Introduction of some species of *Parastagonospora* on poaceous plants in Iran

Received: 07.06.2017 / Accepted: 14.10.2017

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Abstract

The main purpose of this study was to identify species of *Parastagonospora* in association with poaceous plants in Iran. Some species of *Parastagonospora* like *P. nodorum* causes a major loss to wheat production in the world. In the present research, two species including *P. nodorum*, and *P. avenae* were identified from different poaceous species. *Parastagonospora nodorum* was identified on *Phalaris arundinacea*. However, *P. avenae* was divided into two formae specials i.e. *P. avenae* f. sp. *avenaria* (Paa) on *Phalaris arundinacea* and *Avena sativa*, and the identified *P. avenae* f. sp. *tritici*5 (Pat5) on *Phalaris arundinacea*, *Bromus hordeaceus* and *Aegilops tauschii*. Species identification was done according to morphology of anamorph and molecular confirmation was performed on the basis of ß-xylosidase gene sequence. Pat5 and Paa are reported from Iran for the first time and also all of the identified hosts are new to the world.

Keywords: ß-xylosidase, glum blotch, morphology, new species, phylogeny

معرفی گونه‌هایی از جنس *Parastagonospora* روی گیاهان تیره گندمیان در ایران*

دریافت: ۱۳۹۶/۰۳/۱۷ / پذیرش: ۱۳۹۶/۰۷/۲۲

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خلاصه

این مطالعه، با هدف شناسایی گونه‌های *Parastagonospora* مرتبط با گیاهان تیره گندمیان در ایران انجام شده است. برخی گونه‌های جنس *Parastagonospora* مانند *P. nodorum*، خسارت قابل ملاحظه‌ای به این محصول در دنیا وارد می‌کنند. در این تحقیق، دو گونه از جنس *Parastagonospora* شامل *P. nodorum* و *P. avenae* روی گندمیان شناسایی شدند. گونه *P. nodorum* از روی *Phalaris arundinacea* شناسایی شد. گونه *P. avenae* نیز به دو گروه تقسیم شد که گروه اول آن، *P. avenae* f. sp. *avenaria* (Paa) از روی *Phalaris arundinacea* و *Avena sativa* و گروه دوم، *P. avenae* f. sp. *tritici*5 (Pat5) از روی *Phalaris arundinacea*، *Bromus hordeaceus* و *Aegilops tauschii* شناسایی شد. شناسایی این گونه‌ها براساس مشخصات ریخت‌شناسی مرحله غیرجنسی و تایید مولکولی آن‌ها براساس توالی‌یابی ژن بتا زایلوسیداز انجام گرفت. گروه‌های Pat5 و Paa برای نخستین بار از ایران گزارش می‌شوند و همچنین تمامی گندمیان مورد مطالعه در این تحقیق، به عنوان میزبان‌های جدید برای گونه‌های گزارش شده معرفی می‌شوند.

واژه‌های کلیدی: بتا زایلوسیداز، ریخت‌شناسی، سوختگی خوشه، فیلوژنی، گونه جدید

* بخشی از رساله دکترای نگارنده اول به راهنمایی دکتر بهرام شریف‌نبی و دکتر محمد جوان نیکخواه ارایه شده به دانشکده کشاورزی دانشگاه صنعتی اصفهان

Introduction

Parastagonospora Quaedvlieg, Verkley & Crous is haploid fungal pathogen within the class *Dothideomycetes*, order *Pleosporales* (Solomon *et al*. 2006). The primary hosts are domesticated wheats (*Triticum aestivum*, and *Triticum durum*) and *Triticale* (Solomon *et al*. 2006, Zhang *et al*. 2009).

Parastagonospora was originally described as *Septoria* (Weber 1922). Based on conidial morphology, *Septoria* and *Stagonospora* were grouped as two different genera and *Stagonospora* was recognized as the correct designation in place of *Septoria.* The conidia of *Stagonospora* species are more than 10 times smaller than those of *Septoria* (Sprague 1950). Quaedvlieg *et al*. (2013) introduced a new genus *Parastagonospora*. The teleomorphic stage of *Stagonospora* was assigned to the genus *Leptosphaeria* (Weber 1922). Miyake (1909) completely illustrated *Stagonospora* based on ascocarps and ascospores morphology but his results were not recorded. Hedjaroude reclassified it as *Phaeosphaeria* in the late 1960's (Shoemaker & Babcock 1989 Cunfer & Ueng 1999).

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 (Norvell 2011, Wingfield *et al*. 2012). Therefore, the name *Phaeosphaeria* is no more applied but instead the name *Parastagonospora* has been accepted as the valid name (Quaedvlieg *et al*. 2013).

The genus *Parastagonospora*, has immersed ascocarps, globose, becoming depressed, medium brown to black; wall of 3–6 layers of thick-walled, brown textura angularis; ostiole slightly papillate. Asci clavate, cylindrical or curved, shortly stipitate, 8-spored; ascus wall thick, bitunicate. Ascospores fusoid, subhyaline to pale brown, transversely euseptate (–3), constricted at the septa, penultimate cell swollen. Pseudoparaphyses filiform, hyaline, septate. Conidiomata black, immersed, subepidermal, pycnidial, subglobose with central ostiole, exuding creamy conidial mass; wall of 2–3 layers of brown textura angularis. Conidiophores reduced to conidiogenous cells. Conidiogenous cells phialidic, hyaline, smooth, aggregated. Conidia hyaline, smooth, thin-walled, cylindrical, granular to multi-guttulate, with obtuse apex and truncate base, and transversely euseptate (Quaedvlieg *et al*. 2013).

The genus *Parastagonospora* was included as a number of significant plant pathogens with global distribution, and associated with leaf spot diseases on many cultivated and wild plants. Some important plant pathogenic species include *P. nodorum* on wheat, barley, *Triticale* and poaceous plants and *P. avenae* on graminicolous hosts causing major yield losses (Weber 1922, De DeWolf & Francel 2000, McDonald *et al*. 2012).

Parastagonospora nodorum is a heterothallic, with two mating types and the presence of both is required for sexual reproduction (Halama & Lacoste 1992). The sexual stage of the fungus can also be an important inoculum source for early and primary infections. Pseudothecia are produced during the entire growing season and their ascospores are easily spread by wind which infect wheat seedlings in autumn and spring seasons (McFadden & Harding 1989). The conditions for production of both types of fruiting bodies and release and germination of spores are similar since they are dependent on near-UV (NUV) light and moisture, respectively (Eyal *et al*. 1987, Halama & Lacoste 1992).

The morphological characters can not always provide all the necessary data to define a species, therefore, DNA data is useful for precise identification of species. The taxonomy of *Parastagonospora* is extremely complicated and unreliable, hence identification of the species of this genus based on morphological traits 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. Today, many deficiencies and problems of morphological

taxonomy have been solved using molecular techniques. Due to 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). Previous investigations have also indicated that, *P. nodorum* probably resembles a species complex which needs further morphological characterization (McDonald *et al*. 2012).

In the last two decades, the genus *Parastagonospora* was analyzed using six additional single gene phylogenies including mating-type loci (Bennett *et al*. 2003, Ueng *et al*. 2003), ITS sequences (Ueng *et al*. 1998), β-tubulin (*tubA*) (Malkus *et al*. 2005), β-glucosidase (*bgl1*) (Reszka *et al*. 2005), RNA polymerase II (*rpb2*) (Arkadiusz *et al*. 2006) and histidine synthase (*his*) (Wang *et al*. 2007). McDonald *et al*. (2012) examined 300 *Phaeosphaeria* isolates from farmer's fields of five continents over 25 years in addition to more than 50 isolates of wild grasses. They used a three-gene phylogeny (β-xylosidase gene, β-tubulin gene and rDNA-ITS) to determine the relationships among isolates within the *Parastagonospora* sp. complex, using both traditional phylogenetic and newer coalescent methods. Their analysis separated clades i.e. *P. nodorum*, *P. avenae* f. sp. *avenaria* (Paa), and *P. avenae* f. sp. *tritici* (Pat) into five groups, namely, Pat1, Pat3, Pat4, Pat5 and Pat6 (McDonald *et al*. 2012).

So far, only *P. nodorum* report is based on morphological characteristics on wheat from Iran (Aghajani *et al*. 2002). Therefore, this study was conducted in Iran to determine *Parastagonospora* species in association with poaceous plants. Species identification was done according to morphology of anamorph and molecular confirmation using ß-xylosidase gene sequence.

Materials and Methods

- Sampling and fungal isolation

Symptomatic leaves and wheat ears were collected in the field from different provinces of Iran including: Kohgiluyeh and Boyerahmad, Khuzestan, Fars, and Boushehr, and taken to the laboratory. Only one isolate was collected from each plant. 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. Colonies were then transferred into Petri dishes containing Yeast Sucrose Agar (YSA, 10g/L Yeast Extract, 10g/L sucrose, 1.2% agar) amended with 50 µg of kanamycin. Plates were stored at 25 °C for five days. Then, colonies were transferred to YSA medium for the next steps.

- Fungal purification and morphological characterization

a. Asexual form

Pargstagonospora isolates were recovered from poaceous plants and purified by single-spore isolation. Pure cultures of the isolates were stored on lyophilized filter-paper strips at –80 °C.

To examine colony morphology, isolates were grown on plates: YSA-$CaCo_3$ and PDA-V8-$CaCo_3$ (150 ml V8 juice, 3 g $CaCo_3$, 30 g sucrose, 10 g Difco PDA, 16 g agar in 1000 ml water) containing sterilized wheat straws. Cultures were incubated at 20 °C and a 12-h photoperiod near ultraviolet light for perithecial (300 nm <X> 400 nm), and intensities of 400 and 600 pW/cm2 for 14–30 days to promote sporulation (Halama & Lacoste 1991).

Parastagonospora species were identified using their morphological characters such as colony color, conidia and conidiomata morophology, pigmentation, and colony growth rate.

b. Sexual form

Mating type idiomorphs for each isolate was determined using the mating type primers described above (Bennett *et al*. 2003). These primers amplified a specific 510 bp PCR product for *MAT1-2* isolates and a specific 360 bp PCR product for *MAT1-1* (Table 2).

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 denaturation 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 analysed under UV light (GelDoc, Bio-Rad Laboratories). For formation of reproductive form (perithecial) of *Parastagonospora*, isolates grown on 2% WA containing sterilized wheat straws cultures and were then incubated at 10 °C and a 12-h photoperiod near ultraviolet light for perithecial (300 nm <X> 400 nm), and intensities of 400 and 600 pW/cm2 for 30–45 days to promote sporulation (Halama & Lacoste 1991).

Parastagonospora species were identified using their morphological characters such as colony color, ascospore and ascomycota morophology.

- Genomic DNA extraction

Isolates were grown on Petri dishes containing Yeast Sucrose Agar amended with 50 µg of kanamycin. Single colonies were transferred to flasks containing 50 ml Yeast Sucrose Broth (YSB, 10g/L Yeast Extract, 10g/L sucrose) and grown on an orbital shaker for 5–7 days at 120 rpm at 18 °C. Mycelium 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 (cetyltrimethyl ammonium 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 min and centrifuged for 10 min at 13000 × g in a Beckman microfuge (Beckman, USA). Upper phase suspension was transferred to a new microtube. 100 ml of solution of NaCl 5 mM, and 200 ml CTAB 5% were respectively added to each tube which placed at 65 °C for 10 min. According to the volume of material contained in each tube, chloroform/isoamyl alcohol (24:1) was added. After mixing, the tubes were centrifuged for 10 min at 13000 × g. Upper phase of 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).

- Pathogenicity test

Pathogenicity test was conducted in greenhouse on Chamran cutivar, due to the comparison of pathogenicity mean which showed the most disease severity percentage, was related to Chamran Cultivar (Ghaderi *et al*. 2016). Inoculum was prepared by placing a 1 cm^2 piece of agar culture into flasks containing 250 mL YSB and grown on an orbital shaker for 3–5 days at 120 rpm at 18 °C. Inoculum was diluted to 4×10^6 spores mL^{-1} and one drop of Tween 20 per 100 ml solution was added to the inoculum before inoculation. Plants were inoculated by hand sprayer and were covered with a 5 × 10 cm plastic bag for 48 h.

- Phylogenetic analysis

Isolates used in this study, are described in Table 1. A partial sequence of β-xylosidase gene was amplified by PCR using gene specific primers (Table 2). These primers amplified a specific 962 bp PCR product. PCR amplification was 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 30s, annealing at 56 °C for 45 s, and extend at 72 °C for 1 min. A final 7 min extension was applied at 72 °C. Lastly, quantity and quality of PCR products were evaluated and visualized on 1.5% agarose gel. The gels were stained with ethidium bromide and visually analysed under UV light (GelDoc, Bio-Rad Laboratories).

Sequencing reactions mixture were conducted in 10 µl volume using the BigDye® Terminator ver. 3.1 Sequencing Standard Kit (Life Technologies, Applied Biosystems) with both the reverse and forward primers. The PCR cycle parameters were for 2 min at 96 °C followed by 55 or 99 cycles for 10 s at 96 °C, for 5 s at 50 °C and for 4 min at 60 °C. PCR products were cleaned with the illustraTM SephadexTM G-50 fine DNA Grade column (GE Healthcare) according to the manufacturer's recommendations and sequenced with a 3730xl Genetic Analyzer (Life Technologies, Applied Biosystems). Forward and reverse sequences were aligned using the Program Sequencer 5.1 (Gene Code, Ann Arbor, MI).

The consensus regions of ß-xylosidase were blasted against the NCBI's GenBank sequence database using Megablast to identify their closest neighbors. The obtained sequences from GenBank together with the novel generated sequences during this study, were aligned with MAFFT ver. 7 onlineinterface using default settings (http://mafft. cbrc.jp/alignment/server/) (Katoh & Standley 2013). DNA sequence data were analyzed in MEGA (Molecular Evolutionary Genetics Analysis) ver. 6 software (Tamura *et al*. 2007).

Neighbor-Joining method (Saitou & Nei 1987) was used to determine the phylogenetic relationship among isolates. Orthologous sequences from *Pyrenophora tritici-repentis* were used to root the phylogenies. All new sequences generated in this study were deposited in NCBIs GenBank nucleotide database (www.ncbi.nlm.nih.gov) (Table 1). The GenBank accession numbers for the strains obtained from NCBI can be found in McDonald *et al*. (2012) (Table 1).

Results and Discussion

In this study, based on morphological characters, isolates belonged to two *Parastagonospora* species *viz*. *P. nodorum* and *P. avenae* are described below:

Parastagonospora nodorum (Berk.) Quaedvlieg, Verkley & Crous, Studies in Mycology 75: 363 (2013)

= *Phaeosphaeria nodorum* (E. Müll.) Hedjar. Sydowia 22 (1–4): 79 (1969)

Specimen examined: As in Table 1

Parastagonospora nodorum was found on *Phalaris arundinacea* (Gorgan), 2014

Morphology on YSA: Colonies on YSA were flat, undulate and hyaline. The optimum growth temperature was 25 °C, colony growth rate was 10 mm after seven days. There were no significant differences in morphological characters of colonies on YSA. Conidiomata pycnidia, brown to black, subglobose to lenticular, formed mostly on sterilized wheat straws, exuding pale pink conidial cirrhus, 160–210 µm in diameter, with a single ostiolum of 15–20 µm in diameter (Fig. 1, A-B). Conidia hyaline, smooth, thin-walled, subcylindrical, with subobtuse apex and truncate base, 1–2-septate, 14–27 × 2.9–4.5 µm (Fig. 1, C-D).

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

Taxon	Isolate	Source	Location	Host	Iran collection Information	GenBank accession No.
Parastagonospora nodorum	AVR10	McDonald *et al.* 2012	North America	*Leymus cinereus*	-	JQ757531
P. nodorum	AVR2	McDonald *et al.* 2012	North America	*Agropyron* sp.	-	JQ757523
P. avenae f. sp. *avenaria* (Paa)	S258	McDonald *et al.* 2012	Netherlands	*Avena sativa*	-	JQ757854
P. avenae f. sp. *tritici*1 (Pat1)	A2	McDonald *et al.* 2012	Canada	*Triticum aestivum* seed	-	JQ757572
P. avenae f. sp. *tritici*1 (Pat1)	A19	McDonald *et al.* 2012	Canada	*T. aestivum* seed	-	JQ757571
P. avenae f. sp. *tritici*3 (Pat3)	I 34	McDonald *et al.* 2012	Denmark	*Triticum aestivum*	-	JQ757769
P. avenae f. sp. *tritici*3 (Pat3)	I 35	McDonald *et al.* 2012	Denmark	*Triticale*	-	JQ757770
P. avenae f. sp. *tritici*4 (Pat4)	R4	McDonald *et al.* 2012	Iran	*Dactylis glomerata*	-	JQ757546
P. avenae f. sp. *tritici*4 (Pat4)	R3	McDonald *et al.* 2012	Iran	*D. glomerata*	-	JQ757545
P. avenae f. sp. *tritici*5 (Pat5)	AVR3	McDonald *et al.* 2012	North America	*Bromus inermis*	-	JQ757877
P. avenae f. sp. *tritici*5 (Pat5)	AVR4	McDonald *et al.* 2012	North America	*Elymus angustu*	-	JQ757525
P. avenae f. sp. *tritici*6 (Pat6)	R11	McDonald *et al.* 2012	Iran	*Agropyron tauri*	-	JQ757553
P. avenae f. sp. *tritici*6 (Pat6)	R17	McDonald *et al.* 2012	Iran	*Dactylis glomerata*	-	JQ757558
P. nodorum	P1	This study	Golestan (Ali Abad)	*Phalaris arundinacea*	IRAN 3049 C	MF004425
P. avenae f. sp. *tritici*5 (Pat5)	P2	This study	Khuzestan (Dezful)	*P. arundinacea*	IRAN 2840 C	MF004426
P. avenae f. sp. *tritici*5 (Pat5)	P3	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2841 C	MF004427
P. avenae f. sp. *tritici*5 (Pat5)	P4	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2842 C	MF004428
P. avenae f. sp. *tritici*5 (Pat5)	P5	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2843 C	MF004429
P. avenae f. sp. *tritici*5 (Pat5)	P6	This study	Golestan (Gorgan)	*Aegilops tauschii*	IRAN 2844 C	MF004430
P. avenae f. sp. *tritici*5 (Pat5)	P7	This study	Golestan (Gorgan)	*Bromus hordeaceus*	IRAN 2845 C	MF004431
P. avenae f. sp. *tritici*5 (Pat5)	P8	This study	Fars (Noor Abad)	*B. hordeaceus*	IRAN 2846 C	MF004432
P. avenae f. sp. *tritici*5 (Pat5)	P9	This study	Fars (Noor Abad)	*B. hordeaceus*	IRAN 2848 C	MF004433
P. avenae f. sp. *tritici*5 (Pat5)	P10	This study	Fars (Noor Abad)	*B. hordeaceus*	IRAN 2849 C	MF004434
P. avenae f. sp. *avenaria* (Paa)	P11	This study	Fars (Noor Abad)	*Phalaris arundinacea*	IRAN 2850 C	MF004435
P. avenae f. sp. *avenaria* (Paa)	P12	This study	Khuzestan (Dezful)	*P. arundinacea*	IRAN 2851 C	MF004436
P. avenae f. sp. *avenaria* (Paa)	P13	This study	Khuzestan (Dezful)	*P. arundinacea*	IRAN 2852 C	MF004437

Table 1 (contd)

P. avenae f. sp. *avenaria* (Paa)	P14	This study	Khuzestan (Dezful)	*P. arundinacea*	IRAN 2853 C	MF004438
P. avenae f. sp. *avenaria* (Paa)	P15	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2854 C	MF004439
P. avenae f. sp. *avenaria* (Paa)	P16	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2855 C	MF004440
P. avenae f. sp. *avenaria* (Paa)	P17	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2856 C	MF004441
P. avenae f. sp. *avenaria* (Paa)	P18	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2848 C	MF004442
P. avenae f. sp. *avenaria* (Paa)	P19	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2857 C	MF004443
P. avenae f. sp. *avenaria* (Paa)	P20	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2858 C	MF004444
P. avenae f. sp. *avenaria* (Paa)	P21	This study	Fars (Noor Abad)	*Avena sativa*	IRAN 2859 C	MF004445
P. avenae f. sp. *avenaria* (Paa)	P22	This study	Golestan (Gorgan)	*A. sativa*	IRAN 2860 C	MF004446
P. avenae f. sp. *avenaria* (Paa)	P23	This study	Golestan (Gorgan)	*A. sativa*	IRAN 2861 C	MF004447
P. avenae f. sp. *avenaria* (Paa)	P24	This study	Golestan (Gorgan)	*A. sativa*	IRAN 2862 C	MF004448
P. avenae f. sp. *avenaria* (Paa)	P25	This study	Golestan (Gorgan)	*A. sativa*	IRAN 2863 C	MF004449
P. avenae f. sp. *avenaria* (Paa)	P26	This study	Golestan (Gorgan)	*Phalaris arundinacea*	IRAN 2864 C	MF004450
P. avenae f. sp. *avenaria* (Paa)	P27	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2865 C	MF004451
P. avenae f. sp. *avenaria* (Paa)	P28	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2866 C	MF004452
P. avenae f. sp. *avenaria* (Paa)	P29	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2867 C	MF004453
P. avenae f. sp. *avenaria* (Paa)	P30	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2868 C	MF004454
P. avenae f. sp. *avenaria* (Paa)	P31	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2869 C	MF004455
P. avenae f. sp. *avenaria* (Paa)	P32	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2870 C	MF004456
P. avenae f. sp. *avenaria* (Paa)	P33	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2871 C	MF004457
P. avenae f. sp. *avenaria* (Paa)	P34	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2872 C	MF004458
P. avenae f. sp. *avenaria* (Paa)	P35	This study	Golestan (Gorgan)	*P. arundinacea*	IRAN 2873 C	MF004459

Table 2. List of primers used for ß-xylosidase gene and mating type idiomorphs amplification sequenced in this study

Locus	Length of sequencea (pb)	Sequence (5'-3')	Reference
Bxylo_F	962	CAAAGAACCCATTGTCACACAC	McDonald *et al*. 2012
Bxylo_R	962	GCTGTTCTTCAGCCAACTTGAT	McDonald *et al*. 2012
MAT1-1	360	CTTCACGACCGGCCAGATAGT	Bennett *et al*. 2003
MAT1-2	360	CAGAGGCTTGTCGGGTTCAT	Bennett *et al*. 2003
MAT2-1	510	ACCCCGCCCCATGAACAAGTG	Bennett *et al*. 2003
MAT2-2	510	CTAGACCGGCCCGATCAAGACCAAAGAAG	Bennett *et al*. 2003

The PCR amplification of the mating type idiomorphs produced single amplicons in all isolates corresponding to either *MAT1-1* or *MAT1-2* (Fig. 2). Perithecial formation of *Parastagonospora nodorum* is obtained *in vitro* on sterilized wheat straws under strict conditions of light and temperature after 45 days. Ascocarps in longitudinal rows immersed on sterilized wheat straws were subepidermal, globose, glabrous, 120–150 µm wide and 130–160 µm high, but we did not observe ascus and ascospores within perithecia (Fig. 3, A).

Parastagonospora is distinguished from *Stagonospora* as the latter has conidiogenous cells which proliferate percurrently or via phialides with periclinal thickening, and subcylindrical to fusoid-ellipsoidal conidia. Sexual morphs known for species of *Parastagonospora* and *Stagonospora* are *Phaeosphaeria* and *Didymella*, respectively (Quaedvlieg *et al*. 2013).

Historically, *P. nodorum* was often mistaken with *Zymoseptoria tritici*, formerly *Mycosphaerella graminicola* (anamorph: *Septoria tritici*), as both pathogens form brown necrotic lesions on the leaves. Both also have similar looking asexual and sexual fruiting structures. The asexual stage appears as black or brown pycnidia in the leaf surface. Sexual ascospores are produced in pseudothecia and are believed to be the main over-wintering structure. *P. nodorum*, unlike *Z. tritici*, is able to infect and form fruiting structures on the glume and is classified as a necrotroph, feeding exclusively from dead plant tissue (Halama *et al*. 1999, Quaedvlieg *et al*. 2011).

So far, *P. nodorum* has been reported on wheat, barley, *Triticale*, *Poa pratensis*, *Bromus inermis* Leyss., *Elymus repens* Gould, *Aegilops cylindrica* auct, *Cynodon dactylon* Pers, *Hordeum pusillum* Nutt, *Lolium perenne* L., and *Agropyron desertorum* Schultes (Weber 1922, De DeWolf & Francel 2000, McDonald *et al*. 2012). In this investigation, *P. nodorum* identified for the first time on *Phalaris arundinacea* from Iran and it is a new host to the world.

Parastagonospora avenae (A.B. Frank) Quaedvlieg, Verkley & Crous, Studies in Mycology 75: 362 (2013)

= *Phaeosphaeria avenae* (G.F. Weber) O.E. Erikss. Ark. Bot. 6(9): 408 (1967)

Specimen examined: As in Table 1

Morphology on YSA: Colonies on YSA are flat, undulate, and pink with white margins. The optimum growth temperature was 25 °C, colony growth rate was 5.5 mm after seven days. There were partial differences in colonies color on YSA. Conidiomata pycnidial, brown to black, subglobose to lenticular, formed mostly on sterilized wheat straws, exuding pale pink conidial cirrhus, 167–212 µm in diameter, with a single ostiolum, 14–17 µm wide (Fig. 4, A-B). Conidia hyaline, smooth, thin-walled, subcylindrical, with subobtuse apex and truncate base, 1–4-septate, 21–45 × 1.6–2.3 µm (Fig. 4, C-D). Conidia of *P. avenae* are larger than those of *P. nodorum*.

Perithecial formation of *Parastagonospora nodorum* is obtained *in vitro* on sterilized wheat straws, under strict conditions of light and temperature after 45 days. Ascocarps in longitudinal rows immersed on sterilized wheat straws, were subepidermal, globose, glabrous, 120–150 µm wide and 130–180 µm high. We did not observe ascus and ascospores within perithecia (Fig. 3, B).

Parastagonospora avenae, one of the causal agents of Stagonospora leaf blotch diseases in cereals, is composed of two formae specials i.e. *P. avenae* f. sp. *tritici* (Pat), and *P. avenae* f. sp. *avenaria* (Paa). The proof of identity between the two groups i.e. Paa and Pat will require further investigation to resolve, as they both have the same morphology (Quaedvlieg *et al*. 2013).

Three main *Phaeosphaeria*-like species infecting cereals were identified in the 1950's based on spore morphology, spore production, formation of sexual structures and host specialization (Shaw 1957). Isolates that were most pathogenic on wheat and showed heterothallic mating type behavior formed the group now called *P. nodorum.* Second groups of isolates collected from oats or other hosts were initially named *Leptosphaeria avenae*, with the species name reflecting the host preference. These isolates also exhibited heterothallic mating behavior (Shaw 1957). Subsequent molecular studies confirmed that, this group also fell into the *Phaeosphaeriaceae* clade and it was renamed to *Parastagonospora avenae* f. sp. *avenaria.* A third group of isolates was non-pathogenic on oats, weakly pathogenic on wheat and other cereals and homothallic. These isolates were morphologically similar to *P. avenaria* but pathogenic on wheat and were, therefore, named as *Phaeosphaeria avenae* f. sp. *tritici* (Pat) (Shaw 1957, Ueng *et al*. 1995), which later confirmed by Cunfer (2000). The latest researchs were done using a three-gene phylogeny (*tubA*, *βxylo* and rDNA-ITS) to determine the relationships among 355 isolates within the *Phaeosphaeria* sp. complex, using both traditional phylogenetic and newer coalescent methods. They identified genetic differences between Pat groups and led to split Pat into five groups *viz*. Pat1, Pat3, Pat4, Pat5 and Pat6. They reported that, Pat5 isolates have been characterized as non-pathogenic on wheat (McDonald *et al*. 2012). Therefore, to identify Paa and Pat groups, pathogencity test on Chamran cuiltvar was done, as this cultivar was evaluated as a susceptible cultivar to *P. nodorum* (Ghaderi *et al*. 2016).

Pathogencity was observed on Chamran cultivar only by P4, P5 and P6 isolates from Gorgan (Table 1). Therefore, three isolates were named as *P. avenae* f. sp. *tritici* (Pat) and the rest of isolates did not display any pathogenicity on Chamran cultivar as we were not sure that if they are Pat or Paa. To resolve these ambiguities (McDonald *et al*. 2012, Ueng *et al*. 1995), we performed molecular analysis using ß-xylosidase gene sequence for discrimination and precise identification of Pat and Paa groups.

Group 1: ***Parastagonospora avenae*** f. sp. ***avenaria*** (Paa)

Specimen examined: As in Table 1

Parastagonospora avenae f. sp. *avenaria* (Paa) was found on *Avena sativa* (Gorgan, Noor Abad and Dezful), and *Phalaris arundinacea* (Gorgan and Noor Abad), 2014–2015

Paa group identified using sequences of ß-xylosidase gene (refered to Phylogenetic analyses part). This group was already reported from *Avena sativa* in Denmark, North America, Canada and Netherland (Malkus *et al.* 2005, McDonald *et al*. 2012, Reszka *et al*. 2005, Wang *et al*. 2007).

In the present study, Paa group is reported from Iran for the first time (Ershad 2009) and *Phalaris arundinacea* is a new host to the world.

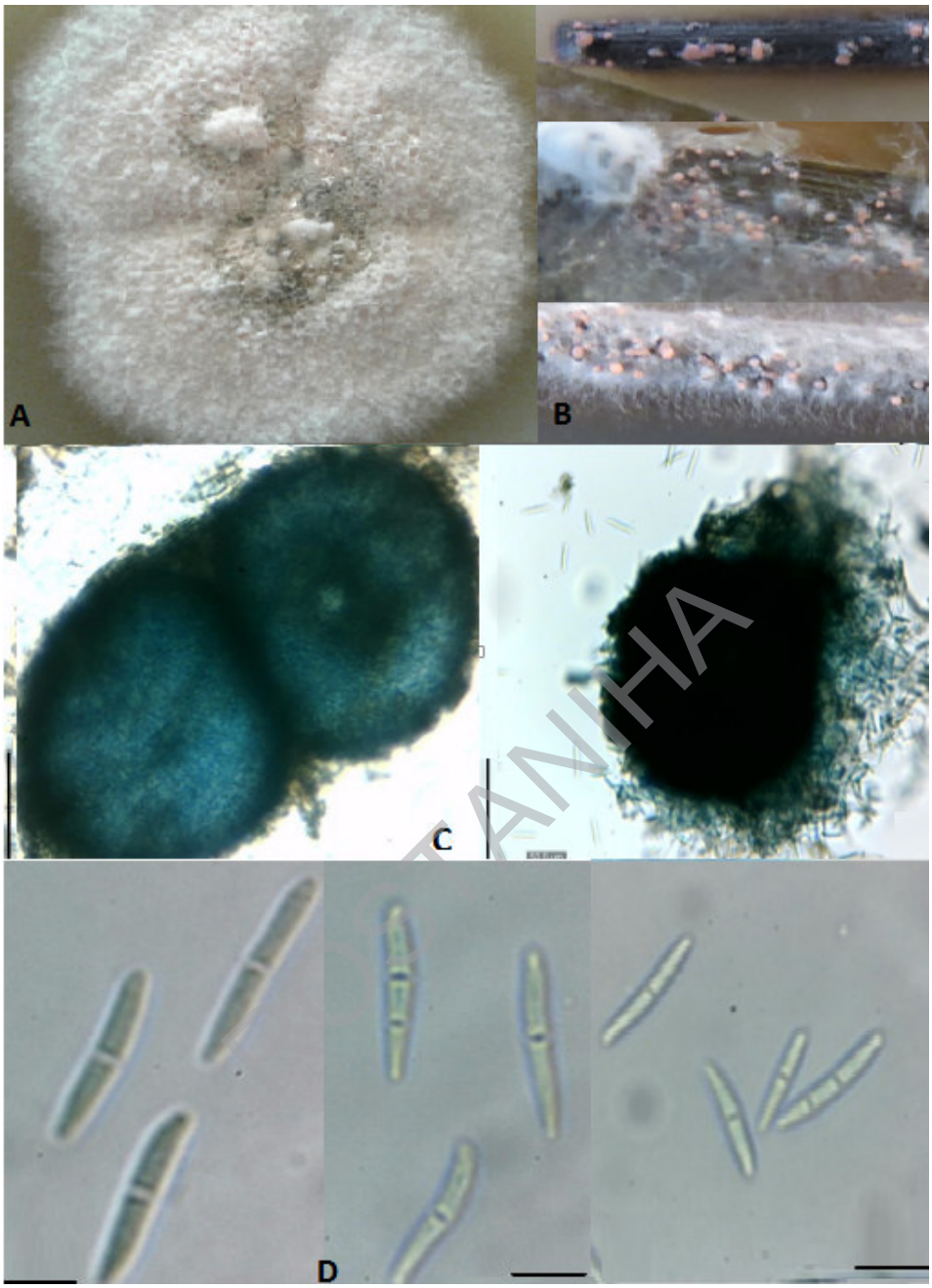

Fig. 1. A. Colony of *Parastagonospora nodorum* on YSA, B. Pycnidia and pycnidiospores formed in culture medium, C. Pycnidium (Bar = 50 µm), D. Pycnidiospores (Bar = 10 µm).

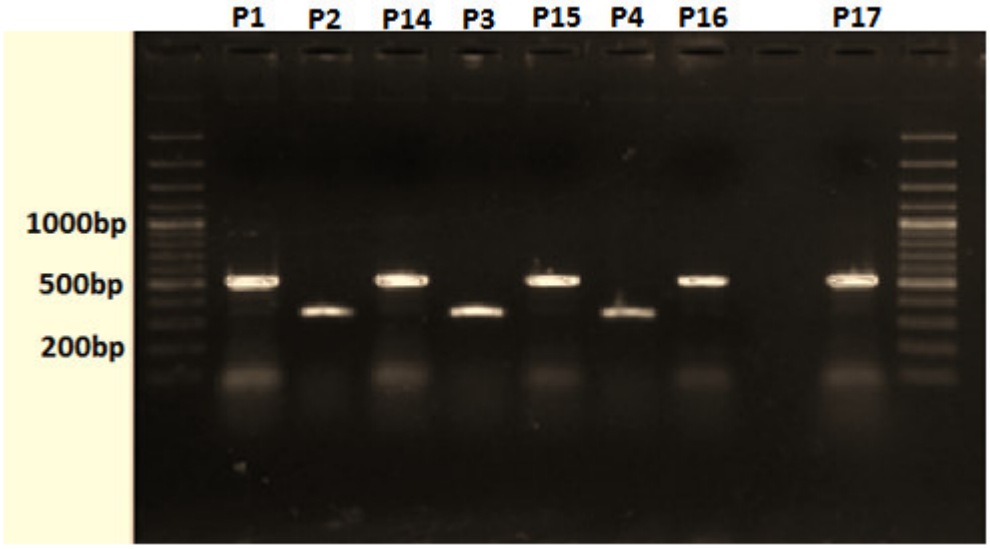

Fig. 2. Amplification of *MAT-1* and *MAT-2* and formation of 510 bp and 360 bp bands in P1 isolate of *Parastagonospora nodorum*, isolates of P2–P4 in *P. avenae* f. sp. *tritici*5 (Pat5) and isolates of P14–P17 in *P. avenae* f. sp. *avenaria* (Paa).

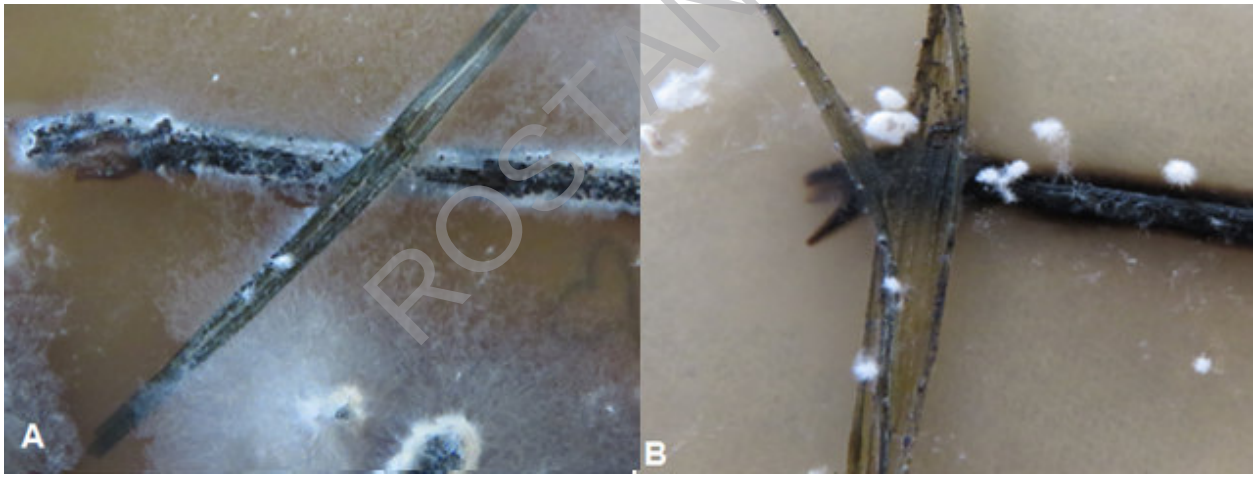

Fig. 3. A. Ascocarp formation of *Parastagonospora nodorum*, B. *P. avenae* on WA supplied with sterilized wheat straws.

Group 2: ***Parastagonospora avenae*** f. sp. ***tritici*5**

Specimen examined: As in Table 1

Parastagonospora avenae f. sp. *tritici*5 was found on *Phalaris arundinacea* (Gorgan and Dezful), *Bromus hordeaceus* (Gorgan and Noor Abad), and *Aegilops tauschii* (Gorgan), 2014–2015

Paa group identified using sequences of ß-xylosidase gene (refered to phylogenetic analyses part).

Pat5 group was already isolated from *Elymus angustus* and *Bromus inermis* in North Dakota (McDonald *et al*. 2012). This group is described from Iran for the first time (Ershad 2009) and identified on *Phalaris arundinacea*, *Bromus hordeaceus*, and *Aegilops tauschii* in our collections that all of the identified hosts are new to the world.

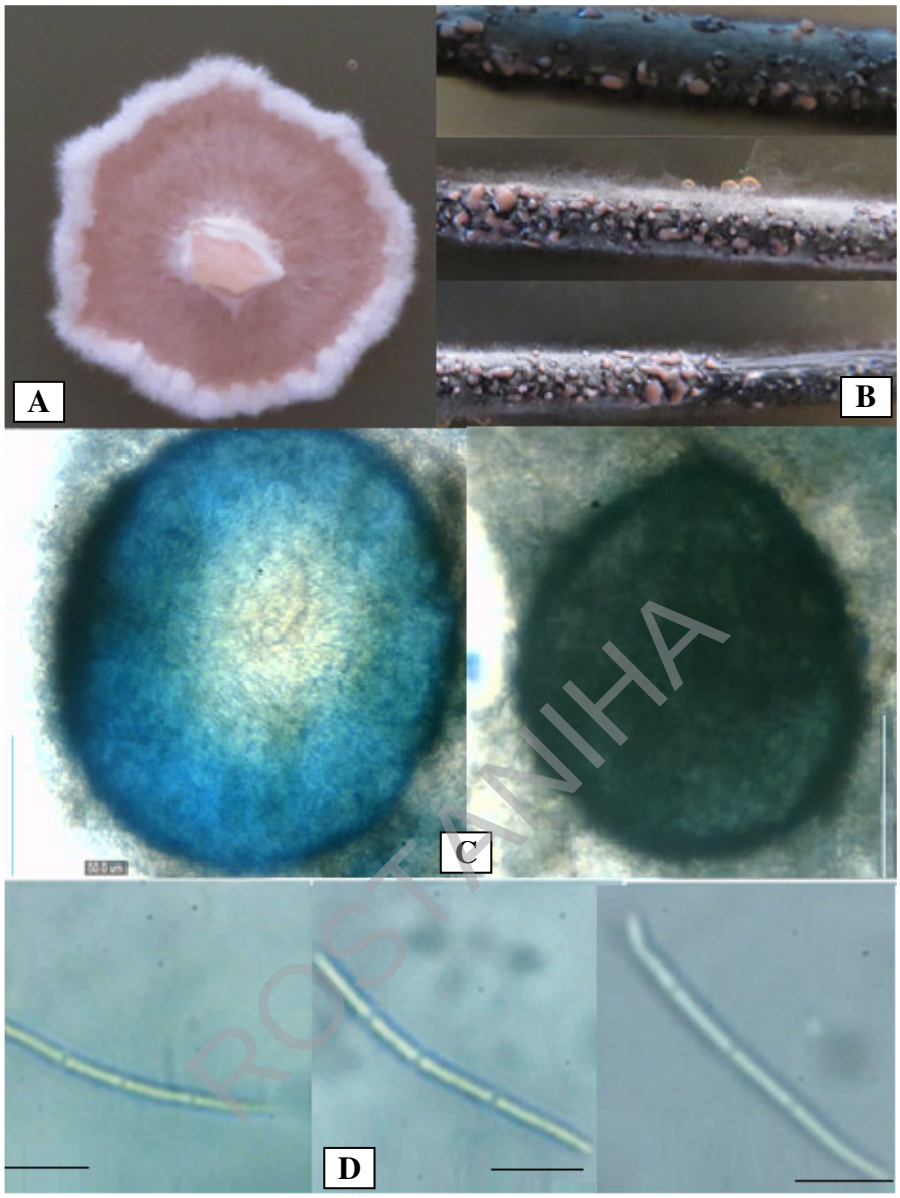

Fig. 4. A. Colony of *Parastagonospora avenae* on YSA, *B.* Pycnidia and pycnidiospores formed in culture medium, C. Pycnidia (Bar = 50 µm), D. Pycnidiospores (Bar = 10 µm).

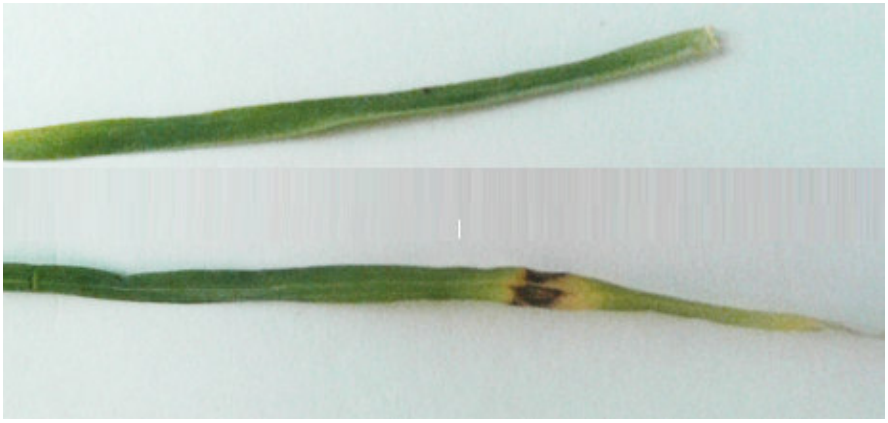

Fig. 5. Weak pathogenicity of *P. avenae* f. sp. *tritici* on wheat Chamaran cultivar (below) and control (above) in greenhouse condition.

- Phylogenetic analysis

The partial nucleotide sequences of β-xylosidase genes (689 bp) were used for phylogenetic analysis of 50 *Parastagonospora* isolates (Fig. 6). We used the closest known pathogenic relatives of *P. nodorum*, *Leptosphaeria maculans*, and *Pyrenophora tritici-repentis*, as outgroups. Based on the gene used in this study, *P. tritici-repentis* is more closely related to the *Parastagonospora* sp. complex than *L. maculans*. Therefore, all further analyses requiring an outgroup, were performed with *P. tritici-repentis*. Phylogenetic result revealed three distinct phylogenetic groups: *P. avenae* f. sp. *avenaria* (Paa), *P. avenae* f. sp. *tritici* 5(Pat5), and *P. nodorum* (Fig. 6). The complete dataset is available for download through GenBank accession numbers MF004425-MF004459.

The sequence similarity was found to be higher than 99.5% between every clade and other reported sequences. In clade *P. avenae* f. sp. *avenaria* (Paa), *P. avenae* f. sp. *tritici* 5 (Pat5) and *P. nodorum* observed 99, 98 and 97% bootstrap, respectively (Fig. 6).

P. avenae f. sp. *avenaria* (Paa) formed the largest clade, found on *Phalaris arundinacea*. Pat5 was the second largest clade, but found on *Bromus hordeaceus*, *Aegilops tauschii*, and *Phalaris arundinacea*. *P. nodorum* found only on *Phalaris arundinacea.* Our results are in correspondence with Phylogenetic analyses by McDonald *et al*. (2012), because they identified nine phylogenetically distinct clades, *P. nodorum* formed the largest clade, found on wheat leaves, ears and seeds, barley, *Triticale*, crested wheatgrass (*Agropyron desertorum*), and other wild grasses. Pat1 was the second largest clade, but found only on wheat leaves, ears and seeds.

Six Pat3 isolates were already isolated from *Triticale* and wheat leaves in Denmark. All Pat4 isolates were isolated from *Elymus tauri* in Iran. Pat6 isolates were found on both *E. tauri* and *Agropyron tauri* in Iran. Pat5 was previously isolated from *E. angustus* and *Bromus inermis* in North Dakota. They found 29 *P. nodorum* isolates, 19 Pat1 isolates and two un-described species, provisionally named as P1 (*Phaeosphaeria* 1, seven isolates) and P2 (*Phaeosphaeria* 2, two isolates) in Iranian wheat fields.

In this study, Pat5 and Paa groups are reported from Iran for the first time. Pat5 identified on *Phalaris arundinacea*, *Bromus hordeaceus*, and *Aegilops tauschii* where all of the recognized hosts are new to the world. Paa found on *Avena sativa* and *Phalaris arundinacea* where only *Phalaris arundinacea* is a new host to the world. *P. nodorum* isolated and identified on *Phalaris arundinacea* which is a new host to the world.

Acknowledgements

We gratefully acknowledge Isfahan University of Technology (Isfahan, Iran) for the financial support and also thank to Patrick C. Brunner and Bruce A. McDonald (Plant Pathology Group, Institute of Integrative Biology, ETH Zurich) for their assistance.

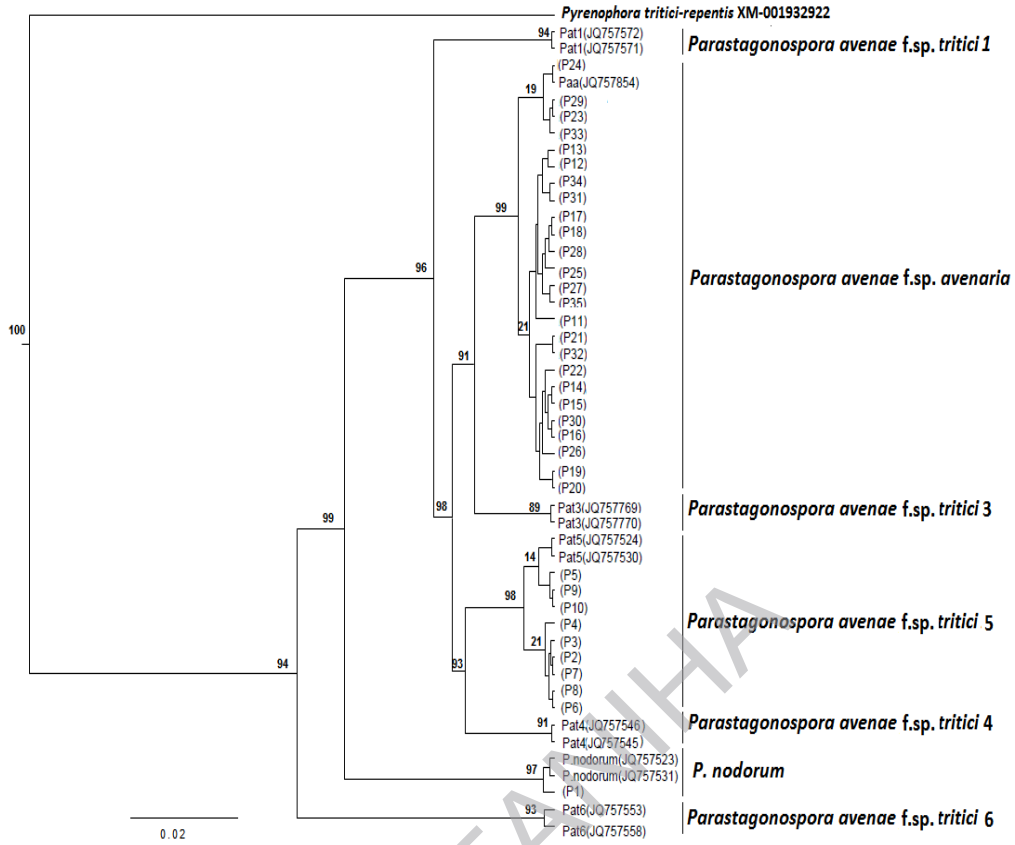

Fig. 6. A phylogenic tree of *Parastagonospora* inferred from ß-xylosidase gene from 52 taxa: Bootstrap values > 50% (1000 replicates) of NJ analysis is shown above the branches (Bar indicates the nucleotide substitution in NJ analysis). *Pyrenophora tritici-repentis* is out group. Accession numbers from gene bank are showen in parenthesis.

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