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Uneven distribution of mating type alleles in Iranian populations of *Cercospora beticola*, the causal agent of *Cercospora* leaf spot disease of sugar beet

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Summary. *Cercospora beticola*, the causal agent of Cercospora leaf spot disease on sugar beet, is thought to be exclusively asexual because no teleomorph has yet been found. The possibility of a clandestine sexual cycle in the Iranian population of *Cercospora beticola* was evaluated by analyzing the distribution and frequency of the mating type alleles on a microspatial and a macrogeographical scale. A total of 89 single-conidial *Cercospora beticola* isolates were obtained from sugar beet fields in the Moghan, the Talesh and the Khoy regions. The isolates were identified using a *Cercospora beticola*-specific primer set in a PCR assay. A multiplex PCR method using previously designed mating type primers was used to study the distribution and the frequency of the mating type alleles. All isolates showed either the 805-bp fragment or the 442-bp fragment of the *MAT1-1* and *MAT1-2* genes, but no isolate had both fragments. The distribution of the mating type genes in the sampled areas was uneven. From three sugar beet fields sampled in the Moghan region, two fields had only *MAT1-1* isolates; while in the third field all isolates had only the *MAT1-2* allele. In the Talesh region only *MAT1-1* isolates occurred, and in the Khoy region the mating type alleles were uniformly distributed amongst the isolates. The skewed distribution of mating type alleles in Northwestern Iran was in line with the lack of a sexual cycle for this species and may also indicate that sugar fields in the Moghan region were infected by *C. beticola* populations of different origins.

Key words: Cercospora leaf spot, multiplex PCR, molecular diagnostics, calmodulin, sexual reproduction.

Introduction

Cercospora beticola causes cercospora leaf spot (CLS) in sugar beet and is the major foliar pathogen of sugar beet world-wide (Holtschulte, 2000). The disease is particularly damaging in warm, humid areas, and significantly reduces root yield, sucrose concentration, and recoverable sugar, while increasing the concentration of impurities, resulting in higher processing costs (Smith and Rupple, 1973; Shane and Teng, 1992). *Cercospora beticola* survives as stromata in crop residues. Conidia produced on these survival structures serve as primary inoculum during the next cropping season. Although wind is the main dispersal factor and the leaf is the primary infection site for *C. beticola* (Khan *et al.*, 2008), CLS symptoms are also produced through root infection (Vereijssen *et al.*, 2004). In addition to sugar beet, several weeds are susceptible to *C. beticola*, including *Chenopodium album* L., *Amaranthus retroflexus* L., *Malva rotundifolia* L., *Plantago major* L., *Arctium lappa* L. and *Convolvulus arvensis* L. (Windels *et al.*, 1998; Jaccobsen *et al.*, 2000). These weed hosts may serve as important reservoirs for *C. beticola* over long periods when sugar beet is not available (Lartey *et al.*, 2003).

Besides *C. beticola*, at least two other species of

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Cercospora, C. apii and *C. apiicola* occur on sugar beet. Identification of these species based solely on cultural and morphological characteristics is difficult (Groenewald *et al.*, 2006). Recently, molecular detection tools have been developed to facilitate accurate and rapid detection of *C. beticola* from both pure culture and naturally infected hosts (Lartey *et al.*, 2003; Groenewald *et al.*, 2005). A *C. beticola*-specific primer set has been developed based on sequence data from the actin gene, which enabled successful detection of *C. beticola* from field samples using a PCR assay (Lartey *et al.*, 2003). Groenewald *et al.* (2005) used sequence data from the calmodulin gene to develop species-specific primers to distinguish *Cercospora* species occurring on sugar beet using a multiplex PCR protocol.

Although no teleomorph has been identified for most of the species in the genus *Cercospora*, including *C. beticola*, a wide array of phenotypic diversity has been described for *C. beticola* isolates, including variation in conidium morphology, cultural characteristics, pathogenicity and resistance to fungicides (Rossi, 1995; Moretti *et al.*, 2004; Weiland and Koch, 2004). In addition, a high genetic variation has been seen in *Cercospora* isolates from sugar beet fields (Moretti *et al.*, 2006; Groenewald *et al.*, 2008). The genus *Cercospora* is a well-established anamorph of *Mycosphaerella* (Crous and Braun, 2003), and phylogenetic analyses of a variety of *Cercospora* species have placed them as a well-defined clade within *Mycosphaerella* (Goodwin *et al.*, 2001). Therefore, if a sexual stage does exist for *C. beticola*, it would be a species of *Mycosphaerella*.

A knowledge of the genetic structure and reproductive capability of a population is essential for developing disease control strategies (Turgeon, 1998). Since there is no known sexual stage, several approaches can be used to test for evidence of sexual reproduction. Populations that regularly undergo sexual recombination should have many more genotypes resulting in higher levels of genotypic diversity than populations with only asexual reproduction (Milgroom, 1996). Another method to test the possibility of sexual reproduction is to establish the occurrence and frequency of mating type genes. Although the presence of mating type idiomorphs in a species is insufficient to prove that species has a sexual stage, it is probable that sexual recombination takes place if the two mating types occur with approximately equal frequency within a population of that species (Milgroom, 1996; Halliday *et al.*, 1999; Waalwijk *et al.*, 2002; Linde *et al.*, 2003).

Sexual reproduction frequently results in genetic recombination and recombination has a major impact on the dynamics and fitness of a species. The teleomorph of *C. beticola* is unknown. As a first step to elucidate the reproductive cycle of *C. beticola* isolates found in Iran, the objectives of this study were: 1) to determine the mating type frequencies of *C. beticola* population on different spatial scales including different regions, fields, plants and lesions in sugar beet fields from northwestern of Iran, and 2) to induce the perfect stage of *C. beticola* in the laboratory.

Materials and methods

Fungal isolation and DNA extraction

Leaf samples with *C. beticola* leaf spot were collected from major sugar beet-producing areas in northwestern of Iran, including three fields in Moghan, one field in the Talesh and one field in the Khoy region. Leaves were randomly sampled from each field to ensure consistency. In each field, four plants and from each plant one leaf and four lesions per leaf were selected. Single-conidial isolates were obtained from symptomatic sugar beet leaves following the method described by Crous (1998), briefly, under a stereomicroscope, a mass of conidia were scraped off from the lesion using a sterile inoculation needle and the conidia were then floated on 2% malt extract agar (MEA, Fluka, Hamburg, Germany) containing 10 mL distilled water and supplemented with 100 mg L^{-1} streptomycin sulphate and 100 mg L^{-1} ampicillin. Plates were incubated in a slanted position overnight, and the germinated conidia were transferred to new MEA plates. A total of 200 isolates were obtained 152 from three fields in the Moghan region, 40 from one field in the Talesh region, and eight from one field in the Khoy region. Five tester isolates (CPC12024 MAT1-2, CPC12025 MAT1-1, CPC12027 MAT1-1, CPC12190 MAT1-2, and CPC12191 MAT1-1) were obtained from the Culture collection of Pedro Crous (CPC), housed at the CBS-KNAW Fungal Biodiversity Centre in Utrecht, the Netherlands. Tester isolates were used as positive controls for the optimization of the ex-

perimental conditions and crossing experiments. Genomic DNA was extracted from eight-day-old fungal isolates grown on 2% MEA at 25°C in dark using the method of Moller *et al.* (1992).

Molecular diagnostics

Primers CercoCal-F, CercoCal-R and CercoCal-beta designed by Groenewald *et al.* (2005) based on the partial DNA sequence of the calmodulin gene, were used in this study. Primers CercoCal-F (CGCGAGGGCAGAGCTAACGA) and CercoCal-R (GTGAGGAATTCGGGGAAATC) acted as outer primers and their amplification functions acted as a positive control. The internal primer CercoCal-beta (GCCCACCCTCTGCGAATGTA) was specific for *C. beticola*. The reaction mixture had a total volume of 12.5 µL and contained 1 µL of diluted gDNA (5–20 ng), 1×PCR buffer (Cinnagen, Tehran, Iran), 1.5 mM $MgCl_2$, 40 µM each of the dNTPs, 1 pmol of CercoCal-F, 3 pmol of each of CercoCal-R and the specific internal primer, and 0.7 units Taq polymerase (Cinnagen, Tehran, Iran). The initial denaturation step was done at 95°C for 5 min, followed by 40 cycles of 95°C (30 s), annealing at 58°C (30 s) and elongation at 72°C (30 s). A final elongation step at 72°C (7 min) was included. The products were separated by electrophoresis at 90 V for 45 min on a 1% (w:v) agarose gel containing 0.1 µg mL^{-1} ethidium bromide in 1×TAE buffer (0.4 M tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV-light.

Mating type determination

A total of 89 isolates (58 from the Moghan, 23 from the Talesh and eight from the Khoy region) were screened for mating type alleles. The two primer sets, CercosporaMat1 and CercosporaMat2, designed by Groenewald *et al.* (2006), were used in a multiplex PCR to screen for the two mating type genes in *C. beticola* populations. The reaction mixture had a total volume of 12.5 µL and contained 1 µL of diluted gDNA (5–20 ng), 1×PCR buffer (Cinnagen), 40 µM of each dNTP, 4 pmol of each primer, 1.5 mM $MgCl_2$ and 0.7 units Taq polymerase (Cinnagen). The initial denaturation step was done at 94°C for 5 min, followed by 40 cycles of 94°C (20 s), 60°C (30 s) and 72°C (50 s); a final elongation step at 72°C (5 min) was included. The products were separated on a 0.8% (w:v) agarose gel and visualized as described above.

Microspatial distribution of mating types (within lesions and leaves)

Two to four lesions from each leaf and two to four isolates per lesion were selected to determine the spatial distribution of *C. beticola* mating types within the leaves and the lesions.

Macrogeographical distribution of mating types (within fields and within regions)

The mating type frequencies of five field populations (three fields from the Moghan, one from the Talesh and one from the Khoy region) were determined using multiplex PCR.

Data analysis

Isolates were tested on different spatial scales in a hierarchy to determine the mating type distribution within lesions, within leaves, within field plots and within field populations. To detect whether any significant deviation from the expected 1 : 1 ratio occurred, the results were analyzed using the χ^2 test. Comparisons were made between fields, between and within plants from the same field and between lesions from the same plant.

Attempts to induce formation of the teleomorph

Various methods were tried to induce a teleomorph of *C. beticola*, including the following. 1) two opposite mating type strains were inoculated on opposite sides of Petri dish (d = 9.0 cm) containing water agar. Autoclaved sugar beet leaf pieces were placed 1.5–2 cm away from both isolates and used as a mating stimulator. The plates were incubated at 25°C in darkness for four weeks. 2) Two opposite mating type strains were inoculated on opposite sides of a Petri dish (diam = 9.0 cm) containing 2% MEA. After 5 days, a 5 mm diameter disc of sterile filter paper immersed in fungicide (carbendazim 60%, 0.04 g 100 mL^{-1} H_2O) suspension was placed on the MEA as a mating stimulator. 3) Two opposite mating type strains were inoculated on opposite sides of a Petri dish (diam = 9.0 cm) containing 2% MEA. After 5 days, a filter paper disc (diam= 2.0 cm) was placed between the two colonies on the MEA, as a physical barrier to stimulate sexual reproduction. 4) Two isolates of C. *beticola* with opposite mating types were used in an *in planta* protocol; 5 mm diameter plugs of mycelium were removed from 8-day-old colonies of

opposite mating type strains grown on 2% MEA using a cork borer. Mycelial plugs were mixed and then suspended in 200 ml sterile distilled water; a hyphal suspension was sprayed on 2-month-old sugar beet plants grown in a greenhouse (27°C, >85% RH). Each test was repeated four times in three replicates.

Results

Species identification

All isolates had both the 234-bp and the176-bp fragment. The 234-bp fragment, served as the positive control and the 176-bp fragment was specific for *C. beticola*, so that all the isolates were identified as *C. beticola* (Figure 1). Our results were consistent with published data (Groenewald *et al.*, 2006).

Multiplex PCR

Mating types of *C. beticola* were readily identified in a multiplex PCR following amplification of a 805 bp *MAT1-1* specific product (part of the α-domain) and a 442 bp *MAT1-2* specific product (part of the HMG-box). A total of 89 *C. beticola* isolates, including 58 from the Moghan, 23 from the Talesh (Table 3), and eight from the Khoy region, which were collected from a sugar beet field (two plants, two lesions per plant and two isolates per lesion) were assayed for mating type. Each isolate showed either the 805-bp fragment or the 442-bp fragment of the respective *MAT1-1* and *MAT1-2* genes, but no isolate showed both fragments (Figure 2).

Microspatial distribution of mating types within lesions and within leaves

Different isolates from the same lesion, different lesion from the same leaf and different leaves from the same field were analyzed for mating-type frequency and tested for deviations from a 1:1 ratio as determined with χ^2 analysis. In the Moghan and the Talesh region, the isolates from the same lesion and from the same leaf had the same mating type (Tables 1 and 2); so that there was a significant deviation from the 1:1 ratio within leaves and within lesions. In the Khoy region, all isolates taken from a single lesion always had the same mating type. However, on different lesions on the same plant both mating types were found.

Macrogeographical distribution of mating types within fields and within regions

The null hypothesis of random mating was rejected ($P<0.01$) in the Talesh and the Moghan regions because of the χ^2 tests and the unequal

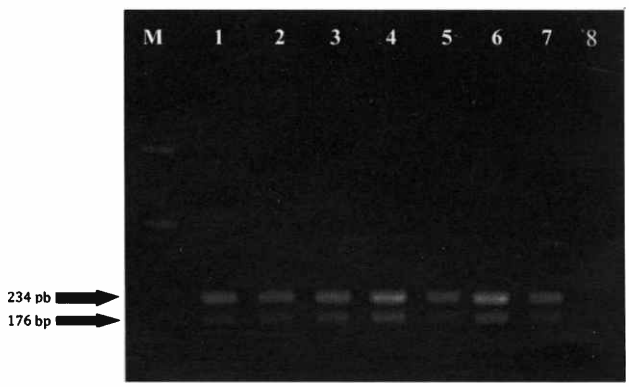

Figure 1. Identification of *Cercospora beticola* using species-specific primers (CercoCal-F, CercoCal-beta) in combination with (CercoCal-R). The primer set (CercoCal-F and CercoCal-R) amplifies an amplicon of 234 bp, which acts as a positive control; the primer set CercoCal-F and CercoCal-beta specifically amplies a 176 bp fragment from *C. beticola*. Lane M, a 100 bp DNA Ladder; Lane 1, positive control (CPC 12024). Lanes 1–7, Iranian *C. beticola* isolates; Lane 8, negative control (master mix).

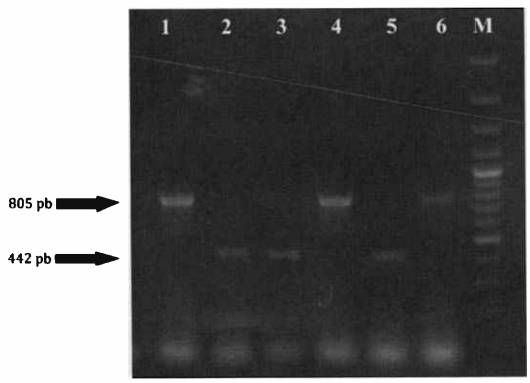

Figure 2. Amplification results with CercosporaMat1 and CercosporaMat2 primer sets using template DNA from Iranian *Cercospora beticola* isolates in a multiplex PCR assay. Lane 1, negative control; lanes 2, 5 and 7, *Mat1-1* isolates, producing an amplicon of 805 bp; lanes 3, 4 and 6, *MAT1-2* isolates producing an amplicon of 442 bp; M, 100 bp DNA ladder.

distribution of mating type genes in those regions. All the 23 isolates from the sugar beet field in Talesh were *MAT1-1* and no *MAT1-2* was found. Among isolates from the three sugar beet fields in the Moghan region only *MAT1-1* isolates occurred in two fields and in the third field only *MAT1-2* isolates occurred (Table 3), while in the Khoy region both mating types were found in the sampled field, which was in agreement with published data (Groenewald *et al.*, 2006).

Attempts to induce the sexual cycle

All methods used to induce formation of the perfect stage by crossing opposite mating types of *C. beticola* isolates were unsuccessful. Inoculation of plants, however, induced the typical lesions.

Discussion

Molecular detection

A total of 89 *C. beticola* isolates (58 from the Moghan, 23 from the Talesh and eight from the Khoy region) were selected to study the distribution of mating-type alleles. Mating-type alleles were screened with a multiplex PCR assay using the primer pairs designed by Groenewald *et al.* (2006); Cercospora MAT1-1F/R (805 bp fragment) and Cercospora MAT1-2F/R (442 bp fragment). Since these primers also amplify the mating-type genes of other *Cercospora* species, they were first screened with *C. beticola*-specific primers. The calmadulin gene has been found very effective in distinguishing the three species described from sugar beet, *C. beticola*, *C. apii* and *C. apiicola*, consequently this area was targeted for the species-specific diagnostic test (Groenewald *et al.*, 2005). We used these primer sets to identify *Cercospora* isolates from sugar beet samples with CLS symptoms. All isolates from sugar beet yielded an amplicon corresponding in size to *C. beticola*, and in line with the cultural and morphological characters of *C. beticola* (Figure 1). In the present study we used DNA from pure cultures of *Cercospora beticola* isolates for molecular detection. *Cercospora beticola* has a wider host range than sugar beet and occurs on a number of weed hosts; therefore, it would be worthwhile to test the

Table 1. Frequencies of mating type distribution of *Cercospora beticola* within and among lesions, plants and fields.

Field- plant	Lesion	No.	*MAT1-1* frequency	*MAT1-2* frequency	χ^2 values (df) within lesions	χ^2 values (df) between lesions
Moghan1 (plant 3)	1	4	1.00	0.00	(1)4.00*	0.00
	2	4	1.00	0.00	(1)4.00*	
	3	4	1.00	0.00	(1)4.00*	
	4	4	1.00	0.00	(1)4.00*	
	Total	16	1.00	0.00	(1)16.00*	
Moghan2 (plant 3)	3	4	0.00	1.00	(1)4.00*	0.00
	4	4	0.00	1.00	(1)4.00*	
	Total	8	0.00	1.00	(1)8.00*	
Talesh1 (plant 4)	1	4	1.00	0.00	(1)4.00*	0.00
	2	4	1.00	0.00	(1)4.00*	
	3	2	1.00	0.00	(1)2.00*	
	Total	10	1.00	0.00	(1)10.0*	

*, Mating type frequencies that differ significantly at $P<0.05$.

Table 2. Frequencies of mating type distribution in *Cercospora beticola* populations within and among plants.

Plants	Total of isolates	*MAT1-1* frequency	*MAT1-2* frequency	χ^2 values (df) within plants	χ^2 values (df) between plants
Moghan (field 1)					
1	8	1.00	0.00	8.00*	0.00
2	3	1.00	0.00	3.00*	
3	16	1.00	0.00	16.00*	
4	5	1.00	0.00	5.00*	
Total	32	1.00	0.00	32.00*	
Moghan (field 2)					
1	7	0.00	1.00	7.00*	0.00
2	8	0.00	1.00	8.00*	
Total	15	0.00	1.00	15.00*	
Moghan (field 3)					
1	4	1.00	0.00	4.00*	0.00
3	6	1.00	0.00	6.00*	
Total	10	1.00	0.00	10.00*	
Talesh (field 1)					
1	5	1.00	0.00	5.00*	0.00
2	8	1.00	0.00	8.00*	
4	10	1.00	0.00	10.00*	
Total	23	1.00	0.00	23.00*	

*, See Table 1.

efficacy and specificity of these primer sets for the amplification of *C. beticola* on DNA extracted from naturally infected host leaves. Lartey *et al.* (2003) developed a PCR protocol based on sequence data from the actin gene, which successfully detected *C. beticola* from naturally infected host plants. We are currently optimizing a PCR protocol for the amplification of *C. beticola* from naturally infected host plants as well as from soil samples of sugar beet fields using the calmodulin primer sets.

Distribution of mating type alleles on microspatial scale

We found only one mating type on either microspatial scale tested. The two mating types were never detected occurring together in the same lesion in any of the sugar beet leaves screened in the study. Both mating types were not detected in the same leaf in any sugar beet plants from the Moghan and Talesh regions; however, both mating types occurred once together on the same leaf from a sugar beet plant in the Khoy region. This is the first report on distribution of *C. beticola* mating types on spatial scales varying from lesions to regions. Groenewald *et al.*(2006) reported that both mating types occur in *C. beticola* populations from Europe, Iran and New Zealand but they did not determine the mating type of isolates from single lesions or from individual leaves. In the case of *Mycosphaerella graminicola*, which regularly undergoes sexual recombination both mating types have been found in the same lesion (Zhan *et al.*,

Table 3. Frequencies of mating types in *Cercospora beticola* from different sampling areas and statistical analysis of the mating type distribution within and among fields.

Field	Total of isolates	*MAT1-1* frequency	*MAT1-2* frequency	χ^2 values (df) within fields	χ^2 values (df) between fields
Moghan (field 1)	33	1.00	0.00	(1)33.00*	(3)48.89*
Moghan (field 2)	15	0.00	1.00	(1)15.00*	
Moghan (field 3)	10	1.00	0.00	(1)10.00*	
Talesh (field 1)	23	1.00	0.00	(1)23.00*	

* See Table 1.

2002). In their study, Groenewald *et al.* (2006), found that mating type alleles were evenly distributed among *C. beticola* isolates from France, Germany, Italy, the Netherlands, Iran and New Zealand. Equal distribution of mating type alleles in *C. beticola* and its high genetic diversity are consistent with the hypothesis that this fungus has a clandestine sexual form (Moretti *et al.*, 2006; Groenewald *et al.*, 2007, 2008). However, the teleomorph still remains to be discovered. No sexual propagation is obviously present in this species in the Moghan and Talesh regions.

Distribution of mating type alleles on a macrogeographical scale

No mating would occur in the Moghan and Talesh populations. *MAT1-2* isolates did not occur in the Talesh fields. The Talesh region is not a big sugar beet cultivation area, such that it appears the population structure of *C. beticola* in this region was shaped by the founder effect, or by contaminated seeds, and *MAT1-2* isolates of *C. beticola* have not been introduced to the region.

Two fields of the Moghan region had only *MAT1-1* isolates and one field had only *MAT1-2* isolates. More sugar beet fields will have to be screened to ascertain whether *MAT1-1* isolates are more frequent in the region as a whole. The *C. beticola* populations of individual fields may well have an independent origin arising from the human introduction of a single genotype to a field (Founder effect). In addition, only one mating type was found to occupy the same lesion, leaf or plant, so that based on chi-square test of mating-type ratios ($P>0.05$), the hypothesis of random mating for *C. beticola* populations in the Moghan and the Talesh regions can be rejected. Mating type genes are frequently used in population studies since their presence, relative frequency and distribution within a population may indicate the reproductive modes of a fungus (Milgroom, 1996). In a sexual population, negative frequency-dependent selection is expected to retain an equilibrium between the two mating-type idiomorphs, while in an asexual population this ratio is skewed (Yun *et al.*, 1999; Richman, 2000). The uneven distribution of mating types in the Moghan and Talesh regions suggests that the sexual recombination of *C. beticola* is rare or non-existent.

The findings for the Khoy region were consistent with Groenewald *et al.* (2006) from France, Germany, Italy, The Netherlands, Iran (Khoy, Pakajik region) and New Zealand. In that study both mating types were fairly evenly distributed among six populations of *C. beticola*, suggesting that the genes may be functional in these populations. The results from the other regions examined in the present study were inconsistent with the findings of Groenewald *et al.* (2006). It is likely that the Moghan and Talesh populations of *C. beticola* reproduce asexually favored by the warm and humid climate of these regions. This would explain why the two mating types of *C. beticola* in these regions differ in their frequency and have an uneven distribution.

Mating type genes play an important role in the biology and evolution of fungal species. A knowledge of these genes can provide insight into the prevalence of sex in species of *Cercospora*, most of which are currently thought to be asexual. The Moghan and Talesh populations of *C. beticola* provide a good model to study its genetic diversity in

absence of one of the mating types, since these population lack the potential of a clandestine sexual cycle. This should be a subject for further study.

Attempts to induce the teleomorph

Various crossing procedures have been attempted to induce the formation of ascomata in fungal species. Some of these methods have been proven to be successful, such as by *in vitro* stimulation, *e.g* using sterilized host leaves in *M. fijiensis* (Etebu *et al.*, 2003), in *M. citri* (Mondal *et al.*, 2004), and in *Setosphaeria turcica* (Yongshan *et al.*, 2007), and by *in planta* stimulation in *Septoria passerinii* (Ware *et al.*, 2007). However, our attempts to cross isolates of *C. beticola* with opposite mating types did not yield any sexual spores, nor have these spores been found in nature, although the high genotypic diversity in natural populations of *C. beticola* (Moretti *et al.*, 2006: Groenewald *et al.*, 2007, 2008) also suggests the possibility of a sexual cycle. Arzanlou *et al.* (2010) were also not successful in their attempt to cross *M. fijiensis* isolates with the opposite mating type and to induce formation of ascomata and ascospores. There are several possible reasons for the failure to induce the sexual stage between opposite mating types. The isolates may have become infertile during culture. Furthermore, the sterility might be attributed to mutations or to the absence of genes, other than MAT genes, that are required for ascoma formation (Sharon *et al.*, 1996).

From the data presented in this study, it is now clear whether the distribution and frequency of mating type alleles in Iranian populations of *C. beticola*, is deviating from the other populations studied from different geographical areas, worldwide. Hence, *C. beticola* populations from Iran may provide a good model to study population dynamism of asexual plant pathogenic fungi in the presences of both mating type alleles or in the absence of one of the mating type alleles in a given population. We are currently screening more *C. beticola* populations from different climatic regions in Iran for mating type distribution and frequency which will further elucidate genetic variation within and amongst these populations.

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