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Phylogenetic Analysis of *Phaeosphaeria* Species Using Mating Type Genes and Distribution of Mating Types in Iran

Fariba Ghaderi [1]*, Azadeh Habibi[2], and Bahram Sharifnabi[3]

[1]*Department of Plant Protection, College of Agriculture, Yasouj University, Yasouj 7591874831, Iran*
[2]*Department of Biodiversity, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman 7631885356, Iran*
[3]*Department of Plant Protection, College of Agriculture, Isfahan University of Technology, Isfahan 8415683111, Iran*

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***Phaeosphaeria* species are pathogenic on wheat, barley and a wide range of wild grasses. To analyze mating type loci of the *Phaeosphaeria* species and investigate mating type distribution in Iran, we sequenced mating type loci of 273 *Phaeosphaeria* isolates including 67 isolates obtained from symptomatic leaves and ears of wheat, barley, and wild grasses from two wheat-growing region in Iran as well as 206 isolates from our collection from other regions in Iran which were isolated in our previous studies. Mating type genes phylogeny was successfully used to determine the species identity and relationships among isolates within the *Phaeosphaeria* spp. complex. In this study, we reported seven new host records for *Phaeosphaeria* species and the *Phaeosphaeria avenaria* f. sp. *tritici* 3 group was first reported from Iran in this study. Mating type distribution among *Phaeosphaeria* species was determined. Both mating types were present in all sampling regions from Iran. We observed skewed distribution of mating types in one region (Kohgiluyeh va Boyer-Ahmad) and equal distribution in the other region (Bushehr). However, when considering our entire dataset of 273 Iranian *Phaeosphaeria* isolates, the ratio of mating types was not deviated significantly from 1:1 suggesting possibilities for isolates of opposite mating type to interact and reproduce sexually, although the sexual cycle may infrequently occur in some regions especially when the climatic conditions are unfavorable for teleomorph development.**

Keywords **:** Iran, *Mat1-1* gene, *Mat1-2* gene, PCR-RFLP, phylogenetic analyses, wild grasses

*Corresponding author.
Phone) +98-7431006000, FAX) +98-9173418661
E-mail) fghaderi2003@yahoo.com
ORCID
Fariba Ghaderi
https://orcid.org/0000-0002-0072-0521

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Phaeosphaeria species are important pathogens of cereals with global distribution. The origin of *Phaeosphaeria* species is in the Fertile Crescent coinciding with their hosts (McDonald et al., 2012). *Phaeosphaeria nodorum* (Muller) Hedjar (anamorph *Parastagonospora nodorum* (Berk.) Quaedvl., Verkley & Crous) is the causal agent of *Septoria nodorum* leaf and glume blotch (SNB) on wheat, a widespread and yield-reducing disease in many of wheat-growing regions of the world (Shipton et al., 1971; Sommerhalder et al., 2006). *P. nodorum* is also pathogenic on barley and a wide range of wild grasses (Solomon et al., 2006). *Phaeosphaeria avenaria* f. sp. *avenaria* (Paa), (Weber) Eriksson (anamorph *Stagonospora avenae* f. sp. *avenae* Frank) is a major leaf pathogen of oat and other cereals. *Phaeosphaeria avenaria* f. sp. *tritici* (Pat) described by Shaw (1957), is morphologically similar to Paa but not pathogenic on oat while pathogenic on wheat and other cereals (McDonald et al., 2012). Ueng and Chen (1994) and Ueng et al. (1998) studies on genetic differences between biotypes, split Pat into three groups, Pat1, Pat2, and Pat3. Later, McDonald et al. (2012) included over 300 *Phaeos-*

phaeria isolates collected from wild grasses on different continents in a three-gene phylogeny of internal transcribed spacer (ITS), β-tubulin and β-xylosidase and mating type loci to determine the relationships among *Phaeosphaeria* spp. complex and introduced three new groups; Pat4, Pat5, and Pat6.

Different genes have been used to analyze *Phaeosphaeria* species complex including mating type loci, β-tubulin, β-glucosidase, RNA polymerase II; histidine synthase (Bennett et al., 2003; Malkus et al., 2005, 2006; Reszka et al., 2005; Ueng et al., 2003; Wang et al., 2007). In a recent study, whole-genome sequencing data have been used to explore the phylogenetic relationships among *Phaeosphaeria* species (Croll et al., 2021).

Phaeosphaeria species are heterothallic fungi. Sexual reproduction in these species requires the presence of two isolates carrying opposite forms of mating type idiomorphs, called *MAT1-1* and *MAT1-2*, at the same geographic location (Solomon et al., 2004; Sommerhalder et al., 2006). The extent of sexual reproduction and the contribution of airborne ascospores as the source of primary inoculum is important in epidemiology and management of pathogens. Recombination resulting from sexual mating have the potential to give rise to fitter genotypes that are more virulent and fungicide resistant. In asexual reproduction, the main source of the inoculum is pycnidiospores that have a limited increase in genetic diversity comparing to ascospores (Sommerhalder et al., 2006). The presence of both mating types and the mating type ratios have been studied to obtain information about the sexual reproduction by heterothallic fungi (Cowger and Silva-Rojas, 2006; Notteghem and Silué, 1992).

There are arguments about the degree of sexual reproduction in populations of *Phaeosphaeria* species. Studies on *P. nodorum* isolates from North Africa, North America, Australia, Europe, and Near East showed that *MAT1-1* and *MAT1-2* were not evenly distributed (Halama, 2002). Skewed mating type ratios among *P. nodorum* isolates are reported among populations from Central Asia (Vergnes et al., 2006). On the other hand, random mating within populations of *P. nodorum* from Texas, Oregon, and Switzerland have been proved (Keller et al., 1997a, 1997b; McDonald et al., 1994; Sommerhalder et al., 2006). McDonald et al. (2012) studied isolates of Pat from five continents and observed that all Pat1 isolates carried only the *MAT1-1* allele, Pat3 and Pat5 isolates had both *MAT1-1* and *MAT1-2* alleles, and Pat4 and Pat6 isolates were all *MAT1-2*.

The objectives of this study were (1) to collect *Phaeosphaeria* isolates from wheat, barley and wild grasses in Bushehr, Kohgiluyeh va Boyer-Ahmad and Khuzestan Provinces in Iran and identify the species using morphological and molecular data, (2) to analyze mating type loci of the collected isolates in addition to isolates collected from wheat, barley, and wild grasses in Iran from our previous studies and use them in a phylogeny to determine the relationships among 273 isolates within the *Phaeosphaeria* spp. complex; and (3) to investigate mating type distribution among *Phaeosphaeria* species in Iran.

Materials and Methods

Sampling, fungal isolation, and morphological characterization. Symptomatic leaves and ears of wheat, barley, and wild grasses were collected from Bushehr, Kohgiluyeh va Boyer-Ahmad and Khuzestan Provinces in Iran, and taken to laboratory. The diseased leaves and ears showing typical symptoms of SNB were cut into segments of 5-7 mm, sterilized for 2 min in 1% sodium hypochlorite, rinsed in sterile water, placed in glass slides with tape, and kept in high humidity until the pycnidia produced cirri containing pycnidiospores. Purification was carried out using single-spore method on 2% water agar medium in plastic Petri dishes with a flame-sterilized needle. After 2-3 days of incubation, germinated spore was transferred to yeast sucrose agar (YSA, 10 g/l yeast extract, 10 g/l sucrose, 1.2% agar). Pure cultures of each isolate were stored on lyophilized filter-paper strips at –80°C (Adhikari et al., 2008). Only one single-spore strain of *Phaeosphaeria* sp. was isolated from each infected plant and morphological characteristics i.e., colony color, conidia and conidiomata morphology, pigmentation and colony growth rate were used for species identifications (Quaedvlieg et al., 2013).

DNA extraction. For molecular identifications, mycelium plugs from isolates grown on YSA (10 g/l yeast extract, 10 g/l sucrose, 16 g/l agar) for 5 days were transferred to flasks containing 50 ml yeast sucrose broth medium (YSB, 10 g/l yeast extract, 10 g/l sucrose) and incubated on an orbital shaker for 7 days at 120 rpm at 18°C. Harvested mycelia were freeze-dried and stored at –20°C until further use. Lyophilized mycelium was ground into powder and total DNA was extracted using CTAB method according to Murray and Thompson (1980). Genomic DNA was visualized on a 1.2% agarose gel (1.2% agarose, 0.5× TAE) using UV light (GelDoc, Bio-Rad Laboratories, Hercules, CA, USA). Obtained sequences were deposited in GenBank (Tables 1 and 2).

Mating type identification and fertility. Mating type idiomorphs, *MAT1-1* and *MAT1-2*, were amplified and se-

Table 1. *Phaeosphaeria* isolates used in phylogenetic analysis of *MAT1-1* gene

Species	Isolate	Region	Host	GenBank accession no.
Phaeosphaeria nodorum	B10	Western Cape	Wheat	JQ758272
P. nodorum	C5	Arkansas	Wheat Seed	JQ758289
P. nodorum	E31	Russia	Durum wheat	JQ758301
P. nodorum	E2	Kyrgistan	Wheat	JQ758295
P. nodorum	E20	Tadjikistan	Wheat	JQ758296
P. nodorum	AVR 1	North Dakota	Inter. Wheat Grass	JQ758317
P. nodorum	AVR12	North Dakota	Barley	JQ758323
P. nodorum	L1	Oregon	Triticale	JQ758361
P. nodorum	I32	Denmark	Wheat	JQ758345
P. nodorum	H2	North Dakota	Crested Wheatgrass	JQ758327
P. nodorum	M7	Sweden	Wheat	JQ758364
P. avenaria f. sp. *tritici* 1 (Pat1)	A1	Saskatchewan	Wheat Seed	JQ758228
P. avenaria f. sp. *tritici* 1 (Pat1)	A12	Alberta	Wheat Seed	JQ758252
P. avenaria f. sp. *tritici* 1 (Pat1)	A37	Manitoba	Wheat Seed	JQ758253
P. avenaria f. sp. *tritici* 5 (Pat5)	AVR6	North Dakota	Smoothe Brome	JQ758319
P. avenaria f. sp. *tritici* 5 (Pat5)	AVR7	North Dakota	Smoothe Brome	JQ758321
P. avenaria f. sp. *tritici* 5 (Pat5)	AVR8	North Dakota	Smoothe Brome	JQ758320
P. avenaria f. sp. *tritici* 5 (Pat5)	AVR9	North Dakota	Smoothe Brome	JQ758322
P. avenaria f. sp. *tritici* 3 (Pat3)	I36	Denmark	Wheat	JQ758367
P. avenaria f. sp. *tritici* 3 (Pat3)	I37	Denmark	Triticale	JQ758368
P. nodorum	Pn1 to Pn35	Iran (KB)	Wheat	OK000630-OK000664[a]
P. nodorum	Pn36 to Pn57	Iran (Fars)	Wheat	OK000665-OK000687[a]
P. nodorum	Pn58 to Pn89	Iran (Khuzestan)	Wheat	OK000688-K000718[a]
P. nodorum	Pn90 to Pn99	Iran (Golestan)	Wheat	OK000719-OK000728[a]
P. nodorum	Pn100 to Pn112	Iran (Bushehr)	Wheat	OK000729-OK000741[a]
P. nodorum	Pn-grass-1	Iran (KB)	*Dactylis glomerata*	OK000742[a]
P. nodorum	Pn-grass-2	Iran (KB)	*D. glomerata*	OK000743[a]
P. nodorum	P1	Iran (Golestan)	*Phalaris arundinacea*	OK000744[a]
P. avenaria f. sp. *tritici*5 (Pat5)	P3 to P5	Iran (Golestan)	*P. arundinacea*	OK000745-OK000747[a]
P. avenaria f. sp. *tritici*5 (Pat5)	Pt1 to Pt4	Iran (KB)	*P. arundinacea*	OK000748-OK000751[a]
P. avenaria f. sp. *tritici*5 (Pat5)	P2	Iran (Khuzestan)	*P. arundinacea*	OK000752[a]
P. avenaria f. sp. *tritici*5 (Pat5)	Pt5	Iran (Khuzestan)	*P. arundinacea*	OK000753[a]
P. avenaria f. sp. *tritici*5 (Pat5)	P6	Iran (Golestan)	*Aegilops tauschii*	OK000754[a]
P. avenaria f. sp. *tritici*5 (Pat5)	P7	Iran (Golestan)	*Bromus hordeaceus*	OK000755[a]
P. avenaria f. sp. *tritici*5 (Pat5)	Pt6	Iran (Khuzestan)	*Aegilops tauschii*	OK000756[a]
P. avenaria f. sp. *tritici*5 (Pat5)	P10	Iran (Fars)	*Bromus hordeaceus*	OK000757[a]
P. avenaria f. sp. *tritici*5 (Pat5)	P11	Iran (Fars)	*P. arundinacea*	OK000758[a]
P. avenaria f. sp. *tritici*3 (Pat3)	Pt7 to Pt8	Iran (Bushehr)	Wheat	OK000759-OK000760[a]
P. avenaria f. sp. *tritici*1 (Pat1)	Pt9 to Pt15	Iran (Bushehr)	Wheat	OK000761-OK000767[a]
P. avenaria f. sp. *tritici*1 (Pat1)	Pt16 to Pt18	Iran (Bushehr)	Barley	OK000768-OK000770[a]
P. avenaria f. sp. *tritici*3 (Pat3)	Pt19 to Pt20	Iran (KB)	Barley	OK000771-OK000772[a]

[a]Sequences generated in this study.

quenced for 67 *Phaeosphaeria* isolates that were obtained in this study and 201 isolates from our collection, which were isolated in Ghaderi et al. (2017, 2020) (Tables 1 and 2).

The amplifications were carried out using a multiplex polymerase chain reaction (PCR) with primers (Table 3) designed by Bennett et al. (2003). PCR amplifications were performed in 25 µl reactions containing 2 µm primers, 0.4 mM dNTPs (Fermentas Inc., Waltham, MA, USA), 8 pg

Table 2. *Phaeosphaeria* isolates used in phylogenetic analysis of *MAT1-2* gene

Species	Isolate	Region	Host	GenBank accession no.
Phaeosphaeria nodorum	B14	Western Cape	Wheat	JQ758369
P. nodorum	E30	Russia	Durum wheat	JQ758401
P. nodorum	F2	Switzerland	Wheat	JQ758407
P. nodorum	I27	Denmark	Triticale	JQ758442
P. nodorum	H26	North Dakota	Crested wheatgrass	JQ758425
P. nodorum	AVR13	North Dakota	Barley	JQ758493
P. avenaria f. sp. *tritici* 6 (Pat6)	R11	Iran	*Dactylis glomerata*	JQ758509
P. avenaria f. sp. *tritici* 6 (Pat6)	R12	Iran	*D. glomerata*	JQ758510
P. avenaria f. sp. *tritici* 6 (Pat6)	R16	Iran	*D. glomerata*	JQ758511
P. avenaria f. sp. *tritici* 6 (Pat6)	R17	Iran	*D. glomerata*	JQ758512
P. avenaria f. sp. *tritici* 5 (Pat5)	AVR3	North Dakota	Smoothe brome	JQ758489
P. avenaria f. sp. *tritici* 5 (Pat5)	AVR4	North Dakota	Altai wild rye	JQ758490
P. avenaria f. sp. *tritici* 4 (Pat4)	R1	Iran	*Dactylis glomerata*	JQ758499
P. avenaria f. sp. *tritici* 4 (Pat4)	R2	Iran	*D. glomerata*	JQ758500
P. avenaria f. sp. *tritici* 4 (Pat4)	R3	Iran	*D. glomerata*	JQ758501
P. avenaria f. sp. *tritici* 4 (Pat4)	R4	Iran	*D. glomerata*	JQ758502
P. avenaria f. sp. *tritici* 3 (Pat3)	I34	Denmark	Wheat	JQ758485
P. avenaria f. sp. *tritici* 3 (Pat3)	I35	Denmark	Wheat	JQ758486
P. avenaria f.sp. *avenaria* (Paa)	s258	Netherlands	Oat	JQ758487
P. nodorum	Pn113 to Pn127	Iran (KB)	Wheat	OK000773-OK000787[a]
P. nodorum	Pn128 to Pn156	Iran (Fars)	Wheat	OK000788-OK000816[a]
P. nodorum	Pn157 to Pn183	Iran (Khuzestan)	Wheat	OK000817-OK000843[a]
P. nodorum	Pn184 to Pn197	Iran (Golestan)	Wheat	OK000844-OK000857[a]
P. nodorum	Pn198 to Pn214	Iran (Bushehr)	Wheat	OK000858-OK000874[a]
P. nodorum	Pn-grass4 to Pn-grass5	Iran (Bushehr)	*Aegilops tauschii*	OK000875-OK000876[a]
P. nodorum	Pn-grass6 to Pn-grass7	Iran (Bushehr)	*Avena sativa*	OK000877-OK000878[a]
P. avenaria f. sp. *avenaria* (Paa)	P15 to P18	Iran (Golestan)	*P. arundinacea*	OK000879-OK000882[a]
P. avenaria f. sp. *avenaria* (Paa)	P21	Iran (Fars)	*Avena sativa*	OK000883[a]
P. avenaria f. sp. *avenaria* (Paa)	Paa1	Iran(KB)	*P. arundinacea*	OK000884[a]
P. avenaria f. sp. *avenaria* (Paa)	Paa2 to Paa3	Iran(KB)	*Convolvulus arvensis*	OK000885-OK000886[a]
P. avenaria f. sp. *avenaria* (Paa)	P22 to P25	Iran (Golestan)	*A. sativa*	OK000887-OK000890[a]
P. avenaria f. sp. *avenaria* (Paa)	P12 to P13	Iran (Khuzestan)	*P. arundinacea*	OK000891-OK000892[a]
P. avenaria f. sp. *avenaria* (Paa)	Paa4	Iran (Khuzestan)	*Aegilops tauschii*	OK000893[a]
P. avenaria f. sp. *tritici* 5 (Pat5)	Pt21	Iran(KB)	*A. tauschii*	OK000894[a]
P. avenaria f. sp. *tritici* 5 (Pat5)	P8 to P9	Iran (Fars)	*Bromus hordeaceus*	OK000895-OK000896[a]
P. avenaria f. sp. *tritici*3 (Pat3)	Pt22-Pt23	Iran (Bushehr)	Wheat	OK000897-OK000898[a]
P. avenaria f. sp. *tritici*3 (Pat3)	Pt24-Pt27	Iran(KB)	Barley	OK000899-OK000902[a]

[a]Sequences generated in this study.

DNA, 0.05 U Taq DNA polymerase (MBI Fermentas), 3 mM $MgCl_2$ and corresponding reaction Dream Taq buffer (MBI Fermentas) (Sommerhalder et al., 2006). The PCR condition was set up in 2 min initial denaturation at 96°C, 35 cycles of 30 s at 96°C, annealing at 55°C for 30 s, extension at 72°C for 1 min and a final 5 min extension at 72°C (Sommerhalder et al., 2006). The PCR products were visualized on 1.5% agarose gel (1.5% agarose, 0.5× TBE). Sequencing was performed by Macrogen (Seoul, Korea). The ratio of *MAT1-1* to *MAT1-2* alleles was evaluated. Deviations from a 1:1 ratio of the two mating types within fields was tested using chi-square statistics.

To confirm the development of sexual phase, genetic crosses were carried out between opposite mating types for 67 *Phaeosphaeria* isolates collected in this study following the procedure of Halama and Lacoste (1992). Opposite mating types were grown on 2% water agar medium encompassing sterilized wheat straws and incubated at 10°C

Table 3. List of primers used in this study

Locus	Length of product	Sequence (5'-3')	Reference
MAT1-1	360	CTTCACGACCGGCCAGATAGT	Bennett et al. (2003)
MAT1-2		CAGAGGCTTGTCGGGTTCAT	Bennett et al. (2003)
MAT2-1	510	ACCCCGCCCCATGAACAAGTG	Bennett et al. (2003)
MAT2-2		CTAGACCGGCCCGATCAAGACCAAAGAAG	Bennett et al. (2003)
Bxylo9Fcod	962	CAAAGAACCCATTGTCACACAC	McDonald et al. (2012)
Bxylo970Rco		GCTGTTCTTCAGCCAACTT	McDonald et al. (2012)

with a 12-h photoperiod, near ultraviolet light (300-400 nm) and intensities of 400 and 600 pW/cm^2 for 50 days. Isolates were paired with themselves as control.

Genetic data analyses. *Mat1-1* and *Mat1-2* sequences were generated for 273 isolates. The obtained sequences of 273 isolates of this study and some sequences from McDonald et al. (2012) which were obtained from GenBank, were used in phylogenetic analyses to determine the taxonomic status of *Phaeosphaeria* species and identifications (Tables 1 and 2).

The phylogenetic analyses of *Mat1-1* and *Mat1-2* alleles were performed separately. Sequences were edited manually and aligned by Geneious version 7 (Biomatters Ltd., Auckland, New Zealand). Phylogenetic analyses were performed using heuristic searches in PAUP v. 4.0a133 (Swofford, 2002) for parsimony, neighbour-joining and maximum likelihood analyses. For maximum likelihood analyses, models of sequence evolution were evaluated for both datasets by JModeltest v.2.1.4 (Posada, 2008) using the Akaike information criterion and model parameter estimates were implemented in PAUP v. 4.0a133. The resulted trees were midpoint rooted. The resulted trees were observed and edited in FigTree v1.4.0. Mating Type polymorphism within each species assessed using DnaSP v5 (Librado and Rozas, 2009).

PCR-restriction fragment length polymorphism technique. In order to confirm identification of *P. avenaria* f. sp. *tritici* 1 (Pat1) isolates, we used PCR–restriction fragment length polymorphism (PCR-RFLP) assay (McDonald et al., 2012) to differentiate between *P. nodorum* and Pat1. Isolates of *Phaeosphaeria* sp. were surveyed using this method.

An isolate obtained from earlier studies of McDonald et al. (2012) was used as positive control. Partial sequence of β-xylosidase gene (962 bp) was amplified using specific primers (Table 3) and was used as template DNA. Species-specific restriction enzyme recognition sites were distinguishing by NEB Cutter v2.0. Digestion of β-xylosidase gene PCR amplicons was carried out with 2 units of the restriction enzyme *Sca*I (MBI Fermentas) at 37°C for 90 min. A 15-min treatment at 65°C was applied for inactivation. Digested PCR products were visualized on 2% agarose gels with ethidium bromide staining.

Results

Fungal isolation and identification. In total, 67 *Phaeosphaeria* isolates were obtained from symptomatic leaves and ears of wheat, barley and wild grasses from Bushehr, Khuzestan and Kohgiluyeh va Boyer-Ahmad Provinces. Based on morphological characterization and molecular analysis, isolates were identified as *P. nodorum,* Paa, Pat1, Pat3, and Pat5. Paa, Pat1, Pat3, and Pat5 were identified based on molecular phylogeny of MAT genes.

In Bushehr province, 48 isolates were collected from wheat and wild grasses (Tables 1 and 2). We obtained 30 isolates of *P. nodorum* from wheat ears, two from *Aegilops tauschii* ears and two from *Avena sativa* leaves. *A. tauschii* and *A. sativa* are new hosts for *P. nodorum* to the world. Four isolates from wheat ears were identified as Pat3. Pat3 was first isolated from Iran in this study. Ten pat1 isolates were obtained from wheat and barley. Barley is a new host for pat1 to the world.

In Kohgiluyeh va Boyer-Ahmad Province, 16 isolates were collected from leaves and ears of wild grasses (Tables 1 and 2). Three Paa isolates, one from *P. arundinacea* and two from *Convolvulus arvensis* leaves were obtained. *Convolvulus arvensis* is a new host for Paa to the world. Five isolates of Pat5 were identified from *P. arundinacea* and *A. tauschii*. Two *P. nodorum* isolates were identified from *Dactylis glomerata* ears, which is a new host to the world. Six Pat3 isolates were obtained from barley ears. Barley is a new host for Pat3 to the world. Pat3 is isolated for the first time from Iran.

In Khuzestan Province, three isolate were collected. One Paa was obtained from ear of *A. tauschii*, which is a new host for Paa to the world and two Pat5 were isolated from *A. tauschii* and *P. arundinacea*.

Phylogenetic analyses. Amplification of *MAT1-1* and *MAT1-2* gene fragments of 510 bp and 360 bp from all isolates (Tables 1 and 2) was conducted successfully. The obtained sequences were deposited in GenBank and accession numbers were obtained (Tables 1 and 2). The aligned data sets of *MAT1-1* and *MAT1-2* gene consisted of 269 and 396 characters of which 52 and 55 characters were parsimony informative, respectively. The three phylogenetic analy-

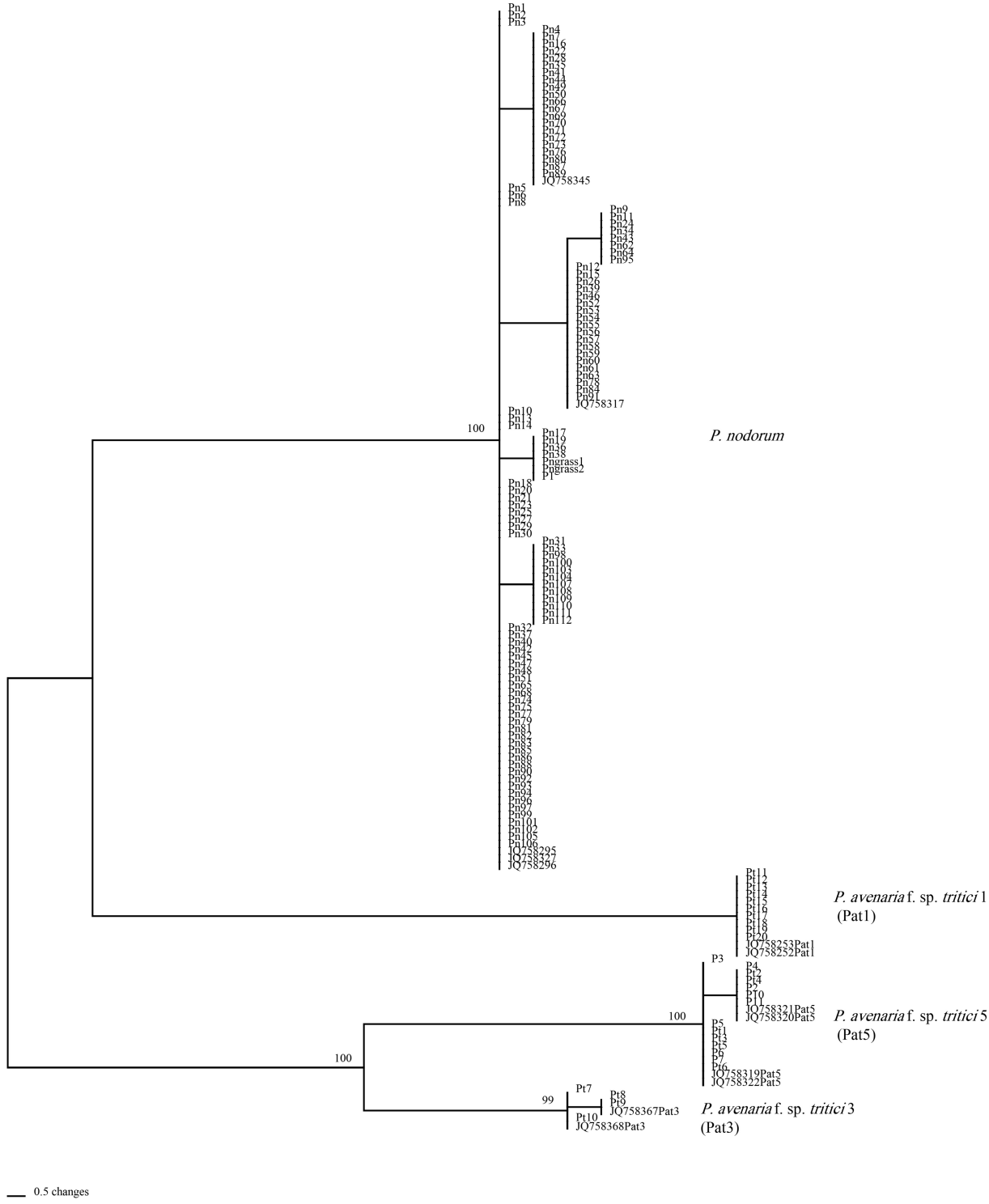

Fig. 1. The parsimony tree constructed using *MAT1-1* gene from 151 *Phaeosphaeria* isolates. Branch length shows the substitution rate. Bootstrap values are labeled on the branches. The tree is midpoint rooted.

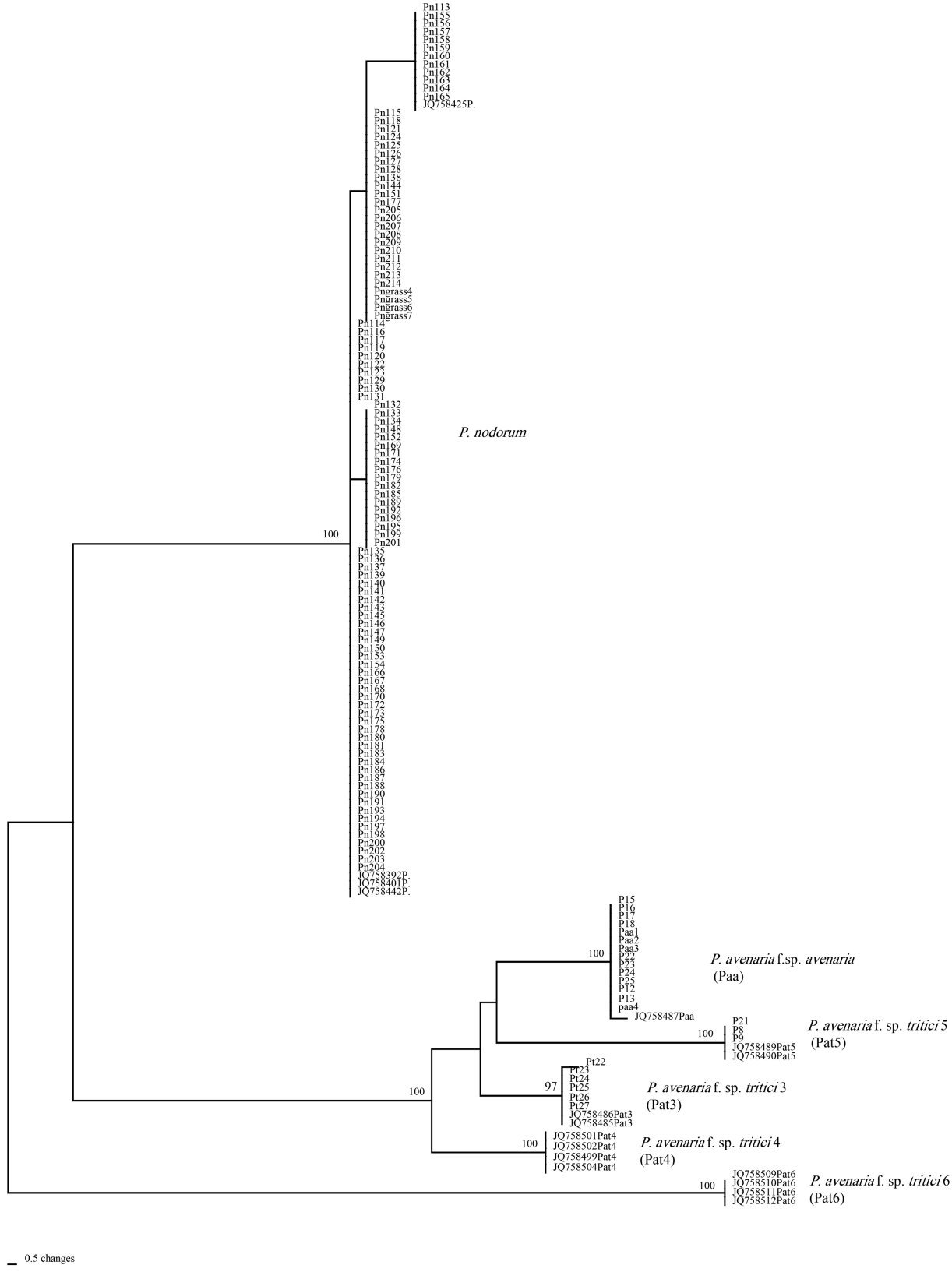

Fig. 2. The parsimony tree constructed using *MAT1-2* gene from 143 *Phaeosphaeria* isolates. Branch length shows the substitution rate. Bootstrap values are labeled on the branches. The tree is midpoint rooted.

Table 4. Summary of mating type polymorphism within each species

	Mat1-1 polymorphism				*Mat1-2* polymorphism			
	Phaeosphaeria nodorum (*n* = 115)	Pat3 (*n* = 4)	Pat5 (*n* = 14)	Pat1 (*n* = 10)	*P. nodorum* (*n* = 106)	Pat3 (*n* = 6)	Pat5 (*n* = 3)	Paa (*n* = 15)
N haplotype	6	2	2	1	4	2	1	1
Haplotype diversity (Hd)	0/7599	1	0.5275	0	0/6748	1	0	0
Intron	1	1	1	0	1	1	1	1
Total no. of mutations	6	1	1	0	5	1	0	0
Synonymous	4	0	0	0	4	0	0	0
Non-synonymous	2	1	1	0	1	1	0	0

Pat, *Phaeosphaeria avenaria* f. sp. *tritici*.

sis methods parsimony, neighbor-joining and maximum likelihood generated trees with similar topologies amongst species. The topology and branch lengths of the parsimony phylogenetic trees are shown in Figs. 1 and 2.

To elucidate phylogenetic relationships among 273 *Phaeosphaeria* species from wheat, Barley and wild grasses and accurate species identifications, separate parsimony trees were created using *MAT1-1* and *MAT1-2* gene sequences (Figs. 1 and 2). Fig. 1 shows the phylogenetic position of *Phaeosphaeria* isolates using *MAT1-1* sequences. The phylogenetic reconstruction revealed four highly supported clades corresponding to *P. nodorum* (containing 6 nucleotide haplotypes, 4 with synonymous mutations, 2 with non-synonymous mutations) (Table 4) and three formae speciales of *P. avenaria* including Pat5 (containing 2 nucleotide haplotypes, 1 with non-synonymous mutations), Pat3 (containing 2 nucleotide haplotypes, 1 with non-synonymous mutations) and Pat1. Fig. 2 shows the phylogenetic position of *Phaeosphaeria* isolates using *MAT1-2* sequences. The tree contains six highly supported clades corresponding to *P. nodorum* (containing 4 nucleotide haplotypes, 4 with synonymous mutations, 1 with non-synonymous mutations) and five *P. avenaria* formae specials including Pat1, Pat3 (containing 2 nucleotide haplotypes, 1 with non-synonymous mutations), Pat5 (with 1 nucleotide haplotype), *P. avenaria* f. sp. *tritici* 6 (Pat6), and Paa (with 01 nucleotide haplotype).

PCR-RFLP technique. We used a PCR-RFLP technique to distinguish *P. nodorum* isolates from Pat1 isolates based on fixed species polymorphisms. The 962-bp PCR products were amplified from genomic DNA with β-xylosidase gene-specific primers and digested using *Sca*I enzyme. PCR products from Pat1 isolates had a specific restriction site and 695-bp and 267-bp fragments were produced. There was no restriction site in PCR products of 221 *P. nodorum* isolates, which produced 962-bp amplicons (Fig. 3).

Mating type distribution and fertility. The ratio of *MAT1-1* to *MAT1-2* alleles and the results of chi-square statistics for testing deviations from a 1:1 ratio of the two mating types within fields are presented in Table 5. The mating type ratio was not significantly different from 1:1

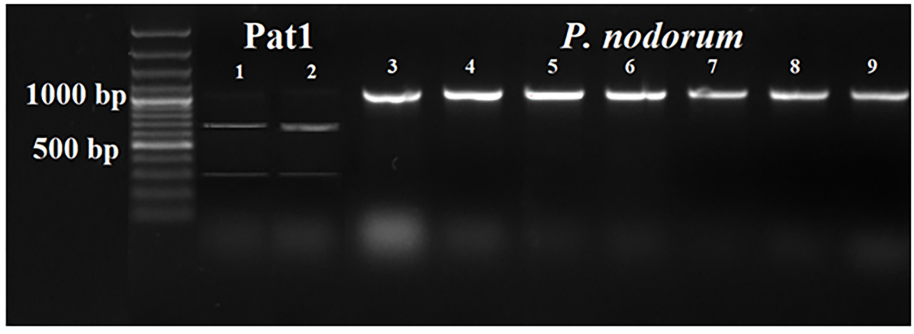

Fig. 3. PCR-RFLP assay used to differentiate between *Phaeosphaeria nodorum* and *P. avenaria* f. sp. *tritici* 1 (Pat1). The PCR products from β-xylosidase gene amplifications were digested with enzyme *Sca*I. *P. nodorum* and Pat1 displayed different patterns of DNA fragments. The Pat1 isolate (lane 1) digestion using enzyme *Sca*I produced two bands of approximately 695 bp and 267 bp. Lane 2 is positive control. There was no cutting site for *P. nodorum* isolates (3-9) which produced 962 bp amplicons. PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Table 5. Measures of mating type ratios using the chi-square test in Iranian wheat fields from two sampling regions

Region	Sample size		*MAT1-1/MAT1-2*	Chi-square value
	Wheat	Wild grass		
Kohgiluyeh va Boyer-Ahmad	-	16	8:8	0^{ns} P=1
Bushehr	30	18	25:23 (13w+12g:17w+6g)	0.0417^{ns} P=0 .83822

Chi-square value for deviation from a 1:1 mating type ratio.
Significant at $P < 0.05$; ns, not significant.

Fig. 4. Ascocarp formation of *Phaeosphaeria* sp. on water agar media supplied with sterilized wheat straws.

ratio for Kohgiluyeh va Boyer-Ahmad and Bushehr.

The mating ability of *Phaeosphaeria* species obtained in this study was examined. None of isolates formed *ascocarp* (pseudothecia) when grown alone. The pseudothecia were obtained after 50 days of incubation on sterilized wheat straws. Pseudothecia were recognized from pycnidia by the absence of cirrhi. We could not observe mature pseudothecia containing asci and ascospores in any of the crosses in laboratory conditions (Fig. 4).

Discussion

In this study, 67 *Phaeosphaeria* spp. were obtained from symptomatic leaves and ears of wheat, barley and wild grasses collected in Bushehr, Kohgiluyeh va Boyer-Ahmad and Khuzestan Provinces in Iran. Based on morphological characteristics and molecular data, *P. nodorum*, Paa, Pat5, Pat3, and Pat1 were identified. These included seven new host records including *A. tauschii*, *A. sativa* and *D. glomerata* for *P. nodorum*, barley for Pat1 and Pat3, *C. arvensis* and *A. tauschii* for Paa. *Phaeosphaeria* species complex have reported to have the ability to infect several grass hosts (McDonald et al., 2012; Solomon et al., 2006). However, the host range for *Phaeosphaeria* species is yet unknown. Pat3 is reported in this study for the first time from Iran.

P. nodorum isolates were wildly distributed in the sampled areas. McDonald et al. (2012) observed the same trend in the distribution of *Phaeosphaeria* species in global scale. Ghaderi et al. (2010) studied the diversity of *Phaeosphaeria* species associated with poaceous plants in Iran and identified *P. nodorum,* Paa, and Pat5. In another study, Ghaderi and Razavi (2018) reported *Phaeosphaeria dactylidis* from wild grasses. Species richness of *Phaeosphaeria* in Iran is consistent with the hypothesis of the origin in the Fertile Crescent.

Since morphological characters between *Phaeosphaeria* species often overlap and cultural characteristics are in many cases variable (Bennett et al., 2003; Cunfer, 2000; Shoemaker and Babcock, 1989), species relationships and taxonomy need further molecular characterization. We used molecular phylogeny of mating type genes to elucidate relationships among the *Phaeosphaeria* species. *Mat1-1* and *Mat1-2* sequences of 67 isolates collected in this study and 206 isolates which were collected in our previous studies (Ghaderi et al., 2017, 2020) were generated (Tables 1 and 2). The obtained sequences were combined with some sequences of *Mat1-1* and *Mat1-2* published previously by McDonald et al. (2012), and were used in the phylogenetic analyses to infer relationships between *Phaeosphaeria* species. In the resulting trees, all clades were separated with high bootstrap support. Isolates were grouped as *P. nodorum* clade, and four clades corresponding to different formae speciales including Paa, Pat5, Pat3, and Pat1. According to Turgeon (1998) within species variations is low for *MAT* genes while between-species variation is high making them a useful region to test the biological and phylogenetic species concepts for outcrossing fungi. Turgeon (1998) suggested *MAT* sequences are more useful in phylogenetic resolution than ITS rDNA and GPD sequence regions. Ueng et al. (2003) studied the potential use of mating type genes in phylogeny and molecular classification of *Pha-*

eosphaeria species. They observed that phylogenetic relationships in cereal *Phaeosphaeria* isolates based on mating type gene sequences were consistent with those based on RFLP fingerprints and rDNA ITS sequences. Bennett et al. (2003) observed between-species *MAT* variations in the genus *Phaeosphaeria*. They suggested *MAT* genes as a reliable diagnostic procedure to elucidate species relationships in the *Phaeosphaeria* species pathogenic to cereal crops. In addition to *MAT* phylogeny, we successfully used a PCR-RFLP technique developed by McDonald et al. (2012) to distinguish *P. nodorum* isolates from Pat1 isolates based on fixed species polymorphisms and 221 *P. nodorum* isolates were identified.

Both mating types were present in all sampling regions from Iran. Mating type ratio for Khuzestan was not calculated because sample size was small. Mating type ratio of the sampled areas in Bushehr was not significantly different from 1:1. In Kohgiluyeh va Boyer-Ahmad, the ratio of mating types for 16 isolates obtained from wild grasses in this study was 1:1. However, when we added the data of 62 isolates which were previously obtained from wheat in Ghaderi et al. (2020) (45 *MAT1-1* vs. 17 *MAT1-2*), the overall mating types ratio showed a significantly skewed distribution in this area. A possible explanation for this skewed distribution is that part of our sampling have been done within a pycnidial clone. However, obtaining a robust estimate of the mating type distribution requires large number of isolates and samplings that are more extensive (Solomon et al., 2004). The ratio of mating types was not deviated significantly from 1:1 when considering our entire dataset of Iranian *Phaeosphaeria* population in Tables 1 and 2 (The chi-square statistic = 0.3091 and $P = 0.578213$). However, *MAT1-1* isolates were predominant. The same results were obtained by Solomon et al. (2004) in populations of *P. nodorum* in Western Australia. They observed that the ratio of mating type alleles was not significantly different from equal proportions while *MAT1-1* isolates were predominant. They tested different hypotheses regarding the predominance of *MAT1-1* alleles including greater virulence and higher asexual fitness of *MAT1-1* strains. None of these hypotheses could explain the prevalence of *MAT1-1* strains.

We did not observe sexual structures in the sampled areas. One possible explanation would be the dry springs in the sampling years, which have substantially decreased the frequency of ascocarps. Another explanation would be that we have collected plant materials at wrong timepoint in the disease cycle. Mutations in other genes and sex barriers such as female sterility have also been proposed as possible explanations for inability to find the teleomorphs (Bennet et al., 2003; Sommerhalder et al., 2006).

Mating type ratios have been explored for *Phaeosphaeria* species to study these pathogens biology in order to reach evidence on the extent of sexual reproduction in populations. Halama (2002) observed that *MAT1-1* alleles predominated in all of the populations sampled from different parts of the world. Bennett et al. (2003) observed skewed distribution of mating types in one population of *P. nodorum* and equal distribution in another population from a different field in New York, USA. Sommerhalder et al. (2006) tested a comprehensive collection of *P. nodorum* isolates from six countries on five continents and reported that this pathogen has even distribution of both mating types among all field populations. Vergnes et al. (2006) examined Central Asia populations of *P. nodorum* and reported the presence of both mating types in Kazakh and Russian origins while no *MAT1-2* isolates were found in Tajikistan population. Mating type ratios data would be used to infer interesting information about population genetics, epidemiology, and control strategies of *Phaeosphaeria* species.

Our observations of the presence of both mating types and a 1:1 mating type ratio for our entire data set indicate that the Iranian *Phaeosphaeria* population have the opportunity to interact and undergo regular sexual reproduction resulting high genetic diversity. This hypothesis is consistent with McDonald et al. (2012) and Ghaderi et al. (2020) who showed that Iranian populations of *Phaeosphaeria* species had high levels of genetic diversity. It is likely that the main primary inoculum of *Phaeosphaeria* diseases is airborne ascospores in years with favorable climatic conditions. In the years with high sexual reproduction, clean seed, and crop rotation techniques are not preventive enough. The presence of a mixed reproductive system in these pathogens should be considered in plant breeding and fungicide screening programs. Quantitative resistance via polygenic control would be useful to overcome the possible break up of co-adapted gene complexes in the sexual reproducing periods.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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