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Pyrenophora trichostoma (Pleosporaceae, Pleosporales): an overview of the species and first record on *Bromopsis inermis* from Russia

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Abstract

Pyrenophora consists of endophytes, pathogens and saprobes that are widely distributed on various cereal crops and grass hosts. *Pyrenophora trichostoma* was found on *Bromopsis inermis* in Russia (Rostov region) and it is described as the first record of this species from this host. The fungus, a sexual morph, was isolated using the single spore isolation technique. The morphology is illustrated and a description has been provided. Sequence data of the LSU and ITS gene regions were analysed to confirm the phylogenetic placement of the species. The history of *P. trichostoma*, its distribution and economic impact as a pathogen that causes leaf spots on cereals and wheat, has been discussed.

Key words – grass fungi – pathogen – phylogeny – saprobe – taxonomy

Introduction

History

Pyrenophora is a distinct genus in Pleosporales as it lacks pseudoparaphyses in the hamathecium and have asci with large apical rings and visible ocular chambers (Sivanesan 1984, Ariyawansa et al. 2014). The genus was introduced by Rebentisch (1804) based on the type *Pyrenophora phaeocomes* (Rebent.) Fr. *Drechslera* has been confirmed as the asexual morph (Zhang & Berbee 2001, Ariyawansa et al. 2014, Marin-Felix et al. 2019, Hyde et al. 2020). *Pyrenophora* species are widespread and a few are well-known plant pathogens (Tekauz 1983, Lamari & Bernier 1989, Kingsland 1991, Gupta & Loughman 2001, Ariyawansa et al. 2014). Marin-Felix et al. (2019) accepted 27 species in *Pyrenophora* based on a multi-gene analysis consisting of ITS, LSU, GAPDH and RPB2 sequence data.

Pyrenophora trichostoma was originally described as *Sphaeria trichostoma*, a species introduced by Fries (1823) in Hypoxylaceae and later synonymized to this name (Fries 1870). However, no holotype is designated in the original description. Wehmeyer (1961) re-introduced *Pyrenophora trichostoma* as the sexual morph of *Helminthosporium* species that were reported on

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several grasses such as *Bromus*, *Hordeum*, *Poa*, *Secale* and *Triticum*. This name was then widely used to identify the tan spot or yellow leaf spot pathogen on wheat (Wiese 1977, Hosford 1982). Later, the species was synonymized under *P. tritici-repentis* for having similar morphological characters, such as producing ascospores with mucilaginous sheaths (Ciuffetti & Tuori 1999, Sivanesan 1984). Based on phylogenetic analysis, Kodsueb et al. (2006) considered them as two distinct species, citing differences in the base pairs of *P. tritici-repentis* and *P. trichostoma* sequences. In the recent study by Marin-Felix et al. (2019) *P. trichostoma* was accepted as a phylogenetically distinct species.

Morphological characters of the sexual/asexual morph

During a revision of *Drechslera*, *Pyrenophora tritici-repentis* was proposed as the sexual morph of the hyphomycete *Drechslera tritici-repentis* (Died.) Shoem. by Shoemaker (1959, 1962). Subsequent studies have confirmed the link between the sexual and asexual morphs of these genera based on phylogeny (Zhang & Berbee 2001, Ariyawansa et al. 2014).

The *Pyrenophora* sexual morph has immersed to semi-immersed ascomata with necks surrounded by brown to reddish brown setae. The hamathecium comprises no pseudoparaphyses, clavate to saccate shaped asci with large apical rings, and muriform, terete ascospores (Ariyawansa et al. 2014, Marin-Felix et al. 2019, Hyde et al. 2020). *Pyrenophora trichostoma* has 330 μm long and 40 μm wide, 8-spored asci, with broad-oblong, asymmetric, muriform, yellow, 52 × 20 μm sized ascospores that are 4–6 septate, with constrictions at the mid septum (Fuckel 1870, translated from Latin). There is no asexual morph reported yet.

In pure culture this species usually forms flat or umbonate colonies, greenish, grey to white, or olivaceous black, which later develop an orangish, brownish or pinkish tint. For sporulation, several media have been proposed as suitable: sterilized rice or wheat straw on PDA, PNA, OA and MEA for asexual morphs, and Sach's agar for the sexual morphs (Marin-Felix et al. 2019).

Diversity of the species

Pyrenophora trichostoma is widely distributed with most occurrences being from Europe and USA (Far & Rossman 2020). A compilation of all records of the species from various hosts and regions is listed in Table 1.

Table 1 Reports of *Pyrenophora trichostoma* on various hosts worldwide.

Host	State/Country	References
Agropyron repens	Canada	Conners (1967)
A. smithii	USA	Krupinsky (1982)
Avena sp.	Italy	Greuter et al. (1991)
*Bothriochloa ischaemum**	Russia	Hyde et al. (2020)
*Bromopsis inermis**	Russia	This study
Bromus sp.	England	Dennis (1978)
Calamagrostis arundinacea	Poland	Chlebicki (1993)
Dactylis sp.	Portugal	Unamuno (1941), Checa (2004)
	Spain	Checa (2004)
Elymus junceus	USA	Krupinsky (1982), Krupinsky & Berdahl (1984), Checa (2004)
Festuca nigricans	Portugal	Checa (2004)
	Spain	Checa (2004)
Hierochloe alpina,	Alaska	Cash (1953)
Hordeum vulgare	China	Tai (1979)
H. vulgare var. *nudum*	China	Tai (1979)
Juncus balticus var. *montanus*	USA	Cooke (1955)
Luzula sylvatica	Austria	Scheuer (1988)
Poa pratensis	Alaska	Cash (1953)
Puccinellia borealis	Alaska	Cash (1953)

Table 1 Continued.

Host	State/Country	References
P. distans	Poland	Chlebicki (1993)
Secale cereal	Portugal	Checa (2004)
	Spain	Checa (2004)
	USA	Index of Plant Diseases in the US (1960), Hanlin (1963)
Triticum aestivum	Bolivia	Farr & Stevenson (1963)
	Brazil	Mendes et al. (1998)
	USA	Index of Plant Diseases in the US (1960), Hanlin (1963)

*Molecular data available in GenBank

Molecular data

The LSU and ITS genes are used as DNA barcodes for *Pyrenophora* for classification at genus level, while ITS, GAPDH and TEF1 are effective in species identification (Marin-Felix et al. 2019). There is no molecular data available in GenBank for the reports mentioning *P. trichostoma* as a pathogen. Yet, the pathogenicity of *Pyrenophora* species is widespread and established, and as a result, researchers have looked into developing molecular genetic markers for rapid identification of the species (Moreno et al. 2012).

Economic importance and pathogenicity

A form of *Pyrenophora trichostoma* that severely infected wheat of North Dakota was reported by Hosford (1971) in 1968 and 1969. The disease symptoms showed light-brown lesions with yellow halos on leaves, and many ascostromata present on wheat stubble. The pathogenicity of the fungus varied, as well as the resistance of different wheat cultivars towards the pathogen. This depended on how long the leaves were exposed to free moisture (Hosford 1971). The species was also found pathogenic causing leaf spots on *Elymus arenarius* and *Elymus cinereus* from North Dakota (Krupinsky & Berdahl 1984).

Due to these diseases, cereal crops have faced a high reduction in grain yield and quality leading to the decrease in crop productivity (Porta-Puglia et al. 1986, Wiese 1987, Sivanesan 1987).

Materials & Methods

Literature survey

Published articles in journals, books, web-based resources such as reports on host plants or disease management, USDA database (Farr & Rossman 2020) and research theses were referred for the literature review. Host names given in the original citation were checked according to The Plant List (http://www.theplantlist.org).

Sample collection, observation and study

Samples were collected in Russia, into paper bags and brought to the laboratory. A Motic SMZ 168 dissecting microscope was used to observe the fungal morphological features as described in Goonasekara et al. (2019). Photographs were obtained through a Canon EOS 600D digital camera fixed to a Nikon Ni compound microscope. Adobe Photoshop CS5 Extended version 12.0 software (Adobe Systems, USA) was used to process the photos. Measurements were taken with The Tarosoft (R) Image Frame Work program v. 0.9.7 software. The fungus was isolated using single spore isolation, in a petri-dish containing 2% water agar (Chomnunti et al. 2014) and incubated at 16–18°C, overnight. Germinating spores were transferred aseptically onto fresh potato dextrose agar (PDA), and further incubated at 16–18°C. Pure colonies were subcultured into new PDA plates. Herbarium material is deposited in the herbarium of Mae Fah Luang University, Chiang Rai, Thailand (MFLU) and living cultures are deposited at in Kunming Culture Collection (KUMCC) and duplicated at Mae Fah Luang University Culture Collection (MFLUCC). The

Facesoffungi and Index Fungorum numbers were obtained according to Jayasiri et al. (2015) and Index Fungorum (2020).

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 processed using the primer pairs 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). The amplification reaction was completed in a 25 µl total volume containing 1µl DNA, 9.5 µl Taq polymerase and PCR buffer mix, 12.5 µl double distilled water and 1 µl of each primer. The PCR thermal cycling program for LSU and ITS gene regions were as follows: an initial denaturing step of 94°C for 3 mins, 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. PCR products were verified by running 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 generated in this study are deposited in GenBank.

Phylogenetic analysis

Sequence results obtained were subjected to BLASTn (NCBI) searches to obtain matching sequences from GenBank (Table 1) and from recent publications (Marin-Felix et al. 2019). An analysis of the combined LSU and ITS sequences of accepted *Pyrenophora* species were included to confirm the phylogenetic placement of our strain, with *Bipolaris panici-miliacei* (CBS 199.29) and *B. yamadae* (CBS 202.29) as the outgroup taxa. Single gene sequences were assembled in BioEdit v. 7.0.9.0 (Hall 1999). 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). Phylogenetic reconstructions of combined gene trees were performed using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) criteria.

The ML 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 (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (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 setup 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. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications resulting from maximum parsimony 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 (BYPP) (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. First The burn-in phase of 20% was set and 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: 27122, http://www.treebase.org/).

Table 2 GenBank accession numbers of the strains used for phylogenetic analysis. Sequences generated in this study are in blue. Type strains are in bold.

Taxon	Culture code	GenBank accession numbers	
		ITS	LSU
Bipolaris panici-miliacei	**CBS 199.29^{LT}**	MH855043	MH866507
B. yamadae	**CBS 202.29^{ET}**	MH855044	MH866508
Pyrenophora avenicola	**CBS 307.84^{T}**	MK539972	MK540042
P. biseptata	CBS 319.69	MK539974	MK540044
	CBS 108963	MK539975	JN712532
P. bromi	CBS 311.68	MK539976	MH870851
	DAOMC 127414	JN943666	JN940074
P. chaetomioides	CBS 195.31	MK539978	MH866633
P. cynosuri	**CBS 127918^{T}**	MK539980	MK540047
P. dactylidis	DAOMC 92161	JN943667	JN940087
P. dictyoides	DAOMC 63666	JN943653	JN940080
	CBS 258.80	MK539981	MK540048
P. erythrospila	CBS 312.69	MK539983	MK540051
	CBS 108941	MK539984	MK540052
P. fugax	CBS 509.77	MK539985	MK540053
P. grahamii	CBS 315.69	MK539986	MK540054
	CBS 128043	MK539987	MH876230
P. japonica	CBS 281.31^{RS}	MK540004	MK540067
P. leucospermi	**CBS 111083^{T}**	JN712467	JN712533
	CBS 111505	MK539989	JN712542
	CBS 114493	MK539990	JN712545
P. lolii	CBS 240.48	MK539991	MK540055
P. nisikadoi	**CBS 190.29^{ET}**	KM257054	KM243296
	CBS 119213	EU552124	MK540056
P. nobleae	CBS 259.80	MK539994	MK540058
	CBS 127936	MK539996	MK540060
P. novozelandica	**CBS 127934^{T}**	MK539997	MK540061
P. phaeocomes	DAOMC 222769	JN943649	JN940093
P. poae	CBS 319.68^{RS}	MK539998	MK540062
	CBS 128045	MK539999	MH876232
P. pseudoerythrospila	**CBS 127931^{T}**	MK540000	MK540063
P. semeniperda	DAOMC 213153	JN943665	JN940088
P. sieglingiae	CBS 127930	MK540002	MK540065
P. teres f. *maculata*	**CBS 228.76^{T}**	MK540003	MK540066
P. teres	CBS 336.29	MK540007	MH877692
P. tetrarrhenae	DAOMC 171966	JN943663	JN940090
	CBS 127924	MK540011	MH877965
P. trichostoma	CBS 328.53	MK540012	MK540072
	CBS 391.54	MK540013	MK540073
	CBS 392.54	MK540014	MK540074
	KUMCC 16-0120	MN736436	MN733192
	KUMCC 16-0123	MW041570	MW041254
P. triseptata	CBS 128047	MK540015	MH877983
P. tritici-repentis	CBS 259.59	MK540017	MK540075
P. tritici-repentis	CBS 191.29	MK540018	MK540076
P. tritici-repentis	CBS 127922	MK540019	MK540077
P. variabilis	**CBS 127920^{T}**	MK540020	MK540078
P. wirreganensis	CBS 109896	MK540021	MK540079

(ET = epitype, LT = lectotype, RS = reference strain, T = type)

Results & Discussion

Phylogeny

A combined dataset of the LSU and ITS sequences of 46 *Pyrenophora* strains were subjected to the phylogenetic analyses, with *Bipolaris panici-miliacei* CBS 199.29 and *B. yamadae* CBS 202.29 as the outgroup taxa (Fig. 1). Bootstrap values equal to or greater than 60% obtained for MP and ML analyses and BYPP values equal to or greater than 0.90 resulting from the BI analysis are given at each node (Fig. 1).

The MP dataset consisted of 1780 characters, of which 1465 characters were constant, 266 were parsimony-informative and 49 were parsimony-uninformative. The most parsimonious tree with a tree length of 900, had the following values: CI = 0.563, RI = 0.777, RC = 0.438, HI = 0.437 and is presented in Fig. 1. The tree topology of the ML and BI analysis did not differ significantly from that of the MP analysis. The RAxML analysis of the combined dataset yielded a best scoring tree with a ML optimization likelihood value of -7078.475836. The matrix had 461 distinct alignment patterns, with 13.71% of undetermined characters or gaps. Parameters for the GTR model of the combined ITS and LSU were as follows: estimated base frequencies; A = 0.245334, C = 0.229315, G = 0.279163, T = 0.246188; substitution rates AC = 1.521880, AG = 1.485545, AT = 1.366202, CG = 0.879594, CT = 3.736353, GT = 1.000000; gamma distribution shape parameter $\alpha = 0.104747$. The BI analysis resulted in 40,002 trees after 1,000,000 generations. The first 2000 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. The average standard deviation of split frequencies was 0.010067.

Our isolate KUMCC 16-0123 together with other *Pyrenophora trichostoma* isolates CBS 328.53, CBS 391.54, CBS 392.54 and KUMCC 16-0120 formed a clade with strong bootstrap support (100% MP/100% ML/1 BYPP), sister to *P. bromi*.

Taxonomy

Pyrenophora trichostoma (Fr.) Fr., Jb. nassau. Ver. Naturk. 23-24: 215 (1870) [1869-70] Fig. 2

Saprobic on dead stems of *Bromopsis inermis* (Leyss.) Holub Sexual morph: *Ascomata* 320–420 μm high 300–345 μm diam. ($\bar{x}$ = 400 × 320 μm, n = 10), black, immersed to semi-immersed or becoming erumpent, solitary or scattered, uniloculate, globose to subglobose, or conical, visible as shiny black dome shaped structures on the host surface, conspicuous, surrounded by setae, glabrous, non-papillate, black. *Ostiole* short, immersed, round, with a pore-like opening. *Setae* dark brown, darker towards the base, septate, tapering towards the apex. *Peridium* 16–26 μm wide, thin, multiple-layered cells of outer dark brown *textura angularis,* and inner cells of pale brown to hyaline flattened *textura angularis* to *textura prismatica*. *Hamathecium* consisting of irregular shaped cellular matter, pseudoparaphyses absent. *Asci* 250–350 × 45–65 μm, ($\bar{x}$ = 310 × 50 μm, n = 20), 8-spored, bitunicate, fissitunicate, slightly curved, broadly cylindric-clavate, smooth-walled, with a bi-lobed, short pedicel, apex rounded, with a distinct ocular chamber. *Ascospores* 35–70 × 15–35 μm, ($\bar{x}$ = 55 × 20 μm, n = 40), overlapping biseriate, ellipsoidal, asymmetrical, muriform, with 3 transverse septa, 1–2 longitudinal septa, constricted at the septa, thick-walled, cells with longitudinal septa larger or swollen, hyaline when immature, pale to golden brown when mature, end cells slightly lighter, conical and broadly to narrowly rounded at the ends, surrounded by a thick mucilaginous sheath, guttulate. Asexual morph: Undetermined.

Culture characteristics – Colonies on PDA reaching approximately 2.5 cm diam after 7 days at 16–18°C, pinkish white, circular, flat, with dense mycelium, smooth margins, becoming orangish red and reverse brown.

Material examined – RUSSIA, Rostov region, Shakhty city district, 20th anniversary of Red Army microdistrict, Solyonaya Balka (Salty gully), at margin of artificial forest, on dead stems of *Bromopsis inermis* (Leyss.) Holub (Poaceae), 21 May 2015, Timur S. Bulgakov, T-431 (MFLU 15-2135, new host record); living culture KUMCC 16-0123.

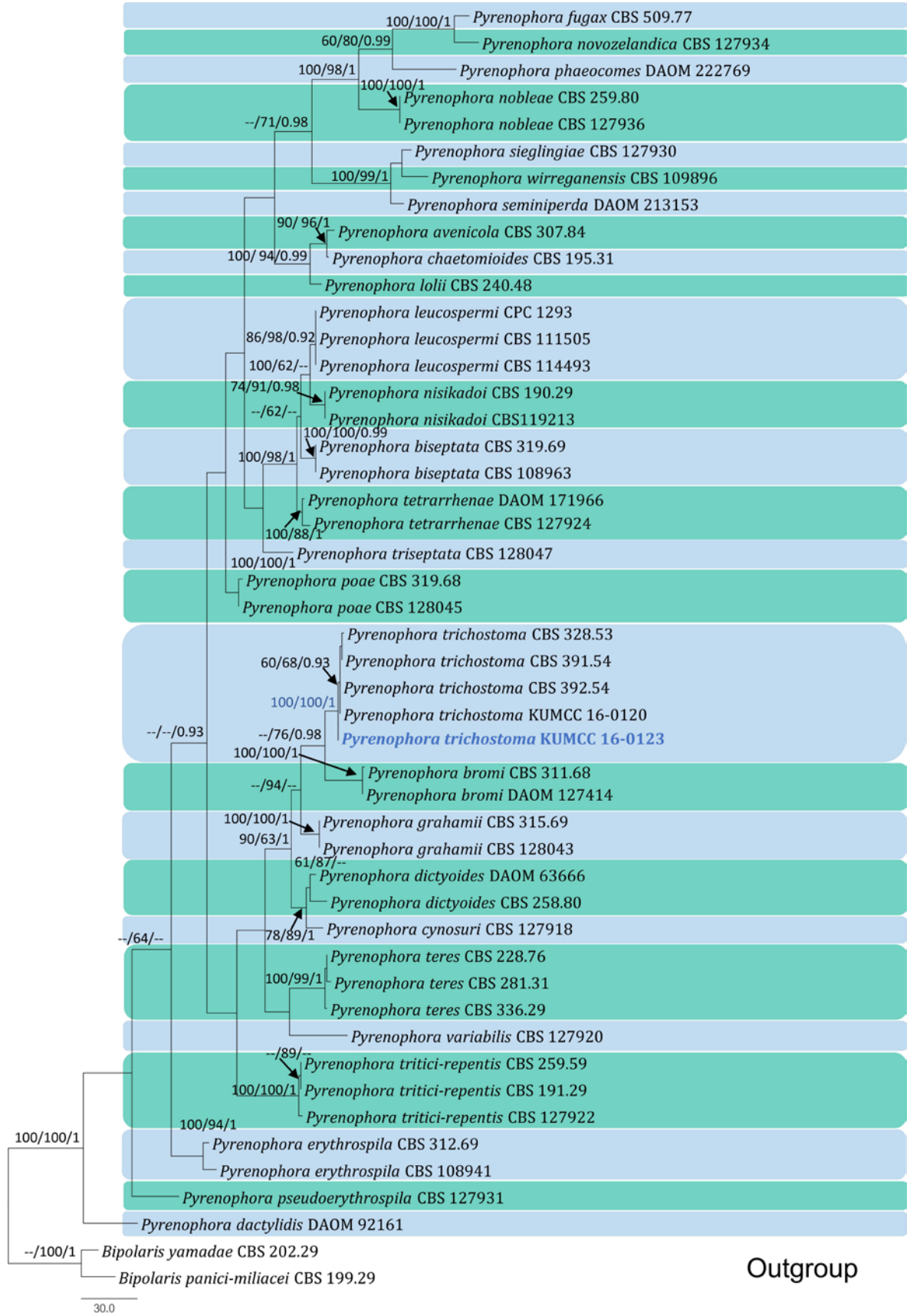

Fig. 1 – Phylogram generated from maximum parsimony (MP) analysis based on combined LSU and ITS sequence data. Bootstrap values for MP and maximum likelihood (ML) analyses equal to or greater than 60% and Bayesian posterior probabilities (BYPP) greater than 0.9 are given at the nodes, respectively. The tree is rooted to *Bipolaris panici-miliacei* CBS 199.29 and *B. yamadae* CBS 202.29. Ex-type strains are in bold. The newly generated isolate is indicated in blue.

Fig. 2 – *Pyrenophora trichostoma* (MFLU 15-2135, new host record). a Appearance of ascomata on host. b Close up of ascoma. c Section of ascoma. d Setae. e Peridium. f Contents of the hamathecium. g Ocular chambers of asci. h–k Immature to mature asci. l–o Ascospores. p Ascospore surrounded in a sheath (stained with Indian Ink). – Scale bars: c, h–k = 100 µm, d, f, g = 20 µm, e, l–p = 10 µm.

GenBank Numbers – SSU: MW048786, LSU: MW041254, ITS: MW041570

Notes – Our species illustrated here has 310 × 50 µm sized, broadly cylindric-clavate and slightly curved asci containing broad, asymmetric, muriform, 55 × 20 µm sized ascospores. These morphological characters resemble *P. trichostoma* as described by Fuckel (1870). Phylogenetically, our isolate KUMCC 16-0123 clusters with three strains CBS. 328.53, CBS 391.54 and CBS 392.54 identified as *P. trichostoma* (Marin-Felix et al. 2019) and another isolate KUMCC 16–0120, a host record on *Bothriochloa ischaemum* and first record from Russia (Hyde et al. 2020). There were no

base pair differences present in the LSU or ITS gene regions in all these strains. Therefore, we identify our species is *P. trichostoma*.

Conclusions

Pyrenophora trichostoma is a known severe pathogen on cereal crops, especially wheat. There are numerous records of the species as a pathogen, but there is a lack of molecular data related to these reports, in GenBank. A proper taxonomic revision based on morphological identification and classification is needed. Therefore, further recollections and establishing a suitable type herbarium for future studies is necessary. The addition of more molecular data will help to confirm its phylogenetic placement and aid in developing modern DNA techniques for plant disease identification.

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