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ORIGINAL ARTICLE

Genetic diversity and population structure of *Ascochyta rabiei* from the western Iranian Ilam and Kermanshah provinces using MAT and SSR markers

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Abstract Knowledge of genetic diversity in *A. rabiei* provides different levels of information that are important in the management of crop germplasm resources. Gene flow on a regional level indicates a significant potential risk for the regional spread of novel alleles that might contribute to fungicide resistance or the breakdown of resistance genes. Simple sequence repeat (SSR) and mating type (MAT) markers were used to determine the genetic structure, and estimate genetic diversity and the prevalence of mating types in 103 *Ascochyta rabiei* isolates from seven counties in the Ilam and Kermanshah provinces of western Iran (Ilam, Aseman abad, Holaylan, Chardavol, Dareh shahr, Gilangharb, and Sarpul). A set of 3 microsatellite primer pairs revealed a total of 75 alleles; the number of alleles varied from 15 to 34 for each marker. A high level of genetic variability was observed among *A. rabiei* isolates in the region. Genetic diversity was high (He=0.788) within populations with corresponding high average gene flow and low genetic distances between populations. The smallest genetic distance was observed between isolates from Ilam and Chardavol. Both mating types were present in all populations, with the majority of the isolates belonging to *Mat1-1* (64%), but within populations the proportions of each mating type were not significantly different from 50%. Results from this study will be useful in breeding for Ascochyta blight-resistant cultivars and developing necessary control measures.

Keywords Ascochyta blight · *Ascochyta rabiei* · Genetic diversity · Gene flow

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Introduction

Ascochyta blight, caused by the fungus *Ascochyta rabiei* (Pass.) Labr., is the most destructive disease of aerial parts of chickpea (*Cicer arietinum* L.) in chickpea-producing regions of the world (Nene and Reddy 1987). Iran is the fourth largest chickpea producer in the world (http://faostat.fao.org) of which Kermanshah and Ilam are important chickpea growing areas that suffer severe epidemics every year. *Ascochyta rabiei* is a haploid, heterothallic dothideomycete fungus within the class Pleosporales (Trapero-Casas and Kaiser 1992; Wilson and Kaiser 1995). The life cycle of this fungus involves several asexual generations during the growing season of the crop, and a sexual or perfect stage (*Didymella rabiei* (Kovachevski) v. Arx.) which develops on infected plant debris during the winter (Navas-Cortes et al. 1998; Trapero-Casas and Kaiser 1992; Trapero-Casas et al. 1996). Although the teleomorph's influence on disease epidemiology and pathogen diversity is still unclear, it may be more widespread than previously thought, and ascospores

may serve as a primary inoculum (Kaiser and Hannan 1987; Trapero-Casas et al. 1996).

The disease appears in the field firstly as small infected foci and then spreads quickly when cool and wet conditions prevail. The severity of disease can be reduced through the use of resistant cultivars, fungicide applications, and cultural practices such as crop rotation, removal of debris, and sowing clean seed. Periodic appearances of new and more virulent strains of the pathogen are a major problem in resistance breeding (Grewal 1984; Singh et al. 1981).

The genetic structure of a population is the amount and distribution of genetic variation within and among populations of a species and reflects the evolutionary history of populations (McDonald and Linde 2002). The amount of genetic variation within a population indicates how rapidly a pathogen can evolve and thus provides valuable information in predicting the effectiveness of control measures such as host resistance genes or chemical control (McDermott and McDonald 1993). The main evolutionary forces that contribute to the genetic structure within and between populations are gene flow, genetic drift, reproduction/mating systems, population size, and selection. Molecular markers have been widely deployed to detect and identify *A. rabiei* isolates and to examine genetic diversity, genetic structure, and virulence in populations of this fungus (Morjane et al. 1994; Peever et al. 2004; Phan et al. 2003; Santra et al. 2001; Udupa et al. 1998). Significant genetic variation was shown in populations of *A. rabiei* in Italy (Fischer et al. 1995), Tunisia (Geistlinger et al. 2000; Morjane et al. 1994), Syria and Lebanon (Udupa et al. 1998), Spain (Navas-Cortés et al. 1998), Pakistan (Jamil et al. 2000) and India (Santra et al. 2001). In some regions of the world where chickpea has only recently been introduced, for example Australia and California, only modest genetic diversity and a single mating type has been reported (Kaiser 1997; Phan et al. 2003).

To examine genetic diversity and population structure among 103 *A. rabiei* isolates from western Iran, we deployed simple sequence repeat (SSR) markers developed by Geistlinger et al. (2000). Amongst different genetic markers available for such studies, SSRs are routinely used because they are abundant, distributed over the euchromatic part of the genome and are highly polymorphic (Schlötterer and Ellegren 1998; Zane et al. 2002). The presence, distribution and frequency of *A. rabiei* mating types in regions of Tunisia have been used to shed light on recent introductions as opposed to stable, randomly mating populations (Rhaiem et al. 2007). To examine the relative proportions of *MAT 1-1*or *MAT 1-2* allelic groups in this study, we applied the MAT-specific multiplex PCR assay developed by Barve et al. (2003).

Fig. 1 Geographical origins of the seven *A. rabiei* populations in western Iran used in this study

Materials and methods

Sampling, isolation and maintenance of fungal isolates

Chickpea plants with brown or black lesions on stem, leaves and pods were randomly collected in 2007 from seven different regions in the western Iranian Ilam and Kermanshah provinces. For the purposes of this study, isolates belonging to these regions are considered as populations (Fig. 1; Table 1). While these regions are geographically juxtaposed, they vary in altitude and climate, are separated by substantial mountain ranges

Table 1 Geographical origin of *A. rabiei* populations from western Iran

Province	Origin site	No. of isolates	Population number
Ilam	Ilam	16	1
Ilam	Aseman abad	12	2
Ilam	Holaylan	12	3
Ilam	Chardavol	19	4
Ilam	Dareh shahr	16	5
Kermanshah	Gilane gharb	15	6
Kermanshah	Sarpul	13	7

Table 2 SSR primer sequences used in this study (Geistlinger et al. 2000). The sequences include the generic tag sequences (forwards 5'-3' ACGACGTTGTAAAA and reverse 5'-3' CATTAAGTTCCCATTA) described by Hayden et al. (2008)

Expected size (bp)	Primer sequence (5'→3')	Repeat motif	Primer locus
197	ACGACGTTGTAAAACTGTCACAGTAACGACAACG CATTAAGTTCCCATTAATTCCAGAGAGCCTTGATTG	$(CAA)_9(CAG)_7(CAA)_{21}$	*Ar*H06T
409	ACGACGTTGTAAAATAGGTGGCTAAATCTGTAGG CATTAAGTTCCCATTACAGCAATGGCAACGAGCACG	$(GAA)_{31}$	*Ar*A03T
227	ACGACGTTGTAAAACATTGTGGCATCTGACATCAC CATTAAGTTCCCATTATGGATGGGAGGTTTTTGGTA	$(CTT)_{18}$	*Ar*H05T

(largely but not exclusively along a north-west to south-east axis) and most rural areas have limited road access. Diseased samples were cut into 2- to 5-mm-long pieces, surface sterilized by dipping into domestic bleach solution (1% NaOCl) for 2–3 min, washed three times with sterile distilled water, dried with sterile filter paper and plated on potato dextrose agar (PDA) or chickpea seed meal agar (CSDA). Samples were incubated for 3 days in an incubator at 23°C with a 12-h photoperiod to induce production of conidia. Isolates were single-spored by harvesting conidia from a single pycnidium with a sterile needle and streaking them on a 2% water agar plate. Conidia were incubated as above and a single germinated conidium sub-cultured onto PDA or CSDA plates and incubated with a 12-h photoperiod for 2–3 weeks. Single-conidial isolates were stored on sterile filter paper at 20°C.

DNA extraction

Liquid cultures were initiated by adding 2–4 mm^2 pieces of filter paper to 250-mL Erlenmeyer flasks containing 100 mL PDB medium (potato dextrose broth plus 2 g yeast extract per liter). Flasks were incubated at room temperature approximately 25°C on a rotary shaker for 6–8 days. Mycelium was collected by filtration through sterile filter paper with a vacuum funnel. Mycelia were harvested, frozen and stored at −20°C. DNA was extracted using a modified hexadecyl trimethyl-ammonium bromide (CTAB) procedure (Doyle and Doyle 1987). Mycelia were ground in liquid nitrogen and suspended in 2% CTAB extraction buffer (1.4 M NaCl, 0.1 M Tris-HCl, pH 8.0, 20 mM EDTA, 0.2% β-mercaptoethanol). Samples were treated with 5 units RNAse at 37°C for 30 min., then extracted with chloroform-isoamyl alcohol 24:1 (v/v). DNA in the supernatant was precipitated with isopropanol, rinsed with ethanol, and adjusted to a final concentration of 20 ng/μl in TE (pH 7.4).

SSRs and mating type (MAT) amplification and analysis

We selected three SSR primer pairs (Table 2) on the basis of their high PIC as described by Geistlinger et al. (2000). SSRs were amplified using the Multiplex Ready Technique (Hayden et al. 2008). The optimal concentration of locus-specific primer required in the multiplexed PCR reactions was determined as 10 nM for each primer. Fragment analysis for visualization of SSRs was performed on ABI3730 DNA analyzer (Applied Biosystems) and SSR allele sizing and calling was performed using GeneMapper v. 3.7 (Applied Biosystems). To determine mating types of the isolates, PCR with the allele specific primers Com1, Tail5, and SP21 was performed as described by Barve et al. (2003). PCR products were separated electrophoretically on 1.5% agarose gels.

Statistical analysis

To characterize genetic variation, observed number of alleles (*Na*), effective number of alleles (*Ne*), Nei's gene diversity (*He*), and Shannon's information index (*I*) were calculated for each locus and population. All calculations were performed using POPGENE program v. 1.31 (Yeh et al. 1999). Mean values of gene diversity of total popula-

Table 3 Number of alleles at each locus across the seven populations of *A. rabiei* in western Iran

Number of alleles							Total alleles	SSR primer
Sarpul	Gilane gharb	Dareh shahr	Chardavol	Holaylan	Aseman abad	Ilam		
7	5	10	8	6	8	8	26	*Ar*H06T
9	9	11	14	6	8	8	34	*Ar*A03T
6	4	7	6	4	6	5	15	*Ar*H05T
7.33	6	9.33	9.33	5.33	7.33	7	25	Average

Table 4 Genetic diversity estimates for *A. rabiei* based on three microsatellite loci

I[a]	*He*[b]	*Ne*[c]	*Na*[d]	Sample size	SSR primer
2.28	0.78	4.7	26	103	*Ar*H06T
2.94	0.9	10.85	34	103	*Ar*A03T
1.77	0.67	3.05	15	103	*Ar*H05T
2.33	0.78	6.2	25	103	Average

[a] *I* Shannon's Information index

[b] *He* Nei's (1973) gene diversity

[c] *Ne* Effective number of alleles

[d] *Na* Observed number of alleles

tions (*Ht*), gene diversity between populations (*Hs)*, proportion of gene diversity attributable to differentiation among populations (*Gst*), and estimates of gene flow from *Gst* (*Nm*) were obtained across loci (McDermott and McDonald 1993). A dendrogram of genetic relationships between populations was estimated from the SSR data using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method on the basis of Nei's (1978) unbiased genetic distance.

Results

Distribution of alleles at polymorphic SSR loci

All three microsatellite loci were highly informative with a total of 75 alleles detected between them. *Ar*H06T, *Ar*A03T, and *Ar*H05T showed a total of 26, 34, and 15 alleles per locus, respectively. The average number of alleles per locus was the highest in the two populations of Chardavol and Dareh shahr (9.33 alleles) and the lowest in the Holylan population (5.33) (Table 3). A summary of the genetic diversity data of three microsatellite locus is given in Table 4. Gene diversity (*He)* and Shannon's information index (*I)* were the highest in the locus *Ar*A03T (*He*=0.9; *I*=2.94) while the lowest values were estimated for the locus *Ar*H05T (*He*=0.67; *I*=1.77).

Genetic variability of population

Genetic parameters for the three microsatellite loci in the seven *A. rabiei* populations are given in Table 5. Observed (*Na*) and effective (*Ne*) numbers of alleles were higher in Chardavol and Dareh shahr compared to other populations. The gene diversity (*He*) and the Shannon's information index (*I*) indices were also higher in Dareh shahr, but lower in Holaylan. Based on the SSR data, the average genetic distance was calculated among the seven populations. The lowest genetic distance was found between Chardavol and Sarpul, while the highest genetic distance was revealed between Ilam and Darah shahr (Table 6).

The total gene diversity (*Ht*) and gene diversities between subpopulations (*Hs*) were estimated to be 0.78 and 0.73, respectively. Moreover, gene diversity attributable to differentiation among populations (*Gst*) was 0.07, while gene flow (*Nm*) was 6.64.

Genetic relationships among populations and distribution of mating types

Nei's pairwise genetic distances between the populations varied from 0.15 to 0.36. Cluster analysis (UPGMA) was used to produce a dendrogram showing the genetic relationships between the populations based on the SSR data (Fig. 2). The lowest genetic distance was between Chardavol and Sarpul and then between Aseman abad and Holaylan. Moreover, the dendrogram showed a distinction between the Dareh shahr population and the six remaining populations.

Both mating types were found in all seven populations and the frequencies of mating types were variable between populations. The majority of the isolates belonged to *Mat1-1* (64.08%) with the remainder (35.92%) being *Mat1-2,* but no population was significantly different from a 50:50 ratio.

Discussion

This study reveals for the first time the levels of genotypic diversity among *A. rabiei* isolates occurring in the

Table 5 Genetic diversity estimates for seven populations of *A. rabiei* based on three microsatellite loci (*Ar*H06T, *Ar*A03T, and *Ar*H05T, Geistlinger et al. 2000)

I[a] (SD)	*He*[b] (SD)	*Ne*[c] (SD)	*Na*[d] (SD)	Sample size	Population
1.55 (0.38)	0.69 (0.12)	3.77 (1.66)	7 (1.73)	16	Ilam
1.75 (0.25)	0.77 (0.07)	4.77 (1.65)	7.33 (1.15)	12	Aseman abad
1.34 (0.44)	0.64 (0.20)	3.40 (1.48)	5.33 (1.15)	12	Holaylan
1.83 (0.58)	0.75 (0.12)	5.51 (4.15)	9.33 (4.16)	19	Chardavol
2.00 (0.30)	0.82 (0.06)	6.13 (2.01)	9.33 (2.08)	16	Dareh shahr
1.43 (0.42)	0.69 (0.09)	3.47 (1.18)	6.00 (2.64)	15	Gilangharb
1.68 (0.26)	0.74 (0.07)	4.13 (1.09)	7.33 (1.52)	13	Sarpul
1.65	0.72	4.45	7.37	14.71	Average

[a] *I* Shannon's Information index

[b] *He* Nei's (1973) gene diversity

[c] *Ne* Effective number of alleles

[d] *Na* Observed number of alleles

Table 6 Nei's original measure of genetic identity and genetic distance between pairs of *A. rabiei* populations. Measures of genetic identities are shown above the diagonal and genetic distances are shown below the diagonal. A maximum genetic identity value of 1.0 occurs when the same alleles occur at the same frequencies for each pair of populations

Sarpul	Gilangharb	Dareh shahr	Chardavol	Holaylan	Aseman abad	Ilam	Population
0.7688	0.7629	0.7431	0.8518	0.7668	0.7560	–	Ilam
0.7293	0.7337	0.667	0.8037	0.8193	–	0.2797	Aseman abad
0.807	0.7686	0.6399	0.829	–	0.1993	0.2655	Holaylan
0.8621	0.7958	0.7294	–	0.1876	0.2186	0.1604	Chardavol
0.6795	0.7236	–	0.3155	0.4465	0.405	0.2969	Dareh shahr
0.8161	–	0.3235	0.2283	0.2631	0.3097	0.2706	Gilangharb
–	0.2032	0.3864	0.1484	0.2145	0.3157	0.2629	Sarpul

provinces of Ilam and Kermanshah in western Iran. The region sampled comprised seven counties over a maximum distance of 350 km. In comparison to other studies using the same SSRs markers (Geistlinger et al. 2000; Rhaiem et al. 2008; Varshney et al. 2009), this area may represent the highest level of allelic diversity detected to date. Although sample sizes are not comparable, at locus *Ar*H06 over similar or larger areas, those studies identified 2, 9, and 15 alleles, respectively, compared to 26 here. Geographically, the SSR data indicated that all seven populations had high levels of gene diversity, with Dareh shahr and Aseman abad showing the highest diversity. This is perhaps unsurprising given western Iran's central position in the original geographic range of chickpea, which comprises the Fertile Crescent and Asia Minor (van der Maesen 1987).

Over 93% of the diversity in this study was distributed on a local level within populations and correspondingly a low level of gene diversity (*Gst*=0.07) was detected among all seven populations. This indicates the majority of variation is within populations with low genetic differentiation among the populations and little evidence for geographical subdivision. Individuals within such populations are likely to be genetically different, with each population containing the same or similar complement of alleles in similar frequencies. This compares to Tunisia where 123 *A. rabiei* isolates representative of five regions were analyzed to estimate genetic diversity and where a high level of genetic differentiation was detected among subpopulations (*Gst*=0.33; Rhaiem et al. 2007). In contrast, on a smaller scale in Tunisia, Morjane et al. (1994) examined the genetic variation among 50 isolates sampled from one single chickpea field. Most of the genetic variability was attributable to diversity within locations rather than between locations. In Australia, a study based on STMS fingerprints found none of the alleles showed significant differences in allele frequencies, and a very low level of gene diversity (*Ht* = 0.02) was found with the majority of the diversity (92%) distributed within subpopulations. This reflects a relatively recent introduction of a single mating type of *A. rabiei* with modest genetic variability (Phan et al. 2003).

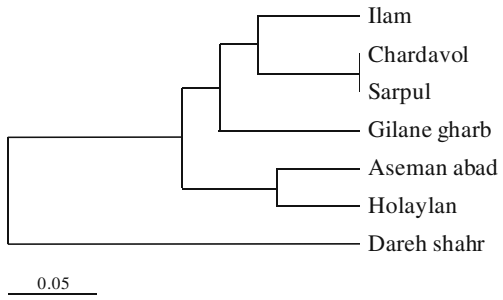

Fig. 2 Dendrogram of genetic relationships between seven populations of *A. rabiei* as reconstructed by UPGMA using Nei's genetic distance

Gene flow is one of the evolutionary forces that can have a significant force on the genetic diversity of a population. In the absence of gene flow, genetic drift causes different allele frequencies at neutral loci, leading to differentiation in isolate populations (Keller et al. 1997). The low genetic differentiation in this study has several possible explanations. Geographic distances between populations are modest, allowing movement of isolates through infected plant debris, infected seeds, and agricultural vehicles. The exchange of chickpea seeds is possibly the commonest cause, because farmers in the region often purchase seed from other farmers. This undermines practices that that might be expected to promote population differentiation, such as the sale of certified seed from central stores in each county and the propagation of seed by some farmers for their own use. Other factors affecting selection and population differentiation include the deployment of different cultivars and fungicides. In western Iran, the number of cultivars is limited to about six Kabuli types, occasionally augmented by resistant cultivars by agricultural research centers, together with a local Kabuli-Desi mixture propagated by individual farmers. Fungicide use is limited or absent.

On the assumption that the high degree of population similarity is due to gene flow and that the populations are at

equilibrium, we can use *GST* to estimate *Nm*, the average number of migrants that would need to be exchanged among populations in each generation to account for their present lack of subdivision. *Nm* averaged 6.64 in all loci and populations, suggesting a level of gene flow that was 6 times greater than needed to prevent populations from diverging by genetic drift (Keller et al. 1997). This result may help explain why resistance in new cultivars, periodically released by agricultural research centers and including material from the International Centre for Agricultural Research in the Dry Areas (INCARDA), has proven ineffective after 2–3 years (data not shown).

The results also showed different allele frequencies between the SSR loci and populations, with isolates from Dareh shahr, the eastern most county, appearing most different. Farmers in the region usually cultivate cereals in rotation with chickpea and the stubble is either burnt or grazed by livestock. However, chickpea debris may also be ploughed in after harvesting, only a limited number of cultivars are farmed, sequential cultivation of more than one chickpea crop occurs in regions with mild climates, and infected seed is exchanged locally between farmers. These practices would promote the asexual spread of *A. rabiei* asexually, which in combination with these cultivation practices may contribute to different allele frequencies. The sample sizes per population and number of SSRs primers were too small in this experiment for a statistically powerful test of gametic equilibrium.

Moreover, infected seed can lead to persistence of genotypes and may explain the different allele frequencies diversity of genotypes found at the end of the growing season in natural populations. If the main source of primary inoculum was asexual and from seeds, we might expect to find some clones that were distributed among different locations in a field. After a while, genetic drift and selection would limit the number of genotypes present in field populations, unless sexual reproduction generated new genotypes and wind dispersal distributed ascospores among populations every year (Shah et al. 1995). Pycnidiospores are likely to play an important role in epidemiology over small distances (1–2 m), but ascospores probably play a more important role in the population biology and perhaps in the epidemiology of disease on a regional scale (tens to hundreds of kilometers; Keller et al. 1997).

The high genetic diversity of *A. rabiei* detected in this study has important implications for farming practices and breeding efforts in the area. Gene flow in the region suggests a significant potential risk for the spread of novel alleles that might contribute to fungicide resistance or the breakdown of resistance genes. Approaches such as using different combinations of pyramided resistance genes or mixtures of cultivars may offer more durable resistance.

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