Two novel species of Parastagonospora (Phaeosphaeriaceae, Pleosporales) on grasses from Italy and Russia

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Abstract

Phaeosphaeriaceae comprises many fungal species occuring mainly on grasses and cereal crops as endophytes, saprobes and especially pathogens. Parastagonospora is an important genus in Phaeosphaeriaceae that includes pathogens causing leaf and glume blotch on cereal crops. In this study, a sexual morph species and an asexual morph species, occurring as saprobes on Poaceae are introduced based on morphology and a combined molecular analysis of the LSU, ITS and RPB2 gene sequence data. The sexual morph of a new Parastagonospora species, P. elymi was isolated from dead stems of Elymus repens in Russia. Parastagonospora elymi is similar to the sexual morph of P. avenae in having cylindrical asci, bearing eight, overlapping biseriate, fusiform ascospores but can be distinguished by its subglobose to conical-shaped, wider ascomata. In addition, no sheath was observed surrounding the ascospores. An asexual morph of Parastagonospora was isolated from dead stems of Dactylis glomerata in Italy and is introduced as P. macrouniseptata. Parastagonospora macrouniseptata is a coelomycete and bears a close resemblance to P. allouniseptata and P. uniseptata in having globose to subglobose, pycnidia and hyaline, cylindrical, 1-septate conidia. However, the new species is morphologically distinct in its conidiomata characteristics and phylogenetic affinity.

Key words – 2 new species – Dothideomycetes – multi-gene analysis – Poaceae – saprobes – taxonomy

Introduction

Phaeosphaeriaceae M.E. Barr is an important family of the order Pleosporales and was introduced by Barr (1979) with Phaeospharia I. Miyake as the generic type (Zhang et al. 2009, 2012, Hyde et al. 2013, Phookamsak et al. 2014, 2017). It consists of species that are mostly

Among the pathogenic microfungi occurring on grasses, asexual morph genera in the Phaeosphaeriaceae are commonly reported and are of great importance (Lamprecht et al. 2011, Phookamsak et al 2014, 2017, Bakhshi et al. 2015). Parastagonospora Quaedvl., Verkley & Crous is one such genus. Parastagonospora avenae (A.B. Frank) Quaedvl., Verkley & Crous and P. nodorum (Berk.) Quaedvl., Verkley & Crous (the generic type), are both pathogens that cause leaf and glume blotch on cereal crops such as barley, wheat and rye, leading to heavy losses of yield (Cunfer 2000, Stukenbrock et al. 2006, Vergnes et al. 2006, Quaedvlieg et al. 2013, Phookamsak et al. 2014).

Elymus repens (L.) Gould, commonly known as couch grass, is important for grazing animals, used in traditional Austrian medicine and is considered an invasive weed in some parts of the world (Werner & Rioux 1977, Klein 2011, Vogl et al. 2013). Dactylis glomerata L. (cocksfoot) is a commonly found grass distributed in temperate regions throughout Asia, Europe and North Africa (Sánchez Márquez et al. 2007). It is widely used as fodder or for pastures (Hackney & Dear 2007). Five fungal species have been recorded from Elymus spp. in Russia (Farr & Rossman 2019) while more than ten species have been recorded on D. glomerata from Italy (Farr & Rossman 2019). In this study two novel species, one on E. repens from Russia and the other on D. glomerata from Italy are introduced based on phylogenetic analyses of ITS, LSU and RPB2 sequence data complete with descriptions and illustrations.

Materials & Methods

Sample collection and specimen examination
Samples were collected in Italy and Russia, and brought back to the laboratory in paper bags. Morphological features of the fungi were examined using a Motic SMZ 168 dissecting microscope. Free-hand sections of the fungal fruiting structures were obtained and mounted in water on a slide to observe their microscopic features. Micro-morphologies were examined using a Nikon ECLIPSE 80i compound microscope and photographed using a Canon EOS 600D digital camera fitted to the microscope. The images were processed using Adobe Photoshop CS5 Extended version 12.0 software (Adobe Systems, USA). The Tarosoft (R) Image Frame Work program v. 0.9.7 was used for taking measurements. Isolates were obtained by single spore isolation as detailed in Chomnunti et al. (2014) in a petri dish containing 2% water agar and incubated overnight at 16–18°C. Germinating spores were then transferred aseptically onto potato dextrose agar (PDA) and further incubated at 16–18°C to obtain pure colonies. Herbarium material is deposited in the herbarium of Mae Fah Luang University, Chiang Rai, Thailand (MFLU) and Herbarium of Cryptogams, Kunming Institute of Botany Academia Sinica (HKAS). Living cultures are deposited at Kunming Culture Collection (KUMCC) and in Mae Fah Luang University Culture Collection (MFLUCC). Facesoffungi and Index Fungorum numbers were obtained as described in Jayasiri et al. (2015a) and Index Fungorum (2019).
DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was extracted from fresh mycelia grown on PDA, following the manufacturer’s standard protocol described in the DNA extraction kit (Biospin Fungus Genomic DNA Extraction Kit). Polymerase chain reactions (PCR) were carried out using the primer pairs of ITS5 and ITS4 to amplify the internal transcribed spacers (ITS) (White et al. 1990), and LROR and LR5 for large subunit rDNA (LSU) (Vilgalys & Hester 1990) and fRPB2-5F and fRPB2-7CR to amplify the partial RNA polymerase II second largest subunit (RPB2) (Liu et al. 1999). The amplification reaction was performed in a 25 μl reaction volume containing 1μl DNA, 12.5 μl Taq polymerase and PCR buffer mix, 9.5 μl double distilled water and 1 μl of each primer. The PCR thermal cycling program for ITS and LSU gene regions were as follows: an initial denaturing step of 94°C for 3 min, followed by 35 amplification cycles of 94°C for 30 s, annealation at 57°C for 45 s, elongation at 72°C for 60 s and a final extension step of 72°C for 10 min. For the RPB2 gene region an initial step of 95°C for 5 min, followed by 40 cycles of amplification at 95°C for 1 min, annealation at 52°C for 2 min, elongation at 72°C for 90 s, and final extension at 72°C for 10 min was followed. PCR products were verified using 1% agarose gel electrophoresis, stained with 4S Green Stain. Purification and sequencing of PCR products were carried out at Shanghai Sangon Biological Engineering Technology and Services Co., China. Sequences derived from this study are deposited in GenBank. Small subunit rDNA (SSU) sequence data for both *Parastagonospora elymi* and *P. macrouniseptata*, and TEF for *P. elymi* were obtained but not used in the phylogenetic tree and can be obtained from the corresponding author on request.

Phylogenetic analyses

Sequences generated from this study were subjected to BLAST (NCBI) searches to obtain the closest matches in GenBank (Table 1), from recently published sequences (Li et al. 2015, Tennakoon et al. 2016, Thambugala et al. 2017, Karunarathna et al. 2017) The single gene sequences alignments were initially aligned using BioEdit v. 7.0.9.0 (Hall 1999) and MEGA version 6 (Tamura et al. 2013). Multiple sequence alignments were generated with MAFFT v. 7 (http://mafft.cbrc.jp/ alignment/server/ index.html) and manually improved using MEGA v.6 for maximum alignment and minimum gaps. Evolutionary models for phylogenetic analyses were independently selected for each gene region following the Akaike Information Criterion (AIC) of the MrModeltest v. 3.7 (Nylander 2004), implemented using both PAUP v. 4.0b10 and MrBayes v. 3. Phylogenetic reconstructions of combined gene trees were performed using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) criteria.

Maximum likelihood analysis was performed using RAxML-HPC2 on XSEDE (8.2.8) (Stamatakis et al. 2008, Stamatakis 2014) in the CIPRES Science Gateway platform (Miller et al. 2010) with GTR+I+G as the model of evolution and bootstrap support obtained by running 1000 pseudo replicates. The MP analysis was performed using PAUP v. 4.0b10 (Phylogenetic Analysis Using Parsimony) (Swofford 2002). Ambiguously aligned regions were excluded and gaps were treated as missing data. The trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Maxtrees were set up to 1000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics for parsimony tree length [TL], consistency index [CI], retention index [RI], relative consistency index [RC] and homoplasy index [HI] were calculated for the maximum parsimonious tree. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications resulting from MP analysis, each with ten replicates of random stepwise addition of taxa (Felsenstein 1985). The Kishino-Hasegawa tests (KHT) (Kishino & Hasegawa 1989) were performed to determine whether the trees were significantly different. The BI analysis was conducted using MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001) to evaluate posterior probabilities (BYP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) by Markov chain Monte Carlo sampling (BMCMC). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation. The distribution of log-likelihood scores were examined to determine the stationary phase for each search and to decide if extra data runs were required to achieve
convergence, using Tracer v. 1.6 program (Rambaut et al. 2014). First 10% of generated trees representing the burn-in phase were discarded and the remaining trees were used to calculate posterior probabilities of the majority rule consensus tree (critical value for the topological convergence diagnostic set to 0.01).

Phylograms were visualized using FigTree v1.4.0 program (Rambaut 2012) and re-edited and formatted using Microsoft Power Point (2013) and Adobe Photoshop CS6 extended version 13.1.2 software. The final tree alignment was submitted to TreeBASE (Submission ID: 24486, http://www.treebase.org/).

Table 1 GenBank accession numbers of the strains used for phylogenetic analysis. Sequences generated in this study are in blue.

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*Ex-type strains are in bold

Results

As a result of the current study, a fungal sexual morph from *Elymus repens* and an asexual morph from *Dactylis glomerata* were isolated. Based on morphological characters and molecular analyses presented below, two new species of *Parastagonospora* are described.

Phylogenetic analyses

A combined analysis of the ITS, LSU and RPB2 sequence data of 45 strains consisting of *Parastagonospora* and other representative genera in Phaeosphaeriaceae were used to confirm the phylogenetic placement of our strains, with *Neosetophoma samarorum* (CBS 138.96) as the outgroup taxon (Fig. 1). This combination of sequence data was performed after confirming that the topologies of the trees obtained from each gene were overall congruent. Bootstrap values obtained for MP and ML analyses and PP values resulted in BI analysis are given at each node (Fig. 1).

The Bayesian analysis resulted in 10,001 trees after 1,000,000 generations. The first 1000 trees, representing the burn-in phase of the analyses, were discarded, while the remaining 9001 trees were used for calculating posterior probabilities in the majority rule consensus tree presented in Fig. 1. The average standard deviation of split frequencies was 0.010067. The RAxML analysis of the combined dataset yielded a best scoring tree with a ML optimization likelihood value of -11145.503449. The matrix had 796 distinct alignment patterns, with 37.7% of undetermined characters or gaps. Parameters for the GTR + I + G model of the combined ITS, LSU and RPB2 were as follows: estimated base frequencies; A = 0.249573, C = 0.222647, G = 0.273922, T = 0.253857; substitution rates AC = 1.771863, AG = 3.436302, AT = 1.824852, CG = 0.770295, CT = 7.693174, GT = 1.000000; proportion of invariable sites I = 0.001000; gamma distribution shape parameter α = 0.146118. The maximum parsimony dataset consisted of 2584 characters, of which 1906 characters were constant, 505 were parsimony-informative and 173 were parsimony-uninformative. The most parsimonious tree showed values as follows: CI = 0.620, RI = 0.707, RC = 0.438, HI = 0.380.

Both KUMCC 16–0125 and KUMCC 16–0111 strains clustered within *Parastagonospora* with KUMCC 16–0111 forming a sister clade to *P. allouniseptata* with strong statistical support (96% ML/ 88% MP/ 1.00 PP). KUMCC 16–0125 grouped closely with *P. avenae, P. caricis, P. forlicesenica* and *P. italica* with moderate support (61% ML/ 49% MP/ 0.93 PP).
Fig. 1 – Bayesian majority consensus tree based on the combined ITS, LSU and RPB2 sequence dataset. Maximum likelihood bootstrap values (ML) and maximum parsimony values (MP) equal or greater than 60% are given at the nodes. Bayesian posterior probabilities (PP) of more than 0.90 are given at the nodes. Ex-type strains are in bold; the new isolates are in blue. The tree is rooted to Neosetophoma samarorum CBS 138.96.
Taxonomy

Parastagonospora elymi Goonas., Bulgakov & McKenzie, sp. nov. Fig. 2

Index Fungorum number: IF556608, Facesoffungi number: FoF06260

Etymology – Named after the host genus *Elymus* from which it was collected.

Holotype – MFLU 15–2243

*Saprobic* on dead stems of *Elymus repens*, appearing as black, raised, shiny, oval lesions on host surface. Sexual morph: Ascomata perithelial, solitary, scattered to gregarious, glabrous, semi-immersed to superficial, 100–150 μm high, 145–200 μm diam. (n = 10), subglobose to conical, dark brown, with central ostiole. *Peridium* 15–20 μm wide, composed of several layers, outer layers; brown, thick-walled cells of *textura prismatica* to *textura angularis* and inner layers, hyaline to lightly pigmented, thin-walled cells of *textura angularis*. *Hamathecium* composed of many filamentous, septate, 1.5–2 μm wide, cellulae *pseudoparaphyses*, periphyses present. *Asci* 55–95 × 7–13 μm (x̅ = 80 × 10.5 μm, n = 20), cylindrical, 6–8-spored, bitunicate, fissitunicate, rounded at the apex, with bi-lobed pedicel. *Ascopores* 21.5–25.5 × 3.5–5 μm (x̅ = 22.5 × 4.5 μm, n = 20), uniseriate to overlapping bi-seriate, hyaline, fusiform, with acute ends, straight or slightly curved, 3-septate at maturity, cells near the mid septum, especially second cell from apex slightly swollen, constricted at the middle septum, smooth-walled. Asexual morph: Undetermined.


Culture characteristics – Colonies reaching 15–20 mm diam. in 4 weeks at 16–18°C on PDA, woolly and raised, margins irregular, from above grey, reverse grey to white.

Notes – In the phylogenetic analyses, *Parastagonospora elymi* groups closely with the two strains of *P. avenae* (CBS 289.69 and CBS 290.69). Morphologically, *P. elymi* is similar to the sexual morph of *P. avenae* (= *Phaeosphaeria avenaria*) in the characteristics of their asci and ascospores (cylindrical asci, with 8, overlapping biseriate, fusiform ascospores), however their dimensions of the ascomata vary. *P. elymi* ascomata are subglobose or conical, 145–200 μm wide and 100–150 μm high, while *P. avenae* has smaller, globose ascomata that are 120–150 μm wide, 120–150 μm high (Shoemaker & Babcock 1989, this study). The ascospores of *P. avenae* are guttulate and surrounded by a sheath, while those of *P. elymi* have neither guttules nor a sheath (Shoemaker & Babcock 1989, this study).

Parastagonospora macrouniseptata Goonas., Camporesi & McKenzie, sp. nov. Fig. 3

Index Fungorum number: IF556607, Facesoffungi number: FoF06261

Etymology – “uniseptata” referring to its similarity to *P. uniseptata*; and “macro” to its larger conidiomata.

Holotype – MFLU 15–0774

*Saprobic* on dead stem of *Dactylis glomerata* L. (Poaceae) appearing as black dots on the host surface. Sexual morph: Undetermined. Asexual morph: Coelomycetous. *Conidiomata* 150–180(–190) μm wide, (120–)130–160 μm high (x̅ = 167 × 145 μm, n = 10), dark brown to black, pycnial, solitary to gregarious, immersed, globose to subglobose, unilocular, glabrous, glabrous, ostiolate. *Ostiole* central, short papilla. *Conidiomatal wall* 8–18 μm wide (x̅ = 13.7 μm), composed of thin, dark brown, pseudoparenchymatous cells, gradually merging with hyaline cells of *textura angularis*. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* 4.2 × 3 μm, hyaline, phialidic, ampulliform to lageniform, discrete, determinate, aseptate, smooth, arising from the inner layers of conidioma. *Conidia* (14–)15–18(–20) × (1–)2–2.5 μm (x̅ = 17.3 × 2 μm, n = 30), hyaline, cylindrical to subcylindrical, rounded at apex, slightly truncate at base, 1-septate, slightly constricted at the septum, smooth-walled, guttulate.

Culture Characteristics – Colonies reaching 15–20 mm diam. in 4 weeks at 18–20°C on
PDA, dense, margins undulate to irregular, from above white, slightly raised and umbonate in the center, becoming grey at the margins, reverse grey to white.

Fig. 2 – *Parastagonospora elymi* (MFLU 15–2243, holotype) a Appearance of ascomata on host. b Close up of ascomata. c, d Vertical section of ascoma. e Peridium. f Pseudoparaphyses. g–j Asci. k–n Ascospores. Scale bars: c, d = 100 µm, e = 50 µm, f = 10 µm, g, h, j–n = 15 µm, i = 20 µm.

Notes – *Parastagonospora macrouniseptata* is morphologically similar to *P. allouniseptata* in having globose to subglobose, pycnidia and hyaline, cylindrical, 1-septate conidia. Both species are described from the same host. However, *P. macrouniseptata* has larger conidiomata ($\bar{x}$ = 167 × 145 µm) than *P. allouniseptata* (60–90 µm high, 70–90 µm diam.) (Li et al. 2015). Phylogenetically, *P. macrouniseptata* forms a sister clade with *P. allouniseptata*, within the *Parastagonospora* clade with high bootstrap support. The differences in base pairs between the two species were 14 for the ITS region, six for LSU and 20 for the TEF gene region.

![Fig. 3](image-url) – *Parastagonospora macrouniseptata* (MFLU 15–0774, holotype) a Specimen from above. b Conidiomata on host. c Squash mount of conidioma. d Section through conidioma. e Wall of conidioma. f, g Conidiogenous cells. h–k Conidia. l Colony on PDA. Scale bars: c, d = 50 µm, e = 20 µm, f, g = 10 µm, h–k = 5 µm.

**Discussion**

*Parastagonospora* was introduced by Quaedvlieg et al. (2013) to accommodate several species previously classified as *Leptosphaeria*, *Phaeosphaeria*, *Septoria* and *Stagonospora*. This genus is characterized by a sexual morph having immersed ascomata with slightly papillate ostiole, bitunicate, shortly stipitate asci, fusoid, subhyaline to pale brown, septate ascospores and a
coelomycetous asexual morph with hyaline, cylindrical, granular to multi-guttulate, transversely euseptate conidia (Quaedvlieg et al. 2013, Li et al. 2015, Thambugala et al. 2017). *Parastagonospora* species are widespread occurring mostly on wheat, grasses such as *Dactylis* sp. and *Poa* sp. as well as other cereal crops (Quaedvlieg et al. 2013, Li et al. 2015, Ghaderi et al. 2017). Apart from *Parastagonospora*, the genera *Neosetophoma*, *Phaeosphaeria*, *Phaeosphaeriopsis*, *Setophoma*, *Wojnowicia* and *Xenoseptoria* in Phaeosphaeriaceae have been associated with leaf spots from various hosts (Carson 2005, Arzanlou & Crous 2006, Quaedvlieg et al. 2013, Phookamsak et al. 2014).

As most sexual morphs in Phaeosphaeriaceae are morphologically similar, it is their asexual morph characteristics, coupled with molecular data, that are essential in classification (Phookamsak et al. 2017). In most cases, the addition of protein coding genes is necessary to clearly distinguish species in Phaeosphaeriaceae (Phookamsak et al. 2014). Based on the phylogenetic results of this study, the combined use of ITS, LSU and RPB2 sequence data satisfactorily segregated the species of *Parastagonospora*, although this may not be sufficient for all genera in the family. There is a need for broader collections, and more importantly, the re-collection of type species, in order to firmly establish the phylogenetic placements of certain genera, especially those with poor species representation (Phookamsak et al. 2014, 2017, Tennakoon et al. 2016). It is doubtful whether most species of this family are host specific (Phookamsak et al. 2014) as initially thought, hence more fresh collections are necessary.

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