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Identification of the species *Parastagonospora dactylidis* on poaceous plants in Iran

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Abstract: The purpose of this study was to identify new species of *Parastagonospora* in association with poaceous plants in Iran . Symptomatic leaves and ears were collected from different poaceous species in the field from provinces including Fars, Khuzestan and Kohgiluyeh and Boyerahmad .In the present research, 12 isolates based on morphological characteristics and sequencing of LSU and ITS-rDNA regions were recognized as *P. dactylidis* on *Phalaris arundinacea* (five isolates), *Bromus hordeaceus* (three isolates) and *Aegilops tauschii* (four isolates). The species of *P. dactylidis* is reported from Iran for the first time and also all of the identified hosts are new host to the world.

Key words: Host, phylogeny, *Parastagonospora dactylidis*, ITS-rDNA, LSU

INTRODUCTION

Parastagonospora (Berk.) Quaedvlieg, Verkley & Crous belonged to the class Dothideomycetes, order Pleosporales and family Phaeosphaeriaceae (Quaedvlieg et al. 2013). Some species of the genus *Parastagonospora* are responsible for significant crop losses in wheat, barley and rye worldwide (Quaedvlieg et al. 2013). *Parastagonospora* was first defined as *Septoria (*Sacc) (Weber 1922). Based on conidial morphology, *Septoria* and *Stagonospora* (Sacc.) were grouped as two different genera and *Stagonospora* was accepted as the correct description in place of *Septoria*. The conidia of *Stagonospora* species were more than 10 times smaller than those of *Septoria* (Sprague, 1950). Quaedvlieg et al. (2013) introduced and used a new genus *Parastagonospora* (Quaedvlieg, Verkley & Crous) instead of *Stagonospora* genus. Since January 2013, following new rules for naming of pleomorphic fungi outlined in the International Code of Nomenclature for Algae, Fungi and Plants (ICN) as the pleomorphic fungus may have only one name (Wingfield et al. 2012). Therefore, the name *Parastagonospora* has been established as the valid name instead of *Phaeosphaeria* (I. Miyake) (Quaedvlieg et al. 2013).

Today, there are many deficiencies and problems of morphological taxonomy that have been solved using molecular techniques. Because of the overlapping observed in the morphological systematic studies within the genus, molecular techniques based on multilocus DNA sequencing analysis have enabled researchers to solve these problems (Quaedvlieg et al. 2013). In the last two decades, the genus *Parastagonospora* was analyzed using different genes including mating-type loci (Bennett et al. 2003, Ueng et al. 2003), ITS sequences (Ueng et al. 1998), *ß-tubulin* gene (Malkus et al. 2005), *ß-glucosidase* gene (Reszka et al. 2005), RNA polymerase II (Arkadiusz *et al*. 2006), *histidine synthase* gene (Wang et al. 2007) and *ß-xylosidase* gene (McDonald et al. 2012).

So far, three species of the genus *Parastagonospora* including *P. nodorum* (Berk.), *P. avenae* f. sp. *avenaria* (A.B. Frank) (Paa) and *P. avenae* f. sp. *tritici* 5 (A.B. Frank) (Pat5) have been reported based on molecular and morphological characteristics on poaceous plants in Iran (Ghaderi et al. 2017). Therefore, this study was conducted to determine other species of *Parastagonospora* in association with poaceous plants in Iran. Species identification was done according to the morphology of anamorph and molecular confirmation using ITS and LSU sequences.

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MATERIALS AND METHODS

Sampling and morphological characterization

During surveys from 2016 to 2017, symptomatic leaves and ears were collected from different poaceous species in the field from three provinces of Iran including Fars, Khuzestan and Kohgiluyeh and Boyerahmad, and taken to the laboratory. Fresh samples were photographed before collection to save distinctive characters using camera EOS 1300D.

For the isolation of causal agents, the diseased leaves and ears were cut into segments of 4–8 cm, surface-sterilized in 70% ethanol for 1 min, rinsed in sterile water and placed in glass slides with tape and kept under high humidity conditions until the pycnidia produced cirri containing pycnidiospores. Then, colonies were transferred into Petri dishes containing Yeast Sucrose Agar (YSA, 10 $g.L^{-1}$ Yeast Extract, 10 $g.L^{-1}$ sucrose, and 1.2% agar) amended with 50 µg of kanamycin. Plates were stored at 25 °C for five days and transferred to YSA medium for the next steps. To examine colony morphology, isolates were grown on YSA plate containing sterilized poaceous straws. Cultures were incubated at 20 °C and a 12-h photoperiod near ultraviolet light for pycnidia (300 nm <X< 400 nm), and intensities of 400 and 600 pW/cm^2 for 14–30 days to promote sporulation (Halama & Lacoste 1999). *Parastagonospora* species were identified using their morphological characters such as conidia and conidiomata morphology, pigmentation, and colony color and growth rate.

Mating type determination and fertility

Mating type idiomorphs for each isolate was determined using the mating type primers described by Bennett et al. 2003. Multiplex PCR amplifications were also performed as described previously (Sommerhalder *et al*. 2006). PCR amplifications were performed in 20 µl reactions containing 0.05 µM of each primer, 1 × Dream *Taq* Buffer (MBI Fermentas), 0.4 µM dNTPs (MBI Fermentas) and 0.5 units of Dream *Taq* DNA polymerase (MBI Fermentas). The PCR cycle parameters were: 2 min initial denaturantion at 96 °C followed by 35 cycles at 96 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min. A final 7 min extension was made at 72 °C. Negative controls were included in every PCR. The PCR products were separated on 1% agarose gel. The gels were stained with ethidium bromide and visually analyzed under UV light (GelDoc, Bio-Rad Laboratories).

For formation of reproductive form (pseudothecia) of *Parastagonospora*, based on mating type idiomorphs, isolates grown on 2% WA containing sterilized wheat straws cultures at 10 °C and a 12-h photoperiod near ultraviolet light for pseudothecia (300 nm <X> 400 nm), and intensities of 400 and 600 pW/cm^2 for 30–45 days to promote sporulation (Halama & Lacoste 1991).

DNA extraction

Single colonies were transferred to flasks containing 50 ml Yeast Sucrose Broth (YSB, 10$g.L^{-1}$ Yeast Extract, 10$g.L^{-1}$ sucrose) and grown on an orbital shaker for 7 days at 120 rpm at 18 °C. Mycelia were harvested using sterile gauze and rinsed with sterile distilled water. Dried mycelia were crushed into fine powder in liquid nitrogen. Total genomic DNA was isolated using a modified CTAB (cetyltrimethylammonium bromide) technique. Approximately 100 mg mycelium powder was suspended in 900 µL of extraction buffer (100 mM Tris- HCl, 100 mM EDTA, 250 mM NaCl), and then 100 µL of 10% N-Lauroylsarcosine (Sigma-Aldrich, Germany) was added. The suspension was incubated at 60 °C for 60 each tube, chloroform/isoamyl alcohol (24:1) was added. After mixing, the tubes were centrifuged for 10 min at 13000 × g. Upper phase of the suspension was transferred to a new 1.5 ml tube and the equal volume of isopropanol was added where the DNA was precipitated at –20 °C. To precipitate DNA, tubes were centrifuged for 10 min at 13000 × g then the supernatant was discarded gently. The sediment was washed with about 100 ml of 75% ethanol and DNA was precipitated. The pellet was diluted in 100 ml of deionized double-distilled sterile water (Murray & Thompson 1980).

Phylogenetic analysis

The primer pairs LROR-LR5 (Vilgalys and Hester 1990) and ITS4-ITS5 (White et al. 1990) were used to amplify a segment of the large subunit rDNA (LSU) and the internal transcribed spacers (ITS) respectively. PCR amplifications were performed in 20 µl reactions containing 0.05 µM of each primer, 1 × Dream *Taq* Buffer (MBI Fermentas), 0.4 µM dNTPs (MBI Fermentas) and 0.5 units of Dream *Taq* DNA polymerase (MBI Fermentas). The PCR cycle parameters were: 2 min initial denaturation at 96°C followed by 35 cycles of 96°C for 30 s, annealing at 57°C for 45 s, and extension at 72°C for 1 min. A final 10 min extension was applied at 72°C. Finally, the quantity and quality of PCR products were evaluated and visualized on 1.2% agarose gel. The gels were stained with ethidium bromide and visually analyzed under UV light (GelDoc, Bio-Rad Laboratories). The sequencing of PCR products were carried at Macrogen, Korea.

The consensus regions of the LSU and ITS-rDNA loci were blasted separately against the NCBI's GenBank sequence database using Megablast to identify their closest neighbors. LSU and ITS-rDNA sequences were obtained from GenBank mostly following previous literature (Liu et al. 2015) and new sequences described in this manuscript are listed in Table 1.

Multiple sequences were aligned using Bioedit v. 7.0.9 (Hall, 1999) and MAFFT v.7 online interface using default settings (http://mafft. cbrc.jp/alignment /server/) (Katoh & Standley 2013). The alignments were checked visually and improved manually

wherever necessary. Maximum likelihood (ML) methods were used to determine the phylogenetic relationship among isolates using MEGA v. 6 software (Molecular Evolutionary Genetics Analysis) (Tamura et al. 2007). Maximum likelihood bootstrap (BS) values (Reeb et al. 2004) and Bayesian posterior probabilities (PP) values using BEAUti and BEAST v1.6.1 software (Drummond & Rambaut 2007) were used as criteria for strongly supported clades. An isolate of *Pyrenophora tritici-repentis* was used as an outgroup for phylogenetic analyses because *P. tritici-repentis* is closely related to the *Parastagonospoa* species complex (McDonald et al. 2012).

RESULTS AND DISCUSSION

The complete ITS region (949bp) and a segment of the large subunit rDNA (LSU) (869 bp) sequences were concatenated using BEAUti and BEAST v1.6. for phylogenetic analysis of 32 *Parastagonospora* isolates. The best scoring RAxML tree was chosen as the backbone tree and is shown in Fig 1. A maximum likelihood tree was inferred by combined analysis of two loci (Fig 2). Based on the phylogenetic analyses, the twelve isolates from various plants in the present investigation grouped with *P. dactylidis* (Li et al. 2015) in a well-supported clade (ML-BS: 93%, PP: 100%). In fact, phylogenetic analysis confirmed the morphological identification.

In the present research, based on morphological characters, all isolates belonged to *Parastagonospora dactylidis* which is described below:

***Parastagonospora dactylidis* W.J. Li, Camporesi, D.J. Bhat & K.D. Hyde, Mycosphere 6 (6): 691 (2015)**

Specimens examined. IRAN, Fars province, Noorabad, on leaves of *Phalaris arundinacea*, 3 May 2016, F. Ghaderi; IRAN, Fars province, Noorabad, on leaves of *Bromus hordeaceus*, 3 May 2016; IRAN, Golestan province, Aliabad, on ears of *Aegilops tauschii*. 20 April 2017.

Colonies on YSA, reaching 17 mm after 2 weeks at 25 ℃, with dense, white, flat, aerial mycelium, with rounded, smooth, margins. There were partial differences in colonies color on YSA (Fig. 1. a-b). Conidiomata pycnidial, brown to black, subglobose to lenticular, immersed, formed mostly on sterilized wheat straws, exuding pale pink conidial cirrhus, 100–130µm in diameter, with a single ostiole, circular, papillate 14–17 µm wide (Fig. 1. c-f). Conidia hyaline, smooth, thin-walled, fusiform, curved, rounded at both ends, slightly narrower at the base, 3-septate, 21–42 × 4–5.3 µm (Fig. 1. g-h).

Table 1. Characteristics of *Parastagonospora* isolates used in phylogenetic analysis

Species name	strain	Host	Country	GenBank accession no.	
				ITS	LSU
Parastagonospora nodorum	CBS 110109	*Lolium perenn*	Denmark	KF251177 [a]	KF251681[a]
P. uniseptata	MFLUCC 13–0387 [T]	*Daucus* sp.	Italy	KU058715 [a]	KU058725 [a]
P. nigrans	CBS 307.79	*Zea mays*	Switzerland	KF251184 [a]	KF251687 [a]
P. poae	CBS 135091	*Poa* sp.	Netherlands	KF251179 [a]	KF251683 [a]
P. poae	CBS 135089T	*Poa* sp.	Netherlands	KF251178 [a]	KF251682 [a]
P. poagena	CBS 136776T	*Poa* sp.	Netherlands	KJ869116 [a]	KJ869174[a]
P. italic	MFLUCC 13–0377 [T]	*Dactylis* sp.	Italy	KU058714[a]	KU058724[a]
P. caricis	CBS 135671/S615 [T]	*Carex acutiformis*	Netherlands	KF251176[a]	KF251680[a]
P. avenae	CBS 290.69	*Lolium perenne*	Germany	KF251175[a]	KF251679[a]
P. avenae	CBS 289.69	*Lolium perenne*	Germany	KF251174[a]	KF251678[a]
P. dactylidis	MFLUCC 13–0375 [T]	*Dactylis* sp.	Italy	KU058712[a]	KU058722[a]
P. allouniseptata	MFLUCC 13–0386 [T]	*Dactylis glomerata*	Italy	KU058711[a]	KU058721[a]
P. typharum	CBS 296.54	*Nardus stricta*	Switzerland	KF251192[a]	KF251695[a]
P. alpine	CBS 456.84	*Phleum alpinum*	Switzerland	KF251181[a]	KF251684[a]
P. papaya	CBS 135416/S528 [T]	*Carica papaya*	Brazil	KF251187[a]	KF251690[a]
P. oryzae	CBS 110110T	*Oryza sativa*	South Korea	KF251186[a]	KF251689[a]
P. thysanolaenicola	MFLUCC 10–0563 [T]	*Thysanolaena maxima*	Thailand	KM434266[a]	KM434276[a]
P. chiangraina	MFLUCC 13–0231 [T]	*Oryza sativa*	Thailand	KM434270[a]	KM434280[a]
P. musae	CBS 120026T	Unknown	Unknown	DQ885894[a]	GU301862[a]
P. minima	MFLUCC 13–0376 [T]	*Dactylis* sp.	Italy	KU058713[a]	KU058723[a]
P. dactylidis	IRAN-1	***Phalaris arundinacea***	Iran	MK032162[b]	MK078104[b]
P. dactylidis	IRAN-2	***P. arundinacea***	Iran	MK032163[b]	MK078105[b]
P. dactylidis	IRAN-3	***P. arundinacea***	Iran	MK032164[b]	MK078106[b]
P. dactylidis	IRAN-4	***P. arundinacea***	Iran	MK032165[b]	MK078107[b]
P. dactylidis	IRAN-5	***P. arundinacea***	Iran	MK032166[b]	MK078108[b]
P. dactylidis	IRAN-6	***Bromus hordeaceus***	Iran	MK032167[b]	MK078109[b]
P. dactylidis	IRAN-7	***B. hordeaceus***	Iran	MK032168[b]	MK078110[b]
P. dactylidis	IRAN-8	***B. hordeaceus***	Iran	MK032169[b]	MK078111[b]
P. dactylidis	IRAN-9	***Aegilops tauschii***	Iran	MK032170[b]	MK078112[b]
P. dactylidis	IRAN-10	***A. tauschii***	Iran	MK032171[b]	MK078113[b]
P. dactylidis	IRAN-11	***A. tauschii***	Iran	MK032172[b]	MK078114[b]
P. dactylidis	IRAN-12	***A. tauschii***	Iran	MK032173[b]	MK078115[b]

[T] signifies epitype isolates; [a]. Cited from Carris et al. 2007; [b]. From this study

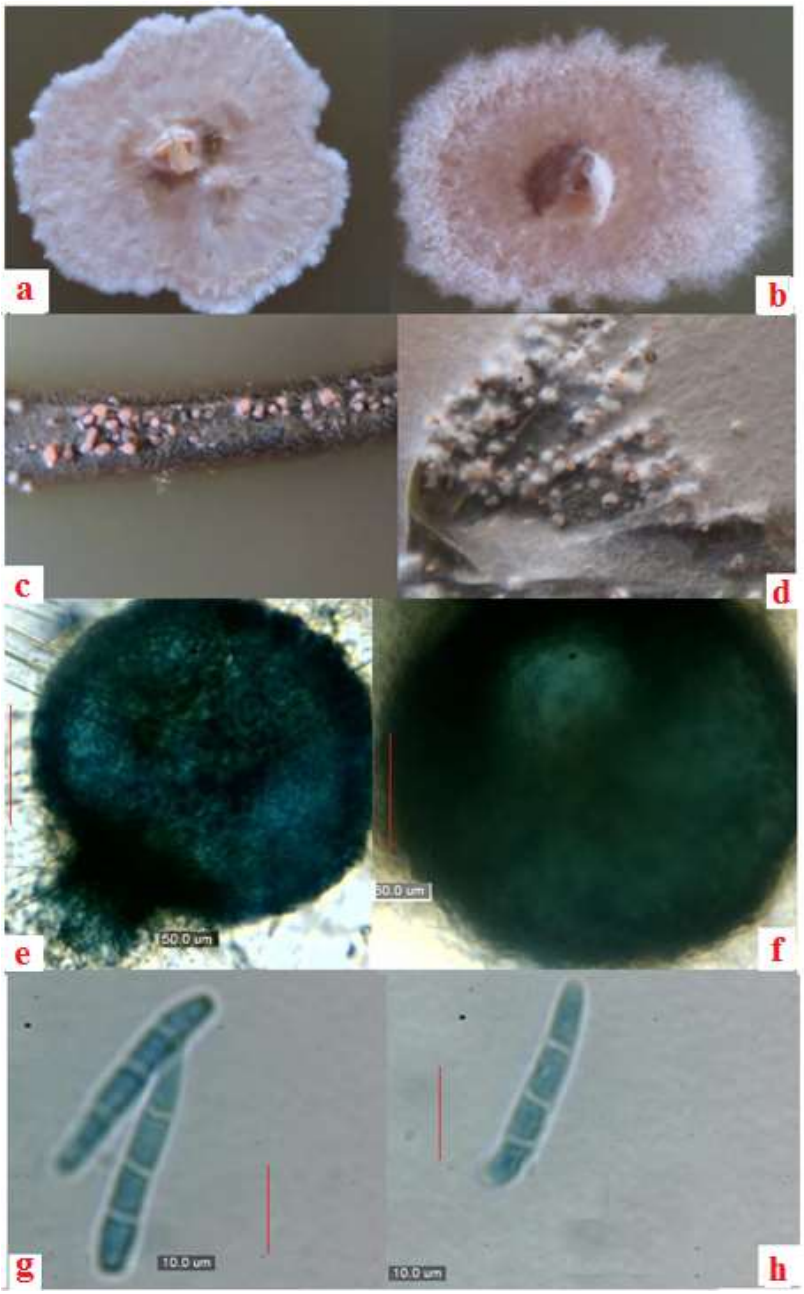

Fig. 1. Colony of *Parastagonospora dactylidis* on YSA (a–b), Pycnidia and pycnidiospores formed in culture medium (c–d), Pycnidium (e–f) (Bar = 50 µm), Pycnidiospores (g–h) (Bar = 10 µm).

This fungus is diallelic heterothallic. Sampled isolates from different places Iran, has indicated both *Mat1-1* and *Mat1-2*. So, the sexual phase formation is related to both of mating type idiomorphs. Pseudothecia formation of *P. dactylidis* is obtained *in vitro* on sterilized straws under strict conditions of light and temperature after one month. Ascocarps in longitudinal rows immersed on sterilized straws were subepidermal, globose, glabrous, 120–150 µm wide and 136–162 µm high, but we did not observe ascus and ascospores within pseudothecia.

Notes. *Parastagonospora dactylidis* is closely related to *P. minima*. However, these two species can be easily distinguished based on conidia morphology. *P. dactylidis* has fusiform conidia with a slightly narrower base, and distinctly granular cytoplasm, whereas *P. minima* has subcylindrical conidia which are wider in the basal half, and narrow at the apex. In addition, the conidiomata and conidia of *P. dactylidis* are larger and longer than those of *P. minima* (conidiomata: 40–70 µm high, 50–100 µm diam., conidia: 20–28 × 3.5–4.5 µm). Furthermore, in the present study, we also clarified the phylogenetic positions of *P. dactylidis* and *P. minima.*

The morphological characters cannot always provide all the necessary data to define a species; therefore, DNA data is useful for precise identification of the fungal species. The taxonomy of *Parastagonospora* is extremely complicated and unreliable (Quaedvlieg et al. 2013), hence

identification of the species of this genus based on morphological characters is unclear, and specific morphological features to describe and identify *Parastagonospora* species are rather limited so that, further research is required to resolve this issue (Quaedvlieg et al. 2013). Overall, the genus *Parastagonospora* is pleomorphic and propagate through sexual or asexual reproduction in field, but it is almost difficult to produce both of sporulating structures (asexual morph adjacent to the sexual morph) on artificial media in laboratory that could help distinguish species of the genus *Parastagonospora* (Quaedvlieg et al. 2013, Shoemaker and Babcock, 1989).

In this study, none of the isolates produced pseudothecia when grown alone. The sexual stage is induced in the laboratory and controlled by mating type idiomorphs (Bennett et al. 2003). But pseudothecia formation obtained *in vitro* and we could not observe ascus and ascospores within pseudothecia to distinguish species.

Consequently, DNA sequence analysis and phylogenetic inference can irrefutably establish asexual and sexual morph connections, for example, the *Mycosphaerella arbuticola* / *Septoria unedonis*, *Phaeosphaeria papaya* / *Phaeoseptoria oryzae*, *Leptosphaeria maculans* / *Plenodomus lingam*, and also provide phylogenetic placements for asexual taxa within the modern taxonomic classification schemes (Quaedvlieg et al. 2013, Ariyawansa et al. 2015).

In the present report, ML consensus tree was supported by Bayesian posterior probabilities and bootstrap values. We could separate the morphological species for genus *Parastagonospora* using combined gene (LSU and ITS). In fact, sequence data together with morphology are used to delimit and propose one new species for the mycobiota of Iran that is named *P. dactylidis*. The correlation between phylogenetic and morphological species are in correspondence with phylogenetic analyses by Li et al. (2015) examining the relationship among species of *Parastagonospora* spp. They carried out a revision of *Phaeosphaeriaceae* based on multigene (LSU and ITS) sequence analyses coupled with morphological data and introduced the new species *Parastagonospora dactylidis*, *P. minima*, *P. italica*, *P. uniseptata* and *P. allouniseptata* in Italy.

In this research, *P. dactilitis* is reported for the first time from Iran that identified on *Phalaris arundinacea*, *Bromus hordeaceus*, and *Aegilops tauschii* where all of the recognized hosts are new to the world.

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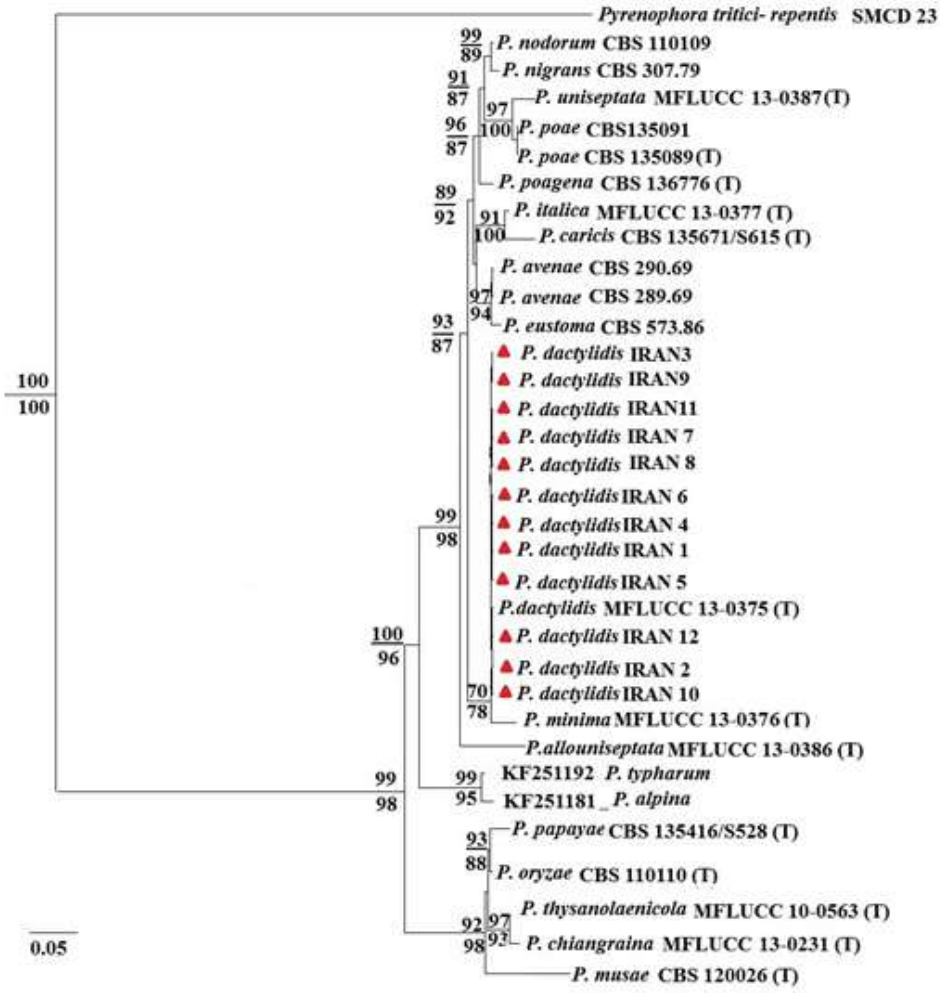

Fig 2. Maximum likelihood tree of Parastagonospora isolates obtained from analysis of ITS and LSU sequence data. Branch length indicates the substitution rate. Maximum likelihood bootstrap (BS) values are indicated on branches, and Bayesian posterior probabilities (PP) values are indicated under branches. The ex-type strains are in parenthesis. The Iranian specimens are shown with bold triangle labels .The tree is rooted to Pyrenophora tritici-repentis.

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شناسایی گونه *Parastagonospora dactylidis* روی تیره گندمیان در ایران

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چکیده: هدف از این مطالعه، شناسایی گونه‌های جدیدی از جنس *Parastagonospora* مرتبط با گیاهان گرامینه در ایران می‌باشد. در این پژوهش، نمونه‌برداری از برگ و خوشه‌های علف‌های هرز مزارع گندم در مناطق مختلف استان فارس، خوزستان و بوشهر صورت گرفت. بر اساس خصوصیات ریخت‌شناسی و توالی ناحیه rDNA-ITS و LSU، ۱۲ جدایه مربوط به گونه *P. dactylidis* از روی *Phalaris arundinacea* (پنج جدایه)، *Bromus hordeaceus* (سه جدایه) و *Aegilops tauschii* (چهار جدایه) شناسایی گردید. این نخستین گزارش از گونه *P. dactylidis* برای ایران می‌باشد و همچنین تمامی گونه‌های گندمیان مورد مطالعه در این تحقیق، به عنوان میزبان‌های جدیدی در دنیا برای گونه *P. dactylidis* معرفی می‌شوند.

کلمات کلیدی: میزبان، فیلوژنی، *Parastagonospora dactylidis*، ITS-rDNA، LSU

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