Characterization of *Pyrenophora* Species Causing Brown Leaf Spot on Italian Ryegrass (*Lolium multiflorum*) in Southwestern China

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Abstract

Drechslera leaf spot (DLS) caused by *Pyrenophora* (*Drechslera*) species is one of the most serious diseases affecting Italian ryegrass (*Lolium multiflorum*) in China. Between 2015 and 2018, this disease was observed in three Italian ryegrass fields in the province of Sichuan, China. Average leaf disease incidence was approximately 1 to 12% but could range up to 100%. Symptoms appeared as brown or tan spots surrounded by a yellow halo, or brown to dark brown net blotch; subsequently, spots increased in number and size, and they later covered a large area of leaf, eventually causing leaf death. In this study, 86 strains of *Pyrenophora* fungi were isolated from leaf lesions of Italian ryegrass. Coupled with phylogenetic analysis of the internal transcribed spacer region, partial 28S ribosomal RNA gene, and glyceraldehyde-3-phosphate

dehydrogenase gene, morphological characteristics showed that *Pyrenophora dictyoides* and *P. nobleae* are associated with Italian ryegrass in southwest China. Pathogenicity tests confirmed that both species can infect Italian ryegrass, causing leaf spot, whereas the virulence of the two species differed; *P. nobleae* showed lower pathogenicity to Italian ryegrass. This is the first time that these two *Pyrenophora* species were formally reported on Italian ryegrass based on both morphological and molecular characters. Overall, this study improves knowledge of the *Pyrenophora* species associated with Italian ryegrass and provides a foundation for control of this disease in the future.

Keywords: fungi, field crops, pathogen diversity, pathogen detection

Italian ryegrass (*Lolium multiflorum*) is one of the most important forage crops in several countries, including Argentina (Gundel et al. 2006), China (Shao et al. 2005), Japan (Inoue et al. 2004), the United Kingdom (White et al. 2005), and the United States (Rauch et al. 2010). It is widely cultivated in temperate, tropical, and subtropical zones because of its high productivity (Ikeda 2005), nutritional value (Shao et al. 2002), palatability, and digestibility (Baldinger et al. 2011; Sharma and Sahi 2005; Takahashi et al. 2014). In addition to use for fresh forage, Italian ryegrass is also widely utilized for hay and silage (Miura et al. 2007; Shao et al. 2005; Takahashi et al. 2014).

The genus *Pyrenophora* (asexual morph: *Drechslera*) is known as a pathogen of gramineous plants (Ariyawansa et al. 2014; Evidente et al. 2006; Hampton and Matthews 1978; Zhang and Berbee 2001). *Pyrenophora dictyoides* causes net blotch of ryegrass in New Zealand (Latch 1966); *P. teres* results in serious foliar disease of barley in Western Australia (Gupta and Loughman 2001); and *P. tritici-repentis* has been reported as the causal agent of tan spot

on wheat in many countries (Lepoint et al. 2010). Characters of conidia are essential for the identification and further research of *Pyrenophora* species (Pratt 2006a; Shoemaker 1962); however, many studies showed that it was difficult to produce conidia of *Pyrenophora* species in culture conditions (Lepoint et al. 2010; Varshney 2001). For instance, because the conidia are rarely formed in culture conditions, the biological control agents against *P. graminea* have not been studied in detail (Varshney 2001); although the conidia of *P. dictyoides* were induced by Pratt (2006a), there were not enough to carry out the inoculation of plants or other experiments. Lepoint et al. (2010) also found that some isolates of *P. tritici-repentis* were incapable of producing conidia in their experiments. An effective method to produce sufficient conidia of *Pyrenophora* species is imperative for identification and other studies.

Drechslera leaf spot (DLS, or brown leaf spot) on Italian ryegrass is a common and widespread plant disease. However, there have been few studies on DLS of Italian ryegrass in the last decade. *Pyrenophora* species associated with Italian ryegrass were reported from many places, such as *P. dictyoides* in Mississippi (Pratt 2006b), *P. biseptata* in Germany (Crous et al. 2011), *P. dematioidea* and *P. nobleae* in New Zealand (Pennycook 1989), *P. lolii* and *P. dictyoides* in Virginia (Roane 2004, 2009), *P. teres* in Florida (Schubert 1991), and *Drechslera poae* in Florida (Alfieri et al. 1984). From 2015, DLS has been frequently observed on Italian ryegrass around the surrounding areas of Chengdu city, Sichuan Province of China. Main objectives of the present study are (i) to accurately identify (from morphological, cultural, and phylogenetic features) these *Drechslera* species isolated from Italian ryegrass and (ii) to determine their ability to cause leaf spots by pathogenicity assays of mycelial plugs, mycelial suspensions, and conidial suspensions on detached leaves or whole plants of Italian ryegrass.

Materials and Methods

Sample collection and isolation. In order to identify the pathogens causing DLS of Italian ryegrass in the province of Sichuan, China, we performed successive sampling between 2015 and 2018. Three experimental locations including Xinjin (30.47785°N,

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103.75878°E), Mengyang (30.96925°N, 104.10223°E), and Hongya (29.89173°N, 103.36937°E) counties were investigated. Symptoms of leaf spot and leaf morbidity were observed and recorded, and samples were collected. Three pieces (1 × 0.5 cm²) of symptomatic leaves were cut from each plant of 10 to 15 random plants collected from each location (Xinjin, 10 plants; Mengyang, 12 plants; Hongya, 15 plants). Leaf pieces were surface sterilized in 70% ethanol for 60 s followed by immersion in 5% of a commercial bleach (NaClO, active chlorine content ≥5.5%; free alkali content [NaOH], 5.0 to 6.0%) for 3 min, rinsed three times with sterilized distilled water, air dried, and plated onto potato dextrose agar (PDA) supplemented with 50 mg/liter of ampicillin sodium salt and incubated at 25°C for 2 to 7 days. Any fungal hyphae that grew from the leaf tissues were immediately transferred to fresh PDA. Referring to the single-spore isolation technique of *Colletotrichum* genus (Cai et al. 2009), the mycelial suspensions (described as below) were diluted to a reasonable concentration, spread onto the surface of PDA agar, and incubated at 25°C in the dark for 24 to 48 h. Single germinated cultures (considered as pure cultures in this study) were transferred onto a new PDA plate for further study. Representative pure isolates were deposited at the Mycological Herbarium of Lanzhou University and China General Microbiological Culture Collection Center (CGMCC).

Morphological and cultural characterization. Mycelial plugs (6 mm diameter) were transferred from the edge of growing colonies to fresh potato carrot agar (PCA, Abrusci et al. 2005) or PDA and subsequently incubated at 25°C in the dark. Colony characteristics on PDA and PCA were recorded after 5 to 16 days. Isolates were cultured on PCA under near-ultraviolet (UV) light (24 h light) to induce sporulation. Microscopic preparations were made in sterile water and observed with a Nikon ECLIPSE Ti microscope (Nikon, Japan) or an LCD-Digital NSZ-808 (NOVEL). Thirty measurements were performed for each type of structure (conidia, conidiophore, etc.). Relevant data were compared by Tukey's test ($P \leq 0.05$) using IBM SPSS Statistics 19.0.

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing. For each pure culture, mycelia (about

60 mm diameter) were scraped from one fresh colony on PDA using a sterile spoon. Genomic DNA was then extracted with the use of a Fungal DNA Kit (Omega Bio-Tek, Doraville, CA). DNA samples were stored at -20°C for further studies. DNA concentrations were determined with a Nanodrop 2000 spectrophotometer (Thermo Scientific) and manually adjusted at 100 ng/μl. The rDNA internal transcribed spacer (ITS) region, partial 28S ribosomal RNA gene (LSU), and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) were amplified using the primer pairs ITS1/ITS4, LR5/LROR, and *gpd*1/*gpd*2, respectively (Manamgoda et al. 2012). PCR amplifications were performed in a T100TM Thermal Cycler (Bio-Rad, Hercules, CA) in a total volume of 20 μl containing 1 μl of genomic DNA, 1 μl of forward and reverse primers (10 μM), 10 μl of I-5 2× High-Fidelity Master Mix (MCLAB, South San Francisco, CA), and 7 μl of sterile water. DNA sequencing was finally performed by Sangon Biotech (Chengdu, China).

Phylogenetic analysis. Sequences obtained from the isolates (in bold) and other reference sequences from GenBank are displayed in Table 1. ITS, LSU, and *gpd* from *Pyrenophora* species were individually aligned using Clustal X as implemented in MEGA version 6 (Tamura et al. 2013) and manually adjusted to allow maximum sequence similarity. Then the three loci were combined together with SequenceMatrix 1.8 (Vaidya et al. 2011). MEGA version 6 was used to determine the best DNA model. Aligned sequences were analyzed using maximum likelihood (ML) with MEGA version 6. For Bayesian inference (BI) analysis, the best-fit nucleotide substitution models of each gene were assessed by MrModeltest 2.3 software (Nylander 2004). Then BI analyses were performed on MrBayes version 3.2.6 (Ronquist et al. 2012). In order to better distinguish these species, the single-locus trees of the three genes in both ML and BI analyses were also compared with the same methods. *Drechslera* species were recognized if the clade is strongly supported in both ML and BI analyses (ML/posterior probability [PP]; ≥70% and ≥0.95, respectively). Trees were decorated in Microsoft Office PowerPoint 2016. The alignments and trees were deposited in TreeBASE (<http://www.treebase.org>).

Pathogenicity of *Pyrenophora* isolates. Strains were cultivated at 25°C for 5 days, and then 4-mm PDA plugs were cut from the edge of

Table 1. GenBank accession numbers of isolates included in this study

Species	Strain	GenBank accessions ^z			References
		ITS	LSU	<i>gpd</i>	
<i>Pyrenophora bromi</i>	DAOM 127414	AY004809	JN940074	AY004839	Zhang and Berbee (2001)
	SM101	EF452456	...	EF513218	Andrie et al. (2008)
<i>P. cynosuri</i>	CBS 127918*	MK539980	MK540047	MK540188	Marin-Felix et al. (2019)
<i>P. dictyoides</i>	DAOM 63666	AY004806	JN940080	AY004836	Zhang and Berbee (2001)
	DAOM 75616	JN943654	JN940079	...	Schoch et al. (2012)
	CBS 967.87	MK539982	MK540049	MK540190	Marin-Felix et al. (2019)
	CBS 258.80	MK539981	MK540048	MK540189	Marin-Felix et al. (2019)
<i>Drechslera andersenii</i>	DAOM 229292	JN943646	JN940084	...	Schoch et al. (2012)
	HMCD-2	MH321434	MN023192	MH321438	This study
	CGMCC 3.19765, = HMCD-9	MH321436	MN023193	MH321440	This study
	HMCD-20	MH321437	MN023194	MH321441	This study
<i>P. fugax</i>	CBS 509.77	MK539985	MK540053	MK540194	Marin-Felix et al. (2019)
<i>P. grahamii</i>	CBS 315.69	MK539986	MK540054	MK540195	Marin-Felix et al. (2019)
<i>P. nobleae</i>	CBS 127936	MK539996	MK540060	MK540208	Marin-Felix et al. (2019)
	CBS 966.87	MK539995	MK540059	MK540207	Marin-Felix et al. (2019)
	CBS 259.80	MK539994	MK540058	MK540206	Marin-Felix et al. (2019)
	DAOM 229296	JN943647	JN940095	...	Schoch et al. (2012)
	CGMCC 3.19764, = HMCD-3	MH321435	MN023197	MH321439	This study
<i>P. novozelandica</i>	CBS 127934*	MK539997	MK540061	MK540209	Marin-Felix et al. (2019)
<i>P. phaeocomes</i>	DAOM 222769	JN943649	DQ499596	unpublish data	
<i>P. tritici-repentis</i>	DAOM 208990	AF071348	AY544672	AF081370	Zhang and Berbee (2001)
	DAOM 226213	JN943670	JN940071	AY004838	Ariyawansa et al. (2014)
	10692	EF452475	...	EF513237	Andrie et al. (2008)
<i>Bipolaris zeae</i>	NBC1	KU571464	KY047114	KU571467	This study

^z ITS = internal transcribed spacers 1 and 2 together with 5.8S nrDNA; *gpd* = partial glyceraldehyde-3-phosphate dehydrogenase gene; and LSU = partial 28S ribosomal RNA gene. Sequences generated in this study are indicated in bold. Asterisk (*) indicates an ex-type culture.

fresh colonies for pathogenicity tests. Conidia were produced by incubating the isolates on PCA for 7 to 15 days under continuous near-UV light; conidial suspensions were adjusted to 1×10^5 conidia/ml in sterile water with 0.01% (vol/vol) Tween 80. Fungal mycelia (5 days old) were scraped from three fresh colonies using a sterile spoon and then were homogenized in sterile water, using an UltraTurrax (IKA T25 digital, Germany); the final inoculum (or mycelial suspensions) was adjusted to 500 ml with 0.01% (vol/vol) Tween 80.

Inoculation of detached leaves. Pathogenicity assays were performed on young healthy detached leaves (8 weeks old) of Italian ryegrass (cv. Splendor). For each isolate, 20 leaves were inoculated by placing 20 μ l of conidial suspension or 4-mm PDA plugs onto the middle of the right side of the leaves. Twenty leaves were inoculated with sterile water containing Tween 80 and 4-mm fungus-free PDA plugs to be used as controls. Leaves were then placed in transparent plastic boxes to maintain high relative humidity at 25°C in an incubator. Disease symptoms were observed and recorded 6 days after inoculation. The length of the lesions was measured to evaluate fungal virulence.

Inoculation of whole plants. Five 8-week-old healthy plants obtained by growing Italian ryegrass (cv. Splendor) in pots were spray-inoculated separately with conidial and mycelial suspensions of each species, respectively. Plants of the same number were sprayed with sterilized distilled water containing Tween 80 as controls. Then all pots were individually covered with transparent polyethylene bags for 5 days to maintain high relative humidity and placed in a greenhouse at 18 to 25°C.

Referring to the method of assessing severity of Drechslera leaf blotch of oats and wheat (James 1971), the disease index (DI) of DLS of Italian ryegrass was graded on a scale of 0 to 4, where 0 indicated none of leaf area affected on individual top leaves, 1 indicated 0 to 1% of leaf area, 2 indicated 1 to 5% of leaf area, 3 indicated 5 to 25% of leaf area, and 4 indicated 25 to 50% of leaf area. DI was calculated by the following formula: $DI = [(sum\ of\ scores\ for\ all\ leaves\ investigated) \times 100] / [(total\ number\ of\ leaves\ investigated) \times 4]$. Both disease incidence (infected leaves) and DI were assessed 14 days after inoculation. To fulfill Koch's postulates, all fungal isolates included in the pathogenicity tests were reisolated from infected leaves to confirm their identity as described above. All relevant data were compared by Tukey's test ($P \leq 0.05$) using IBM SPSS Statistics 19.0.

Results

Symptoms and separation rate of *Pyrenophora* species. From 2015 to 2018, DLS was successively observed in the three locations between January and June. The average disease incidence was 1 to 12%. Usually, DLS develops seriously at later plant development stages (middle April to June), and the incidence can reach 40%. In 2015, DLS first appeared sporadically in Hongya in early April prior to a sudden outbreak on a large area in late April with incidence reaching up to 100% (Fig. 1A and G). Typical symptoms of DLS corresponded either to a brown or tan spot surrounded by a yellow halo (Fig. 1B to E) or to brown to dark brown net blotch (Fig. 1F). In advanced infection stages, brown (tan) spots increased in number and size, and they further covered a large area of the infected leaves, eventually causing leaf death.

In total, 86 *Pyrenophora* strains were isolated from the three Italian ryegrass fields. Based on the high similarity of cultural characterization on PDA as well as ITS, LSU, and *gpd* sequences, 75 *Pyrenophora* sp. 1 obviously appear as the main species of DLS, representing 87% of all *Pyrenophora* isolates, followed by 11 *Pyrenophora* sp. 2.

Morphological and cultural characteristics. *Pyrenophora* sp. 1 isolates on PDA were cottony, white to light olivaceous on the upper side, and gray to dark brown on the reverse side, and they grew 65 to 70 mm in diameter after 5 days (Fig. 2A and B); conidia induced by culturing colonies on PCA under near-UV light were pale to yellow-brown, straight or slightly curved, tapered, 1 to 13 distoseptate (mostly 6 to 9) (Fig. 2F to L), (32.5 to) 55.8 to 278.0 $\times$ (5.2 to) 8.2 to 15.8 μ m, mean $\pm$ SD = $126.6 \pm 50.3 \times 11.3 \pm 2.3$ μ m ($n = 30$), length/width (L/W) ratio = 2.9 to 23.3 (mostly 8.5 to 16.2), (3.9 to)

5.4 to 9.4 (to 13.2) μ m at the apex, (5.8 to) 10.3 to 20.4 μ m at the base, apical cell width/basal cell width (AW/BW) = 0.3 to 0.9 (mostly 0.5 to 0.7). Conidiophores were hyaline to yellow-brown, septate, flexuous, mainly single, up to 430 μ m (Fig. 2M and N). These morphological features were generally similar to those of *P. dictyoides* (Lam 1985; Roane 2009). Although there was variation from the length range of conidia (32.5 to 278.0 μ m in this study versus 59 to 161 μ m [Lam 1985] [or 92 to 103 μ m; Roane 2009]), by coupling morphology with molecular characteristics, *Pyrenophora* sp. 1 was identified as *P. dictyoides*.

Pyrenophora sp. 2 isolates on PDA were cottony, white to light olivaceous on the upper side, and gray to dark brown on the reverse side (Fig. 3A and B), and they grew 71 to 76 mm in diameter after 5 days; colonies on PCA under near-UV light were dark brown with some conidia produced in the center. Conidia on PCA were gray to light golden brown, straight or slightly curved, obclavate, 0 to 7 distoseptate (mostly 4 to 7) (Fig. 3D to K), (20.8 to) 63.3 to 116.7 (to 175.6) $\times$ 8.2 to 16.6 μ m, mean $\pm$ SD = $88.3 \pm 27.1 \times 11.6 \pm 1.7$ μ m ($n = 30$), L/W ratio = 6.4 to 11.1 (mostly), 6.5 to 9.9 μ m at the apex, and AW/BW = 0.6 to 0.8 (mostly). One of the three cell at the bottom often was slightly inflated. Sometimes light Y-shaped conidia developed (Fig. 3I). Conidiophores were gray to dark brown, septate, unbranched or branched, single or in small groups, geniculate at the upper end, up to 335 μ m (Fig. 3K and L). These features were highly similar to *P. nobleae* (Morrison 1982) but differed in the existence of light Y-shaped conidia and slight inflating on the third cell. According to the molecular and morphological features, *Pyrenophora* sp. 2 was identified as *P. nobleae* in this study.

Phylogenetic analysis. The ITS, LSU, and *gpd* reference sequences from 19 *Pyrenophora* strains were obtained from GenBank (Table 1). Three representative strains from *Pyrenophora* sp. 1 and one strain from *Pyrenophora* sp. 2 were selected for phylogenetic analysis. *Bipolaris zeae* (NBC1) was used as the outgroup. The dataset contained 2,115 characters including gaps after sequence alignment (623 for ITS, 853 for LSU, and 639 for *gpd*). A K2+G model was selected for ML phylogenetic analyses of combined gene data. For BI analysis, the following models were selected by MrModeltest and used: GTR+G for ITS, GTR+I for LSU, and HKY+I for *gpd*. Only the ML tree is showed in this study. The bootstrap support values (1,000 replicates)/Bayesian PP scores above 70%/0.95 are displayed at the tree nodes (ML/PP). Based on that, the phylogenetic tree (Fig. 4; TreeBASE: S25208) showed that HMCD-2, CGMCC 3.19765 (= HMCD-9) and HMCD-20 isolates of *Pyrenophora* sp. 1 cluster well with five typical *P. dictyoides* isolates (Marin-Felix et al. 2019; Schoch et al. 2012; Zhang and Berbee 2001). Moreover, the CGMCC 3.19764 (= HMCD-3) isolate of *Pyrenophora* sp. 2 as well as four reported *P. nobleae* isolates (Marin-Felix et al. 2019; Schoch et al. 2012) formed a well-supported subclade (100/1). In the single gene analysis, the single loci ITS and *gpd* separated *P. dictyoides* and *P. nobleae*, respectively. Furthermore, *P. nobleae* was separated also by LSU. In addition, the NCBI BLAST data showed that the ITS region separated both *P. dictyoides* (= *Drechslera andersenii*) and *P. nobleae* well from the other *Pyrenophora* species, respectively.

Pathogenicity. *On detached leaves.* In order to confirm Koch's postulates, pathogenicity of four representative isolates (three *P. dictyoides* strains and one *P. nobleae* strain) was tested on detached Italian ryegrass leaves. All tested isolates were pathogenic 6 days after inoculation with mycelial plugs or conidial suspensions. For the four *Pyrenophora* sp., both conidial suspension and PDA plugs induced brown to dark brown necrotic spots surrounded by a bright yellow halo (Fig. 5A). No symptoms were observed on the corresponding controls. Pathogenicity tests showed that *P. dictyoides* was more highly pathogenic to detached leaves than *P. nobleae* (Table 2; $P \leq 0.05$).

On whole plants. Healthy plants (about 8 weeks old) were obtained by growing Italian ryegrass (cv. Splendor) in plastic circular pots (10 cm bottom diameter, 15 cm upper diameter, 12 cm height) in a greenhouse at 18 to 25°C. Fourteen days after inoculation, both conidial suspensions and mycelial suspensions could result in necrotic

spots on plants (Fig. 5), with no symptoms developing on the control plants. In the inoculation series using conidial suspensions, the symptoms caused by both *P. dictyoides* and *P. nobleae* developed brown necrotic spots surrounded by a yellow halo after 14 days, and the symptoms were highly similar to those that occur under natural

conditions in the field (Fig. 5B to E). When plants were inoculated with mycelial suspension, symptoms initially appeared as water-soaked, irregular, pale brown necrotic spots, later becoming brown or tan lesions; sometimes, whole leaves withered, and died after 14 days (Fig. 5F).

Fig. 1. Leaf spot of Italian ryegrass caused by *Pyrenophora* species. A to G, Natural symptoms in the field.

Fig. 2. Colony and morphology characteristics of *Pyrenophora dictyoides* on Italian ryegrass. A and B, Colony on potato dextrose agar at 25°C for 7 days in the dark; C to E, conidiophores and conidia on the potato carrot agar surface at 25°C for 9 days under near-ultraviolet light; F to L, conidia; and M and N, conidiophores.

All data on both disease incidence and DI showed that *P. dictyoides* was the most aggressive species; however, *P. nobleae* was weakly pathogenic (Table 3; $P \leq 0.05$). Using mycelial suspensions to infect plants, symptoms were more pronounced than those caused by conidia (Table 3). All isolates used in these pathogenicity tests were reisolated from the corresponding symptomatic leaves, and their identification was confirmed by morphology and molecular assays as described above.

Discussion

In the past few decades, *Pyrenophora* species have been identified by using morphological and cultural characteristics (Cromey 1983; Shoemaker 1962; Sivanesan 1987; Sun and Zhang 2004). However, it is difficult to accurately identify *Pyrenophora* species due to instability of its morphological characters under different conditions (Imran et al. 2018; Shoemaker 1962). For example, conidia of one typical isolate of *P. dictyoides* were 70 to 135×14 to $20 \mu\text{m}$, $L/W = 5.7$, and mostly 5 distoseptate; however, conidia of another isolate under moist conditions were 100 to 277×15 to $19 \mu\text{m}$, $L/W = 8.9$, and 5 to 15 distoseptate (Shoemaker 1962); likewise, conidia of isolates (*P. dictyoides*) from Italian ryegrass were given as 92 to 103×12 to $15 \mu\text{m}$, 5 to 6 distoseptate (Roane 2009), but in this study, conidia of *P. dictyoides* produced on PCA under near-UV light were 55.8 to 278×8.2 to $15.8 \mu\text{m}$, average = $127.7 \times 11.3 \mu\text{m}$, 1 to 13 distoseptate. Because of this variability in morphology of conidia (Imran et al. 2018), phylogenetic analysis is indispensable for the identification of *Pyrenophora* species (Ariyawansa et al. 2014; Zhang and Berbee 2001). Zhang and Berbee (2001) showed that the ITS and *gpd* data, coupled with morphology, could improve the standards for identification of *Pyrenophora* species. Ariyawansa et al. (2014) used five loci (ITS, *gpd*, *RPB2*, *SSU*, and *LSU*) for the phylogenetic analysis of *Pyrenophora* genus. In a recently published paper (Marin-Felix et al. 2019), at least 27 species were identified in *Pyrenophora* genus based on four loci (ITS, *gpd*, *RPB2*, and *LSU*) coupled with some morphological characters. Considering these available sequences of *P. dictyoides* and *P. nobleae* in GenBank, three loci

(ITS, *gpd*, and *LSU*) were used in this study. Accordingly, 75 *Pyrenophora* sp. 1, representing 87% of all *Pyrenophora* isolates, were accurately identified as *P. dictyoides*; the other 11 isolates (*Pyrenophora* sp. 2) were identified as *P. nobleae*.

P. dictyoides (anamorph: *Drechslera dictyoides*) was first described as *Helminthosporium dictyoides* Drechsler, a fungus that

Fig. 4. Phylogram generated for the *Pyrenophora* species from a maximum likelihood (ML) analysis based on the combined internal transcribed spacer region, partial 28S ribosomal RNA gene, and glyceraldehyde-3-phosphate dehydrogenase alignment. Bootstrap support values/Bayesian posterior probability (PP) scores above 70%/0.95 are shown at the nodes (ML/PP). Strains in this study are shown in bold. The tree was rooted to *Bipolaris zeae* (NBC1). TreeBASE: S25208.

Fig. 3. Colony and morphology characteristics of *Pyrenophora nobleae* on Italian ryegrass. **A** and **B**, Colony on potato dextrose agar at 25°C for 7 days in the dark; **C**, conidiophores and conidia on the potato carrot agar surface at 25°C for 9 days under near-ultraviolet light; **D** to **K**, conidia; and **K** and **L**, conidiophores.

caused net blotch on leaves of *Festuca elatior* (Drechsler 1923). Later, it was placed in genus as *Drechslera dictyoides* (Drechsler) Shoemaker (Shoemaker 1962). Paul and Parbery (1968) successfully induced the teleomorph of *H. dictyoides* by pairing two isolates (one from *Lolium perenne*, and another from *Vulpia myuros*). Because of the high-supported clade (100/1) in phylogenetic analysis (ITS, LSU, and *gpd*) and the similar morphology of the asexual morph, *Drechslera andersenii* belonged to a synonymy of *P. dictyoides* (Marin-Felix et al. 2019). *P. dictyoides* has previously been reported on Italian ryegrass in Australia (Paul and Parbery 1968), New Zealand (= *D. andersenii*; Pennycook 1989), Poland (Mulencko et al. 2008), Virginia (Roane 2009), Denmark, Germany, and the United Kingdom (Sivanesan 1987). In China, *P. dictyoides* was recorded on *Phleum pratense* (Tai 1979) and *L. perenne* (Hu et al. 2018; Li 2018; Sun 2003). Although *P. dictyoides* was described from seeds of Italian ryegrass (Shang 1990), pathogenicity tests and DNA sequence data from multiple loci were not done. To our knowledge, this is the first formal report of *P. dictyoides* causing DLS of Italian ryegrass in China.

P. nobleae, teleomorph of *D. nobleae*, was first recorded as an unknown species on ryegrass seed (Matthews 1971) and then was formally described in 1977 on seeds and yellowing leaves of *Lolium*

spp. in New Zealand (McKenzie and Matthews 1977): conidia were pale straw-colored, 3 to 8 distoseptate, and 55 to 125×9 to $19 \mu\text{m}$. In 1982, Morrison described *P. nobleae* again (isolated from blighted leaves of Italian ryegrass in Alabama, U.S.A.): conidia were pale

Table 2. Pathogenicity of *Pyrenophora* isolates on detached leaves of Italian ryegrass 6 days after inoculation^x

Species	Isolates	Lesion length (mm) ^y	
		Conidial suspension ^z	PDA plugs (4 mm diam.)
Control check		0 a	0 a
<i>P. dictyoides</i>	HMCD-2	16.9 ± 3.7 c	10.8 ± 2.2 c
	HMCD-9	17.8 ± 3.7 c	11.3 ± 3.1 c
	HMCD-20	17.1 ± 3.2 c	9.5 ± 1.9 c
<i>P. nobleae</i>	HMCD-3	10.4 ± 2.0 b	5.5 ± 1.7 b

^x Columns with the same letter do not differ significantly according to Tukey's test ($P \leq 0.05$).

^y Lesion length following inoculation by conidial suspension and potato dextrose agar (PDA) plugs. Data represent the mean of three replications, each with 10 leaves, on detached leaves.

^z Conidial suspension of *Pyrenophora* strains was 1×10^5 conidia/ml.

Fig. 5. The symptoms of *Drechslera* leaf spot in inoculated leaves of Italian ryegrass. **A**, Symptoms on detached leaves inoculated with 1×10^5 conidial suspension of *Pyrenophora dictyoides* at 25°C for 6 days; **B**, symptoms on whole plants inoculated with conidial suspension of *P. nobleae*; **C** to **E**, symptoms on whole plants inoculated with conidial suspension of *P. dictyoides*; and **F**, symptoms when inoculated with mycelial suspension for 14 days. CK = control check.

Table 3. Pathogenicity of *Pyrenophora* isolates on whole plants of Italian ryegrass 14 days after inoculation*

Species	Disease incidence/infected leaves (%)		Disease index (DI)	
	Conidial suspension ^y	Mycelial suspension ^z	Conidial suspension ^y	Mycelial suspension ^z
Control check	0 a	0 a	0 a	0 a
<i>P. dictyoides</i>	43.0 ± 5.9 c	73.5 ± 6.1 c	23.6 ± 4.8 c	60.7 ± 7.4 c
<i>P. nobleae</i>	19.7 ± 3.8 b	34.0 ± 7.6 b	7.6 ± 2.7 b	24.1 ± 4.4 b

* Columns with the same letter do not differ significantly according to Tukey's test ($P \leq 0.05$).

^y Conidial suspension of *Pyrenophora* strains was 1×10^5 conidia/ml.

^z Mycelial suspension of *Pyrenophora* strains was obtained according to the method described in the text.

to mid-golden brown, straight, obclavate, 3 to 9 distoseptate, 55 to 125 × 9 to 17 µm, L/W = 6.5 to 8.3, and AW/BW = 0.66 to 0.75. Lam (1984) showed that the morphological characteristics of *D. nobleae* (conidia: 4 to 11 distoseptate, 62 to 175 × 9 to 16 µm) on perennial and Italian ryegrass in England were consistent with those of McKenzie and Matthews (1977) and Morrison (1982). These features were highly similar to those of *P. nobleae* isolated from Italian ryegrass in this study but differed in the absence of light Y-shaped conidia. In 2015, *D. nobleae* was named *P. nobleae* (McKenzie & D. Matthews) Rossman & K.D. Hyde, comb. nov. (Rossman et al. 2015). *P. nobleae* was also recorded on Italian ryegrass in Australia (Sivanesan 1987). To our knowledge, this is the first report of *P. nobleae* causing brown leaf spot on Italian ryegrass in China.

DLS on Italian ryegrass was previously attributed to *P. nobleae* in North America (Morrison 1982) and England (Lam 1984). However, in southeastern America, *P. dictyoides* was the most frequently observed species and was considered as an established ryegrass pathogen (Pratt 2006b). In New Zealand, DLS of Italian ryegrass caused by *P. lolii* was found throughout the year; although *P. lolii* was less common than *P. dictyoides*, it led to more harm to individual plants (Latch 1966). In this study, both *P. dictyoides* and *P. nobleae* could form tan spots on leaves, but no clear lesions caused by these two species were observed (Fig. 5). Coupled with the isolation rate, pathogenicity tests showed that *P. dictyoides* (87% of all *Pyrenophora* isolates) was the most important causal agent, followed by *P. nobleae* (Tables 2 and 3). To some degree, mycelia of the two *Pyrenophora* species could cause more damage to Italian ryegrass than conidia (Table 3).

In 1966, DLS caused by *P. dictyoides* was considered to be the second most important foliar disease of ryegrass, after crown rust, in New Zealand (Latch 1966); however, in 1981, Lam suggested that DLS of ryegrasses was more serious than crown rust in Britain (Lam 1981). In this study, we accurately identified two *Pyrenophora* species isolated from leaf lesions of Italian ryegrass based on the morphological and molecular characteristics, and pathogenicity tests showed that *P. dictyoides* was the dominant species and the most important causal agent of DLS, followed by *P. nobleae*. These data provide a better understanding of *Pyrenophora* species associated with *L. multiflorum* and will be useful for the accurate identification and effective control of DLS in the future.

Literature Cited

- Abrusci, C., Martín-González, A., Del Amo, A., Catalina, F., Collado, J., and Platas, G. 2005. Isolation and identification of bacteria and fungi from cinematographic films. *Int. Biodeterior. Biodegradation* 56:58-68.
- Alfieri, S. A., Jr., Langdon, K. R., Wehlburg, C., and Kimbrough, J. W. 1984. Index of Plant Diseases in Florida (Revised). Bull. 11. Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, FL.
- Andrie, R. M., Schoch, C. L., Hedges, R., Spatafora, J. W., and Ciuffetti, L. M. 2008. Homologs of *ToxB*, a host-selective toxin gene from *Pyrenophora tritici-repentis*, are present in the genome of sister-species *Pyrenophora bromi* and other members of the Ascomycota. *Fungal Genet. Biol.* 45:363-377.
- Ariyawansa, H. A., Kang, J. C., Alias, S. A., Chukeatirote, E., and Hyde, K. D. 2014. *Pyrenophora*. *Mycosphere* 5:351-362.
- Baldinger, L., Baumung, R., Zollitsch, W., and Knaus, W. F. 2011. Italian ryegrass silage in winter feeding of organic dairy cows: Forage intake, milk yield and composition. *J. Sci. Food Agric.* 91:435-442.
- Cai, L., Hyde, K. D., Taylor, P. W. J., Weir, B., Waller, J., Abang, M. M., Zhang, J. Z., Yang, Y. L., Phouliwong, S., Liu, Z. Y., Prihastuti, H., Shivas, R. G., McKenzie, E. H. C., and Johnston, P. R. 2009. A polyphasic approach for studying *Colletotrichum*. *Fungal Divers.* 39:183-204.
- Cromey, M. G. 1983. Studies on net blotch of ryegrass caused by *Drechslera dictyoides*. Ph.D. thesis, University of Canterbury, Christchurch, New Zealand.
- Crous, P. W., Summerell, B. A., Swart, L., Denman, S., Taylor, J. E., Bezuidenhout, C. M., Palm, M. E., Marinowitz, S., and Groenewald, J. Z. 2011. Fungal pathogens of Proteaceae. *Persoonia* 27:20-45.
- Drechsler, C. 1923. Some graminicolous species of *Helminthosporium* 1. *J. Agric. Res.* 37:473-492.
- Evidente, A., Andolfi, A., Cimmino, A., Vurro, M., Fracchiolla, M., Charudattan, R., and Motta, A. 2006. Ophiobolin E and 8-epi-ophiobolin J produced by *Drechslera gigantea*, a potential mycoherbicide of weedy grasses. *Phytochemistry* 67:2281-2287.
- Gundel, P. E., Maseda, P. H., Ghersa, C. M., and Benech-Arnold, R. L. 2006. Effects of the *Neotyphodium* endophyte fungus on dormancy and germination rate of *Lolium multiflorum* seeds. *Austral Ecol.* 31:767-775.
- Gupta, S., and Loughman, R. 2001. Current virulence of *Pyrenophora teres* on barley in Western Australia. *Plant Dis.* 85:960-966.
- Hampton, J. G., and Matthews, D. 1978. *Drechslera* spp. on New Zealand certified cereal seed. *N. Z. J. Agric. Res.* 21:539-542.
- Hu, J., Yang, J. Y., Li, J., Gao, T., Yang, G. W., and Ren, H. Y. 2018. First report of *Drechslera andersenii* causing leaf spot on perennial ryegrass in Jiangsu, China. *Plant Dis.* 102:1174-1175.
- Ikedo, S. 2005. Isolation of disease resistance gene analogs from Italian ryegrass (*Lolium multiflorum* Lam.). *Grassl. Sci.* 51:63-70.
- Imran, Z. K., Hasan, K. M. A., and Ali, Z. H. 2018. A novel taxonomic study of the *Helminthosporium* Link ex Fries status and closely related genera. *Biochem. Cell. Arch.* 18:927-935.
- Inoue, M., Gao, Z., and Cai, H. 2004. QTL analysis of lodging resistance and related traits in Italian ryegrass (*Lolium multiflorum* Lam.). *Theor. Appl. Genet.* 109:1576-1585.
- James, W. C. 1971. An illustrated series of assessment keys for plant diseases, their preparation and usage. *Can. Plant Dis. Surv.* 51:39-65.
- Lam, A. 1981. The effects of *Drechslera* spp. and other fungal pathogens in ryegrass swards. Doctoral dissertation, University of Reading, Reading, U.K.
- Lam, A. 1984. *Drechslera nobleae* on ryegrass in England. *Trans. Br. Mycol. Soc.* 83:339-341.
- Lam, A. 1985. *Drechslera andersenii* sp. nov. and other *Drechslera* spp. on ryegrass in England and Wales. *Trans. Br. Mycol. Soc.* 85:595-602.
- Latch, G. C. M. 1966. Fungous diseases of ryegrasses in New Zealand. *N. Z. J. Agric. Res.* 9:394-409.
- Lepoint, P., Renard, M.-E., Legrève, A., Duveiller, E., and Maraite, H. 2010. Genetic diversity of the mating type and toxin production genes in *Pyrenophora tritici-repentis*. *Phytopathology* 100:474-483.
- Li, M. 2018. Occurrence and control of major diseases of ryegrass. *Mod. Agric. Sci. Technol.* 18:108-110.
- Manamgoda, D. S., Cai, L., McKenzie, E. H., Crous, P. W., Madrid, H., Chukeatirote, E., Shivas, R. G., Tan, Y. P., and Hyde, K. D. 2012. A phylogenetic and taxonomic re-evaluation of the *Bipolaris-Cochliobolus-Curvularia* complex. *Fungal Divers.* 56:131-144.
- Marin-Felix, Y., Hernández-Restrepo, M., Iturriza-González, I., García, D., Gené, J., Groenewald, J. Z., Cai, L., Chen, Q., Quaedvlieg, W., Schumacher, R. K., Taylor, P. W. J., Ambers, C., Bonthond, G., Edwards, J., Krueger-Hadfield, S. A., Luangsa-ard, J. J., Morton, L., Moslemi, A., Sandoval-Denis, M., Tan, Y. P., Thangavel, R., Vaghefi, N., Cheewangkoon, R., and Taylor, P. W. 2019. Genera of phytopathogenic fungi: GOPHY 3. *Stud. Mycol.* 94:1-124.
- Matthews, D. 1971. A survey of certified ryegrass seed for the presence of *Drechslera* species and *Fusarium nivale* (Fr.) Ces. *N. Z. J. Agric. Res.* 14:219-226.
- McKenzie, E. H. C., and Matthews, D. 1977. *Drechslera nobleae* sp. nov. on ryegrass. *Trans. Br. Mycol. Soc.* 68:309-311.
- Miura, Y., Hirata, M., and Fujimori, M. 2007. Mapping of EST-derived CAPS markers in Italian ryegrass (*Lolium multiflorum* Lam.). *Plant Breed.* 126:353-360.
- Morrison, R. H. 1982. *Drechslera nobleae* on *Lolium multiflorum* in North America. *Mycologia* 74:391-397.
- Mulencko, W., Majewski, T., and Ruskiewicz-Michalska, M. 2008. A Preliminary Checklist of Micromycetes in Poland. W. Szafer Institute of Botany, Polish Academy of Sciences, Warsaw, Poland.
- Nylander, J. A. A. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.

- Paul, A. R., and Parbery, D. G. 1968. *Pyrenophora dictyoides* sp. nov. the perfect state of *Helminthosporium dictyoides*. Trans. Br. Mycol. Soc. 51:707-710.
- Pennycook, S. R. 1989. Plant Diseases Recorded in New Zealand. Vol. 3. Plant Diseases Division, D.S.I.R., Auckland, New Zealand.
- Pratt, R. G. 2006a. Enhancement of sporulation in species of *Bipolaris*, *Curvularia*, *Drechslera*, and *Exserohilum* by growth on cellulose-containing substrates. Mycopathologia 162:133-140.
- Pratt, R. G. 2006b. Frequency and pathogenicity of dematiaceous hyphomycetes on annual ryegrass overseeded on bermudagrass in Mississippi. Plant Dis. 90:1085-1090.
- Rauch, T. A., Thill, D. C., Gersdorf, S. A., and Price, W. J. 2010. Widespread occurrence of herbicide-resistant Italian ryegrass (*Lolium multiflorum*) in Northern Idaho and Eastern Washington. Weed Technol. 24:281-288.
- Roane, C. W. 2004. Graminicolous Fungi of Virginia: Fungi in Collections 1995-2003. Va. J. Sci. 55:139-157.
- Roane, C. W. 2009. Graminicolous fungi of Virginia: Fungi in Collections 2004-2007. Va. J. Sci. 60:13-50.
- Ronquist, F., Teslenko, M., Van Der Mark, P., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., and Huelsenbeck, J. P. 2012. MrBayes v. 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61:539-542.
- Rossman, A. Y., Crous, P. W., Hyde, K. D., Hawksworth, D. L., Aptroot, A., Bezerra, J. L., Bhat, J. D., Boehm, E., Braun, U., Boonmee, S., Camporesi, E., Chomnunti, P., Dai, D.-Q., D'Souza, M. J., Dissanayake, A. J., Gareth Jones, E. B., Groenewald, J. Z., Hernandez-Restrepo, M., Hongsanan, S., Jaklitsch, W. M., Jayawardena, R., Jing, L. W., Kirk, P. M., Lawrey, J. D., Mapook, A., McKenzie, E. H. C., Monkai, J., Phillips, A. J. L., Phookamsak, R., Raja, H. A., Seifert, K. A., Senanayake, I., and Slippers, B. 2015. Recommended names of pleomorphic genera in Dothideomycetes. IMA Fungus 6:507-523.
- Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., and Chen, W., and Fungal Barcoding Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc. Natl. Acad. Sci. U.S.A. 109:6241-6246.
- Schubert, T. S., Ed. 1991. Tri-ology 30:5-6. Technical Report. Division of Plant Industry, Florida Department of Agriculture, Gainesville, FL.
- Shang, H. 1990. Blotter test for four seeds-borne species of *Drechslera* and *Bipolaris* in ryegrass. Pratacultural Sci. 6:30-32.
- Shao, T., Ohba, N., Shimojo, M., and Masuda, Y. 2002. Dynamics of early fermentation of Italian ryegrass (*Lolium multiflorum* Lam.) silage. Asian-Australas. J. Anim. Sci. 15:1606-1610.
- Shao, T., Zhang, Z. X., Shimojo, M., Wang, T., and Masuda, Y. 2005. Comparison of fermentation characteristics of Italian ryegrass (*Lolium multiflorum* Lam.) and guineagrass (*Panicum maximum* Jacq.) during the early stage of ensiling. Asian-Australas. J. Anim. Sci. 18:1727-1734.
- Sharma, N. C., and Sahi, S. V. 2005. Characterization of phosphate accumulation in *Lolium multiflorum* for remediation of phosphorus-enriched soils. Environ. Sci. Technol. 39:5475-5480.
- Shoemaker, R. A. 1962. *Drechslera* Ito. Can. J. Bot. 40:809-836.
- Sivanesan, A. 1987. Graminicolous species of *Bipolaris*, *Curvularia*, *Drechslera*, *Exserohilum* and their teleomorphs. Mycol. Pap. 158:1-261.
- Sun, B. 2003. Taxonomic Studies on the Hyphomycetes genera of *Drechslera* Ito and *Exserohilum* Leonard and Suggs in China. M.A. thesis, Shandong Agricultural University, Taishan, China.
- Sun, B., and Zhang, T. 2004. One new species and two new Chinese records of *Drechslera*. Mycosystema 23:20-23.
- Tai, F. L. 1979. Sylloge Fungorum Sinicorum. Science Press, Academica Sinica, Peking, China.
- Takahashi, W., Miura, Y., Sasaki, T., and Takamizo, T. 2014. Identification of a novel major locus for gray leaf spot resistance in Italian ryegrass (*Lolium multiflorum* Lam.). BMC Plant Biol. 14:303.
- Tamura, K., Stecher, G., Peterson, D., Filipowski, A., and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30:2725-2729.
- Vaidya, G., Lohman, D. J., and Meier, R. 2011. SequenceMatrix: Concatenation software for the fast assembly of multi-gene datasets with character set and codon information. Cladistics 27:171-180.
- Varshney, V. 2001. Effect of plant extracts on *Drechslera graminea*, the causal agent of stripe disease of barley. Indian Phytopathol. 54:88-90.
- White, G. M., Moss, S. R., and Karp, A. 2005. Differences in the molecular basis of resistance to the cyclohexanedione herbicide sethoxydim in *Lolium multiflorum*. Weed Res. 45:440-448.
- Zhang, G., and Berbee, M. L. 2001. *Pyrenophora* phylogenetics inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. Mycologia 93:1048-1063.