Research

Phylogenetic and Morphological Reassessment of *Mycosphaerella nawae*, the Causal Agent of Circular Leaf Spot in Persimmon

Oliul Hassan and Taehyun Chang,[†] Department of Ecology & Environmental System, College of Ecology & Environmental Sciences, Kyungpook National University, Sangju-si, Gyeongsangbuk-do, 37224, Korea (Republic of)

Abstract

Persimmon (*Diospyros kaki*) fruit production is severely affected by circular leaf spot worldwide. *Mycosphaerella nawae* causes circular leaf spot of persimmon (CLSP) and can result in leaf spot, defoliation, early fruit maturation, and subsequent softening and abscission. The morphology and phylogenetic position of *M. nawae* within the family *Mycosphaerellaceae* is, therefore, of utmost importance given its impact on persimmon production. Based on previous morphological and molecular studies, the phylogenetic position of the anamorphic genera associated with *M. nawae* remain in confusion. In the present study, 15 isolates of *M. nawae* were collected from the tissue of living leaves exhibiting leaf spot symptoms. A subsample of three isolates was characterized phylogenetically and morphologically.

Isolates were compared based on DNA sequence data for the internal transcribed spacer region (ITS1-5.8S ITS2), part of the 28S nrDNA including domains D1-D3 (LSU), actin (Act), translation elongation factor 1- α (EF-1 α), and RNA polymerase II second largest subunit (rpb2). The anamorph and teleomorph structures, ascospore germination patterns, as well as host specificity were used to describe the isolates. The phylogenetic and morphological analyses revealed that *M. nawae* requires a new holomorphic genus within *Mycosphaerellaceae*, described herein as *Plurivorosphaerella* gen. nov. A host specificity test revealed that *Plurivorosphaerella nawae* comb. nov. (*M. nawae*) can superficially colonize, but not infect, apple, peach, cherry, and plum.

Mycosphaerella comprises one of the largest generic complexes of plant-pathogenic ascomycetes. Although most of the *Mycosphaerella* species are known as foliicolous plant pathogens, some are responsible for stem cankers and fruit lesions (Cortinas et al. 2006; Crous 2009; Crous et al. 2001; Pretorius et al. 2003). Being saprobic, plant pathogens to hyperparasites, endophytes, and symbionts, *Mycosphaerella* spp. are able to survive in various ecosystems (Crous et al. 2001; Quaedvlieg et al. 2012). *Mycosphaerella* spp. are plant pathogens that cause significant economic losses of both temperate and tropical crops worldwide, including banana, cereals, sugar beet, strawberry, soybean, citrus, eucalyptus, acacia, pines, guava, and persimmon (Crous 2009; Crous and Braun 2003; Jacobs et al. 2014; Kwon et al. 1998a). Teleomorph morphology of *Mycosphaerella* is relatively simple and conserved, but 30 anamorphic genera have been associated with this generic complex (Crous 2009; Crous et al. 2007b). The phylogenetic relationships of numerous *Mycosphaerella* species and their anamorphs based on multigene sequences revealed that the *Mycosphaerella* complex is in fact poly- and paraphyletic (Crous 2009; Crous et al. 2007a; Hunter et al. 2006). For this reason, taxonomic redistribution is needed for most of the phylogenetic clades within the complex (Crous 2009; Quaedvlieg et al. 2012). The dual nomenclature within the *Mycosphaerella* complex based on teleomorphs and anamorphs is confusing and complicated. Videira et al. (2017) recently split the whole complex into numerous genera based on results of phylogenetic multigene analyses reflecting that traditional morphological approaches are not tenable for taxonomic purposes within *Mycosphaerellaceae*. *Mycosphaerella nawae*, the

causal agent of circular leaf spot of persimmon (CLSP), is a great threat to the persimmon industry. Fruit production is affected by early fruit maturation and subsequent softening and abscission, along with foliar symptoms and defoliation (Bassimba et al. 2017; Berbegal et al. 2010). The morphology of *M. nawae* in different pathosystems has been the focus of many studies (Berbegal et al. 2010; Ikata and Hitomi 1929; Kwon et al. 1998a, b; Lee et al. 2016). All authors agree that the teleomorph of *M. nawae*, consists of small, black, spherical fruiting bodies (pseudothecia), cylinder- or banana-shaped asci, and uniseptate and mostly spindle-shaped ascospores (Berbegal et al. 2010; Kwon et al. 1998c; Lee et al. 2016). Kwon et al. (1998a, b) detailed the anamorph of *M. nawae* and the ability of asexual conidia to infect persimmon leaves. The authors suggested that the anamorph of *M. nawae* is linked to the genus *Ramularia* based on its morphological characteristics. Asexual conidia were of various shapes and sizes and were formed solitarily or in chains on potato dextrose agar (PDA) and on infected leaves (Kwon et al. 1998a, b). Multigene sequence data revealed that the morphological characteristics used to define the anamorph are not always informative (Verkley et al. 2004). Lee et al. (2016) analyzed the phylogenetic relationships of *M. nawae* and allied species by using multilocus gene sequences and identified *M. nawae* as the closest species to members of the genus *Phaeophleospora*, especially *P. scytalidii*, although *M. nawae* belongs to a clade distinct from *P. scytalidii*. These authors were not able to show sufficient evidence in the characteristics of the anamorph to support the phylogenetic relationships. The dilemma regarding the taxonomic positioning of the anamorph of *M. nawae* remains unresolved. Persimmon (*Diospyros kaki*) has been reported as the only host for *M. nawae* (Kwon et al. 1998c). However, there is little data available to support this hypothesis. Recent studies based on molecular data indicate that some species of *Mycosphaerella* have narrow host ranges, while others have wider host ranges (Crous and Groenewald 2005; Crous et al. 2006, 2009d). Host-specific *Mycosphaerella* species may jump from a dying host to a nonhost and produce progeny that then may infect another favorable host (Crous and Groenewald 2005). Therefore, *M. nawae* could have a secondary host or may switch to secondary hosts in the absence of persimmon leaves.

The aims of this study were to (a) re-evaluate the morphological (teleomorph and anamorph) and molecular characteristics of

[†]Corresponding author: T. Chang; E-mail: thchang@knu.ac.kr

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M. nawae, (b) clarify the phylogeny and generic taxonomy, and (c) evaluate the pathogenicity and host specificity of *M. nawae*.

Materials and Methods

Isolation and preliminary identification of *M. nawae*. A total of 15 isolates associated with CLSP symptoms in persimmon leaves were collected from five different orchards located in Sanju, Korea. The isolates were recovered from the margins of leaf lesions. Diseased leaves were surface disinfected with 95% ethanol and washed using distilled water. Small sections (1 cm²) were cut from the affected leaf tissue and disinfested with 0.5% sodium hypochlorite solution for 2 min. The sections were then washed three times in sterile distilled water and dried by blotting. Small sections of lesion margins were placed on water agar (WA) supplemented with streptomycin (0.05 g/liter). Plates were incubated at 25°C until fungal hyphae emerged from necrotic tissue. Individual hyphal tips were transferred to fresh PDA after 6–7 days of incubation. To achieve a pure culture, all isolates were hyphal-tipped and cultured on PDA. *M. nawae* isolates were initially identified by morphology, and nested-PCR for specific identification as described by Berbegal et al. (2013). The nested-PCR methods comprised two rounds of amplification. The first round PCR was performed with the universal fungal primers, ITS1F/ITS4, and the second round was performed with the specific primer pair, MNf/MNr (Berbegal et al. 2013). A subsample of three isolates (MN6-1, MN7, and MN10) identified as *M. nawae* were used for further studies.

Genomic DNA extraction, PCR amplification, purification, and sequencing. Total genomic DNA was extracted from the three isolates using a HiGene Genomic DNA Prep Kit (Yuseong-Gu, Daejeon, Korea) following the manufacturer's instructions. Mycelium from actively growing cultures was used to isolate genomic DNA. The isolation process was confirmed by visualizing DNA in a 1% agarose gel (wt/vol), stained with loading STAR(6X) (DYENE BIO), and viewed under ultraviolet light. Isolated genomic DNA was used as a template to amplify and sequence the ITS (ITS1–5.8S ITS2) region, part of the 28S nrDNA, including domains D1–D3 (LSU), actin (ACT), RNA polymerase II second largest subunit (rpb2), and translation elongation factor 1- α (EF-1 α). The details of the primers used for PCR amplifications are shown in Table 1. PCRs were carried out in a simpliAmp thermal cycler (Thermo Fisher Scientific Inc.). Each 25 μ l PCR mixture was made with 18.2 μ l of UV-sterilized ultra-filtered water, 2.5 μ l 10 $\times$ F-star Taq buffer, 0.5 μ l dNTP Mix (each 10 mM), 1 μ l forward primer (10 pmol), 1 μ l reverse primer (10 pmol), 1 μ l genomic DNA, and 0.4 μ l F-star Taq DNA polymerase (BIOFACT, Korea). PCR cycling conditions were largely identical for selected genes except for the primer annealing step. All of the PCR cycles were run with an initial denaturation step at 95°C for 4 min, followed by 34 cycles of denaturation at 95°C for 35 s, primer extension at 72°C for 35 s, and a final elongation step at 72°C for 7 min. The annealing step was adjusted to 52°C for ITS, 61°C for Act, 62°C for LSU, 72°C for EF-1 α (Hunter et al. 2006), and 60°C for rpb2 (Videira et al. 2017). All of the PCR products were examined by electrophoresis in 1% agarose gels, and the product of successful reactions was purified using a HiGene PCR Purification Kit (Yuseong-Gu, Daejeon, Korea). Purified

double-stranded PCR fragments were sequenced directly using their respective primers at Macrogen, Inc. (Seoul, Korea). The resulting consensus sequences were deposited in GenBank (accession numbers are listed in Table 2).

Phylogenetic analysis. All of the generated sequences (ITS, Act, LSU, EF-1 α , and rpb2) were compared with other fungal DNA sequences previously deposited in GenBank using a BLAST search (<https://blast.ncbi.nlm.nih.gov/>). Gene sequences of *M. nawae* and selected reference type strains belonging to the Mycosphaerellaceae, Teratosphaeriaceae, and Davidiellaceae were retrieved from GenBank (Berbegal et al. 2010; Burgess et al. 2007; Crous et al. 2009d; Hunter et al. 2006; Lee et al. 2016; Videira et al. 2017). Gene sequences of reference type anamorphs linked to *Mycosphaerella* spp. (Crous et al. 2009c) also were retrieved from GenBank (Table 2). Multiple sequence alignments of sequences generated from the present isolates and those retrieved from GenBank (Table 2) were done using muscle multiple sequence alignment programs of MEGA v. 6.0 (Tamura et al. 2013) and edited manually where necessary. The aligned sequences of each gene were concatenated with Mesquite v. 2.75 (Maddison and Maddison 2011). Maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) phylogenetic analyses were conducted for the aligned sequence data. ML and MP were performed using MEGA v. 6.0 (Tamura et al. 2013). ML trees were constructed using a Tamura-Nei substitution model with Gamma distributed with Invariant (G+I) rates among the site. The ML Heuristic search option with Nearest-Neighbor-Interchange (NNI) was used to infer the tree. The 1,000-bootstrap replicates were implemented to assess the relative stability of the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1, in which the initial trees were obtained by the random addition of sequences (10 replicates). BI analysis was performed using MrBayes v. 3.2.2 (Ronquist et al. 2012). The Markov Chain Monte Carlo (MCMC) algorithm was used to regenerate the phylogenetic trees. Four Markov chains were run from trees for 2 million random generations. Trees were sampled every 1,000th generation. The initial 25% of trees in the burn-in phase of each analysis was discarded, and the remaining trees in each analysis were used to calculate the posterior probabilities in the 50% majority rule consensus tree. The robustness of each node was examined by posterior probability analysis. The generated phylogenetic trees were viewed in FigTree v 1.3.1 (Rambaut and Drummond 2009).

Morphological analysis. Isolate MN10 was selected for morphological description. To observe colony morphology and the effects of temperature, light, and relative humidity (RH) on mycelial growth, mycelial discs (5 mm diameter) were removed from the actively growing edge of 4-day-old cultures, and each mycelial disc was placed on a 90 mm PDA plate. Plates were incubated at 25°C in the dark for morphological observation. To evaluate the effects of temperature, light, and RH on mycelial growth, the plates were incubated under the following conditions: 1) at 5, 10, 15, 20, and 25°C in the dark; 2) at 5, 10, 15, 20, and 25°C under 16 h of light; and 3) at 5, 10, 15, 20, and 25°C with >75% RH in the dark. A relative humidity of over 75% was maintained by keeping the plates with mycelial discs open inside a sterile plastic box containing moist tissue paper. Morphological characteristics were observed and recorded after

Table 1. Details of the primers used in this study, with sequences and sources

Locus	Primer name	Direction	Primer sequence (5'-3')	References
ITS	ITS-1F	Forward	CTTGTCATTTAGAGGAAGTAA	White et al. 1990
	ITS-4	Reverse	TCCTCCGCTTATTGATATGC	White et al. 1990
LSU	LR0R	Forward	ACCCGCTGAACCTTAAGC	Moncalvo et al. 1995
	LR7	Reverse	TACTACCACCAAGATCT	Vilgalys and Hester 1990
Actin	ACT-512F	Forward	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn 1999
	ACT-783R	Reverse	TACGAGTCCTTCTGGCCCAT	Carbone and Kohn 1999
EF-1 α	EF1-728F	Forward	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn 1999
	EF1-986R	Reverse	TACTTGAAGGAACCCCTTACC	Carbone and Kohn 1999
rpb2	RPB2-5F2	Forward	GGGGWGAYCAGAGAAGGC	Sung et al. 2007
	RPB2-7cR	Reverse	CCCATRGCTTGYTTRCCCAT	Liu et al. 1999

2 weeks of incubation, and colony diameter was recorded for three replicate cultures of isolate MN10, with two measurements per replicate. The diameter of each colony was measured by subtracting the diameter of the 5-mm plug from the two perpendicular diameters, and

the obtained measurements were averaged. The effects of different media, including oatmeal agar (OMA), malt agar (MA), low carbon agar (LCA), PDA, V8, and corn meal agar, on the mycelial growth of isolate MN10 were also examined. Mycelial discs (5 mm diameter)

Table 2. Collection details and GenBank accession numbers of the fungal isolates used in this study for molecular data analyses. Ex-type cultures are indicated in bold text.

Current name	Previous name (if different)	Isolate no. ^a	GenBank accession number				
			LSU	ITS	Actin	EF-1α	rpb2
<i>Mycosphaerellaceae</i>							
<i>Plurivorosphaerella nawae</i>	<i>Mycosphaerella nawae</i>	MN6-1	LC380932	LC194865	LC380938	LC380935	LC380941
<i>P. nawae</i>	<i>M. nawae</i>	MN7	LC380933	LC194866	LC380939	LC380936	LC380942
<i>P. nawae</i>	<i>M. nawae</i>	MN10	LC380934	LC194867	LC380940	LC380937	LC380943
<i>M. nawae</i>		SJ01	LC121140	LC121109			
<i>M. nawae</i>		Jap 01	LC121154	LC121123			
<i>M. africana</i>		CBS 116154	DQ246257	AF309602	DQ147609	DQ235099	
<i>M. africana</i>		CBS 116155	DQ246258	DQ267577	DQ147608	DQ235098	
<i>M. ambiphylla</i>		CBS 110499	DQ246219	AY725530	DQ147669	DQ235103	
<i>M. aurantia</i>		CBS 110500	DQ246256	AY725531	DQ147610	DQ235097	
<i>M. communis</i>		CBS 114238	DQ246262	AY725541	DQ147655	DQ235141	
<i>M. communis</i>		CBS 110976	DQ246261	AY725537	DQ147654	DQ235140	
<i>M. crystallina</i>		CMW 3042	DQ204746	DQ267578	DQ147637	DQ211662	
<i>M. crystallina</i>		CMW 3033	DQ204747	AY490757	DQ147636	DQ211663	
<i>M. ellipsoidea</i>		CMW 4934	DQ246253	AF309592	DQ147647	DQ235129	
<i>M. ellipsoidea</i>		CMW 5166	DQ246254	AF309593	DQ147648	DQ235127	
<i>M. endophytica</i>		CBS 111519	DQ246255	DQ267579	DQ147646	DQ235131	
<i>M. endophytica</i>		CMW 5225	DQ246252	DQ267580	DQ147649	DQ235128	
<i>M. fori</i>		CMW 9095	DQ204748	AF468869	DQ147618	DQ211664	
<i>M. fori</i>		CMW 9095	DQ204749	DQ267581	DQ147619	DQ211665	
<i>M. gracilis</i>		CBS 243.94	DQ204750	DQ267582	DQ147616	DQ211666	
<i>M. grandis</i>		CMW 8557	DQ246241	DQ267583	DQ147644	DQ235108	
<i>M. grandis</i>		CMW 8554	DQ246240	DQ267584	DQ147643	DQ235107	
<i>M. gregaria</i>		CBS 110501	DQ246251	DQ267585	DQ147650	DQ235130	
<i>M. intermedia</i>		CBS 114415	DQ246248	AY725547	DQ147627	DQ235132	
<i>M. keniensis</i>		CMW 5147	DQ246259	AF309601	DQ147611	DQ235100	
<i>M. lateralis</i>		CBS 110748	DQ204768	AF173315	DQ147651	DQ211684	
<i>M. lateralis</i>		CBS 111169	DQ246260	AY25550	DQ147652	DQ235139	
<i>M. madeirae</i>		CBS 112895	DQ204756	AY725553	DQ147641	DQ211672	
<i>M. marksii</i>		CBS 682.95	DQ246249	DQ267587	DQ147624	DQ235133	
<i>M. marksii</i>		CBS 110920	DQ246250	AF309588	DQ147625	DQ235134	
<i>M. marksii</i>		CMW 5230	DQ246246	DQ267588	DQ147626	DQ235135	
<i>M. nubilosa</i>		CBS 116005	DQ246228	AF309618	DQ147666	DQ235111	
<i>M. nubilosa</i>		CBS 114708	DQ246229	AF449099	DQ147667	DQ235112	
<i>M. parkii</i>		CBS 387.92	DQ246245	AY626979	DQ147612	DQ235137	
<i>M. parva</i>		CBS116289	DQ246243	AY626980	DQ147645	DQ235110	
<i>M. parva</i>		CBS110503	DQ246242	AY725576	DQ147642	DQ235109	
<i>M. tasmaniensis</i>		CBS111687	DQ246233	DQ267591	DQ147676	DQ235121	
<i>M. tasmaniensis</i>		CBS114556	DQ246234	DQ267592	DQ147677	DQ235122	
<i>Annellostypodiella juniper</i>		CPC 23276	KJ869204	KJ869204			MF951436
<i>Australosphaerella nootherensis</i>	<i>Mycosphaerella nootherensis</i>	CBS 130522	KF901835	MF951293			
<i>Brunswickiella parsoniae</i>		CBS 137979	KJ869188	KJ869131			MF951593
<i>Brunneosphaerella nitidae</i>		CBS 130595	GU214396	GU214625			
<i>B. protearum</i>		CBS 130597^{ET}	GU214397	GU214626			
<i>Chuppomyces handelii</i>	<i>Mycosphaerella handelii</i>	CBS 113302	GU214437	EU167581			
<i>Cytostagonospora martiniana</i>	<i>Septoria</i> sp.	CBS 135102^{ET}	KF251657	KF251153			MF951484
<i>Devonomyces endophyticus</i>	<i>Phaeophleospora gregaria</i>	CBS 111167	KF902058	KF901711			MF951588
<i>D. endophyticus</i>	<i>Mycosphaerella endophytica</i>	CBS 114662	KF902060	KF901713			MF951590
<i>Epicoleosporium ramularioides</i>		CBS 141103	GU214688	GU214688			
<i>Exosporium livistonae</i>	<i>Passalora</i> sp.	CBS 131313	JQ044446	JQ044427			
<i>Hyalinozasmidium sideroxyli</i>	<i>Zasmidium</i> sp.	CBS 142191	MF951169	MF951323			
<i>Madagascaramyces intermedius</i>	<i>Passalora intermedia</i>	CBS 124154	FJ790297	FJ790267			
<i>Microcyclosporella mali</i>		CBS 126136	GU570547	GU570535			
<i>Mycodiella sumatrensis</i>	<i>Mycosphaerella sumatrensis</i>	CBS 118501	JX901872	DQ303049			

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^a ET: ex-epitype.

from the actively growing edge of 4-day-old cultures were placed at three equidistant points on a 90-mm plate containing the respective media. The diameter of each colony was measured as described above after incubation at 25°C in the dark for 2 weeks. This entire experiment was replicated nine times. For enhancing the production

of sexual and asexual spores, the incubation period of the cultures for all growth media was extended for 110 more days. Data related to colony diameter in each treatment were compared using Duncan multiple range tests (DMRT) ($\alpha = 0.05$) using the JMP Pro 10.0.0 software. To examine size and shape of the conidia, the following

Table 2. (Continued from previous page)

Current name	Previous name (if different)	Isolate no. ^a	GenBank accession number			
			LSU	ITS	Actin	EF-1 α rpb2
<i>Mycosphaerelloides madeirae</i>	<i>Mycosphaerella madeirae</i>	CBS 112895	KF902017	AY725553		
<i>Neoceratosperma eucalypti</i>		CBS 142190	KJ869210	KJ869153		
<i>Neomycosphaerella pseudopentameridis</i>		CBS 136407	KF777226	KF777173		
<i>N. legnophorica</i>	<i>Stenella</i> sp.	CBS 142189	MF951183	MF951327		
<i>Neopenidiella nectandrae</i>	<i>Cladosporium ferrugineum</i>	CBS 734.87	KF901982	MF951335		MF951546
<i>Nothopericoniella perseae-macranthae</i>	<i>Periconiella perseae-macranthae</i>	CBS 122282	GU452681	MF951355		MF951584
<i>Nothopericoniella perseae-macranthae</i>	<i>Periconiella perseae-macranthae</i>	CBS 122097	GU452682	MF951354		MF951583
<i>Lecanosticta acicola</i>		CBS 133791 ^{ET}	KC013017	KC012999		MF951507
<i>L. brevispora</i>		CBS 133601	KF902021	JX901763		MF951508
<i>L. longispora</i>		CBS 133602 ^{ET}	JX901858	JX901766		MF951510
<i>Pachyramichloridium pini</i>		CBS 461.82	EU041859	EU041802		
<i>Paramycosphaerella brachystegiae</i>		CBS 136436	KF777230	KF777178		
<i>Passalora zambiae</i>		CBS116289	DQ246264	KF901810	DQ147675	DQ235136
<i>P. wachendorffiae</i>	<i>Mycosphaerella wachendorffiae</i>	CBS 129579	JF951163	JF951143		
<i>Phaeophloeospora eugeniae</i>		CPC 15143	FJ493206	FJ493188		MF951594
<i>P. eugeniae</i>		CPC 15159	FJ493207	FJ493189		MF951595
<i>Polyphialoseptoria tabebuiae-serratifolia</i>		CBS 112650	KF251716	KF251213		
<i>P. terminaliae</i>		CBS 135106	KF251717	KF251214		
<i>Pseudopericoniella levispora</i>	<i>Periconiella levispora</i>	CBS 873.73	EU041837	EU041780		
<i>Pseudozasmidium vietnamense</i>	<i>Paramycosphaerella vietnamensis</i>	CBS 119974	JF700944	DQ632675		
<i>Ruptoseptoria unedonis</i>	<i>Ruptoseptoria unedonis</i>	CBS 755.70	KF251732	KF251229		
<i>Virosphaerella pseudomarksii</i>	<i>Mycosphaerella pseudomarksii</i>	CBS 123241	KF902127	KF901770		
<i>Xenomycosphaerella elongata</i>		CBS 120735	JF700942	EF394833		
<i>Xenosonderhenia eucalypti</i>		CBS 138858	KP004485	KP004457		
<i>Xenosonderhenioides indonesiana</i>	<i>Passalora</i> sp.	CBS 142239	MF951261	MF951396		
<i>Zasmidium angulare</i>		CBS 132094	JQ622096	JQ622088		MF951690
<i>Z. fructigenum</i>		CBS 13962	KP895926	KP896056		MF951704
<i>Z. nocoxi</i>		CBS 125009	KF251788	KF251284		MF951719
<i>Z. xenoparkii</i>	<i>Stenella xenoparkii</i>	CBS 111185	JF700966	DQ303028		MF951732
Schizothyriaceae						
<i>Schizothyrium pomi</i>		CBS 228.57	EF134947	EF134947		
Dissoconiaceae						
<i>Ramichloridium apiculatum</i>	<i>Chloridium apiculatum</i>	CBS 156.59	EU041848	EU041791		
<i>Uwebraunia australiensis</i>	<i>Dissoconium australiensis</i>	CBS 120729	KF442553	KF442513		
<i>U. musae</i>	<i>Dissoconium musae</i>	CBS 122453	JQ739816	EU514225		
Phaeothecodiellaceae						
<i>Exopassalora zambiae</i>	<i>Passalora zambiae</i>	CBS 112971	EU019273	AY725523		
<i>Sporidesmajora pennsylvaniensis</i>		CPC 16112	MF951122	MF951287		
<i>Houjia pomigena</i>		CBS 125224	MF951120	MF951285		
Teratosphaeriaceae						
<i>Acrodontium crateriforme</i>	<i>Chloridium crateriforme</i>	CBS 144.33	KX286952	MF951410		
<i>Batcheloromyces proteae</i>		CBS 110696 ^{ET}	EU019247	JF746163		
<i>Myrtapenidiella corymbia</i>	<i>Penidiella corymbia</i>	CBS 124769	KF901838	GQ303286		
<i>Parapenidiella tasmaniensis</i>		CBS 111687	GU214452	KF901521		
<i>Pseudoteratosphaeria flexuosa</i>	<i>Mycosphaerella flexuosa</i>	CBS 110743	KF902098	DQ302955		
<i>Readeriella nontingens</i>	<i>Readeriella nontingens</i>	CPC 14444	GQ852663	GQ852786		
<i>Stenella araguata</i>		CBS 105.75	EU019250	EU019250		
<i>Teratosphaeria stellenboschiana</i>		CBS 125215	KF937247	KF901733		
Cladosporiaceae						
<i>Cladosporium allcinum</i>		CBS 188.53	MF951115	KT600367		
<i>Cladosporium</i> sp.		CBS 179.47	MF951118	MF951283		
Dothioraceae						
<i>Cylindroseptoria ceratonia</i>	<i>Septoria ceratoniae</i>	CBS 477.69	KF251655	KF251151		MF951419
Botryosphaeriaceae						
<i>Botryosphaeria ribis</i>		CMW 7773	DQ246263	DQ267604	DQ267605	DQ235142

slide culture techniques were followed. A small square block (1 cm²) of the medium was cut from the PDA plate and placed in the middle of the slide. Using a sterilized needle, the four sides of the PDA block were inoculated with mycelium of isolate MN10. A coverslip (18 × 18 mm) was placed on top of the inoculated PDA block, and then the slide was placed inside a petri dish containing moist filter paper on a glass support. After approximately 3 months of incubation at 25°C in the dark, the conidia formed across the underside of the coverslip, and their sizes were measured with a stage micrometer under an Olympus BX43 microscope (Olympus Corporation, Japan) at 400× magnification. To examine the size and shape of the pseudothecia, asci, and ascospores, mature pseudothecia were excised from a lesion of overwintered infected leaves, squashed in lactophenol-acid cotton blue, and examined at 400×. The size of the pseudothecia, asci, and ascospores were measured in the same way as the size of conidia was measured.

Pathogenicity and host specificity analysis. In field studies, the pathogenicity of ascospores and conidia were tested on 2-year-old seedlings of persimmon, apple, peach, cherry, and plum. Seedlings were washed with sterile distilled water. Conidial suspensions were prepared from 75- to 90-day-old cultures in distilled water. Ultra-pure water was added to the petri plate containing the desired *M. nawae* culture. The culture was rubbed until the conidia loosened and became suspended in the solution. The resulting suspension was filtered through four layers of cheesecloth to remove mycelial

fragments. The concentration of the conidial suspension was measured using a hemocytometer. A suspension of 1×10^4 conidia/ml was prepared from a concentrated suspension (using the formula $v_1 \times c_1 = v_2 \times c_2$). To prepare the ascospore suspension (1×10^4 ascospores/ml), the same procedure was used. The ascospore suspension was prepared by soaking overwintered diseased leaves in ultra-pure water. Ascospore or conidial suspensions were sprayed onto seedlings of different fruits. Seedlings sprayed with distilled water were used as the negative control. Seedlings treated with conidia, or ascospores, or water were isolated separately outdoors until symptoms developed. Five seedlings were used per treatment. The inoculation of seedlings was done in mid-July. Disease incidence was estimated as the total percentage of symptomatic (having at least one circular spot) leaves per seedling, and disease severity was estimated according to Berbegal et al. (2012).

In the laboratory, ascospores and conidia were tested for pathogenicity using small branches of apple, peach, cherry, and plum. Small branches (healthy) of the respective trees were brought to the laboratory and cut into small pieces (6–8 cm in length), which were disinfected through both surface and autoclave sterilization. Surface sterilization was done with 70% ethanol for 1 min and 1% NaOCl for 2 min followed by rinsing in sterile distilled water. The sterilized stems were placed in a plastic saucer and allowed to dry before inoculation. The stems were then wounded by removing a small square of tissue and placing a 10-μl droplet of conidial suspension

Fig. 1. Bayesian inference (BI) tree (based on 50% majority rule consensus) of the LSU sequence alignment showing phylogenetic affinities of the present isolates. The tree is rooted to *Lasiodiplodia theobromae* (GenBank accession DQ377858). The Bayesian inference posterior probabilities values are >50% around the branch. The scale bar shows the number of substitutions per nucleotide position. Family clades are delimited in boxes, with the family name indicated on the right.

over the wound. Distilled water was used as a negative control. The plastic saucers with the inoculated stems (positive and negative controls) were transferred to an enclosed sterile plastic box containing moist tissue paper. The stems were incubated at 25°C in high humidity to encourage disease development, and observed periodically.

Scanning electron microscopy of conidia produced on leaves. The fine fungal structures on infected leaves were observed using field emission scanning electron microscopy (JEOL FE-SEM JSM-

6701F; JEOL USA, Inc.). Samples (leaf and culture) were prepared for SEM according to Kwon et al. (1998b). The leaves of inoculated seedlings showing typical CLSP symptoms were collected at the end of October after defoliation. The lesions on infected leaves were cut into small sections (5 × 5 mm) with a surgical blade and fixed in 2.5% glutaraldehyde for 24 h. The specimens were washed with 0.1 M phosphate buffer (pH 7.4), postfixed with 2% osmium tetroxide (Sigma-Aldrich, Korea) for 90 min, and washed again with the same

Fig. 2. Maximum likelihood tree from MEGA 6 showing the phylogenetic relationships based on the combined ITS, LSU, ACT, and EF-1 α DNA sequence alignment of present isolates and *Mycosphaerella* spp. occurring on *Eucalyptus*. The scale bar shows the number of substitutions per site, and bootstrap support values (>50%) from 1,000 replicates are shown at the nodes.

buffer. The specimens were dehydrated in a series of ascending concentrations of ethanol (20, 40, 60, 80, and 90%) for 30 min and 100% for 60 min, followed by a mixture of isoamyl acetate + ethanol (1:1) for 30 min. Specimens were then dried in a fume hood. Finally, specimens were sputter-coated with gold and observed under a JEOL FE-SEM at Central Lab of Kyungpook National University, Sangju campus, Korea.

Results

DNA amplification and identification. The complete amplification of ITS and the partial amplification of LSU, Act, rpb2, and EF-1 α was successful for all isolates (Table 2). The sizes of the resulting sequences were: ITS ~630 pb, LSU ~1,335 pb, Act ~217 pb, rpb2

~993 pb, and EF-1 α ~317 pb. The isolates of the present study were identified as *M. nawae* based on the obtained ITS sequences. A BLAST search showed that the ITS sequences of the present isolates were 100% identical with those of the Spanish, Korean, and Japanese *M. nawae* isolates (GQ465767, GQ465767, LC121124, LC121127, and LC121128).

Phylogenetic analysis. Dataset 1 consisted of the alignment of the LSU loci that contained 57 taxa representing several genera known from culture belonging to *Mycosphaerellaceae*, *Teratosphaeriaceae*, and *Davidiellaceae*. A strain of *Lasioidiplodia theobromae* was used as the outgroup. The Bayesian analyses of the LSU gene alignment generated 16,562 trees, 12,423 of which were used to construct the tree and calculate the posterior probabilities (Fig. 1).

Fig. 3. Phylogenetic tree resulting from the maximum likelihood analysis of the combined LSU and ITS sequence alignment of anamorphic genera belonging to *Mycosphaerellaceae*, *Dissoconiaceae* (D), *Phaeothecoidiaceae* (P), *Schizothyriaceae* (S), *Teratosphaeriaceae* (T), and *Cladosporiaceae* (C). The maximum likelihood bootstrap support values (>50%) are indicated at the nodes. The clades of the genera are delimited by boxes, with the genus name indicated on the right. The tree is rooted to *Cylindroseptoria ceratoniae* (CBS 477.69).

Figure 1 shows the present isolates confidently identified as a member of *Mycosphaerellaceae*, as they clustered together with other genera in *Mycosphaerellaceae* with 0.96 (96%) bootstrap support.

Dataset 2 consisted of the alignment of four loci (ITS, LSU, Act, and EF-1 α) that contained 38 taxa representing several genera known from culture belonging to *Mycosphaerellaceae* occurring on *Eucalyptus*, which were described by Hunter et al. (2006). A strain of *Botryosphaeria ribis* was used as the outgroup. The ML analysis of the concatenated four-locus alignment reached a final ML highest log likelihood of -15859.7540. The initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The topology with the superior log likelihood value was then selected (Fig. 2). A discrete Gamma distribution was used to model the evolutionary rate differences among sites (five categories (+G, parameter = 0.4795)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 42.3% sites). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st + 2nd + 3rd + noncoding. There were a total of 2,580 positions in the final dataset. Figure 2 shows that the present isolates are closely related to *Mycosphaerella endophytica*, which is synonymous to both *Devonomyces endophyticus* and *Pseudocercospora endophytica* (Videira et al. 2017).

Dataset 3 consisted of the alignment of two loci (ITS and LSU) that contained 65 taxa of anamorphic-typified genera belonging to *Mycosphaerellaceae* (related to the present isolates), *Dissoconiaceae*, *Phaeothecoidiaceae*, *Schizothyriaceae*, *Teratosphaeriaceae*, and

Cladosporiaceae (Videira et al. 2017). A strain of *Cylindroseptoria ceratoniae* was used as an outgroup. The ML analysis of the concatenated two-locus alignment reached a final ML highest log likelihood of -32641.0641. The initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the MCL approach, and the topology with the superior log likelihood value was then selected (Fig. 3). A discrete Gamma distribution was used to model the evolutionary rate differences among sites (five categories (+G, parameter = 0.4931)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 36% sites). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st + 2nd + 3rd + noncoding. The final dataset contained a total of 1,110 positions. Figure 3 demonstrates that the phylogenetic positions of *M. nawae* isolates are close to the genus *Devonomyces*. The phylogenetic position of the present isolates is outside the *Phaeo-pleospora* clade, sitting in (Fig. 3) sister position to the clade of *Devonomyces*.

Dataset 4 consisted of 21 taxa belonging to the genera *Zasmidium*, *Nothopericoniella*, *Annelosympodiella*, *Neopenidiella*, *Devonomyces*, *Phaeophloeospora*, *Cytostagonospora*, *Brunswickiella*, and *Lecanosticta* (Fig. 3). *Cylindroseptoria ceratoniae* (CBS 477.69) was used as the outgroup. The Bayesian analyses of combined alignment, based on a total of 1,818 characteristic (ITS: 491, LSU: 557, and rpb2: 770), generated 1,502 trees, 1,126 of which were used to construct the tree and to calculate the posterior probabilities (Fig. 4, first value: PP $\leq$ 1 shown). The ML analysis reached a final

Fig. 4. Phylogenetic tree (50% majority rule consensus) resulting from a Bayesian analysis of the combined ITS, LSU, and rpb2 sequence alignment (dataset 4; representing *Zasmidium*, *Nothopericoniella*, *Annelosympodiella*, *Neopenidiella*, *Mycosphaerella*, *Devonomyces*, *Phaeophloeospora*, *Cytostagonospora*, *Brunswickiella*, and *Lecanosticta* genus of Fig. 3). The Bayesian posterior probabilities (PP), maximum likelihood bootstrap support values (50%; ML-BS), and maximum parsimony bootstrap support values (50%; MP-BS) are given at the nodes (PP/ML-BS/MP-BS). The scale bar represents the expected number of changes per site. The tree is rooted to *Cylindroseptoria ceratoniae* (CBS 477.69). The lengths of two branches with $\times 2$ on them were halved for the layout of the tree.

ML highest log likelihood of -10384.6325. The final dataset contained a total of 1,815 positions.

The bootstrap support values from the best-scoring tree were mapped on the Bayesian tree as the second value in the tree nodes (Fig. 4; ML-BS $\geq 50\%$ shown). The MP analysis generated the most parsimonious tree with length = 1,853, CI = 0.592308, RI = 0.759847, and composite index = 0.477161 (0.450063) for all sites and parsimony-informative sites (in parentheses in Figure 4). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was mapped on the Bayesian tree as the third value in the tree nodes (Fig. 4, MP-BS $\geq 50\%$ shown). Figure 4 shows that the isolates of this study are sitting in a sister clade to *Devonomyces* but outside of *Phaeopleospora* clade.

Morphological analysis. *Teleomorph.* Pseudothecia were observed in the lesions on the undersides of the diseased leaves. Pseudothecia matured in spring (late April) after overwintering and were scattered over lesions. Microscopic analyses revealed the following morphological characteristics: Pseudothecia black, oval, flask- and pear-shaped, $57.17\text{--}131.32 \times 55.35\text{--}116.74 \mu\text{m}$ (average $81.23 \times 74.30 \mu\text{m}$) ($n = 50$) (Fig. 5A, B) with ~ 35 asci per pseudothecia. Asci $30.70\text{--}51.42 \times 8.11\text{--}10.4 \mu\text{m}$ (average, $40.65 \times 9.00 \mu\text{m}$) ($n = 50$), banana-shaped and 8-spored (Fig. 5B). Ascospores spindle-shaped, uniseptate (unequally), hyaline, $10.56\text{--}13.41 \times 2.93\text{--}4.65 \mu\text{m}$ (average, $12.08 \times 3.85 \mu\text{m}$) ($n = 50$) (Fig. 5D, E). During the germination of ascospores, germ tubes emerged from one end or both ends (Fig. 5F, G). Ascospores became slightly swollen or completely distorted after several germ tubes emerged, growing at various angles to the long axis of the spore (Fig. 5F, G). Germ tubes hyaline (Fig. 5F, G).

Anamorph. The cultures grew slowly and reached a diameter of 11–13 mm within 2 weeks on PDA at 25°C in the dark. The colonies were initially dark green and ultimately became dark gray to black on PDA while in incubation at 25°C in the dark. When incubated at 5°C in the dark, the colonies were slightly pinkish in color. In the presence of light or high RH, the growth of mycelium was significantly faster than that of mycelium incubated in the dark at 20°C and 25°C. In all conditions, the growth of mycelium was significantly less at 5°C and 10°C. Mycelial growth at 15°C was significantly faster than that at 5°C and 10°C but less than that at 20°C and 25°C. There was no difference in mycelial growth at 20°C and 25°C in dark. The effect

of culture media on mycelial growth was significant. The cultures grew significantly larger on OMA than on PDA and MA. The least mycelium growth was observed on LCA. Pseudothecial and conidial formation was observed only on OMA and PDA after 120 days of incubation at 25°C in light and high RH. The pseudothecia on OMA were small, black, and spherical or oval (Fig. 6A). The mycelium was dark gray, composed of septate, guttulate hyphae, width on average $\pm \text{SD} = 4.20 \pm 0.46$ ($n = 50$) (Fig. 6B, C). The pseudothecia formed through the aggregation and pigmentation of mycelium (Fig. 6D, E). Two types of conidia were observed: conidia of type I: Conidia formed singly or in chains, single-celled or septate (0–1), and variously shaped (cylindrical, oval, obovoid), guttulate, $9.3\text{--}28.2 \times 4.3\text{--}11.7 \mu\text{m}$ (average $16.5 \times 8.1 \mu\text{m}$) ($n = 50$) in size, and light brown (Fig. 6F); and conidia of type II (rare): 3- to 6-celled, brown in color, walls slightly thickened, guttulate, constricted at the septa, and with swollen cells at the base (Fig. 6G–K). Conidiogenesis cells monoblastic or holoblastic, hila and conidiogenous loci inconspicuous, not thickened and not darkened. Conidiophores mostly reduced to conidiogenous cells, or some erect, light brown, size $17\text{--}44.86 \times 3.09\text{--}7.6 \mu\text{m}$ (average, $25.16 \times 5.3 \mu\text{m}$) ($n = 20$). Conidiogenous cells integrated, terminal and lateral, forming a subcylindrical chain, the walls slightly thickened, straight to variously curved, septate and light to dark brown (Fig. 6L). Conidia germinated to form branches of mycelium (Fig. 6M, N).

Pathogenicity and host specificity analysis. The CLSP symptoms were observed on persimmon seedlings after approximately 2 months (>50 days) of artificial inoculation with conidia and ascospores. The resulting symptoms from spraying the conidial suspension were identical to the typical symptoms caused by both natural and artificial ascospore infection. Disease incidence and disease severity after inoculation with conidia were 53.48% and 24.81%, respectively (Table 3). Disease incidence and severity after inoculation with ascospores was 87.23% and 65.23%, respectively (Table 3). No visual symptoms were observed on apple, peach, cherry, or plum seedlings inoculated with conidia or ascospores. In the laboratory experiment, *M. nawae* (*P. nawae* comb. nov.) was able to grow superficially on the stems of apple, peach, cherry, and plum. Extent of colonization was higher in peach stems followed by apple, pine, and plum. The mycelium grew beyond the wound site. Necrosis on these hosts did not occur.

Fig. 5. Observed teleomorphic characteristics of *Plurivorosphaerella nawae* comb. nov. (*Mycosphaerella nawae*) in overwintered, infected leaves. **A**, Leaf spot with black pseudothecia; **B**, mature pseudothecia and asci; **C**, asci and ascospores; **D–E**, ascospores; and **F–G**, germinating ascospores (scale bars: C–D = 15 μm , E = 5 μm).

Electron scanning microscopy of conidia produced on leaves. Conidia and conidial chains were observed on the lesions of leaves inoculated with both conidia and ascospores under FE-SEM. The conidia and conidial chains were identical to those found in culture media (PDA and OMA) (Fig. 7A–C).

Taxonomy. *Plurivorosphaerella* O. Hassan & T. Chang, **gen. nov.** MycoBank, MB825548.

Etymology. The generic name *Plurivorosphaerella* composed of plurivorus (plurivorous) and sphaerella (from *Mycosphaerella*), referring to the wider host range of the type species.

Diagnosis. Phylogenetically closely allied to *Devonomyces* but forming a separate clade in sister position. The anamorph was different from the closely related species (Table 4), dematiaceous, conidigenous cells producing chains of spores and catenate (true chain) dimorphic conidia, either 0–1-septate (amero- to didymosporous) or 3- to 6-celled (phrago- to scolecosporous), constricted at the

septa, and with swollen cells at the base, conidiogenesis holoblastic (monoblastic or polyblastic), conidogenous loci neither thickened nor darkened-refractive, rather inconspicuous. Sexual morph mycosphaerella-like (pseudothecia flask- to pear-shaped, asci banana-shaped, with eight colorless unequally uniseptate ascospores, germinating from one or both ends).

Type species. *Plurivorosphaerella nawae* (Hiura & Ikata) O. Hassan & T. Chang, **comb. nov.** MycoBank, MB825619.

Basionym. *Mycosphaerella nawae* Hiura & Ikata, Research Bulletin of the Faculty of Agriculture, Gifu University 5: 1, 1929.

Discussion

The genus *Mycosphaerella* is one of most important genera among mycologists and plant pathologists for their diverse morphological characteristics and destructive effects on crops. Although this genus has a long taxonomic history researched over many decades and

Fig. 6. Micrograph of the *Plurivorosphaerella nawae* comb. nov. (*Mycosphaerella nawae*) grown on PDA and diseased leaves. **A**, Pseudothecia in 120-day-old culture on Oatmeal Agar (OA); **B**, young mycelium (7-days-old); and **C**, old mycelium (>40-days-old); **D–E**, pseudothecia in culture; **F**, conidia type I, single and varying size and shape; **G–J**, conidia type II, constricted at the septa and swollen cells at the base; **K**, conidiophore and conidia type II collected from a diseased leaf; **L**, conidigenous cell which give rise to the conidia; and **M–N**, germinating conidia (scale bars: G = 15 µm).

published in numerous publications, major taxonomic challenges remain unresolved (Crous 2009). Recently, Videira et al. (2017) provided a phylogenetic framework for the taxonomic work within *Mycosphaerella* as a genus within the family *Mycosphaerellaceae*. It opened the door to resolving the phylogenetic position of many *Mycosphaerella* species, such as *M. nawae*. *M. nawae* was first identified based on morphological characteristics (Ikata and Hitomi 1929). In 1998, the anamorph of *M. nawae* was reported as being similar to *Ramularia* sp. based on morphological characteristics, and it was shown to be able to induce CLSP (Kwon et al. 1998a,

b). The traditional identification (which relies on morphological characteristics) of fungal species within the *Mycosphaerella* complex is not reliable because many species are morphologically indistinguishable from one another (Groenewald et al. 2005; Crous 2009). Phylogenetic and genomics studies based on DNA sequence data became indispensable among mycologists owing to the accuracy of identification. Phylogenetic analyses using multigene sequence data revealed that *Mycosphaerella* was polyphyletic and that previously well-defined anamorph-typified genera clustered in several clades (Crous et al. 2006, 2007a, b; Hunter et al. 2006;

Table 3. Disease incidence and disease severity of *Plurivorosphaerella nawae* comb. nov. (*Mycosphaerella nawae*) species inoculated with ascospores and conidia in the seedlings of different fruits

Types of inoculum	Disease incidence				Disease severity			
	Persimmon	Apple	Pears	Peach	Persimmon	Apple	Pears	Peach
Ascospores	87.23%	-	-	-	65.23%	-	-	-
Conidia	53.48%	-	-	-	24.81%	-	-	-

Fig. 7. Conidia observed using SEM. **A–B**, Conidia on leaf lesion inoculated with the conidial suspension; **C–D**, conidia on leaf lesion inoculated with the naturally released ascospores.

Videira et al. 2017). Lee et al. (2016) attempted to delineate the phylogenetic position of *M. nawae* using multigene sequence data and morphological characteristics. They suggested a close affinity of *M. nawae* to the genus *Phaeophleospora*, despite clustering in distinct clades. However, the reported morphological data, especially for the anamorph, do not support a close affinity to *Phaeophleospora*. The genus *Phaeophleospora* seems to be polyphyletic according to phylogenetic data presented by Crous et al. (2009a). Some previously known *Phaeophleospora* spp. were renamed and classified as new genera, e.g., *Brunswickiella* (Videira et al. 2017). Hence, the phylogenetic position of *M. nawae* must be viewed with some circumspection. For this reason, the present study aimed to clarify the phylogenetic positioning of *M. nawae*. The present study integrated anamorph and teleomorph morphologies, pathogenicity, and host specificity with four phylogenetic datasets based on partial sequences of the ITS, LSU, ACT, rpb2, and EF-1 α gene sequence data. Based on the ITS, LSU, ACT, and EF-1 α sequence data presented here, it is clear that *M. nawae* belongs to the family *Mycosphaerellaceae* (Fig. 1; Fig. 2). ITS and LSU sequences retrieved from identified *M. nawae* isolates formed a distinct clade with *Devonomyces* in sister position (Fig. 3), which was confirmed by phylogenetic analysis with ITS, LSU, and rpb2 sequence data (Fig. 4). ITS has been extensively used as a molecular marker for the successful identification and subgeneric phylogenetic inference of fungi, and it was formally proposed as the primary barcode marker for fungi (Nilsson et al. 2008; Schoch et al. 2012; Videira et al. 2017). As with *Colletotrichum* spp., distinguishing between closely related *Mycosphaerella* species based on ITS sequence data is not reliable (Stielow et al. 2015; Weir et al. 2012). The combination of multilocus sequence data is not only reliable for the recognition of fungal species, but it is also fast, relatively cost-effective, and does not require specific morphological characteristics associated with life stages (Quaedvlieg et al. 2012). For this reason, a combination of ITS and a secondary barcode (LSU and rpb2) has been proposed for the recognition of many genera in *Mycosphaerellaceae* (Videira et al. 2017). We also found that LSU and rpb2 loci, in addition to the ITS sequence data, are sufficient for confirming the phylogenetic position of *M. nawae*. Using rpb2 gene sequence data has some benefits over the use of other genes, in that it: i) resolves higher levels of classification; ii) is easy to align, as the sequence has no introns; and iii) has a higher level of variability within sequence data, which helps to discriminate at the species level (Videira et al. 2017). However, it is difficult to amplify rpb2 (Videira et al. 2017). The difficulty regarding less available rpb2 sequence data in the public databases is overcome with the increasing amount of newly generated sequence data (Videira et al. 2017).

In this study, the *M. nawae* isolates were described by multilocus phylogenetic analysis and morphological characteristics. The comparison among the morphological characteristics of the present isolates and previously described *M. nawae* isolates has been presented in Table 4. The sexual and anamorph of the present *M. nawae* isolate was well-supported with those described by Kwon et al. (1998a, b). The conidia of the present isolate were smaller than those of the reference *M. nawae*. Conidia of the present isolate were light brown while conidia of the reference isolate were colorless to light olive. The slight morphological differences can be explained by the influence of different growing conditions and different geographic isolates. The anamorph of the present *M. nawae* was further confirmed through FE-SEM using leaves artificially inoculated with both ascospores and conidia. Conidial chain and conidia of type II were observed from the lesions of infected leaves, which were identical with those of found in culture media. To our knowledge, we have described the germination pattern of ascospores and conidia of type II for the first time. The morphological characteristics of *M. nawae* are not similar to those of the genera *Phaeophleospora* and *Devonomyces*. Fungal species within *Phaeophleospora* are morphologically characterized by comparing them with the species *P. eugeniae* (Quaedvlieg et al. 2014). The conidia of *P. eugeniae* are characterized as being predominantly sigmoid (Crous et al. 1997). However, the recently described conidia of type II of *M. nawae* are characterized by being phragmo- to scolecosporous, while conidia of type I are aseptate or septate and vary in shape and size. The anamorph of *M. nawae* is similar to that of most *Phaeophleospora* species, such as *P. destructans*, (Burgess et al. 2006), *P. faureae* (Taylor and Crous 1999), *P. stonei* (Crous et al. 2009b), and *P. vohchysiae* (Savi et al. 2018), but the morphology of *M. nawae* does not correspond to that of any described *Phaeophleospora* species. *Phaeophleospora* species are readily distinguishable from the anamorph of *M. nawae* by having vermiform, long obclavate, bases truncate, widest in the middle region, versicolored (basal cell light brown, apical cell pale brown), vermiform conidia (Andjic et al. 2007; Crous et al. 1997). The morphological characteristics of *M. nawae* are also comparable with those of the genus *Devonomyces*, as described by Videira et al. (2017). The pseudocercospora-like morph of *Devonomyces endophyticus* is the important morphological feature, which is distinct from the anamorph of *M. nawae* (Videira et al. 2017). Ascospores of *Devonomyces* are fusoid-ellipsoidal medianly 1-septate (Videira et al. 2017), while ascospore of *M. nawae* are spindle shaped and unequally 1-septate.

M. nawae isolates used in the present study are morphologically and molecularly clearly distinct from both *Phaeophleospora* and *Devonomyces* and must therefore be accommodated in a different

Table 4. Comparison of asexual morph of the present isolates (*Plurivorosphaerella nawae* comb. nov.) and previously described strains of *Mycosphaerella nawae* and closest species of *Devonomyces* and *Phaeophleospora* as reported in the literature

Morphological characteristics	<i>P. nawae</i> comb. nov. (<i>M. nawae</i>) (present isolates)	<i>M. nawae</i> (Kwon et al. 1998a, b)	<i>Phaeophleospora eugeniae</i> (Crous et al. 1997)	<i>Devonomyces endophyticus</i> (Videira et al. 2017)
Conidiophore	Mostly reduced to conidiogenous cells, or some erect, light brown, 17–44.86 $\times$ 3.09–7.6 μ m (average, 25.16 $\times$ 5.3 μ m), aseptate	Erect, short, colorless or light brown, 20.4–102.0 $\times$ 3.1–10.2 μ m (average, 49.2 $\times$ 5.1 μ m), 0–1 septate (mostly aseptate)	Absent or reduced to conidiogenous cells	Cylindrical, straight to irregularly curved, rarely pigmented and verruculose in lower part, mostly hyaline and smooth throughout, thick-walled, septate
Conidiogenous cell	Integrated, terminal and lateral, light to dark brown, forming a subcylindrical chain, straight to variously curved, septate	Integrated, thickened walled, brown	Terminal, discrete, light brown, subcylindrical to ampulliform, percurrent	Terminal, hyaline, smooth, unbranched, straight or slightly curved
Conidia	Conidia of type I: solitary or in chain, light brown, various shaped (cylindrical obovoid), guttulate 9.3–28.2 $\times$ 4.3–11.7 μ m (average, 16.52 $\times$ 8.1 μ m), 0–1 septate. Conidia of type II: 3- to 6-celled, brown in color (uniformly), constricted at the septa, and with swollen cells at the base	Conidia of type I: solitary or in chain, colorless to light brown, various shaped (cylindrical obovoid), 12.2–32.6 $\times$ 6.1–10.2 μ m (average, 21.0 $\times$ 9.4 μ m), 0–3 septate. Conidia of type II: Not reported	Single type of conidia, solitary, pale brown to pale olivaceous brown, darker in the center and paler toward both ends, vermiform, long-obconical, 30–150 $\times$ 5–8 μ m, (16–) 24–27 (–30) euseptate, generally not constricted at septa, bases truncate with frills	Single type of conidia, solitary, hyaline, smooth, narrowly obclavate, septate, irregularly curved, rarely straight, apex obtuse, base long obconic-truncate, lateral branches common, secondary conidia forming on most mature primary conidia

genus within *Mycosphaerellaceae*. A previous study reported that *M. nawae* is phylogenetically distinct from *Ramularia* spp. (Lee et al. 2016). *Ramularia* species are readily distinguishable from the anamorph of *M. nawae* by having conspicuous conidiogenous loci and conidial hila, thickened and darkened-refractive, with coronate, cladosporium-like structure (Braun 1998; Videira et al. 2016). Furthermore, *Ramularia* species are colorless (mucedinaceous), whereas the anamorph of *M. nawae* is characterized by being pigmented (dematiaceous).

We also found that *M. nawae* is responsible for CLSP on persimmon. Wounded, inoculated excised stems of apple, peach, cherry, and plum did not become infected, but growth occurred on top of the wood and beyond the wounded region of each stem. *M. nawae* was recently isolated from the leaves of the traditional Chinese medicinal plant *Smilax china* as an endophyte (Wang et al. 2017). The colonization of *M. nawae* of plants other than persimmon is due to the endophytic or opportunistic nature of *M. nawae*. Some fungal species such as *Colletotrichum salicis* are pathogenic on one host and can opportunistically colonized another host (Johnston 2000; Weir et al. 2012). The ecology and hosts of *M. nawae* should be further investigated.

In conclusion, it can be said that *M. nawae* represents a new holomorphic genus within *Mycosphaerellaceae*, introduced in the present paper as *Plurivorosphaerella*, and it appears to have a restricted host range.

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