African Journal of Biotechnology Vol. 12(12), pp. 1344-1349, 20 March, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2299
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

High genetic diversity of *Mycospaherella graminicola* (*Zymoseptoria tritici*) from a single wheat field in Tunisia as revealed by SSR markers

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Accepted 11 December, 2012

Microsatellite markers were used to assess genetic diversity of *Mycosphaerella graminicola* at a micro geographical scale in Tunisia. Forty five (45) isolates were sampled and assessed using seven pairs of single-locus microsatellite primers not previously tested on populations of *M. graminicola* in Tunisia. Genetic diversity of the field population ranged from 0.403 to 0.555 with an average of 0.484. A high level of genetic diversity was found at a sharp scale throughout the pathogen population tested. Among 45 isolates sampled, 39 different multi locus genotypes (MLG) were identified. Cluster analysis (UPGMA) showed that 86% of the isolates tested were distinct. The high degree of DNA polymorphism, the large number of different molecular genotypes and the pattern of cluster analysis suggest that sexual ascospores and/or asexual spores of a highly mutable local population could have contributed to the genetic diversity of *M. graminicola* in Tunisia.

Key words: Durum wheat, genetic diversity, microsatellites, *Mycosphaerella graminicola*, micro geographical scale, Tunisia.

INTRODUCTION

Septoria tritici blotch (STB), caused by the pathogen *Mycosphaerella graminicola* (Fückel) J. Schröt. in Cohn (anamorph , *Zymoseptoria tritici* (Desm.) Quaedvlieg et al., 2011) is the most important leaf blight disease in major wheat-growing regions worldwide (Eyal et al., 1985). In Tunisia, *M. graminicola* is more virulent on durum than on bread wheat (Djerbi and Ghodhbane, 1975; Kamel et al., 1984). Gilchrist and Skovmand (1995) attributed this fact to a specialization of the pathogen to durum wheat species. STB has been a major constraint limiting wheat production in north Tunisia. Under favorable growing conditions with high relative humidity (85%) and optimum temperature (22°C), this disease could reduce yield by 30 to 70% (King et al., 1983; Eyal et al., 1987; Chungu et al., 2001). Changes in farming practices (higher sowing densities and nitrogen fertilization), monocropping system and limited number of cultivars contributed significantly to the increase of the disease incidence (Camacho et al., 1995). Integrated management, including resistant varieties, crop rotation, appropriate fertilizer and fungicide applications, proper seeding rates

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and dates would be an alternative to control STB disease (Rezgui et al., 2008). Knowledge of the genetic diversity of the pathogen and of the relative contribution of sexual and asexual reproduction could have direct implications for development of effective and durable disease-management strategies (Schnieder et al., 2001; McDonald and Linde, 2002).

Genetic diversity of *M. graminicola* populations has been intensively analysed using different sample sizes and different molecular markers (Schnieder et al., 2001; Linde et al., 2002; Zhan et al., 2003; Razavi and Hughes, 2004 a, b). Molecular markers are being increasingly used to characterize fungal plant pathogen populations and were efficiently used to comprehensively describe the pathogen related traits (Majer et al., 1996) and used for population genetic studies (McDonald and McDermott, 1993; McDonald et al., 1999). In this context, AFLP markers were used to study the genetic structure of the Kansas populations of *M. graminicola* at different spatial scales: micro-plot, macro-plot and state-wide (Kabbage et al., 2008). Medini and Hamza (2008), using amplified fragment length polymorphism (AFLP) analysis, reported a high level of genetic diversity in populations of *M. graminicola* isolates collected from Tunisia, Algeria, and Canada. No clones were obtained and each isolate showed a unique haplotype. Boukef et al. (2012) studied the genetic structure of a large population of *M. graminicola*, collected from different farmers' fields in Tunisia; and reported that STB populations in Tunisia were characterized by high genotype diversity attributed particularly to sexual recombination and most importantly to gene flow.

Previous investigations showed that sampling *M. graminicola* populations on a microgeographical scale (from a single field) of the wheat leaf blotch pathogens was associated with a high level of genetic diversity (McDonald et al., 1989, 1999). Using this approach in the USA, McDonald and Martinez (1990) reported a mosaic pattern of *M. graminicola* distribution at a micro geographical sampling area. A similar approach was used in western Canada by Razavi and Hughes (2004a, b) who analyzed 90 isolates of *M. graminicola* collected from a single field. They found a high degree of DNA polymerphism, suggesting that the primary source of inoculum was due to airborne ascospores that would be dispersed uniformly within the field. High levels of gene diversity of field populations were also reported in other fungal pathogens. For instance, McDonald et al. (1994) assayed the genetic structure of *Phaeosphaeria nodorum* within a field population in Texas, using nuclear restriction fragment length polymorphism (RFLP) markers. The population tested was characterized by high gene and genotype diversity, providing evidence for the regular occurrence of sexual reproduction. Besides, Kiros-Meles et al. (2005) studied 40 isolates of *Rhyncosporium secalis* collected from one single field in Syria. Using AFPL markers, they could reveal a high genetic diversity originating from the studied population.

In the present study, we attempted to further study the diversity of *M. graminicola* populations collected from a single durum wheat field to better understand the inherent genetic variability of the STB disease. The assessment of the genetic diversity of *M. graminicola* using simple sequence repeats (SSR) markers would eventually provide us with valuable information on genetic variability of the pathogen to explain the high level of incidence and severity, and would support the occurrence of sexual stage of the pathogen. Such information is needed to enhance durum wheat breeding in Tunisia to eventually attain greater disease resistance, leading to higher and stable yield.

MATERIALS AND METHODS

Sampling and isolation of *M. graminicola*

Samples were collected from a single field grown to a susceptible durum wheat variety, cv. Karim, in Béja (36° 43' 30" N 9° 10' 55" E) during the 2008 growing season. Karim is a widely grown durum wheat variety in Tunisia, Morocco, Algeria, Spain and Italy. Béja is a major wheat-growing area located at the north west of Tunisia and is a primary hot spot for STB. The sampled durum wheat field was at the main experimental station (Centre Régional de Recherches des Grandes Cultures de Béja: CRRGC). This wheat field was isolated and surrounded by bread wheat and triticale fields. The sampling method was carried out within 4 m width and every 10 m along the field length using hierarchical sampling method described by McDonald et al. (1999). 60 infected leaves from different plants were collected from eight different sites in the field. Leaf tissue were placed in paper envelopes, air-dried at room temperature and stored at 4°C until evaluated. Wheat leaves with STB lesions were placed on wet filter paper at room temperature (20 to 25°C) for 24 h. When cirri were visible, cirrus from one pycnidium was isolated from each leaf and was transferred to a Petri dish containing fresh potatoes dextrose agar (PDA) containing 0.5% of yeast extract, then streaked across the agar surface to separate individual pycniospores. One single spore was collected from each pycnidium. Plates were incubated for five to seven days at 22°C. Colonies that developed from single pycnidiospores were stored in 50% glycerol.

A total of 45 isolates of *M. graminicola* were then selected from distinct lesions and used to study the genetic diversity in the entire field population. This double sampling scheme has been adopted to accommodate the unfavourable weather conditions during the season.

DNA extraction

Each isolate was transferred to yeast-sucrose broth (YSB) (1% sucrose, 1% yeast extract) and incubated at 20°C on an orbital shaker (180 rpm) for five to seven days. The spores were harvested by centrifugation, frozen overnight at -80°C and lyophilised for two to three days followed by DNA extraction. DNA from each isolate was extracted using a modified CTAB protocol (Dubois et al., 1998), re-suspended in 30 µl of TE buffer and stored at -20°C until use.

PCR amplification

Seven pairs of single locus microsatellite primers described by Owen et al. (1998) were used to study the genetic variability of the pathogen (Table1). Polymerase chain reactions (PCR) were performed in a total volume of 10 µl containing approximately 30 ng

Table 1. Optimum annealing temperatures, range of fragment size, and the number of alleles detected by six SSR primers in the population of *M. graminicola.*

Locus	Reported repeat motif	Optimum annealing temperature (°C)	Range of fragment size (bp)	Number of alleles	Number of isolates with null allele	Genetic diversity*
ST1E3	$(CGG)_5$	65	74-77	2	5	0.555
ST1E7	$(CGG)_5$	65	105-111	3	7	0.514
ST1G7	$(TG)_9$	65	101-103	2	2	0.521
ST2E4	$(GGC)_5$	58	96-102	2	5	0.409
ST1A4	$(CGG)_7$	58	116-118	2	7	0.505
ST1D7	$(AC)_{22}$	65	102-114	4	31	0.403

*****Genetic diversity was calculated for each locus by including "nulls" as a separate allele. The formula for calculating genetic diversity is the same as for expected heterozygosity in diploid species [$(1 - \sum x_i^2)$, where x_i is the frequency of allele i]. In the case of haploids this represents the probability of two individuals, sampled at random from a population, having different alleles at that locus (Owen et al., 1998).

of genomic DNA, 1 unit of Taq DNA polymerase (New England BioLabs, Ipswich, MA), 200 µM of each dNTP, 10 mM KCl, 10 mM $(NH4)_2SO4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, 3 nM IRD700-labeled M13 forward primer, and 125 nM for each SSR primer. The forward primer of each pair was extended with an M13 tail to allow labelling. PCR amplification was conducted on a Biometra thermocycler (Göttingen, Germany) programmed as follows: 60 s at 95°C for initial denaturation followed by 30 cycles involving a denaturation step at 95°C for 40 s, an annealing step at 65°C for 60 s, and extension at 72°C for 60s. The final extension cycle was for 10 min at 72°C. For ST2E4, and ST1A4 the annealing temperature was 58°C. Amplification products were resolved on 6.5% polyacrylamide denaturing gels on a LI-COR IR4200 DNA sequencer (LI-COR Inc. Lincoln, NE). Gel images were analyzed and the genotypes were automatically scored using the SAGA software (LI-COR Inc. Lincoln, NE). For each locus, amplified fragments with different molecular sizes were considered as different alleles.

Data analysis

The SAGA 2.1 software was used to determine the size of each allele at a locus and the produced SSR bands were scored based on their presence/absence in each isolate. The banding patterns were transformed into a binary data matrix ("1" for the presence and "0" for the absence of a band at each particular locus). For each locus, the number of alleles detected was determined. The frequencies of different alleles of each locus were calculated and the gene diversity of the population was estimated using Nei's formula (Nei, 1973) according to the formula $H = 1 - \sum x_i^2$, where, H is the gene diversity of the population and x_i is the frequency of different alleles revealed by the primer. Genetic similarity between isolates was performed with Dice similarity index in the SIMQUAL program of NTSYS-pc ver. 2.02 software (Rohlf 1998).The generated similarity index matrix was used to cluster groups of isolates in the SAHN program with options UPGMA.

RESULTS

Molecular diversity of 45 isolates of *S. tritici* was assessed by using seven SSR markers. Among the primers, one (ST2C10) consistently failed to produce an amplicon under all of the amplification conditions that were tested. Another (ST1D7) yielded an amplicon in only 14 of 45 isolates. The remaining isolates were considered to carry a null allele. The five other SSR loci resulted in successful amplification in most isolates. The number of alleles produced by the different primers ranged from 2 to 4 and the size range of the observed alleles was between 96 and 114 bp. Polymorphism among alleles of the SSR loci ST1E3, ST1E7, ST1G7, ST2E4 and ST1D7 seemed to be due to the number of repeats. However, for locus ST1A4, differences between allele 1 and 2 was attributed to one nucleotide.

The gene diversity of the population was calculated for all the SSR markers. The average genetic diversity was 0.484, ranging from 0.403 to 0.555. The highest value was recorded by the primer ST1E3 (0.555) and it was found to be the lowest for the primer ST1D7 (0.403).

Using the primer pairs to screen the 45 isolates, 39 different molecular genotypes were identified within the relatively small sampling geographical area. This indicated that only a small proportion (about 14%) of the population was clonal, whereas 86% were genetically different.

The genetic relationship among *M. graminicola* isolates was described by generating a dendrogram from the similarity matrix based on Dice similarity index and the UPMGA clustering method (Figure 1). The Dice coefficient ranged from 0.39 to 1.0, and among the 45 isolates studied, the highest similarity index (GS 1.0) was recorded for the samples st2 and st3, st14 and st15 and between st9 and st10 and the minimum was found between isolates st33 and st43 (GS 0.40).

DISCUSSION

The aim of this study was to evaluate the genetic diversity of single field population of *M. graminicola* in Tunisia using seven single-locus microsatellite markers. The results show that the *M. graminicola* population sampled from a single durum wheat field had a high level of genetic diversity at sharp scale. Among the seven used SSR markers, six were polymorphic. Number of repeat units at the SSR loci ST1E3, ST1E7, ST1G7, ST2E4 and

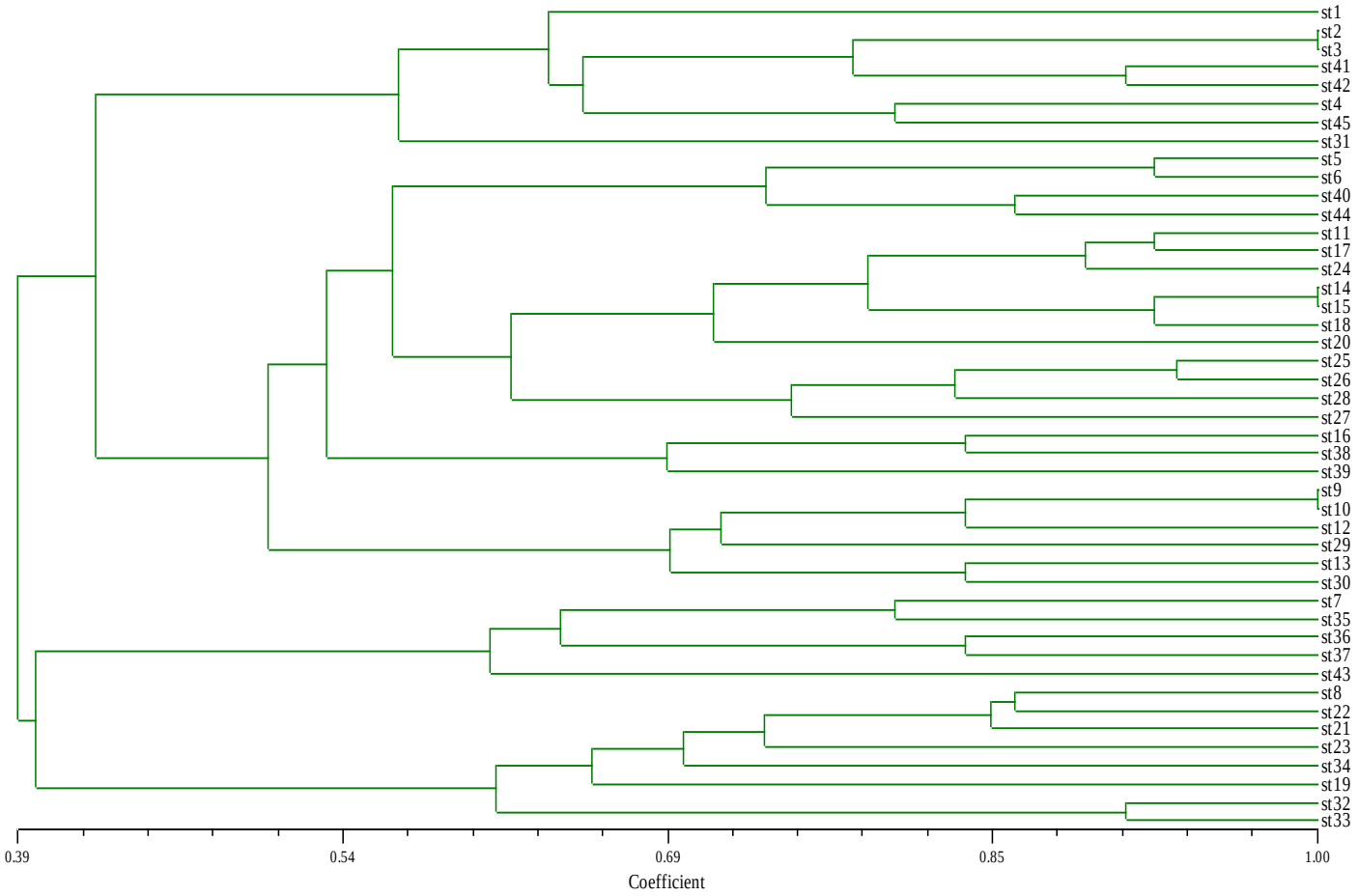

Figure 1. SSR based dendrogram of genetic relationship among 45 isolates of *M. graminicola.*

ST1D7, explained the polymorphism noted among the different alleles. However the difference noted between alleles at the SSR locus ST1A4 was likely due to one nucleotide and may have resulted from mutation during DNA replication. This hypothesis was based on the molecular weight of the alleles (Table 1). Similar results were reported by Owen et al. (1998) and Razavi and Hughes (2004 b). In this study, some isolates in the population did not produce any amplification product. Owen et al. (1998) reported that the lack of amplification of expected DNA fragments was the result of mutation in the primer binding sites of the genome.

Genetic diversity of this field population ranged from 0.403 to 0.555 with an average of 0.484. Previous investigations on one single field and using similar SSR markers indicated that an average genetic diversity of 0.49 and 0.441 was noted for 12 UK isolates (Owen et al., 1998) and for 90 Canadian isolates (Razavi and Hughes, 2004 b), respectively. These authors reported that genetic recombination attributed to the occurrence of sexual reproduction, is one of the most important factors that explain diversity. Cordo et al. (2006) reported that micro geographical observations showed both high degrees of gene and genotype diversity of *M. graminicola* populations and a higher variation on type and number of genotypes at a very small spatial scale in Argentina.

Genetic similarity between isolates was obtained by generating a dendogram from the similarity matrix, based on Dice's coefficient and the UPGMA clustering method (Figure 1). The dendrogram showed that six isolates tended to cluster together with the highest similarity index (GS 1.0). These isolates were collected from the same sampling sites and their similarity might have originated from the same clonal lineage. 39 isolates were different and associated with a close genetic similarity; this observation would imply that each single isolate could be perceived as a unique genotype as outlined by Chen and McDonald (1996). This trend suggests that sources of inoculum were originated from genetically diverse *M. graminocola* local population spores that conserved a limited familial structure. Razavi and Hughes (2004a) showed that the maximum similarity among the 90 isolates collected from one single field was approximately 81%. They reported the maximum dissimilarity between some isolates which had been collected from different lesions of the same leaf, to an infection induced by genetically different spores.

Results from this investigation would imply that isolates

of *M. graminicola* collected from a single field were genetically diverse suggesting the occurrence of the potential sexual stage of the pathogen. This hypothesis is supported by the studies of Hamada et al. (2008) and Boukef et al. (2012) who reported the coexistence of the two mating types (MAT1-1 and MAT1-2) in Tunisia. This genetic recombination may favour the expression of new emerging virulent genes reducing yielding ability of the most durum wheat cultivars in Tunisia. Therefore, knowledge of the magnitude of the genetic variability of pathogen could promote the alternative use of disease management strategy. This strategy includes the use of tolerant cultivars, early fungicide application (at three leaves growth stage to ensure delayed disease establishment), late sowing date and limited nitrogen applications. Previous investigations indicated that septoria variability was more pronounced in a local sampling scheme using 40 x 40 m section from a single field rather than on a macro geographical one (McDonald and Martinez, 1990). Considerable genetic variability of *M. graminicola* was also reported within lesions sampled from a single leaf and within a single lesion (Boerger et al., 1993; Cordo et al., 2006)

This study shows high genetic diversity within a single field. This high level of genetic diversity could be attributed to sexual recombination, spontaneous mutation and possibly as yet undescribed teleomorph ascospores of *M. graminicola*. These factors might be considered as potential mechanisms explaining this high genetic diversity within a single field.

Further field sampling and pathogen population analyses are needed to assess samples from different durum or bread wheat fields. It is expected that greater genetic variability would be attributed to the physical barriers between fields and wheat species and could provide information regarding host specificity. The appearance of new emerging virulent genes in *M. graminicola* would contribute in designing breeding programs for introgression and deployment of genes for resistance to STB disease.

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