Some evidence for skewed mating type distribution in Iranian populations of *Rhynchosporium commune*, the cause of barley scald disease

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Abstract: *Rhynchosporium commune* (formerly known as *Rhynchosporium secalis*), the causal agent of scald disease on barley, is known to spread asexually by splash dispersed conidia. However, there are multiple lines of evidence for the possibility of a clandestine sexual cycle occurrence in this species including extensive genotypic diversity, equal distribution of mating type alleles across the world and expression of mating type genes. In the current study, the potential for the occurrence of a sexual cycle amongst the Iranian population of *R. commune* was assessed by analyzing distribution and frequency of the mating type alleles at both micro and macro-spatial scales. A total of 95 single-conidial *R. commune* isolates were obtained from different barley fields in Kurdistan province. Previously designed primers were applied in a multiplex PCR assay to study distribution and frequency of the mating type alleles within and between populations. Totally, 67 isolates were determined as *MAT1-1* and the remaining 28 isolates as *MAT1-2* throughout the sampling counties. The results obtained at a macro-spatial scale revealed that unlike Kamyaran county (both *MAT1-1* and *MAT1-2* at an equal ratio), an unequal distribution of mating type genes was dominant among *R. commune* isolates in both Mariwan and Dehgolan counties. Our findings support a predominantly asexual reproduction for Mariwan and Dehgolan counties and the possibility of sexual stage occurrence in Kamyarna county.

Key words: clonal propagation, distribution, Kurdistan, mating types, multiplex PCR

Introduction

Rhynchosporium commune Zaffarano, McDonald & Linde, the pathogen that causes 'rhynchosporium', 'barley leaf blotch' or 'scald' on *Hordeum vulgare* L. (barley), is one of the most economically important barley pathogens worldwide, causing yield and grain quality reduction (Zhan *et al.* 2008). Yield loss normally ranges from 1 to 19%, although in Iran yield losses around 35% occasionally occur on barley landraces, and can reach up to 65% in epidemic years (Bouajila *et al.* 2006; Beigi *et al.* 2013). *Rhynchosporium commune* is present in all barley-growing areas from northern and central Europe to the Middle East, Central Asia, North and South Africa, the Americas, Australia and New Zealand (Shipton *et al.* 1974; Robbertse *et al.* 2001; von Korff *et al.* 2004; Abang *et al.* 2006; Brunner *et al.* 2007; Beigi *et al.* 2013). The disease is particularly significant in cool, semi-humid areas, where crop canopies are aggregated and leaves are exposed to prolonged wet conditions (Shipton *et al.* 1974).

Rhynchosporium commune is splash dispersed and mainly overwinters on crop debris, seed and soil. It infects barley plants during the next growing season (Shipton *et al.* 1974; Stefansson *et al.* 2013). Infected seeds might play an important role in the introduction of scald disease to new geographical areas (Fountaine *et al.* 2010).

The pathogen was previously known as *Rhynchosporium secalis*, however recent phylogenetic and pathogenic analyses led to the description of *R. commune* as the taxon associated with *Hordeum* and *Bromus* spp. (Zaffarano *et al.* 2011). *Rhynchosporium commune* has recently been described on Italian ryegrass as well (King *et al.* 2013). This species has long been considered to be exclusively asexual and is known only from its conidial morph and host specialization nature. The development of a sexual phase under field conditions or in the laboratory has never been reported. However, there is increasing evidence of a sexual cycle in *R. commune* including the discovery of isolates of both *MAT1-1* (with alpha domain) and *MAT1-2* (with HGM-box region) in field populations (Foster and Fitt 2003; Linde *et al.* 2003; King *et al.* 2015), expression of mating type genes (King *et al.* 2015) and high levels of genotypic diversity amongst *R. commune* populations. These have been demonstrated using DNA markers, such as isozymes (Burdon *et al.* 1994), restriction fragment length polymorphism (RFLP) (McDonald *et al.* 1999; Salamati *et al.* 2000), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) (von Korff *et al.* 2004; Kiros-Meles *et al.* 2005). Based on phylogenetic analyses of the ITS-rDNA region, it was predicted that, if a sexual morph of *R. commune* exists, it

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would be a species of *Oculimacula* (Goodwin 2002; Foster and Fitt 2003).

Analyzing distribution and frequencies of mating type alleles within populations are good indications of the occurrence of a sexual cycle in fungal species. In populations with approximately equal frequencies of mating alleles, it is possible that sexual recombination does take place (Milgroom 1996; Halliday *et al.* 1999; Waalwijk *et al.* 2002; Linde *et al.* 2003; Arzanlou *et al.* 2010; Arzanlou and Narmani 2014). Mating type idiomorphs have been cloned and characterized in *R. commune* (Foster and Fitt 2003) and a multiplex polymerase chain reaction (PCR) method has been developed for one-step determination of its mating type identity in *R. commune* (Linde *et al.* 2003). Analyses of distribution and frequencies of mating type alleles among *R. commune* populations have revealed the occurrence of *MAT1-1* and *MAT1-2* types at broadly similar ratios in most barley growing regions (Linde *et al.* 2003).

Although, scald is a major disease of barley in Iran, very little is known about the population biology of the causal agent in the region and there is no data for the possibility of a clandestine sexual cycle in Iranian populations of *R. commune*. Therefore, the objective of this research was to determine the mating type frequencies of the *R. commune* population across spatial scales including different counties, fields and plants in barley fields in Kurdistan province.

Materials and Methods

Fungal isolation and DNA extraction

Leaf samples with typical scald symptoms were collected from major barley-producing areas in Kurdistan province, including seven fields in Mariwan, two fields in Dehgolan and one field in Kamyaran counties (Fig. 1). Sampling was designed in a X figure across each field to ensure consistency. *Rhynchosporium* isolates were recovered from symptomatic barley leaves using a moist chamber technique described by McDonald *et al.* (1999). A small piece from each mycelial tuft was transferred to a hand-made Potato Dextrose Agar (PDA) culture medium (200 g of potatoes, 20 g dextrose, 20 g agar, 1,000 ml distilled water). Plates were incubated at 15°C for 1 to 2 weeks in darkness until abundant sporulation occurred and pure cultures were subsequently established using a single-spore technique (Arzanlou *et al.* 2015). Cultures were preserved in 2 ml tubes containing Lima Bean Agar (LBA) culture medium at 4°C and sterile water plus glycerol at –80°C for long-term secure storage. Total genomic DNA was extracted following the protocol of Möller *et al.* (1992). The quality and quantity of DNA samples were checked on 1% agarose gel and nanodrop spectrophotometer. DNA samples were diluted in Tris-EDTA (TE) buffer (pH 8.0) and stored at –20°C for subsequent use.

Mating type determination

Mating type gene-specific primers designed by Linde *et al.* (2003) were used for amplification of the *R. commune* mating type genes. For this purpose, the primer set RsMAT1R/RsMAT1F was used for the amplification of 590 bp sized fragment from *MAT1-1* isolates and RsMAT2R/RsMAT2F set for the amplification of a 360 bp sized fragment from *MAT1-2* isolates (Linde *et al.* 2003). The reaction mixture contained 1 µl of diluted gDNA (5–20 ng), 1X PCR buffer (Cinnagen, Iran), 40 µM of each dNTP, 4 pmol of each primer, 1.5 mM $MgCl_2$ and 0.5 units Taq polymerase (Cinnagen, Iran). The reaction volume was adjusted to a total of 12.5 µl by adding sterilized distilled

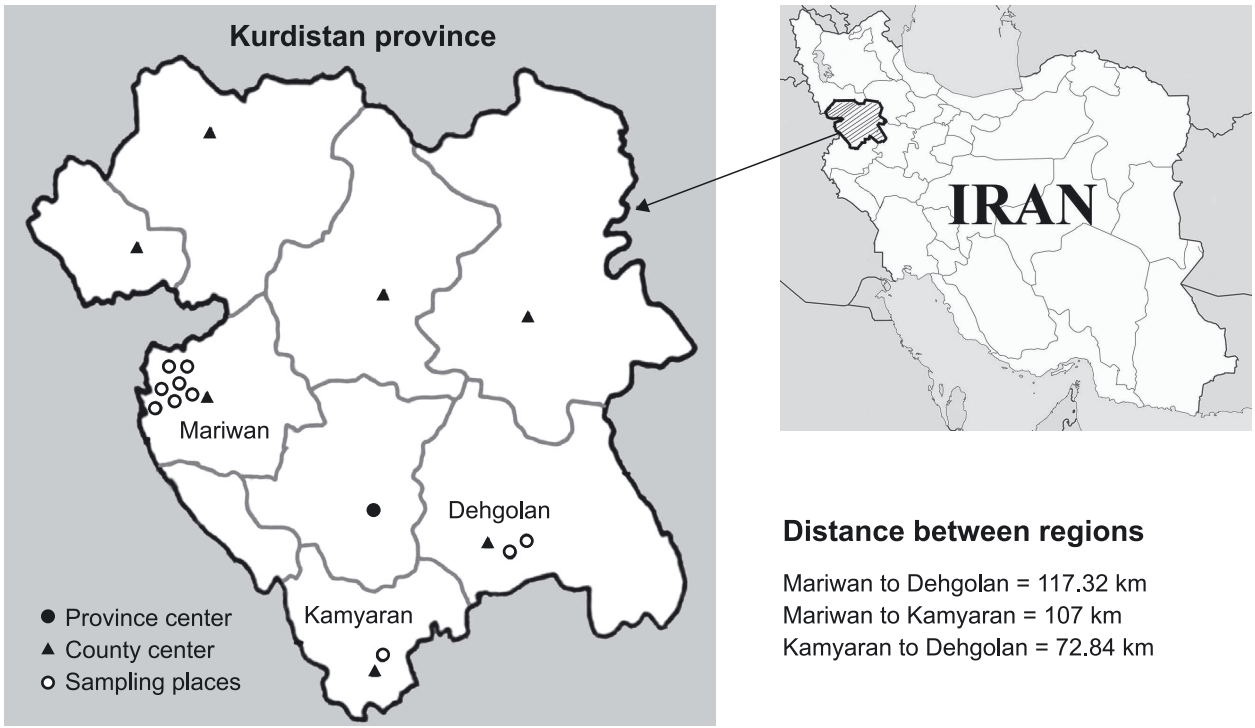

Fig. 1. The location of sites and the number of barley fields sampled in this study

water (nuclease free). The thermal cycling conditions consisted of an initial denaturation for 2 min at 96°C, followed by 40 cycles of 60 s at 96°C, 60 s at 58°C and 60 s at 72°C, with a final extension of 5 min at 72°C. The PCR products were separated by electrophoresis at 80 V for 45 min on 1% (w : v) agarose gel containing 0.1 μg · ml^{-1} ethidium bromide in 1X TAE (Tris-acetate-EDTA) buffer and visualized under UV-light.

Micro-geographical and macro-geographical distribution of mating types

The spatial distribution of *R. commune* mating types was micro-geographically determined on a single plant, different lesions and even within the same lesion. While for macro-geographical distribution of mating types (within fields and regions), the mating type frequencies of 10 field populations from Kurdistan province were determined.

Data analysis

The frequency of each mating type was separately determined at different levels (counties, fields, plants and lesions) for the *R. commune* isolates collected in Mariwan, Dehgolan and Kamyaran counties. Then, observed frequency of each mating type was calculated by χ^2 test and contingency χ^2 analysis in departures and subpopulations within micro and macro-spatial scales, respectively (Everitt 1977). All analyses were performed using the SAS software package (SAS Institute, Inc., USA, 2003).

Results

Species identification

A total number of 95 *Rhynchosporium* isolates were recovered from barley plants with typical scald symptoms. *Rhynchosporium* isolates were identified as *R. commune* based on morphological features (beaked conidia) and host plant association. According to recent molecular studies on *Rhynchosporium* species infecting grasses, *R. commune* is the only species found on cultivated barley (*H. vulgare*) (Zaffarano *et al.* 2011).

Multiplex PCR

Each primer set was first applied in separate PCR assay in order to confirm their efficacy in amplification of relevant mating type gene and to further optimize amplification conditions for the multiplex PCR test. Our results showed that RsMAT1-1F/RsMAT1-1R primer set amplified a 590 bp sized product only from the corresponding *MAT1-1* isolates. Likewise, RsMAT1-2F/RsMAT2R primer set amplified a 360 bp sized product only from *MAT1-2* isolates (Fig. 2). In multiplex PCR assay, amplicons characteristic for *MAT1-1* (590 bp) and *MAT1-2* (360 bp) genes were produced for all 95 isolates (Fig. 3). A total number of 95 isolates belonging to Mariwan (seven fields, 54 isolates), Dehgolan (two fields, 23 isolates) and Kamyaran (one field, 18 isolates) counties were subjected to the multiplex PCR assay. Each tested isolate showed either the 590-bp fragment or 360-bp fragment of the respective *MAT1-1* and *MAT1-2* genes, and none of the isolates showed both fragments (Fig. 3).

Micro-geographical distribution of mating types (within lesions and leaves)

Different isolates from the same lesion, different lesions from the same leaf and different leaves from the same field were analyzed for mating type frequencies and tested for deviations from a 1 : 1 ratio as determined with χ^2 analyses. In Mariwan county and the first field from Dehgolan county all isolates from the same lesion (n = 54 and 8) were exclusively of the *MAT1-1* and *MAT1-2* genotypes, respectively (Table 1). This phenomenon appeared both within and between leaves (Table 1). In the second field in Dehgolan and the single field in Kamyaran counties, both mating types were present in some single lesions as well as in different lesions on the same plant (Table 1).

A comparison of plants from each field confirmed unequal distribution of mating type genes for all isolates obtained from Mariwan and Dehgolan counties (Table 1).

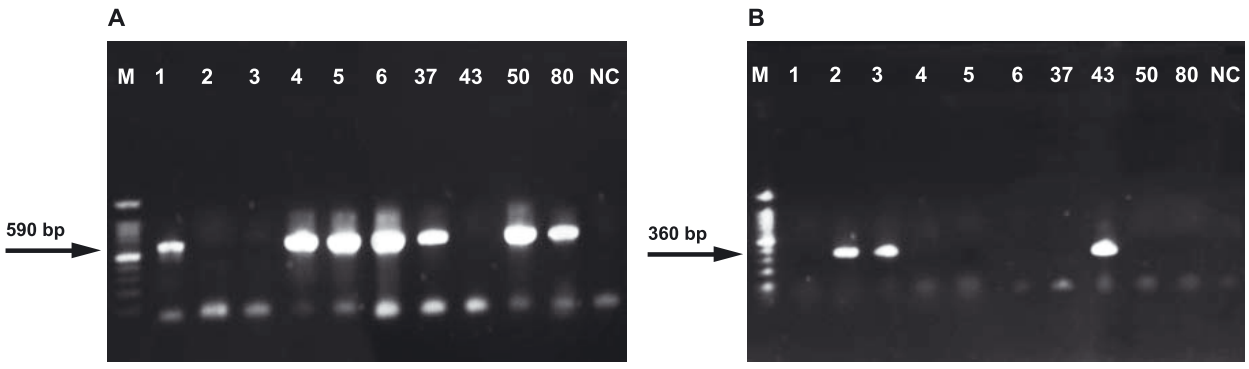

Fig. 2. Polymerase chain reaction (PCR) products resulting from *Rhynchosporium commune* DNA amplified with mating type-specific primers. Lanes 1, 4, 5, 6, 37, 50 and 80 *MAT1-1* isolates produced an amplicon of 590 bp in size (A); lanes 2, 3 and 43 *MAT1-2* isolates produced an amplicon of 360 bp (B); lane M – 100 bp DNA ladder; NC – negative control

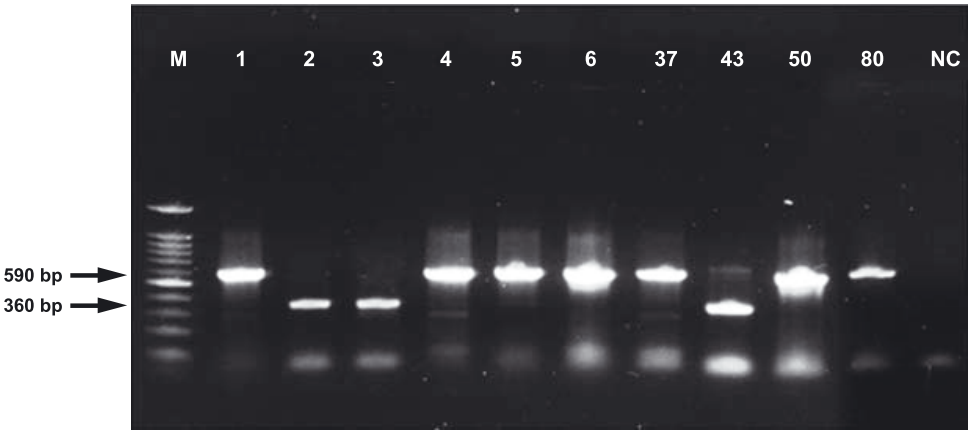

Fig. 3. Polymerase chain reaction (PCR) products resulting from *Rhynchosporium commune* DNA amplified with mating type-specific primers in multiplex assay. Lanes 1, 4, 5, 6, 37, 50, 80 *MAT1-1* isolates produced an amplicon of 590 bp in size; lanes 2, 3, and 43, *MAT1-2* isolates produced an amplicon of 360 bp; lane M – 100 bp DNA ladder; NC – negative control

Macro-geographical distribution of mating types (within and between fields and counties)

Null hypothesis (equal distribution of mating type genes) in the present study was rejected in the Mariwan and the Dehgolan counties based on χ^2 tests. Unequal mating type alleles distribution was observed in the sampled areas (Table 2). All of the 54 isolates from seven fields in Mariwan county were *MAT1-1*. In Dehgolan county the first field contained only *MAT1-2* isolates but in the second field, plus Kamyaran county, both mating types were present (Table 2). Significant deviations from the 1 : 1 ratio were recorded within all fields except in Kamyaran (Table 2).

The analysis of mating type frequencies within different counties showed a skewed distribution of mating type alleles in populations of Mariwan and Dehgolan counties (Table 2), while in Kamyaran county the ratio was nearly 60 : 40 *MAT1-1*/*MAT1-2* (Table 2).

Discussion

Identification

In the present study, 95 fungal isolates were isolated from barley leaves with scald disease symptoms. The isolates were identified as *R. commune* based on the host species and the presence of typical beak-shaped conidia (Zaffarano *et al.* 2008). The conidial morphology (beaked conidia and dimensions) was in agreement with previous works (King *et al.* 2013).

Distribution of mating type alleles at a micro-spatial scale

At the micro-geographical level: unlike Mariwan county and the first field in Dehgolan county (D1), in Kamyaran and field number 2 in Dehgolan county both mating types were detected (Table 1). This work is the first report of mating type distribution in *R. commune* over spatial scales ranging from lesions to counties regarding a group of Iranian populations of *R. commune*. No previous data are available on mating type distribution of *R. commune* in Iran. Linde and coworkers (2003) have reported the presence of both mating types in *R. commune* populations from Europe, Asia and New Zealand. Both mating types have been found within the same lesion for *Zymoseptoria tritici* (formerly *Mycosphaerella graminicola)*, a fungus known to regularly undergo sexual recombination (Zhan *et al.* 2002). Our results are in line with a lack of sexual cycle for *R. commune* in Mariwan and parts of Dehgolan counties. It can be speculated that the pathogen has entered these counties by infected plant materials or seeds, a founder effect where the corresponding mating partner has not yet entered. Conversely, in the second field in Dehgolan and in Kamyaran both mating types were found, so that a sexual cycle might occur there. The presence of both mating types in lesions and plants in those populations is in full agreement with some cases in the study of Linde *et al.* (2003).

Distribution of mating type alleles at a macro-geographical scale

At a macro-geographical level**,** equal frequency of two mating type genes was recorded only in the field from Kamyaran county, in agreement with some macro-geographical comparisons by Linde *et al.* (2003). In that study, most of the populations from different regions of Australia, Switzerland, Ethiopia, Scandinavia, California, and South Africa had equal frequencies of mating type genes (Linde *et al.* 2003). The presence of two mating type genes in the field, as in Kamyaran, or even on the same lesion, in an equal frequency, provides suitable opportunity for isolates of opposite mating types to interact and reproduce sexually (Linde *et al.* 2003). However, further investigation of genetic variation of *R. commune* populations in different counties and a comparison with the distribution of mating type genes between them using appropriate molecular markers can provide more understanding about the relationship between genetic variation and mating type genes in terms of distribution and frequency.

No sexual cycle has been observed in *R. commune* for many years despite much research (Caldwell 1937). Spore trapping work in the UK indicates that despite

Table 1. Frequencies of mating type distribution in *Rhynchosporium commune* within and between lesions, leaves and plants, respectively

Field	Plant	Leaf	Lesion	*MAT1-1*[a] frequency	*MAT1-2*[b] frequency	χ^{2c}	χ^{2d}	χ^{2e}	χ^{2f}	χ^{2g}	χ^{2h}
M1	P1	1	1	4	0	–	–	–	–	–	–
	P2	1	1	2	0	–	–	–	–	–	
			1	1	0	–					
			1	1	0	–					
M2	P1	1	1	4	0	–	–	–	–	–	–
			1			–					
		1	1	2	0	–	–	–			
	P2	1	1	1	0	–	–	–	–	–	
		1	1	1	0	–	–	–			
		1	1	2	0	–	–	–			
	P3	1	1	2	0	–	–	–	–	–	
M3	P1	1	1	1	0	–	–	–	–	–	–
	P2	1	1	1	0	–	–	–	–	–	
		1	1	1	0	–	–	–			
M4	P1	1	1	3	0	–	–	–	–	–	–
M5	P1	1	1	1	0	–	–	–	–	–	–
		1	1	2	0	–	–	–			
	P2	1	1	2	0	–	–	–	–	–	
M6	P1	1	1	2	0	–	–	–	–	–	–
			1	1	0	–					
	P2	1	1	1	0	–	–	–	–	–	
		1	1	2	0	–		–			
M7	P1	1	1	3	0	–	–	–	–	–	–
			1	3	0	–			–		
		1	1	2	0	–	–	–			
	P2	1	1	2	0	–	–	–	–	–	
			1	1	0	–					
		1	1	2	0	–	–	–		–	
	P3	1	1	3	0	–	–	–	–		
			1	1	0	–					
D1	P1	1	1	0	2	–	–	–	–	–	–
	P2	1	1	0	2	–	–	–	–	–	
			1	0	2	–					
	P3	1	1	0	2	–	–	–	–	–	
D2	P1	1	1	0	3	–	–	–	–	–	–
			1	0	2	–					
		1	1	0	1	–	–	–			
		1	1	1	1	–	–	–			
	P2	1	1	0	3	–	–	–	–	–	
	P3	1	1	1	1	–	–	–	–	–	
		1	1	0	2	–	–	–			
K1	P1	1	1	0	2	–	–	–	–	–	–
			1	0	2	–		–			
	P2	1	1	1	0	–	–	–	–	–	
			1	1	1	–					
		1	1	1	1	–	–	–			
	P3	1	1	3	0	–	–	–	–	–	
			1	0	1	–					
			1	0	1	–					
	P4	1	1	0	2	–	–	–	–	–	
		1	1	1	1	–	–	–			

M – Mariwan; D – Dehgolan; K – Kamyaran

[a, b]the number of *MAT1-1* and *MAT1-2* isolates

[c, e, g]χ^2 value based on a 1 : 1 ratio with 1 degree of freedom within lesions, leaves and plants, respectively

[d, f, h]χ^2 value based on a contingency χ^2 analyses between lesions, leaves and plants, respectively

(–) indicates χ^2 test performing was not valid where sample sizes were too small

Table 2. Frequencies of mating type distribution in *Rhynchosporium commune* within and between fields and regions, respectively

Regions	Fields	Total of isolates	*MAT1-1*[a]	*MAT1-2*[b]	χ^{2c}	χ^{2d}	χ^{2e}	χ^{2f}
Mariwan	M1	8	100	0.00	–	66.081(9)**	–	65.635(2)**
	M2	12	100	0.00	–			
	M3	3	100	0.00	–			
	M4	3	100	0.00	–			
	M5	5	100	0.00	–			
	M6	6	100	0.00	–			
	M7	17	100	0.00	–			
Dehgolan	D1	8	0.00	100	–			
	D2	15	13.33(2)	86.67(13)	8.0667(1)**		15.6957(1)**	
Kamyaran	K1	18	61.11(11)	38.89(7)	0.8889(1)		0.8889(1)	
Total		95	70.53 (67)	29.47 (28)	16.010(1)**			

[a, b]the frequency of *MAT1-1* and *MAT1-2* isolates; values in parenthesis show the number of isolates

[c, e]χ^2 value based on a 1 : 1 ratio with 1 degree of freedom within fields and regions, respectively

[d, f]χ^2 value based on a contingency χ^2 analyses between fields and regions respectively; values in parenthesis indicate degree of freedom

**indicate significant different of mating type frequencies at $p < 0.01$

the occurrence of both *MAT* types, there was no evidence for airborne ascospores (Fountaine *et al.* 2010). Moreover, a recent study has shown that the expression of the *MAT1-1* gene in *R. commune* is weak (King *et al*. 2015). Although both *MAT1-1* and *MAT1-2* type isolates are present in *R. commune*, *R. agropyri* and *R. secalis* populations; *R. orthosporum* and *R. lolii* appear to harbor only the *MAT1-1* gene (King *et al*. 2015). Therefore, it has been postulated that the genus may be undergoing a shift toward asexuality which helps relevant species to at least develop geographically (King *et al*. 2015). In fact, mating type genes play an important role in population dynamics and the evolution of fungal species (Turgeon *et al.* 1993; Turgeon 1998). Knowledge about structure, evolution and functionality of these genes can provide insight into the potential prevalence of sex in species of *Rhynchosporium*, all of which are currently thought to be asexual. In the present study we analyzed the distribution and frequency of mating type genes among and within *R. commune* isolates collected from 10 barley fields in western Iran (Kurdistan province). The existence of only one mating type gene among populations might imply that founder effect has played a major role in shaping *R. commune* populations in these counties. Even though, both mating types were present in some counties, we think that additional populations from those counties should be screened in order to get a better view of the sexual structure *R. commune*. However, some studies related to fungal species with known sexuality show a remarkable deviation from the 1 : 1 ratio in natural populations (Consolo *et al.* 2005; Rhaiem *et al.* 2008); therefore, the lack of sexual recombination due to the absence of an equal distributions of *MAT* genes cannot be claimed with certainty (King *et al.* 2015).

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