combined internal transcribed spacer, partial 28S rDNA, and translation elongation factor-1α (*TEF-1α*) gene sequences. *Pseudoramichloridium henryi* (CBS 124775) was used as the outgroup. The numbers above the branches represent the bootstrap values obtained for 1,000 replicates (values smaller than 80 are not shown). The scale bar represents a phylogenetic distance of 0.02%

results showed that the Korean and Japanese *M. nawae* isolates were closest to the genus *Phaeophleospora* spp. especially *P. scytalidii* (Fig. 4).

Observation of pseudothecia on DL. The upper and lower surfaces of CLS-DL were observed using a stereoscopic microscope. Pseudothecia were observed on both surfaces

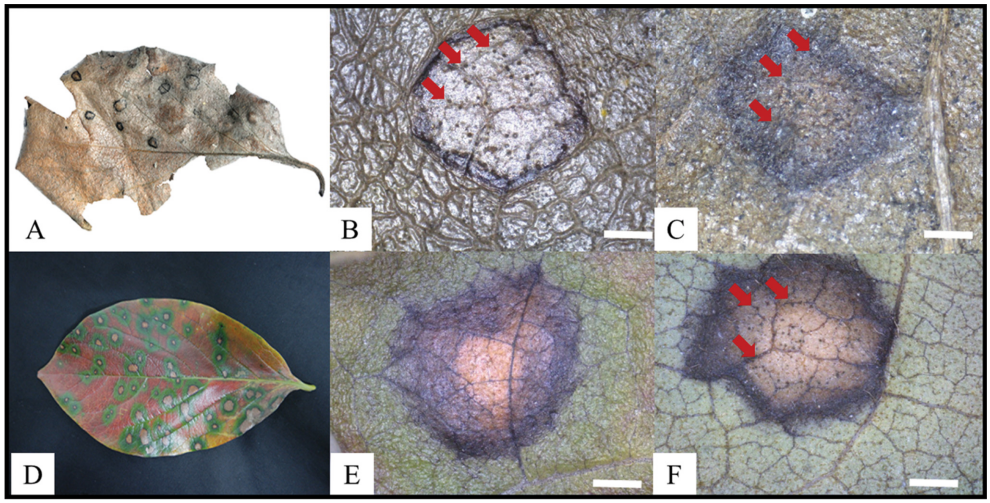

Fig. 5. Photographs of overwintered diseased leaves (ODL) and diseased leaves (DL) along with stereoscopic micrographs of their adaxial and abaxial sides. A, ODL; B, E, Stereoscopic micrographs of the adaxial side of ODL and the abaxial side of DL; C, F, Pseudothecium observed on the adaxial side of ODL and the abaxial side of DL; D, DL, red arrows indicate the observed pseudothecium (scale bars: B, C, E, F = 1 mm).

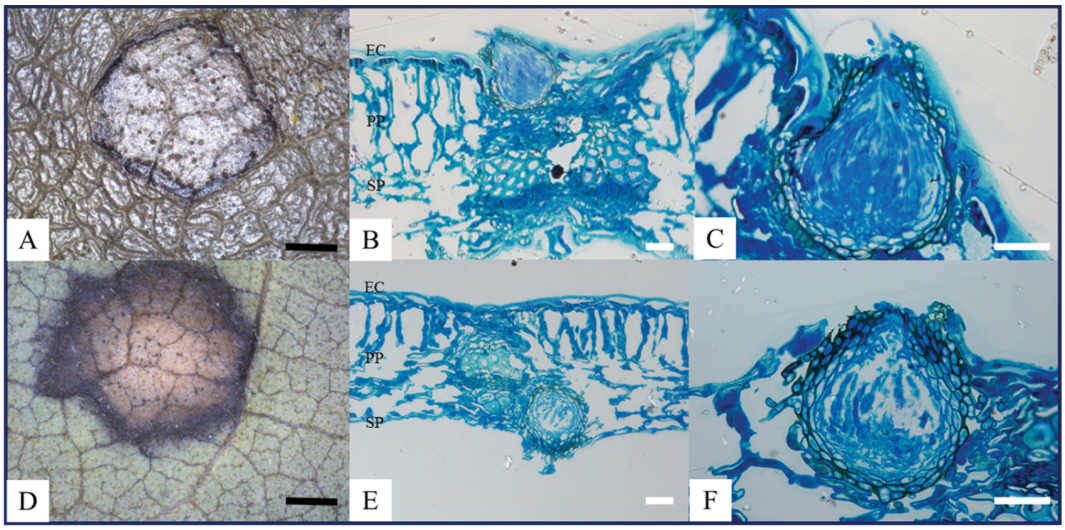

Fig. 6. Stereoscopic micrographs of overwintered diseased leaves (ODL) and diseased leaves (DL) along with cross section analysis. A, Stereoscopic micrographs of ODL; B, E, Pseudothecium observed in a semi-thin section; C, F, Enlarged image of B, E; D, Stereoscopic micrograph of DL (scale bars: A, D = 1 mm, B, C, E, F = 10 μm). EC, epidermal cell; PP, palisade parenchyma; SP, spongy parenchyma.

of ODL, whereas they were only observed on the lower side of DL (Fig. 5). The structures were observed on the cross sections of the leaves. The pseudothecia where located between the epidermal cells and the palisade parenchyma of the ODL. They were mostly flask- and pear-shaped structures, 55.1~62.2 μm wide (average 58.3 μm), and 70.8~80.3 μm high (average 76.0 μm) (Fig. 6). The pseudothecia on the DL were located between the palisade and spongy parenchyma, and were mostly ovoid and flask-shaped, 55.6~69.2 μm wide (average 60.7 μm), and 55.6~69.9 μm high (average 64.8 μm) (Fig. 6). The morphology of the asci and ascospores observed on the structures in the ODL confirmed that these structures represented the pseudothecia of *M. nawae*.

Observation of ascospores and conidia. Mature asci were observed in ODL collected from leaf litter between May and July 2015 (Fig. 7). These were cylindrical to clavate and banana-shaped structures, 8-spored, straight or curved, 4.6~6.8 μm wide (average 5.7 μm), and 25.9~34.1 μm high (average 31.1 μm) (Fig. 7A and 7B). The ascospores were oval to fusiform, hyaline, one-septate or aseptate, mostly tapering at both ends, 1.7~3.1 μm wide (average 2.5 μm), and 8.1~14.1 μm high (average 10.3 μm) (Fig. 7C).

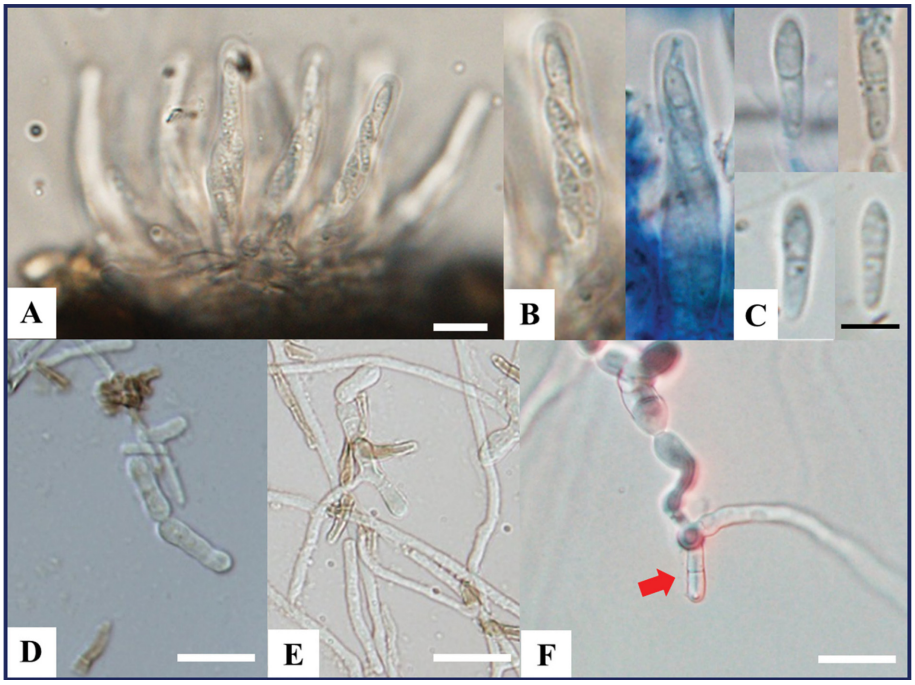

Fig. 7. The observed ascospores and conidia-like structures of *Mycosphaerella nawae*. A, B, Asci and ascospores; C, Ascospore; D, E, Mycelia observed in *M. nawae* cultured for 30 days; F, Conidia. Arrow indicates germinated conidia (scale bars: A, D~F = 10 μm, C = 5 μm).

Furthermore, because conidia were rarely observed in *M. nawae* cultured on PDA, a suspension of the aerial mycelia was spread on the PDA. Thereafter, very few conidia including germinated conidia and hyphae were observed on the PDA. The conidia were oval, guttulate, hyaline, one-septate, 12.8~19.8 μm high (average 17.1 μm), and 3.5~4.9 μm wide (average 4.3 μm) (Fig. 7F).

DISCUSSION

A previous study reported that the *M. nawae* anamorph was similar to that of *Ramularia* spp. in its morphological characteristics [7], whereas the *M. nawae* isolates in the present study were distinct from those of the *Ramularia* spp., as indicated in our phylogenetic analysis of the ITS region (Fig. 2). The phylogenetic placement of *M. nawae* using the combined dataset revealed that the *M. nawae* group was closest to the genus *Phaeophleospora*, especially *P. scytalidii* (Figs. 3 and 4). This suggests that *M. nawae* had a high degree of similarity with the genus *Phaeophleospora*.

Recently, new combinations and genera were introduced in *Mycosphaerellaceae*, based on phylogenetic analysis, such as genus *Amycosphaerella*, *Xenomycosphaerella*, and *Phaeophleospora* [19, 20]. Among these, several species belonged to the genus *Phaeophleospora*, *Xenomycosphaerella*, and *Paramycosphaerella*; they combined the species or changed the genus name based on only phylogenetic analysis results, without morphological comparison [19]. In this study, we constructed a phylogenetic tree comparing the allied species of *M. nawae* and the Korean and Japanese *M. nawae* isolates, based on combined ITS region, 28S rDNA, *Tub*, and *Act* sequences (approximately 2,450 bp) and the combined ITS region, 28S rDNA and *TEF-1α* gene sequences (approximately 1,200 bp). The results showed that the Korean and Japanese *M. nawae* isolates were closest to *Phaeophleospora* spp., and that *P. scytalidii* was the closest species of the genus *Phaeophloeospora* (Figs. 3 and 4). According to Videira et al., *M. nawae* has a *Ramularia*-like anamorph and is close to the genus *Phaeophleospora*, based on the ITS region [27]. Our results confirmed that i) *M. nawae* could be differentiated from *Ramularia* spp. by its morphological characteristics, ii) although it was close to the genus *Phaeophleospora*, it was closest to *P. scytalidii*.

The conidia of *M. nawae* were previously observed only in 1929 [1], and later, Kwon *et al.* [7] reported the anamorph stage of *M. nawae* as that of *Ramularia* spp. because of their similar morphological characteristics. One of the major characteristics of *Ramularia* spp. is the presence of scar structures on conidia [27]. In this study, the structures were not observed during microscopic observation (Fig. 7D~7F). Furthermore, phylogenetic analyses based on the ITS sequence data revealed that the *Ramularia* spp. were not closely related to the *M. nawae* isolates. These results indicate that *M. nawae* is distinct from *Ramularia* spp. Phylogenetic analysis showed that the genus *Phaeophleospora* was closely related to *M. nawae* (Fig. 3 and 4). The morphology of *M. scytalidii* (= *P. scytalidii*) has many similarities with *M. nawae*, such as pseudothecium production, similar size of ascospores, and conidia tapering at both ends, guttulate, and septate, among others

[17]. Interestingly, mycelial structures of *P. scytalidii* and *M. nawae* share similarities (Fig. 7D and 7E), including being solitary or branched, septate, and peanut- or bottle gourd-shaped [7, 17].

Many recent studies have been conducted on the genus *Mycosphaerella* and its anamorph [19, 20, 28-30]. Many species belonging to the genus *Mycosphaerella* have been segregated into other groups based on the morphology of their particular anamorph [31, 32], as well as based on teleomorph features such as asci and ascospores [12]. However, these classifications have not always been correlated with phylogenetic analysis [15, 32]. Recent studies on the genus *Phaeophleospora* indicated that species that were newly transferred into the genus based on phylogenetic inference, including *P. gregaria*, *P. scytalidii*, and *P. stramenti*, reproduce sexually and lack the asexual state [19]. In addition, phylogenetic analysis based on the multi-locus result revealed that most of the heterosynonym or homosynonym species are included in the genus *Mycosphaerella* (Table 1, Fig. 3). Furthermore, these current species names were not reflected in the anamorph stage, except for *M. graminicola* (anamorph: *Zymoseptoria tritici*). Since phylogenetic data revealed that the *M. nawae* cluster was closely related to *Phaeophleospora* spp. and especially to *P. scytalidii*, there is a possibility that *M. nawae* could be accommodated in the genus *Phaeophleospora* according to previous reports [19, 20]. Nonetheless, the common morphological features, cultural characteristics, and classification of the *Phaeophleospora* spp. and *M. nawae* need to be re-evaluated.

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