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

**MATERIALS AND METHODS**

**Isolation of \textit{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 \textit{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 \(\mu\)L) was then added on the back of the symptomatic spots, which were then spread onto 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 \textit{M. nawae} according to the cetyltrimethylammonium bromide method [21]. Using the genomic DNA of \textit{M. nawae} isolates and their allied species, the ITS region, the partial region of 28S rDNA, \(\beta\)-tubulin, and \textit{Act} were amplified using the corresponding primer pairs [22-25]. A total reaction volume of 20 \(\mu\)L contained 1 \(\mu\)L of genomic DNA, 2 \(\mu\)L of 10× \(\text{Taq}\) buffer, 0.4 \(\mu\)L of 10 mM dNTP, 0.5 \(\mu\)L each of 10 PM forward and reverse primer, and 0.2 \(\mu\)L of \text{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, \(\beta\)-tubulin, and \textit{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 \textit{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 leaves (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 \textit{Mycosphaerella nawae} from CLS-DL.** Twenty isolates of \textit{M. nawae} were obtained from collected leaves with CLS-disease from each region of collection. At

![Fig. 1. Morphological characteristics of the isolated \textit{Mycosphaerella nawae} on potato dextrose agar (PDA). A, Isolated \textit{M. nawae} colonies after 4 wk of growth on PDA; B, Reverse side of the 4-week-old colony.](image)
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 nawaee* 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%.
| 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 E. globulus  | Eucalyptus    | LC121129 LC121200 LC121211 KF903115 LC121211 |
| *Lecanosticta acicola*    | CBS 133789                       | *Dothistroma acicola*                          |                  | Pinus sp.     | LC121130 LC121201 LC121212 JX901648 LC121212 |
| *Mycosphaerella graminicola* | CBS 398.52                       |                                                |                  | T. aestivum   | LC121131 LC121202 LC121213 JQ739795 LC121213 |
| *Mycosphaerella musae*    | CBS 121386                       |                                                |                  | Musa sp.      | LC121133 LC121204 LC121215 - LC121215     |
| *Paramycosphaerella blechii* | COAD 1183                       |                                                |                  | Blechnum serratum | KT037544 KT037586 KT037611 KT037503 -       |
| *Paramycosphaerella sticheri* | COAD 1422                       |                                                |                  | Sticherus pennger | KT037528 KT037569 KT037615 KT037488 -       |
| *Paramycosphaerella cyatheae* | CPC 24730                       |                                                |                  | Cyathea delgadii | KT037534 - - - -                     |
| *Paramycosphaerella intermedia* | CBS 114415                      | *Mycosphaerella intermedia*                    | 20 E. globulus  | Eucalyptus    | KF901682 KF902027 KF903468 KF903143 -       |
| *Paramycosphaerella madeirensis* | CBS 112301                      | *Mycosphaerella madeirensis*                   | 21 E. globulus  | Eucalyptus    | KF901688 KF902033 KF903453 KF903108 -       |
|                            | CBS 112895                       |                                                 |                  | E. globulus   | LC121137 LC121203 LC121214 KF903109 LC121214 |
| *Paramycosphaerella marksi* | CBS 110981                      | *Mycosphaerella marksi*                        | 20 E. sp.      | LC121137 LC121209 LC121219 KF903145 LC121219 |
|                            | CBS 110920                       |                                                 |                  | E. globulus   | LC121137 LC121209 LC121219 KF903145 LC121219 |
| *Paramycosphaerella parkii* | CBS 387.92                       | *Mycosphaerella parkii*                        | 21 E. grandis  | LC121139 LC121210 LC121221 KF903392 LC121221 |
| *Paramycosphaerella vietnamensis* | CBS 119974                    | *Mycosphaerella vietnamensis*                  | 21 E. grandis hybrid | LC121136 LC121207 LC121218 KF903142 LC121218 |
| *Paramycosphaerella intermedia* | CBS 114356                      | *Mycosphaerella intermedia*                    | 20 E. saligna   | Eucalyptus    | LC121134 LC121205 LC121216 - LC121216     |
| *Passalora fulva*         | CBS 119.46                       | *Cladosporium fulvum*                          |                  | Protea caffra | FJ493187 FJ493205 - - - -                   |
| *Phaeophleospora concentrica* | CPC 3615                        |                                                |                  | Lyco persicum sculentum | LC121134 LC121205 LC121216 - LC121216  |
| *Phaeophleospora epicocoides* | CMW 22486                       | *Kirramyces epicocoides*                       |                  | E. urophylla  | DQ632706 - - DQ632720 -                    |
| *Phaeophleospora eucalypticola* | CPC 26523                       |                                                |                  | E. robusta    | KX228387 KX228318 - KX228374 -             |
| *Phaeophleospora eugeniae* | CMW 5351                        |                                                |                  | Eugenia uniflora | DQ632710 - - EF011663 -                 |
| *Phaeophleospora eugeniicola* | CPC 2558                        |                                                |                  |                | FJ493191 FJ493209 - - - -                   |
| *Phaeophleospora gregaria* | CBS 114662                      | *Mycosphaerella gregaria*                      | 20 E. sp.      | Eucalyptus    | KF901713 KF902060 KF903470 KF903165         |
|                            | CBS 111519                       |                                                |                  | Eucalyptus    | DQ267579 JX901861 JX902108 JX901655 -       |
|                            | CBS 111167                       |                                                |                  | E. cladocalyx | KF901711 KF902058 KF903434 KF903163 -       |
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| Species                          | Culture collection accession No. | Synonym  | Reference | Isolated host | Genbank accession No. |
|----------------------------------|---------------------------------|----------|-----------|---------------|-----------------------|
| Phaeophleospora hymenocallicola  | CBS 110501                      |          |           | Eucalyptus globulus | LC121135 LC121206 LC121217 |
|                                  | CPC 25014                       |          |           | Fern          | KR476739 KR476772     |
| Phaeophleospora hymenocallichis  | 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 |
| Phaeophleospora stonei           | CBS 118493                      |          |           | Eucalyptus urophylla | KF901631 KP901966 KP903493 KP903167 |
| Phaeophleospora stramenti       | CBS 118909                      | = Mycosphaerella stramenti | 20 Eucalyptus sp. | KP901617 KP901942 KP903506 KP903169 |
| Pseudoramichloridium henryi     | CBS 124775                      |          |           | Corymbia henryi | GQ303289 KF442561 KP903559 KP903227 |
| 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 glemnii                | CPC 18468                       |          |           |               | KJ504775 KJ504734 KJ504439 KJ504690 |
| Ramularia grevillea              | CBS 114732                      | = Ramularia punctiformis | Fragaria ananassa | KP894221 KP894438 KP894328 KP894113 |
| Ramularia inaequalis             | CBS 250.96                      | = Ramularia inaequal | Taraxacum officinale | KP894224 |
| Ramularia lactea                 | CBS 114442                      | = Ramularia violae | Viola hirta | KP894229 KP894122 KP894337 KP894337 |
| Ramularia nyssicola              | CBS 127664                      | = Mycosphaerella nyssicola | Nysaea gene sylvatia 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                       |          |           | Diplozaemia sp. | KT037542 KT037584 KT037627 KT037501 |
| Xenomycosphaerella elongata      | CBS 120735                      | = Mycosphaerella elongata | 20 Triticum aestivum | KP901808 KP902170 KP903528 |
| Xenomycosphaerella yunnanensis   | CBS 119975                      | = Mycosphaerella yunnanensis | 20 Musa cultivar | KP901628 KP901962 KP903515 KP903375 |

*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

![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 mucedora* 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%.

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Fig. 3.
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.
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).
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 *Phaeophloeospora*, 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 *Phaeophloeospora* 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 pustule production, similar size of ascospores, and conidia tapering at both ends, guttulate, and septate, among others.

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**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).
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, inference, including newly transferred into the genus based on phylogenetic analysis, revealed reproductive patterns of 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 *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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