DETERMINATION OF GENETIC DIVERSITY WITHIN *ASCOCHYTA RABIEI* (Pass.) Labr., THE CAUSE OF ASCOCHYTA BLIGHT OF CHICKPEA IN TURKEY

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SUMMARY

Genetic diversity among 64 isolates of *Ascochyta rabiei* obtained from diseased chickpea plants in 18 different provinces of Turkey was characterized by microsatellite-primed PCR using di-, tri- and tetra-nucleotide repeats. Of sixteen primers tested, ten amplified 61 bands, of which 56 were polymorphic. UPGMA (Unweighted Pair Group Method with Arithmetic Average) analysis, performed with the resulting data of SSR (Simple Sequence Repeats) fingerprints clustered Turkish isolates of *A. rabiei* into seven groups. However, these groups did not correspond to their geographic origin. Group 2, the largest group consisting of 35 isolates from 16 different provinces, clustered together with one Syrian isolate belonging to pathotype 3 while isolates belonging to pathotypes 1 and 2 did not cluster with any isolate originating from Turkey. The highest genetic diversity within geographical populations was found in the South Eastern Anatolia region (Shannon index: 0.156). Also, rDNA ITS (Internal transcribed spacer) regions of isolates representative of each group were sequenced. The ITS1-5.8S-ITS2 sequences were highly conserved among all groups of *A. rabiei*. This is the first report on detection of genetic diversity in *A. rabiei* populations in Turkey. The SSR fingerprints (three or four repeats) generated using *A. rabiei* DNA indicated that such microsatellites are useful for population studies in this fungus.

Key words: Ascochyta rabiei, chickpea, microsatellite, ITS sequencing, genetic diversity.

INTRODUCTION

Ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Labr., (teleomorph, *Didymella rabiei* (Kov.) v. Arx) is the most important disease of chickpea in Turkey. The disease mainly affects all the above ground parts of the plants, causing lesions mostly on the stems and breaking them. Frequently, it devastates chickpea crop areas and causes heavy yield losses (up to 100%) in severely affected fields (Singh and Reddy, 1990). Many researchers have used the disease reactions on different chickpea cultivars to determine various pathogenicity groups of *A. rabiei* isolates. Reddy and Kabbabeh (1985) reported six races of *A. rabiei* in Syria and Lebanon on the basis of reactions on different chickpea cultivars while Bedi and Aujla (1969) indicated the existence of 11 races. Dolar and Gürcan (1992) reported the presence races 1, 4, and 6 of *A. rabiei* in Turkey. Udupa and Weigand (1997) identified three pathotype groups among 53 isolates of *A. rabiei* in Syria and Lebanon using three differential chickpea lines. Similarly, Chen *et al.* (2004) used the two-pathotype system in assessing pathogenic variation of US isolates.

Development of ascochyta blight resistant or tolerant cultivars is the most practical and effective means of disease control (Nene and Reddy, 1987). However, the development of resistant cultivars is very difficult. Ascochyta blight resistant cultivars become susceptible over time, resulting from selection of new pathotypes (Katiyar and Sood, 1985).

Many different molecular methods have been used to reveal genetic polymorphism within populations of plant pathogenic fungi such as restriction fragment length polymorphism (RFLP) (Kistler *et al.*, 1991; Elias *et al.*, 1993), PCR-based methods (Hantula and Muller, 1997; Kang *et al.*, 2002) and DNA fingerprinting (Weising *et al.*, 1991). Random amplified polymorphic DNA (RAPD) analysis, using short, arbitrary oligonucleotides to generate amplification products, have been used to assess genetic variation in pathogenic fungi such as *Fusarium oxysporum* f.sp. *pisi* (Grajal-Martin *et al.*, 1993), *F. oxysporum* f.sp. *ciceris* (Kelly *et al.*, 1994), *Phoma lingam* (Schäfer and Wöstemeyer, 1992) and *Pyrenophora teres* (Peever and Milgroom, 1994). Also, different strategies have been developed to exploit microsatellite polymorphism as molecular markers in eukaryote genomes. Meyer *et al.* (1993) used microsatellite primers to differentiate strains and serotypes of *Cryptococcus neoformans*.

Microsatellite-primed PCR (MP-PCR) yielded similar

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banding patterns in amplification by RAPD in the presence of single primers complementary to a target microsatellite. In a closely related strategy, Zietkiewicz *et al*. (1994) used 5' or 3' – anchored microsatellite primers to reveal polymorphism within different eukaryotic taxa. Many investigations have been performed for molecular characterization of *A. rabiei* and detected extensive genetic diversity among the isolates. Morjane *et al*. (1994) reported a high genomic variability among isolates of *A. rabiei* collected from only one field with oligonucleotide fingerprints.

Hybridization of restricted digested genomic DNA was used to detect variation among *A. rabiei* isolates by Geistlinger *et al*. (1997). Udupa and Weigand (1997) described that the DNA markers distinguish variability within and among the pathotypes of *A. rabiei* with a microsatellite sequence and RAPD markers. Santra *et al*. (2001) reported that RAPD markers were useful to detect genetic variation among *A. rabiei* isolates from different geographic origin. Barve *et al*. (2003) developed a multiplex PCR assay for mating type in *A. rabiei* and stated that this assay was useful for population genetic studies of this fungus. However, polymorphisms at the DNA level among Turkish isolates of *A. rabiei* have not been described yet.

The objective of this study was to determine the genetic diversity of *A. rabiei* isolates obtained from different chickpea growing areas of Turkey using a microsatellite based PCR technique and ITS sequencing.

MATERIAL AND METHODS

Fungal material. Single spore isolates of *A. rabiei* were collected from 18 different provinces (Ankara, Eskişehir, Kayseri, Kırşehir, Sivas, Yozgat, Denizli, Uşak, Afyon, Burdur, Kütahya, Diyarbakır, Adıyaman, Çorum, Amasya, Tokat, Kahraman Maraş, and Antalya) in Turkey. An isolate of race 6 as described by Reddy and Kabbabeh (1985) was obtained from the Department of Plant Protection, Faculty of Agriculture, University of Ankara (Dolar and Gürcan, 1992). Ascochyta isolates used in this study and their locations are shown in Table 1. Pathotypes 1, 2 and 3 of *A. rabiei* were obtained from Dr. B. Bayaa and race 4 from Dr. S.P.S. Beniwal (both ICARDA, International Centre for Agricultural Research in the Dry Areas, Aleppo, Syria).

DNA extraction. Mycelial discs from the growing margin of single spore colonies on chickpea seed meal-dextrose agar (CSMDA: 4% chickpea flour, 2% dextrose, and 2% agar) were used to inoculate 100 ml potato dextrose broth (PDB, Difco) in 250 ml flasks and incubated at 21°C on orbitary shaker at 140 rpm for 7 days. Mycelia were harvested, frozen in liquid nitrogen and stored at –80°C until use. Genomic DNA was extracted with 2% hexadecyltrimethyl-ammonium bromide (CTAB) extraction buffer (1.4 M NaCl, 0.1 M Tris-HCl pH: 8.0, 20 mM EDTA, 0.2% β-mercaptoethanol) using the method of Weising *et al*. (1991).

Table 1. Distribution of *A. rabiei* isolates collected from different chickpea production areas in Turkey.

Regions	Provinces	Number of isolates
Central Anatolia	Ankara	10
	Eskişehir	5
	Kayseri	1
	Yozgat	2
	Kırşehir	3
	Sivas	1
Aegean	Denizli	5
	Uşak	8
	Afyon	1
	Burdur	2
	Kütahya	1
South Eastern Anatolia	Diyarbakır	6
	Adıyaman	7
Black sea	Amasya	4
	Çorum	3
	Tokat	1
Mediterranean	Antalya	3
	Kahraman Maraş	1
Total		64

Microsatellite-primed PCR analysis. MP-PCR analysis was performed according to Geistlinger *et al*. (1997). Amplifications were carried out in 25 µl reaction volumes containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.8 % Nonident P40, 2 mM $MgCl_2$, 1 µM primer, 15 ng DNA, 0.2 µM dNTPs and 1 unit *Taq* DNA polymerase (MBI, Fermantas). Amplifications were performed in a thermal cycler with an initial denaturation step for 1 min at 94°C, followed by 37 cycles of 30 sec at 94°C, 1 min at annealing temperature and 1 min at 72°C, with a final extension for 2.5 min at 72°C. Different annealing temperatures for different microsatellites sequences were used according to Table 2. The PCR products were separated electrophoretically in 1.4% agarose gel using 1 x Tris-acetate-EDTA (TAE) buffer (Sambrook *et al*., 1989). The gels were stained with ethidium bromide and visualized by bio-imaging system (Syngene).

ITS sequencing. The rDNA ITS regions of seven isolates (Ank-11, Esk-4, Uşk-7, Uşk-1, Ant-1, Km-1, Adı-2) were amplified by PCR using primers ITS1 and ITS4 (White *et al*., 1990). PCR was carried out as follows: the total volume of the reaction mixture was 25 µl and contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.8 % Nonident P40, 1.5 mM $MgCl_2$, 1 µM each primers, 1 µl DNA, 0.2 µM dNTPs and 1 unit *Taq* DNA polymerase

Table 2. Sequences, G+C content and annealing temperatures for 16 different microsatellite primers.

Primer sequences	G+C content	Annealing temperature
$(GATA)_4$	25	37 °C
$(CAA)_5$	33.3	37 °C
$(CAT)_5$	33.3	37 °C
$(AGT)_5$	33.3	37 °C
$(ATC)_5$	33.3	37 °C
$(GAA)_5$	33.3	37 °C
$(AAC)_8$	33.3	55 °C
$(AAG)_8$	33.3	55 °C
$(GAGA)_5$	50	55 °C
$(CA)_8$	50	45 °C
$(ACTG)_4$	50	45 °C
$(GACA)_4$	50	45 °C
$(GTG)_5$	66.7	45 °C
$(TGTC)_4$	50	45 °C
$(GT)_8$	50	45 °C
$(GGAT)_4$	50	45 °C

(Sigma, RedTaq, Ready mix PCR reaction kit). Touchdown-PCR cycling parameters were: 4 min at 94°C, 10 cycles of 30 sec at 94°C, 30 sec at 65°C, 1 min at 72°C and then 25 cycles of 30 sec at 94°C, 30 sec at 56°C, 1 min at 72°C: with a final extension at 72°C for 7 min. The amplification products were analyzed by running 7 µl of each mixture through a 1 % agarose gel in 1 x TAE buffer. ITS PCR products were purified, sequenced directly using a Bigdye termination kit (Applied Biosystems, Foster City, CA, USA) with universal ITS1 and ITS4 primers and separated on an ABI 377 sequencer.

Data analysis. For each individual isolate, bands were determined and designated 1 for present or 0 for absent. The estimate of genetic similarity among *A. rabiei* isolates was calculated according to Jaccard's coefficient by employing the SIMQUAL program. The matrix similarity was analyzed by the unweighted pairgroup method using the arithmetic average (UPGMA) as suggested by Sneath and Sokal (1973). All data analysis were performed using NTSYS-pc numerical taxonomy package, version 2.0 (Rohlf, 1998). The genetic diversity within geographical populations was tested using Shannon's diversity index with POPGENE32 (Yeh *et al*., 1999). Sequence analysis was performed using DNAstar software (DNASTAR Inc., Wisconsin, WI, USA).

RESULTS

The aim of the present study was to assess the genetic diversity among 69 *A. rabiei* isolates obtained from 18 provinces of Turkey by using 16 microsatellite primers. In a previous study, the disease incidences and severities in these provinces ranged from 0.7% to 20.9% and 0.2% to 14.8%, respectively (Bayraktar *et al*., 2002). Ten of the 16 primers generated reproducible banding patterns, but $(AGT)_5$, $(GAGA)_5$, $(CA)_8$, $(GTG)_5$, $(GT)_8$ and $(GGAT)_4$ did not give amplification products. A total of 61 bands were scorable, out of which 56 were polymorphic. The G+C content of the primer varied between 25.0-66.7%, and 3-10 fragments were amplified from each isolate. $(ACTG)_4$ was the least informative primer (3 bands) while $(AAC)_8$ was the most informative one (10 bands).

The size of amplified DNA fragments ranged from 0.3-2.7 kb. The dendrogram, obtained from the binary matrix (present or absent bands) using NTSYS-pc ver. 2.0. clustered 69 isolates into nine groups at an arbitrary level of 80% similarity (Fig. 1). The similarity index values between these isolates ranged from 37% to 100%. Group 1 consisted of nine isolates from Antalya, Eskişehir, Ankara and Amasya provinces together with race-6 with 87% similarity. In group 2, pathotype 3, together with 35 Turkish isolates from 16 different provinces formed the major group and shared the same genotype with six isolates. In addition, there were three subgroups of 3 (Dez-2, Ank-2, Dez-5), 2 (Diy-1, Diy-3) and 2 (Af-1, Kır-2) isolates with identical banding patterns within this group. Pathotype 1 formed a different branch of the dendrogram with 79% similarity to Turkish isolates. Group 3 formed the second largest group with 14 isolates from 8 provinces at the level of 82% similarity; two isolates, Ank-1 and Çor-3 appeared identical. Group 4 consisted of 3 isolates, Ank-9, Diy-6, Ank-11, and was clustered with race 4. Adı-6 and Uşk-7 isolates formed group 5 with 83% similarity. Group 6 and 7 consisted of only one isolate each, Km-1 and Adı-2.

Syrian pathotypes 1 and 2 constituted two separate branches of the dendrogram. Although Turkey and Syria are neighbouring countries, none of the Turkish isolates clustered with pathotype 2. This isolate was significantly different from all the Turkish isolates of *A. rabiei* with only 42.8% similarity. Also, races 4 and 6 were separately clustered at the level of 73% similarity.

The Shannon index showed that the highest genetic variation accrued in the South Eastern Anatolia population (0.156) while the lowest variation was in the Mediterranean region (0.08). Genetic diversities in Central Anatolia, Aegean and the Black sea populations were found to be 0.131, 0.134 and 0.12, respectively. However, the genetic diversity index of the populations was not significantly different ($P< 0.05$).

PCR amplification of the rDNA of seven *A. rabiei* isolates, representative each genotypic group generated a single fragment of about 553 bp in size. PCR products were sequenced in both directions, trimmed manually, and assembled using the DNAstar software. The sequence analysis of ITS1-5.8S-ITS2 rDNA regions revealed that *A. rabiei* isolates were closely related and have

a high level of similarity along 498 alignments. ITS rDNA sequences showed only 1 bp substitution between the isolates studied. Km-1 isolate had a cytosine at position 290 as measured from beginning of the ITS1 region while the isolates belonging to other groups had a thymine at the same position. When comparing the Turkish isolates sequenced in this work with *A. rabiei* strain CBS237.37 (GenBank accession number AY152550) one cytosine/guanine substitution and one cytosine insertion were detected at position 362 and 363, respectively.

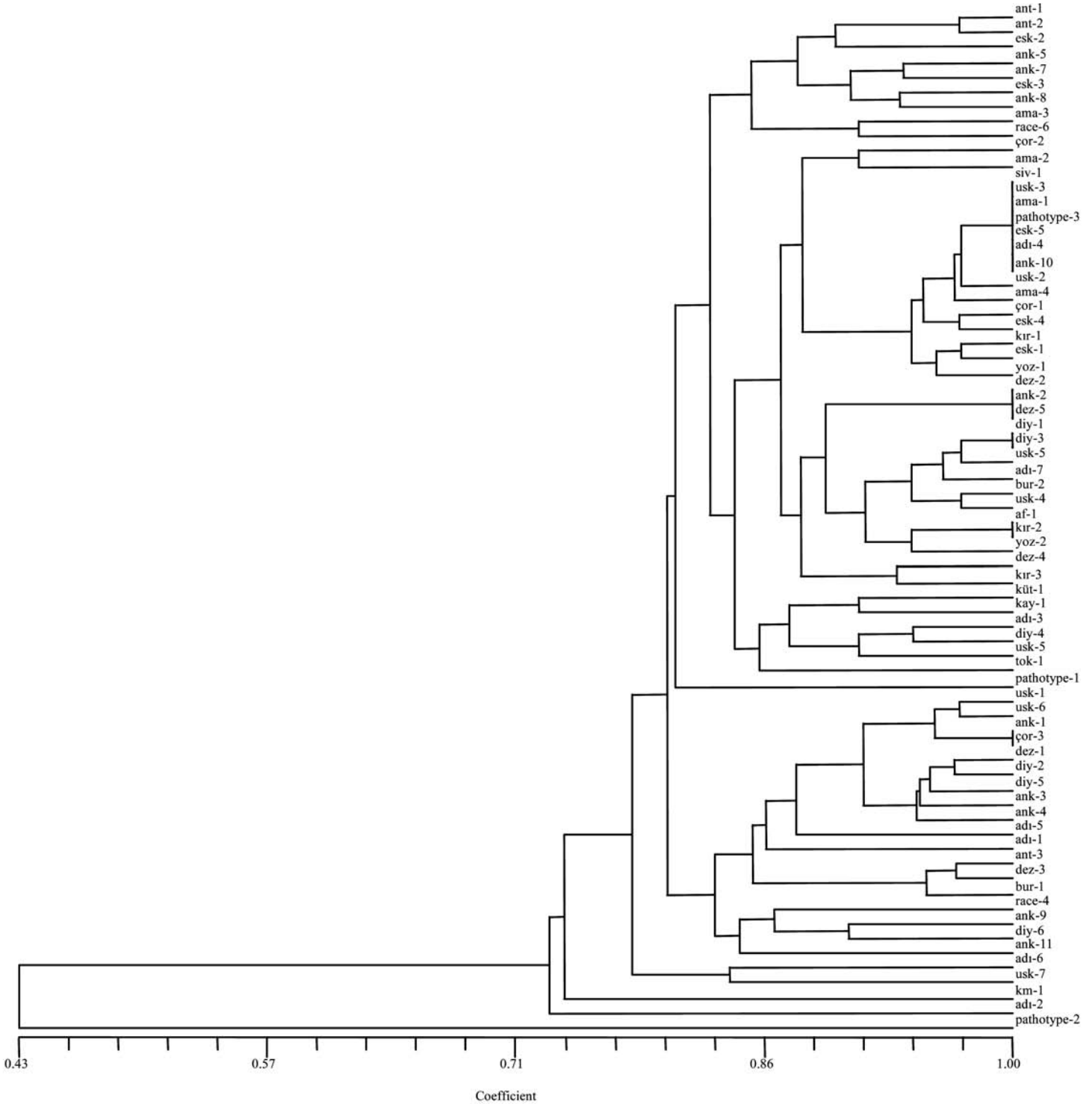

Fig. 1. Dendrogram showing relationships among the 69 *A. rabiei* isolates. Similarity coefficients were calculated by the method of Jaccard's. A dendrogram was constructed from the similarity coefficients by using UPGMA. Abbreviations represent Ank (Ankara), Esk (Eskişehir), Kay (Kayseri), Kır (Kırşehir), Siv (Sivas), Yoz (Yozgat), Dez (Denizli), Uşk (Uşak), Af (Afyon), Bur (Burdur), Küt (Kütahya), Diy (Diyarbakır), Adı (Adıyaman), Çor (Çorum), Ama (Amasya), Tok (Tokat), Km (Kahraman Maraş), and Ant (Antalya).

DISCUSSION

Ascochyta blight, caused by *A. rabiei* is one of the most important factors, causing crop losses in many provinces of Turkey. Thus, genetic characterization of *A. rabiei* is important for efficient disease management of this pathogen in different chickpea growing regions. In the present study, genetic variation among *A. rabiei* isolates from different provinces in Turkey was examined by both microsatellite-primed PCR and ITS sequencing, which detected a high degree of genetical similarity. However, the use of microsatellite markers enabled us to divide *A. rabiei* isolates into different groups at the molecular level.

Six of the sixteen primers used did not produce any amplification patterns among these isolates. The lack of amplification with these primers may be due to unsuitable binding sites, compared to other eukaryotes (Meyer *et al*., 1992; Longato and Bonfante, 1997). Similarly, Geistlinger *et al*. (1997) used some of the primers tested in this study, and detected a low level of polymorphism. In this study, primer $(GGAT)_4$ failed to produce amplification products. Primers $(GATA)_4$, $(GACA)_4$ and $(GAA)_5$ could not discriminate among most of the isolates. However, $(ATC)_5$ and $(TGTC)_4$ clearly displayed differences among isolates of *A. rabiei*.

The dendrogram, constructed from the data of SSR fingerprints clustered all the Turkish isolates into seven groups. Similarity index within each of these clusters was at least 80%. No significant sequence changes were detected in ITS1-5.8S-ITS2 regions of isolates representing each group. Only, the Km-1 isolate had a base pair substitution at position 290.

Similarly, Barve *et al*. (2003) could not detect significant sequence differences within the ITS region of *Ascochyta* isolates associated with legumes. Isolates from the same provinces were genetically different while a highly genetic similarity was observed among isolates of *A. rabiei* from different chickpea growing areas. Our results indicated that most of Turkish isolates were clustered with Syrian pathotype 3, but not pathotypes 1 and 2. The samples collected from 16 of 18 provinces in Turkey were included in the same group as Syrian pathotype 3 while the isolates from Antalya and K. Maraş were not included in this group. More genetic diversity in Ankara and Adıyaman provinces than in other provinces was detected.

Udupa and Weigand (1997) reported that pathotype 3 was found in all chickpea growing areas of Syria, neighbouring to Turkey, and that the resolving power of the pathotypes by microsatellite markers was better than RAPD marker. For the present, this research indicates that there are distinct genetic groups within *A. rabiei* isolates in Turkey. In addition, the high similarity observed among isolates from different provinces suggests that chickpea seeds are important for the spread of *A. rabiei* pathotypes into these regions. Air borne ascospores were reported to play an important role in the development of new pathogenic races, pathogen survival and disease spread in different provinces (Trapero-Casas and Kaiser, 1992; Kaiser, 1997). In this study, there was no clear relationship between the geographically distribution of isolates and MP-PCR polymorphisms, but a higher level of genetic diversity within South Eastern Anatolia region than in other regions. These results suggested that microsatellite markers were suitable to evaluate the genetic variability at DNA levels among isolates of *A. rabiei.*

Fischer *et al*. (1995) analyzed thirty Italian isolates of *A. rabiei* using three decamer primers by RAPD, but found no correlation between RAPD data and pathogenic groups. Using RAPD analysis, Navas-Cortes *et al*. (1998) detected a relatively low level of genetic diversity among *A. rabiei* isolates representing different geographical regions and clustered these isolates into 10 RAPD groups at the level of 7% dissimilarity. Using RAPDs and oligonucleotide fingerprinting, Jamil *et al*. (2000) discriminated *A. rabiei* population in Pakistan into six distinct groups and 46 genotypes at a genetic distance of 0.3 by cluster analysis. Santra *et al*. (2001) clustered 47 *A. rabiei* isolates with a total of 48 RAPD markers according to geographical origin and grouped 37 Indian isolates into two major clusters.

However, they did not find correlation between pathogenicity of these isolates and RAPD groups. Peever *et al*. (2004) screened STMS (sequence-tagged microsatellite site) markers characterized by Geistlinger *et al*. (2000) and detected polymorphic four loci in *A. rabiei* samples from the US Pacific Northwest.

As a preliminary study, this research contributed to our understanding of geographical distribution and extent of genetic variability of *A. rabiei* isolates in Turkey. However, the determination of races and population structure of *A. rabiei* with more isolates representing different provinces in Turkey using more sensitive techniques such as AFLP, DNA fingerprinting will be useful. Results from this study provide an insight to chickpea breeders in disease management and improvement of resistant chickpea cultivars against ascochyta blight in Turkey and contribute to future studies.

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