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Pyrenophora lolii, a new species for the mycobiota of Iran

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Abstract: *Pyrenophora lolii* causes leaf spot on grasses including *Festuca* spp., *Lolium* spp., *Dactylis* spp., *Avena sativa* and wheat (*Triticum aestivum*). Infected oat leaves (*Avena sativa*) showing leaf spot symptoms were collected from the margin of barley fields in Golestan province of Iran during the spring of 2016. A morphological examination of the *Pyrenophora* specimen was carried out using light microscopy. Inoculation of oat leaves with the isolates of *Pyrenophora lolii* in greenhouse induced leaf spot on leaves. In order to confirm the morphological identification, sequences of glyceradehyde-3-phosphate dehydrogenase (*gpd*) gene and Internal transcribed spacer (ITS) regions were amplified using gpd1/2 and ITS1/4 primers, respectively. The phylogenetic analysis based on these sequences showed that the isolated *Pyrenophora* specimen clustered together with sequences of *P. lolii*. Based on result of morphological examination and phylogenetic analysis, it was concluded that the causal agent of leaf spot of *A. sativa* (oat) was *P. lolii*.

Key words: *Dreschlera siccans*, oat, phylogeny, ITS, *GPDH*

INTRODUCTION

Avena sativa L. is an annual grass (Dimberg et al. 1996) and placed in the family Poaceae and its wild ancestor is *A. sterilis* which is endemic to Iran, Iraq, and Turkey. Oat has been cultivated for centuries worldwide (Zhou et al. 1999) for its grain as food and fodder, as well as for medicine (Coffman 1977). *Avena sativa* is infected by two species of fungal pathogens of the genus *Pyrenophora; P. avenae* causing leaf blotch and black leaf spot, and *P. lolii* causing leaf spot (Mehta 2001).

Species in the genus *Pyrenophora* (anamorph: *Drechslera*), cause disease on Poaceous plants (Zhang & Berbee 2001) and is placed in the family Pleosporaceae (Berbee 1996). Some species of this genus are agents of destructive diseases on Poaceae including barley and wheat (Ariyawansa et al. 2014). In previous studies, phylogenetic analysis of *Pyrenophora* was carried out based on sequence data of ITS, *GPDH*, *RPB2*, *nrSSU* and *nrLSU* DNA regions (Ariyawansa et al. 2014, Zhang & Berbee 2001).

Pyrenophora lolii cause leaf spot on grass (Jones 2013) and is found in different parts of the world including Sweden, New Zealand, England and Wales (Jones 2013, Lam 1984). *Pyrenophora lolii* infect *Festuca* spp., *Lolium* spp., *Dactylis* spp., *Avena sativa* (Oat) and *Triticum aestivum* (Wilkins 1973, Gönner et al. 1993, Tonin et al. 2015, Jones 2013) but it is more common on *Lolium* spp. Yield loss of this fungus in mixed infection with *D. catenaria* on *Lolium perenne* can reach up to 15 %. (Jones 2013).

The goal of this study was to characterize and identify a new-found *Pyrenophora* species infecting oat in Iran, using morphological characterization and phylogenetic analyses based on *GPDH* and ITS sequences.

MATERIALS AND METHODS

Sampling and morphological characterization

Infected *A. sativa* leaves exhibiting leaf spot symptoms were collected from margins of barley fields during the spring of 2016 in the Golestan province located in the Northeast of Iran (Sadabad

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village), at an altitude of 160 m above sea level. After sterilization with 1% sodium hypochlorite solution, infected leaves were transferred on 2% water agar and incubated at 20 ˚C and with 12 h darkness and 12 h NUV light (Akhavan et al. 2016). After 10 days, spores emerged on the surface of leaves and these were used for morphological identification. The morphological characteristics of the specimen are summarized in Table 1.

Pathogenicity test

To assess the pathogenicity of the isolated fungal spores, *A. sativa* plants were grown in the greenhouse under 11 h light and 13 h darkness at 20 ˚C. For sporulation of *P. lolii*, it was cultured on Potato Dextrose Agar, where after cubes of 6-day-old colony margin were transferred to 2% WA (water agar) and incubated at 12 h NUV light and 12 h darkness at 20 ˚C. For plant inoculation, the concentration of spore suspension was adjusted to 5×10^5 conidia per mL and sprayed on plant leaves at the third to fourth leaf stage (Akhavan et al. 2016). After inoculation, pots were covered by a plastic bag for 24 h in order to create a 100% humidity condition. The two days following inoculation, leaves were surveyed daily. When symptom developed after 10 days, infected leaves were cultured on 2% WA under 12 h NUV light and 12 h darkness at 20 ˚C for re-isolation (Akhavan et al. 2016).

DNA extraction, PCR amplification

For DNA extraction, one isolate was cultured on PDB (Potato Dextrose Broth) and incubated at 20 ˚C and with 12 h darkness and 12 h NUV light (Akhavan et al. 2016). After 10 days, DNA was extracted using the CTAB method (Murray & Thompson 1980). For molecular identification, ITS region and *gpd* gene were amplified and sequenced.

For amplification of the ITS1 - 5.8S – ITS2 region, forward primer ITS1 (5'-TCCGTAGGTGAA CCTGCGG-3') and reverse primer ITS4 (5'-TCCTC CGCTTATTGATATGC-3') were used (White et al. 1990) and for amplification of the *gpd* gene, forward primer gpd1 (5'-CAACGGCTTCGGTCGCATTG-3') and reverse primer gpd2 (5'-GCCAAGCAGTTGGTTG TGC-3') were used (Berbee et al. 1999).

Reaction conditions for both ITS region and *gpd* gene consisted of an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C, followed by an extra extension at 72 °C for 7 min. PCR analysis was performed in a reaction mixture containing 10 µL master mix, 6 µL H2O, 2 µL DNA (3 ng/µL), 20 pmol of each of the forward and reverse primers, and final volume was 20 µL.

The sequencing was done by Sanger sequencing. For the ITS region, the sequenced fragment was approximately 560 bp including the internal transcribed spacer 1, partial sequence, 5.8 S gene and internal transcribed spacer 2, complete sequence; and 28 S ribosomal DNA gene, partial sequence. The gpd1/2 primers amplified a 600 bp fragment including 417 bp of the coding region and 180 bp of two introns. ITS and *gpd* sequences were deposited in NCBI with accession numbers of MN420825 and MN433343, respectively.

Phylogenetic analyses

To assess the phylogenetic position of the Iranian *Pyrenophora* specimens on *A. sativa*, both their ITS and *gpd* sequences were compared with *Pyrenophora* ITS and *gpd* sequences available in GenBank. The ITS and *gpd* sequences of the *Pyrenophora* specimen were blasted using blast analyses (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (2020). Alignment of sequences for both ITS and *gpd* was conducted by using MEGA7 and the align by clustal W option (Kumar et al. 2016) and the result of alignment were used for phylogenetic analyses utilizing a maximum likelihood (ML) method based on the Tamura-Nei model (Tamura & Nei 1993) and neighbor-joining (Nj) method (Saitou & Nei 1987). The analysis was conducted with MEGA 7 using the bootstrap method option with 1,000 replicates (Kumar et al. 2016). The tree was drawn by using integrated sequences of *gpd* and ITS and rooted with *Pleospora alfalfae*.

RESULTS

Morphological characterization

The results of the morphological analysis of the specimen are included in Table 1 and depicted in Fig.1.

***Pyrenophora lolii* Dovaston, Transactions of the British Mycological Society 31 (3-4): 251 (1948) MycoBank MB 290353**

Symptom on the infected oat leaves was an ellipsoid brown spot form. Red-brown, long and narrow conidiophores usually arise singly. They were enlarged at base. A group of 7-20 conidia grow at the top of conidiophores. The length of the conidiophores was 120-140 µm and the base of conidiophore was enlarged, globose, 10-20 µm wide. Cylindrical and straight conidia are 10-20 µm thick, 48-110 µm long and nearly round at both ends, pale to yellowish-brown, often having 3- 8 pseudosepta. The length and width of each cell of conidium is 10 to 20 µm. The diameter of the colony reaches to 7 cm after 5 days on PDA. The color of the colony is dark brown with the grey aerial mycelium developing later.

Specimens examined. IRAN, Golestan province, Sadabad village, on *Avena sativa*, 15 April 2016, A. Vasighzadeh.

The morphology of specimen on *Avena sativa* from Iran agrees with the description of *P. lolii* on *Lolium perenne* (Shoemaker 1962) and it is shown in Table 1.

Pathogenicity test

Inoculation of the *Pyrenophora* specimens on *A. sativa* leaves induced brown pin-like symptoms after 4 days.

This symptom developed after 10 days as oval and brown leaf spot with chlorotic margin (Fig. 1). By culturing of the symptomatic leaves on 2% WA, fungal spores emerged on the surface of the culture medium.

Phylogenetic analyses

After the blast analysis in NCBI, some of the sequences of *P. lolii* which were identical to the *Pyrenophora* specimen sequence, included in the alignment. The ITS sequence of *Pyrenophora* specimen in this study was 100% similar to previously published *P. lolii* sequences which were included in the phylogenetic analysis. The *gpd* sequence of *P. lolii* in this study was compared to *gpd* sequences of four isolates of *P. lolii* and was also 100% similar to sequences of three isolates of *P. lolii*. We only identified a one-nucleotide difference between *Pyrenophora* specimen sequence and *Pyrenophora lolli* strain HMCI which the sequence differentiation was 0.19 %. The trees obtaining from neighbor-joining (data not shown) and maximum likelihood methods based on *gpd* and ITS sequences showed the same tree topologies.

Based on phylogenetic analysis of integrated sequences of ITS and *gpd*, we found that the *Pyrenophora* specimen on *A. sativa* clustered with *P. lolii* isolates which had been isolated from *L. multiflorum* (Fig 2). The *P. lolii* isolates on *L. multiflorum* and *Avena sativa* formed a sister clade with *P. chaetomioides* and *P. avenae* which is a known pathogen of *A. sativa* (bootstrap values 100% for both methods). *Pyrenophora lolii* was distant to a monophyletic lineage on barley, wheat, and bromegrass (*P. teres*, *P. graminea*, *P. tritici-repentis* and *P. bromi*).

DISCUSSION

In previous studies, the connection between *Pyrenophora* and *Drechslera* which are teleomorph and anamorph, respectively, was proved based on ITS and *gpd* data (Zhang & Berbee 2001). For instance, ITS and *gpd* sequences of *P. lolii* and its related anamorph, *D. siccans*, were identical (Zhang & Berbee 2001). In this study, as it was excepted, *Pyrenophora* spp. and its related anamorph, *Drechslera* spp., clustered together in the phylogenetic tree.

In previous studies, *P. avenae*, *P. chaetomioides*, and *P. lolii* which are related to *D. avenae*, *D. avenacae* and *D. siccans* anamorphs, were proposed to be one taxon based on numerical taxonomy (Ibrahim & Threlfall 1966) but the molecular study based on the ITS and *gpd* data showed that *P. lolii* formed a sister group with a clade comprising *P. chaetomioides* and *P. avenae* and they could not be considered as one monophyletic lineage (Zhang and Berbee 2001). This result was similar to the phylogenic analyses of ITS and *gpd* sequences of these taxa in this study.

According to the phylogenetic analysis based on ITS and *gpd* data, the *Pyrenophora* specimen on *Avena sativa* in this study grouped with *P. lolii.* This species is closely related to *P. chaetomioides* and

Table 1. Comparison of the most important features of the specimen on *Avena sativa* from Iran with *Pyrenophora lolii.*

Features	*Pyrenophora lolii* On *Avena sativa* (this study) µm	*Pyrenophora lolii* On *Lolium perenne* µm
Length of conidiophores	120-140 (250)	(15) 100-150 (270)
Width of conidiophores	10-20	(8) 12-15 (22)
Length of conidia	48-110	(54) 90-110 (154)
Width of conidia	10-20	(12) 16-18 (21)
Septa number of conidia	(3) 4-6 (8)	(3) 4-6 (8)
Size of each cell of conidia	10-20	14-18

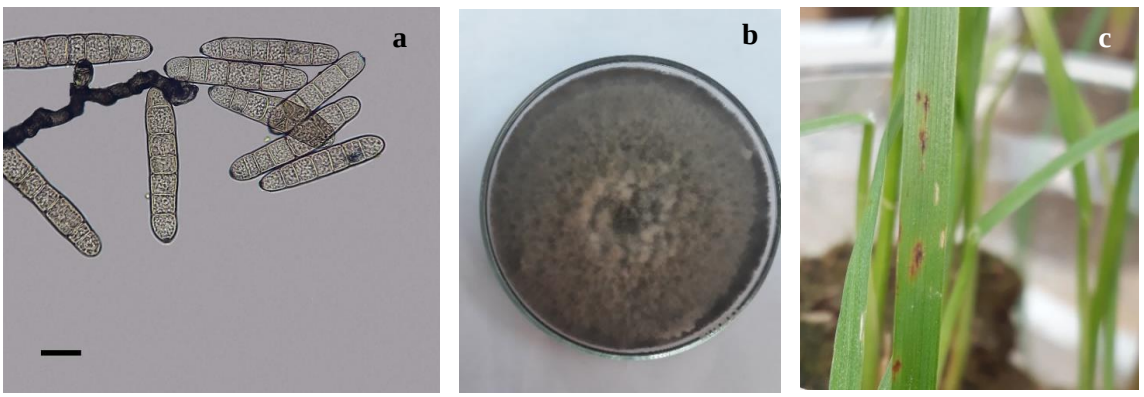

Fig. 1. *Pyrenophora lolii***.** a. Light micrographs of *Pyrenophora lolii* on *Avena sativa*; conidia (on 2 % TWA, at 20 ˚C, 12 h darkness, and 12 h NUV light) —Scale bars = 25 µm. b. A 7-day-old colony of *P. lolii* on PDA. c. The symptom of *P. lolii* on oat leaves, 10 days after inoculation in the greenhouse.

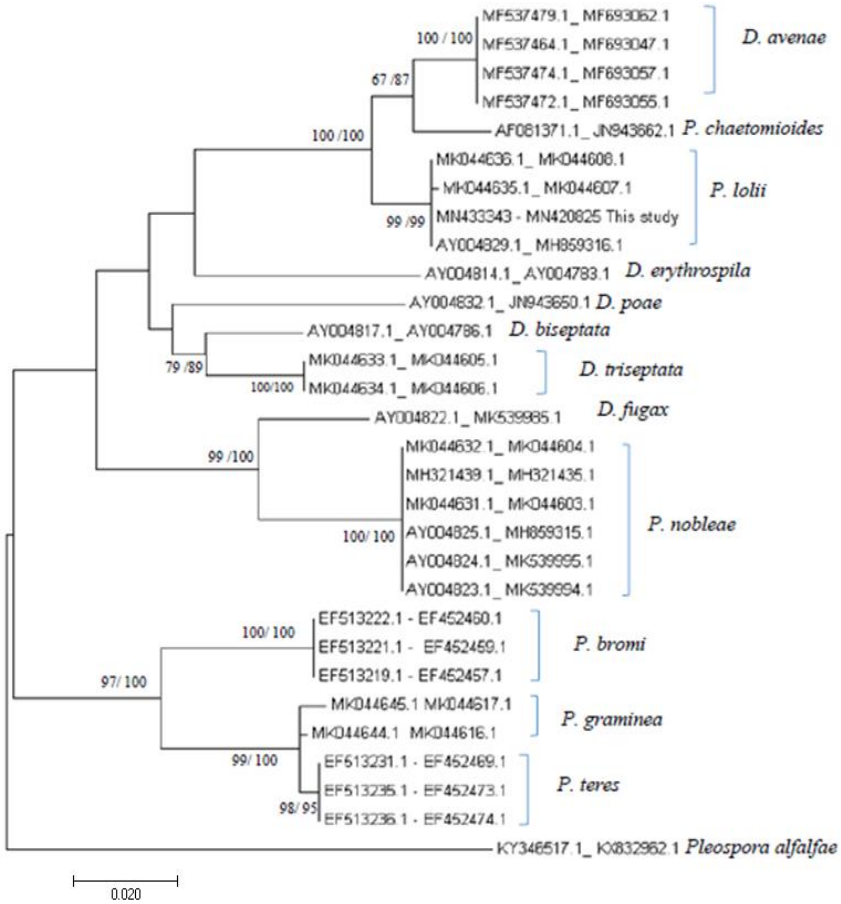

Fig.2. The cladogram was constructed based on integrated ITS and *gpd* sequences of *Pyrenophora* spp. and shows the phylogenetic position of the *Pyrenophora* specimens on *Avena sativa* from Iran among *Pyrenophora* spp. The tree was drawn using a Maximum Likelihood method and rooted with *Pleospora alfalfae.* Bootstrap values were calculated for 1,000 replicates. Number (*/*) on and under branches are bootstrap values for the neighbor-joining and Maximum Likelihood method, respectively. Numbers (*-*) in front of branches are accession numbers for *gpd* and ITS sequences, respectively. *P. = Pyrenophora. D. = Drechslera.*

and *P. avenae* which is host similarity among these species. Results of phylogenetic analysis of both sequences of the *Pyrenophora* specimen agree with the morphological characterization and the specimen was identified as *P. lolii*.

Several species *of Pyrenophora*/*Drechslera* were found in Iran. Some of these species are destructive pathogens on wheat and barley such as *P. tritici-repentis*, *P. graminea* and *P. teres* (Ershad 2009). This study is the first record of *P. lolii* on *Avena sativa* in Iran.

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گزارش جدیدی از گونه *Pyrenophora lolii* برای میکوبیوتای ایران

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چکیده: قارچ *Pyrenophora lolii* (آنامورف *Dreschlera siccans*) عامل لکه برگی گراس‌هایی همچون .spp *Festuca*، *Lolium* .spp، *Dactylis* spp.، *Avena sativa* و *Triticum aestivum* می باشد. برگهای آلوده یولاف (*Avena sativa*) با علایم لکه برگی از حاشیه مزرعه جو در استان گلستان در بهار سال ۹۵ جمع‌آوری شد. بررسی ریخت شناسی نمونه *Pyrenophora* جدا شده از برگ-های یولاف با کاربرد میکروسکوپ نوری انجام شد. مایه زنی برگ‌های یولاف با نمونه *Pyrenophora* در شرایط گلخانه علایم لکه برگی را ایجاد کرد. به منظور تایید بررسی‌های ریخت شناسی، توالی ژن گلیسرآلدهید۳- فسفات دهیدروژناز (*gpd*) و ناحیه ITS به ترتیب با کاربرد آغازگرهای gpd1/2 وITS1/4 تکثیر شدند و در مطالعات فیلوژنی مورد استفاده قرار گرفتند. آنالیزهای فیلوژنی بر اساس هر دو توالی، نمونه *Pyrenophora* جدا شده در این پژوهش را با توالی *P. lolii* گروه‌بندی کرد. براساس نتایج بررسی ریخت شناسی و آنالیز فیلوژنی، قارچ عامل لکه برگی در یولاف در این پژوهش *P. lolii* می باشد.

کلمات کلیدی: ژن *GPDH*، ناحیه ژنومی ITS، یولاف، *Dreschlera siccans*

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