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Frequency of mutations associated with fungicide resistance and population structure of *Mycosphaerella graminicola* in Tunisia

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Abstract The occurrence of fungicide resistance in *Mycosphaerella graminicola* populations from Tunisia was investigated by examining mutations known to be associated with strobilurin and azole resistance. Few mutations associated with fungicide resistance were detected. No evidence for strobilurin resistance was found among 357 Tunisian isolates and only two among 80 sequenced isolates carried mutations associated with azole resistance. A network analysis suggested that these mutations emerged independently from mutations found in previously described European populations. The population genetic structure of *M. graminicola* in Tunisia was analyzed using variation at 11 microsatellite loci. Populations in Tunisia were characterized by high gene and genotype diversity. All populations were in gametic equilibrium and mating type proportions did not deviate from the 1:1 ratio expected under random mating, consistent with regular cycles of sexual reproduction. In combination with a high degree of gene flow among sampling sites, *M. graminicola* must be considered a pathogens with high evolutionary potential. Thus, control strategies against Septoria blotch in Tunisia should be optimized to reduce the emergence and spread of resistant isolates.

Keywords DMI fungicides · Gene flow · QoI fungicides · Parallel evolution · *Septoria tritici*

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Introduction

The ascomycete fungus *Mycosphaerella graminicola* (Fuckel) Schröter in Cohn (anamorph *Septoria tritici*) is the causal agent of Septoria tritici blotch on wheat. *M. graminicola* is distributed worldwide, but significant yield losses are more prevalent in areas with spring and summer rainfall (Eyal et al. 1985) and in humid Mediterranean regions. In Tunisia, Septoria tritici leaf blotch is the most important disease on wheat.

Most widely grown European wheat cultivars are susceptible or only moderately resistant to *M. graminicola.* Therefore, disease control depends primarily on fungicides (Fraaije et al. 2005). Two main classes of fungicides have been used against *M. graminicola* during the last two decades. Quinone outside inhibitors

(QoI) act by inhibiting electron transport involving the cytochrome bc1 enzyme complex III (Bartlett et al. 2002) encoded by the *cytb* gene located in the mitochondrial genome. Launched in Europe in 1996, the strobilurins initially provided good disease control and additional favourable effects on the plant physiology (Ruske et al. 2003). However, a high degree of resistance quickly developed in *M. graminicola* populations and spread throughout many European countries (Fraaije et al. 2005). The major mechanism of QoI resistance is a point mutation at codon 143 in *cytb* causing a single amino acid change from glycine "G" to alanine "A" at the Qo site. Based on sequence variation at three mitochondrial loci, Torriani et al. (2009a) concluded that the G143A substitution in *M. graminicola* emerged independently at least four times in Western Europe in distinct genetic and geographic backgrounds. The resistant haplotypes increased rapidly in frequency due to strong fungicide selection coupled with an absence of fitness costs associated with the G143A substitution and then spread into Eastern Europe through wind dispersal of ascospores. The second main class of fungicides is the azoles. Triazoles or sterol demethylation inhibitors (DMIs) act by inhibiting the target enzyme sterol 14-alpha-demethylase encoded by the nuclear *CYP51*gene in *M. graminicola*. This enzyme is required for the biosynthesis of ergosterol, an essential component of the fungal cell membrane. As a consequence of the spread of QoI resistance, azoles are the only class of systemic fungicides that remain effective against *M. graminicola* (Cools and Fraaije 2008). However, during the last decade azoles have also become less effective in controlling Septoria tritici leaf blotch (Leroux et al. 2005). The reduced azole sensitivity in *M. graminicola* is conferred by different mechanisms, but the most important appear to be amino acid substitutions and deletions in the *CYP51* gene (Zhan et al. 2006; Fraaije et al. 2007; Leroux et al. 2007; Stammler et al. 2008).

A recent evolutionary study based on sequence analysis of the *CYP51* gene showed that reduced sensitivity towards DMIs in European *M. graminicola* populations was associated with a dramatic increase in frequencies of *CYP51* alleles carrying mutations associated with azole resistance (Brunner et al. 2008). Another interesting finding was a gradient of *CYP51* alleles across Europe. Western-most populations of *M. graminicola* showed the highest frequency of alleles possessing mutations associated with azole resistance, whereas the lowest frequency was found in the eastern-most populations. A plausible explanation for this pattern is the local emergence of azole resistance mutations (probably in Denmark or the UK) and the subsequent eastward spread across Europe through wind dispersed ascospores.

In this study, we analyzed fungicide resistance in *M. graminicola* populations from Tunisia. QoI- and azole-associated resistance mutations were assessed using a PCR-RFLP approach and direct sequencing. Since durum wheat is much more common than bread wheat in Tunisia, this allowed us to compare our findings with previous studies based mainly on *M. graminicola* collected from European fields of bread wheat. Knowledge of the genetic structure of a pathogen population in agricultural ecosystems can provide useful information on a pathogen's epidemiology and evolutionary potential (McDonald and Linde 2002). For example, intragenic recombination at *CYP51* combined with gene flow and selection were hypothesized to be the main drivers of rapid fungicide resistance evolution in European populations of *M. graminicola* (Brunner et al. 2008). Therefore, we also investigated the population genetic structure of *M. graminicola* in Tunisia by analyzing variation at 11 microsatellite loci.

During the last decades, an increasing degree of pathogenicity of *M. graminicola* was observed in Tunisian wheat fields. We tested the hypothesis that the increased pathogenicity was due to an accumulation of fungicide resistance mutations by direct sequencing of a fragment of the *CYP51* gene. We compared our findings with previous studies from resistant European *M. graminicola* populations to investigate whether Tunisian alleles carrying resistance mutation were introduced from Europe by gene flow or whether they emerged *de novo* by local mutations.

Materials and methods

Fungal collections and DNA extraction

Mycosphaerella graminicola isolates were collected across Tunisia from naturally infected farmers fields of durum and bread wheat during the 2007/08 growing season. Because durum wheat is much more common in Tunisia than bread wheat, the majority of the collections were from fields of durum wheat. Sampling was either

hierarchical (McDonald et al. 1999) or random (Fig. 1). Only one isolation was obtained from each plant and single spore colonies were produced as described earlier (Linde et al. 2002). Fungal biomass was lyophilized, ground into a powder and total DNA was extracted using the DNeasy Plant Mini DNA extraction kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions.

Diversity in genes associated with fungicide resistance

Presence / absence of the G143A substitution, conferring QoI-resistance was screened using the PCR-RFLP approach of Torriani et al. (2009b). Briefly, a multiplex polymerase chain reaction (PCR) amplification of the mitochondrial *cytb* gene was followed by digestion with the mutation-specific restriction enzyme *Fnu4HI*. Sensitive isolates possessing the G143 allele have two restriction fragments (442 bp and 210 bp, respectively) while the resistant isolates possessing the A143 allele have three fragments (298 bp, 210 bp and 144 bp, respectively).

Potential mutations altering azole sensitivity in *M. graminicola* were detected by direct sequencing of the nuclear *CYP51* gene. Specific PCR primers were designed from reference isolate ST1 (GenBank accession AY730587) and amplification of the gene was carried out in three PCR reactions using the following primer pairs: (a) EBI112F (5-TTCAGCACGCTCGC CATCCTCC-3)/CYP51-766R (5-TGATCGGTGT GAATCCCATA-3) generating a 676-bp fragment, (b) CYP51-601 F (5-AAGAAGTTCGCATCGACCAG-3)/ CYP51-1399R (5-GAAGGGTTTCTTTGACGAC-3) generating a 799-bp fragment and (c) CypFra1F(5-CAT ATG ATG ATT GCG CTG CT- 3)/CypFra1R (5-CGG CTG AAC AAA CTG CTG TA-3) generating an 898-bp fragment. PCR conditions were set as follows: 2 min at 96°C followed by 35 cycles of: 1 min at 95°C, 1 min at 63°C for primers (a), 56°C for primers (b), 55°C for primers (c), and 1 min at 95°C. The final products were incubated for 5 min at 72°C. PCR products were sequenced in both directions on an ABI 3730xl DNA Analyzer. DNA sequences were assembled and edited using Sequencher 6.0 (Gene Code Corp, Ann Arbor, MI).

To address the problems associated with dichotomously branching trees (Posada and Crandall 2001), genealogical relationships among haplotypes was inferred using NETWORK 4.5.1.6. (Bandelt et al., 1999; www.fluxusengineering.com) to construct an unrooted reduced median network. An additional 288 *CYP51* sequences from previously described European populations (Brunner et al. 2008) were included in this analysis to obtain a broader picture of haplotype evolution and relationships.

Neutrality and potential recombination of the *CYP51* sequences were assessed using the HYPHY

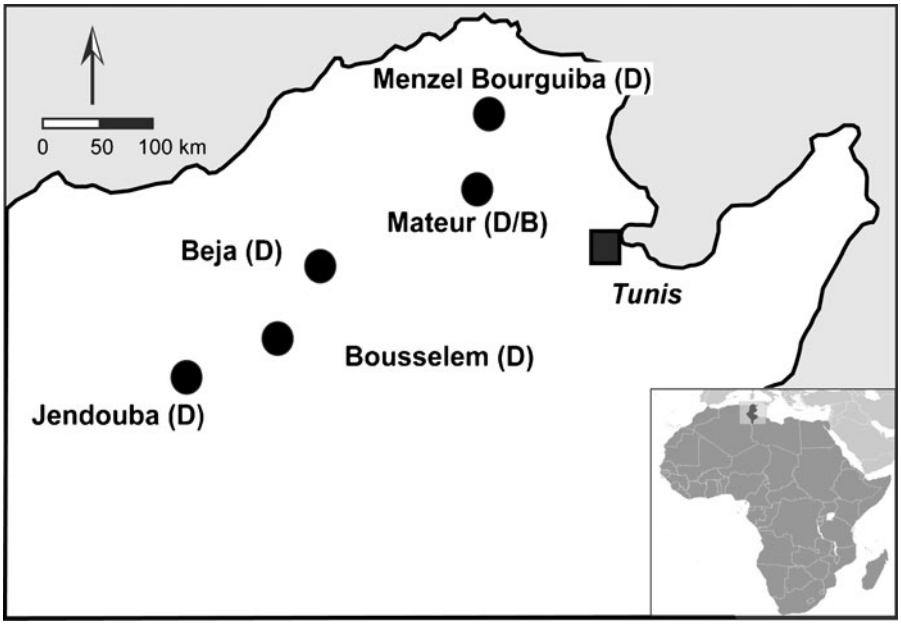

Fig. 1 Geographical location of the five major sampling regions in northern Tunisia. Letters in brackets refer to type of wheat; D, durum wheat (*Triticum durum*); B, bread wheat (*T. aestivum*)

package (Kosakovsky Pond et al. 2005) as implemented in the DATAMONKEY web-server (Delport et al. 2010).

Fungicide resistance essays based on mycelial growth

Isolates possessing mutations potentially associated with reduced sensitivity were subsequently tested using an *in vitro* assay for fungicide resistance. The test was based on mycelial growth on potato dextrose agar (PDA) medium. Five microlitres of calibrated spore suspension (100 spores / ul) for each isolate were inoculated onto PDA plates containing epoxiconazole at a concentration of 0.1 ppm and onto plates without epoxiconazole as controls. Each isolate was replicated three times per plate and every plate was repeated three times. The inoculated PDA plates were incubated in the dark at 22°C for 2 weeks. Mycelial growth was compared to the growth of a Tunisian isolate carrying the sensitive wild-type allele (H111TN) and a German isolate possessing the resistance-conferring deletion mutation ΔY459/G460.

Mating type and microsatellite analyses

M. graminicola undergoes both sexual and asexual reproduction during a wheat growing season. Asexual pycnidiospores are dispersed over short distances by rain splash while the sexual ascospores are air-borne and can be transported over long distances (Shaw and Royle 1989). The mating types of *M. graminicola* are determined by two different alleles (idiomorphs) at a single locus. To gain insight into the reproductive mode of Tunisian *M. graminicola*, the mating type for each isolate was determined with a multiplex PCR using the primer combinations MAT1-1 F / MAT1-1 R, and MAT1-2 F, and MAT1-2 R (Waalwijk et al. 2002). The PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized under UV-light. Isolates possessing the MAT1-1 allele produced a 340 bp long amplicon while MAT1-2 isolates produced a 660 bp amplicon.

The 11 EST-derived microsatellite loci used in this study were: CCA-0003, CAA-0005, GGC-0001, GCA-0003, TCC-0008, TCC-0009, AC-0001, AC-0007, AG-0006, AG-0011 and GT-0003 (Goodwin et al. 2007). PCR reactions were performed in a total volume of 20 µl with 4 µl of genomic DNA (40 ng final concentration), 2 µl of 10X reaction buffer, 0.3 µM of each primer, 0.1 mM of each dNTP, and 0.2 U of Taq polymerase (New England Biolabs). PCR amplifications entailed a 2 min initial denaturation step at 96°C, followed by 35 cycles of 30 s each at temperatures 94°, 53° and 72°C, and a final extension step of 5 min at 72°C. Fluorescent labeled products were analyzed using the GeneScan-500 LIZ size standard on an ABI 3730xl DNA Analyzer and scored using the software GENESCAN (Applied Biosystems).

Isolates with the same multilocus microsatellite genotype (i.e. possessing the same allele at all microsatellite loci) were considered clones. Genetic diversity was measured with several indexes. Clonal fraction was calculated as described earlier (Zhan et al. 2003). Nei's genotypic diversity (G) corrected for sample size was calculated for each population using GenoDive version 1.2 (Meirmans and Van Tienderen 2004). Nei's unbiased gene diversity (H) was estimated and averaged over all loci. Allelic richness standardized to the smallest sample size was estimated as the mean number of alleles per locus averaged over all loci using the rarefaction approach (Petit et al. 1998). Estimates of gene diversity and allelic richness as well as number of private alleles were calculated after clone correction using FSTAT v2.9.3 (Goudet 2001).

Hidden population structure of the Tunisian samples was inferred using several approaches. First, pairwise estimates of population differentiation were based on Nei's G_{ST} values calculated using GenALEX 6 (Peakall and Smouse, 2005). Second, we used two Bayesian-based methods to i) identify the optimal number of clusters (K) for individuals using STRUCTURE 2.2 (Pritchard et al. 2000) and ii) for predefined populations as implemented in BAPS 5 (Corander et al. 2008). In STRUCTURE, a burn-in period of 10^5 iterations, followed by 10^6 iterations was used to estimate K ranging from 1 to 10. We applied the two implemented models "no admixture" and "admixture" and tested the use of "independent" and "correlated" allele frequencies, as we did not know if migration occurred between groups. In BAPS, K was determined by clustering of groups of individuals, i.e. the five sampling locations of *M. graminicola* from Tunisia (Fig. 1). Additionally, data from three European populations (Switzerland, Germany and the United Kingdom; 32 isolates each) were included in this analysis as a likely source of resistant haplotypes. Also included in this analysis were one population each (32

isolates per population) from Algeria, Syria, Israel and Iran to identify levels and patterns of gene flow to and from Tunisia, Evaluated *K*-values ranged from 1 to 12, the total number of sampled locations. We performed the analyses with 10 iterations for each *K* to judge the consistency of the simulation results. These results were then used to estimate the admixture coefficients (i.e. ancestral source of alleles for the individuals) as an indicator of gene flow among identified population clusters. Each simulation included 100 reference individuals/population and 100 iterations (Corander and Marttinen 2006; Tang et al. 2009). We also performed tests for the detection of first generation migrants using GeneClass 2.0 (Piry et al. 2004). We used the implemented Bayesian inference method (Rannala and Mountain 1997) and Monte Carlo resampling computation (10,000 simulated individuals) to infer the significance of assignments (alpha= 0.01).

Random mating was tested using three approaches. First, we tested whether the proportion of *MAT1-1* and *MAT1-2* alleles deviated significantly from the 1:1 ratio expected under the scenario of regular cycles of sexual reproduction using a χ^2 test. Second, the association among loci was tested by measuring the index of association I_A and r_d the unbiased estimate of multilocus linkage disequilibrium, as implemented in MultiLocus version 1.3 (Agapow and Burt 2001). Departure from the null hypothesis (no linkage disequilibrium; I_A=0) was tested by permutation of alleles among individuals for each locus independently (1000 permutations). Third, gametic disequilibrium (GD) was calculated in Genepop 3.4 (Raymond and Rousset 1995). The null hypothesis of genotypes at one locus to be independent from genotypes at another locus was tested with Fisher's exact test using a Markov chain approach with 1000 randomizations, 100 batches and 1000 iterations per batch as implemented in the program.

Results

Diversity in genes associated with fungicide resistance

All isolates were analyzed for the presence/absence of the G143A substitution in the mitochondrial *cytb* gene associated with strobilurin-resistance. The PCR-RFLP method produced two fragments for all isolates, indicating that the G143A resistance substitution was either absent or at a very low frequency in Tunisia. The strobilurin sensitivity of a random subset of 20 Tunisian isolates was confirmed with an *in vitro* assay as described in Torriani et al. (2009b).

A 1,358 bp-fragment of the *CYP51* gene was sequenced and analyzed for the presence of mutations associated with azole resistance. Nucleotide positions were numbered according to the complete gene sequence of strain ST1 (GenBank accession AY730587). Sequences from 80 randomly chosen Tunisian isolates revealed 40 polymorphic sites. Thirty mutations occurred in coding regions and eight mutations occurred in introns. The aligned sequences collapsed into 24 different *CYP51* haplotypes (Supplementary Fig. S1; Genbank accessions: HQ214633–HQ214656).

On the amino acid level 61 isolates (76%) were identical to the GenBank reference sequence of isolate ST1. We classified these isolates as "wild-type" not carrying any amino acid mutations associated with reduced azole sensitivity. Seventeen isolates differed from the ST1 sequence by a single substitution of asparagine (N) to serine (S) at position 188 (S188N). These isolates were also classified as wild-type because S188N has no direct effect on azole sensitivity (Leroux et al. 2007). In contrast, two unique haplotypes were identified, carrying one substitution each (G460D and Y461S respectively) previously associated with reduced azole sensitivity. The *in vitro* test confirmed that these two haplotypes had reduced sensitivity to epoxiconazole (Fig. 2).

No selection was detected among the *CYP51* haplotypes, suggesting a neutral mode of nucleotide evolution. One potential recombination breakpoint was inferred at nucleotide position 423 giving rise to topological incongruence in tree-like reconstructions. However, the NETWORK approach allows for multiple connections between haplotypes (i.e. reticulations), thus, allowing us to investigate the impact of recombination on the inferred topology. The *CYP51* haplotype network including European isolates is shown in Fig. 3. As described in more detail in a previous study (Brunner et al. 2008), the European haplotypes clustered into two groups. One group, which also forms the central part of the overall phylogenetic network, is composed of ancestral *CYP51* haplotypes. These haplotypes were mainly detected in European samples collected in the early 1990s. The second group

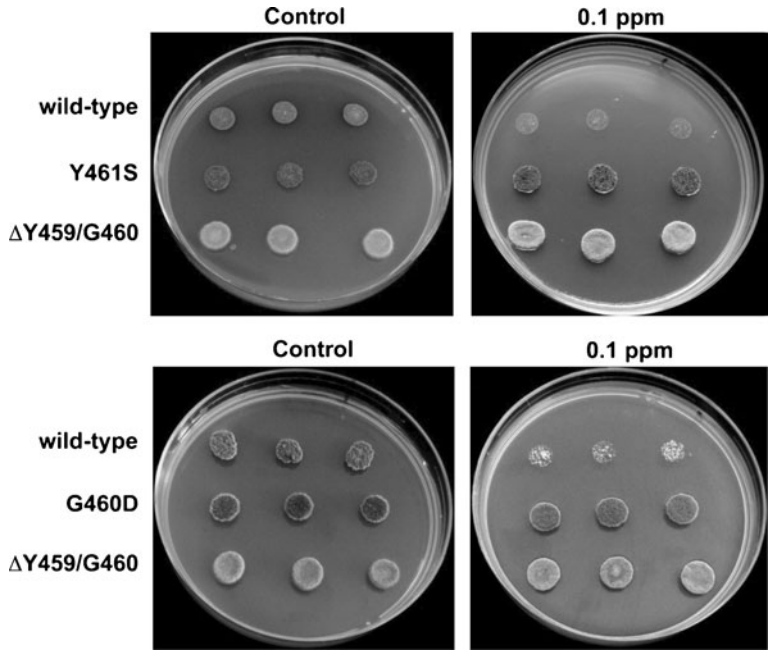

Fig. 2 *In vitro* sensitivity test for isolates carrying the resistance substitutions G460D and Y461S. Sensitive wild-type and resistant isolates carrying the deletion mutation (ΔY459/G460) were added as negative and positive controls, respectively. All isolates (three replicates each) showed normal growth on the standard control medium (*left*). On the medium containing 0.1 ppm epoxiconazole (*right*) only the isolates with resistant *CYP51* haplotypes showed growth

was composed exclusively of derived haplotypes detected in more recent European collections and included all European haplotypes associated with azole resistance.

All haplotypes identified among Tunisian isolates of *M. graminicola* clustered together, forming a distinct part of the network. The most frequent Tunisian wild-type, haplotype H111TN, was found in all populations

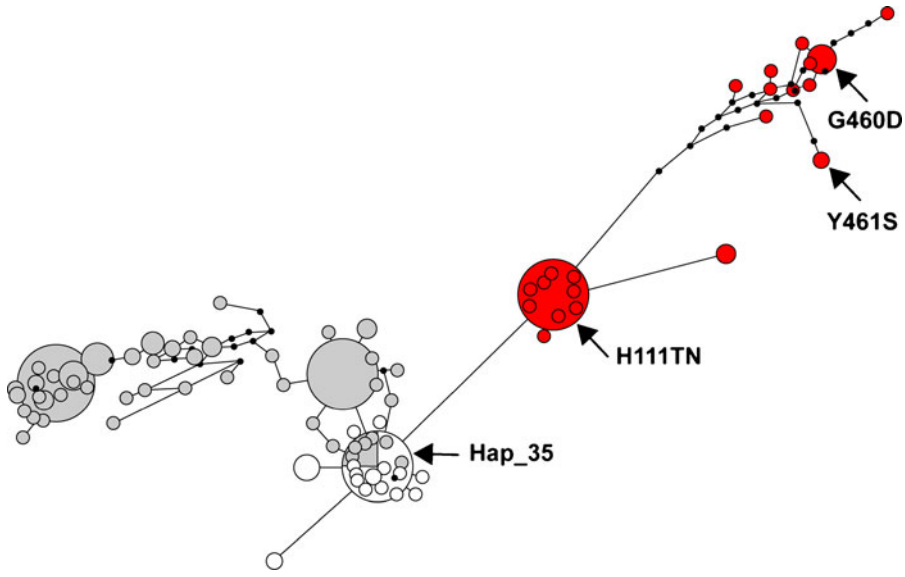

Fig. 3 Phylogenetic relationships of 368 *CYP51* sequences depicted in a reduced median network. Circle size is proportional to haplotype frequency and branch length is proportional to distance between haplotypes. Small black circles represent median vectors, hypothetical mutational steps not detected in the data set. Colours denote the sampled population groups; white, "old" European isolates sampled in the early '90s; grey, "new" European isolates; red, isolates collected from Tunisia. Haplotype 35 (Hap_35) was the most ancient European wild-type identified in a previous study (Brunner et al. 2008). Haplotype 111 (H111TN) is the most widespread wild-type allele in Tunisia (Supplementary Table S1). The arrows indicate the positions of the haplotypes carrying the resistance substitutions G460D and Y461S

(Supplementary Table S1) and formed the centre of a star-like structure, typical for the pattern associated with a recent founder event and subsequent population expansion. A second tail-like cluster that included the two resistant Tunisian haplotypes was separated by several mutational steps, indicating a more distant relationship. None of the nucleotide haplotypes was shared between Tunisia and Europe. However, H111TN is identical on the amino acid level to the most ancestral European wild-type Hap_35. These two haplotypes formed the only network connection between European and Tunisian haplotypes.

Population genetic structure

All microsatellite loci were highly polymorphic. The number of alleles per locus ranged from 4 (TCC-0008) to 19 (AC-0001), with an average of 8.6 alleles per locus. 216 distinct multilocus genotypes were found among 218 assessed Tunisian isolates, resulting in maximum genotypic diversity (G=1) for four out of five populations (Table 1). Each locus had one or two highly frequent alleles with other alleles at low frequencies and Nei's measure of gene diversity per population ranged from 0.44 to 0.53 (Table 1).

Pairwise analyses of population differentiation revealed low G_{ST} values (ranging from 0.007 to 0.018) not significantly different from zero, suggesting a lack of genetic differentiation among all Tunisian sampling sites. This lack of genetic differentiation was supported by both Bayesian clustering methods. The individual-based STRUCTURE analysis failed to detect any population substructure grouping all individuals together (K=1). Similarly, BAPS grouped all five Tunisian populations into a single cluster. However, on a larger scale including all 12 populations, the estimated K was 8 (posterior probability 1.0), as all non-Tunisian populations clustered separately (Fig. 4). The admixture analysis revealed that 23 out of 218 Tunisian isolates (10%) were likely of mixed origin (p<0.01). In contrast, only 7 out of 224 non-Tunisian isolates (3%) were identified as having mixed origins (Fig. 4). Surprisingly, none of these latter isolates had indications of Tunisian origins, suggesting that gene flow occurred only into Tunisia but not out of Tunisia into the other populations. These findings were supported by the subsequent migration analysis. GeneClass confirmed that 20 out of the 23 admixed Tunisian isolates were likely first generation immigrants. With the exception of UK, these migrants were assigned to all included populations. Thus, Tunisia acted as a sink population in this analysis (Table 2).

Mating type frequencies did not deviate from the 1:1 ratio expected for random-mating populations in any field (Table 3). Similarly, the multilocus index of association (I_A) indicated no departures from gametic equilibrium in 4 of the 5 field populations and in the overall Tunisian population. The Beja population had a statistically significant I_A, as 3 out of 55 pairwise locus-by-locus comparisons were significantly associated. Additional analyses at the allele level revealed that the allele-by-allele comparisons that gave rise to disequilibrium involved alleles that were present at very low frequencies. Extreme allele frequencies are known to provide misleading measures of disequilibrium (Asmussen and Basten 1994). Repeating the association test with rare alleles removed (cut-off value for

Table 1 Genetic diversity in Tunisian populations of *M. graminicola* based on the analysis of 11 microsatellite loci

Host population	Type of wheat	Sample size	Nb of genotypes	G[a]	Clonal fraction	H[b]	Allelic richness[c]	Private alleles[d]
Menzel Bourguiba	Durum	30	30	1	0	0.48	4.98	6
Bousselem	Durum	31	30	0.998	0.03	0.44	4.89	5
Jendouba	Durum	27	27	1	0	0.53	4.45	3
Bejà	Durum	73	72	1	0.01	0.53	5.45	12
Mateur	Bread	57	57	1	0	0.50	5.09	5
Total		218	216					

[a] Nei's genotypic diversity corrected for sample size, calculated by GenoDive 1.2

[b] Nei's unbiased gene diversity averaged over all loci, calculated by FSTAT 2.9.3

[c] Average number of alleles corrected for sample size, calculated by FSTAT 2.9.3

[d] Alleles occurring in only one population, calculated by FSTAT 2.9.3

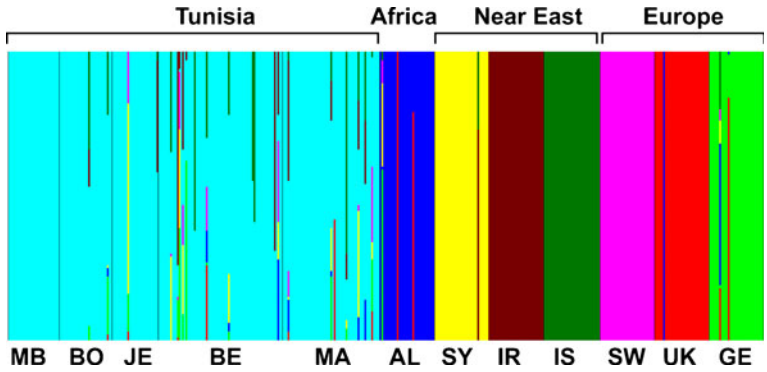

Fig. 4 Groups of individuals assigned based on Bayesian genotypic cluster analysis at the K=8 level using BAPS. Histogram shows the admixture, i.e. the proportion of each individual's genome that originated from each of the 8 identified clusters. Each individual is represented by a vertical line corresponding to its membership coefficient. Abbreviations for sampling sites; MB Menzel Bourguiba, BO Bousselem, JE Jendouba, BE Beja, MA Mateur, SW Switzerland, UK United Kingdom, GE Germany, AL Algeria, SY Syria, IR Iran and IS Israel

allele frequency <5%) resulted in non-significant I_A also for the Beja population.

Discussion

QoI resistance emerged quickly in Europe following the widespread use of strobilurin-based fungicides. For example, in the UK the frequency of resistant *M. graminicola* isolates dramatically increased within a single season (Fraaije et al., 2005). We did not detect the QoI resistance substitution G143A among Tunisian isolates. We speculate that the absence or very low frequency of this substitution in Tunisia was due to restrictions placed on the use of strobilurins on cereals after QoI resistance spread in Europe. Current fungicide treatments in Tunisia rely mainly on DMIs, therefore there is much interest in assessing the possible evolution of resistance to azoles.

Table 2 Detection of first generation migrants using GeneClass. Sampling locations of individuals are on the left and inferred origins are shown along the top. Location abbreviations are the same as given in Figs. 1 and 2

	TU	AL	SY	IS	IR	SW	UK	GE
Tunisia (n=218)	198	2	6	6	3	2	–	1
Algeria (32)	–	25	2	–	–	1	3	1
Syria (32)	–	2	29	1	–	–	–	–
Israel (32)	–	–	2	30	–	–	–	–
Iran (32)	–	–	–	–		–	–	–
Switzerland (32)	–	2	2	1	–	24	–	3
United Kingdom (32)	–	2	1	–	–	–	25	4
Germany (32)	–	2	–	–	–	1	2	27

A screen for mutations associated with DMI resistance detected 24 distinct *CYP51* haplotypes, 22 of which were sensitive wild-types. 21.25% out of 80 sequenced isolates carried the S188N substitution. In two isolates (2.5%) substitution S188N was associated with substitution G460D or Y461S. Both substitutions are associated with reduced azole sensitivity, a finding consistent with our resistance assay (Fig. 2) and can act as a single substitution or in combination with other mutations. Alterations at positions 50, 188, 379 and 513 were reported not to be related to DMI resistance. In several European populations, the substitution S188N was found in combination with L50S and N513K (Leroux et al. 2007; Mullins et al. 2011). Because these two substitutions are located outside of the *CYP51* fragment analyzed in our study, we cannot exclude their presence within Tunisian *M. graminicola* isolates and further investigations might reveal other mutations. Several amino acid changes in *CYP51* affecting azole sensitivity have been found in European populations at high frequencies. For example, the G460D substitution was found in 58% of Danish isolates and the Y461S substitution was found in 16% of Swiss isolates (Brunner et al. 2008). Alterations between codons 459 to 461, located in a highly conserved domain specific to fungal *CYP51*, have shown to be associated with the highest resistance factors (Cools et al. 2010). Interestingly, recent molecular modelling of *M. graminicola* CYP51 and its variants showed how different combinations of mutations affected binding of azoles fungicides (Mullins

Table 3 Mating type frequencies and tests for gametic disequilibrium in Tunisian populations of *M. graminicola*

Location	Gametic disequilibrium						Mating types			
	N[a]	I_A[b]	Pvalue	r_d[c]	Pvalue	GD[d]	N[e]	Mat1-1	Mat1-2	X_1^2[f]
Menzel Bourguiba	30	−0.20	0.924	−0.02	0.924	0/55	46	0.57	0.43	1.76
Beja	73	0.21*	0.008	0.02*	0.008	3/55	86	0.55	0.45	0.91
Mateur	57	−0.01	0.558	−0.01	0.558	0/55	88	0.48	0.52	0.30
Bousselem	31	−0.09	0.701	−0.01	0.701	0/55	48	0.48	0.52	0.08
Jendouba	27	0.07	0.280	0.01	0.280	1/55	89	0.47	0.53	0.42
Total	218	0.05	0.223	0.01	0.223		357	0.51	0.49	0.12

[a,e] Number of isolates

[b,c] Indexes of multilocus association. These indices were computed by MultiLocus software 1.2 (Agapow & Burt, 2001). r_d is a modification of the I_A to remove the dependency on the number of loci. r_d=1 if there is complete linkage and 0 in the absence of linkage. * reflects significance at $P \leq 0.01$

[d] number of pairs of loci showing significant disequilibrium as implemented by Genepop 3.4

[f] Chi-squared value for the deviation from the expected 1:1 ratio

et al. 2011). These authors showed that in addition to the differential effect of mutations combinations, substitutions at positions 459–461 lead to an increase of the protein cavity volume. G460D leads even to double the cavity volume. Whereas S188N was positioned far from the binding site, suggesting that this substitution does not directly affect azole sensitivity.

None of the Tunisian *CYP51* haplotypes was shared with European populations. However, the network connection showed a very close relationship between the most ancestral and widespread European wild-type (Hap_35) and the most frequent Tunisian haplotype H111TN. Given the migration analysis, we hypothesize that a wild-type *CYP51* allele was introduced into Tunisia either from Europe or another country showing high gene flow into Tunisia, for example Syria or Israel (Table 2). Additional sequencing of the CYP51 gene from these other potential source populations is needed to further test this hypothesis.

Interestingly, the European and Tunisian haplotypes carrying the resistance substitutions G460D and Y461S are located at opposite ends of the phylogenetic network. This leads us to hypothesize that both of these resistant haplotypes emerged independently in Europe and Tunisia. Under this scenario, the application of azole fungicides has imposed the same selection pressure on the *CYP51* gene, leading to independent origins for the codon substitutions G460D and Y461S in different *CYP51* genetic backgrounds, i.e. a case of parallel evolution. Parallel evolution of pesticide resistance has been demonstrated previously. Two independent origins of QoI fungicide resistance alleles were identified in the grapevine downy mildew pathogen *Plasmopara viticola* (Chen et al. 2007) and at least four independent origins for the G143A substitution were proposed for European populations of *M. graminicola* (Torriani et al. 2009a).

It is generally assumed that mutations associated with resistance carry a fitness cost, thus providing an advantage only in a highly selective environment. The use of fungicides to control Septoria tritici leaf blotch and other leaf diseases is more intense in Europe than in other parts of the world. Almost 90% of global fungicide use on cereals is in Europe (Oerke et al. 1994). Thus, selection pressure is likely much lower in Tunisia where expensive fungicides are applied at lower doses and less frequently. Despite the differences in degree of selection, our findings indicate that azole-resistant *CYP51* mutations have already emerged in Tunisian wheat fields. We believe that the frequency of these mutant alleles could increase rapidly and they could become widespread in Tunisia through gene flow if azole fungicides are used more intensively, as observed in Europe. Furthermore, sexual recombination demonstrated in this study would further increase the risk of rapid propagation of resistance through the recombination of beneficial mutations.

M. graminicola populations in Tunisia are characterized by high gene and genotype diversity. These findings are in agreement with previous studies revealing high genetic variation in natural populations of *M. graminicola* around the world (Zhan et al. 2003). We found that Tunisian populations were in gametic equilibrium and that mating type proportions did not deviate from the 1:1 ratio expected under random mating. The latter finding is consistent with a global survey showing that *M. graminicola* mating types are present at approximately equal frequencies at all spatial scales tested, from lesions to continents (Zhan et al. 2002). Taken together with the near absence of clonality, these findings indicate that Tunisian populations of *M. graminicola* also undergo regular cycles of sexual reproduction. McDonald and Linde (2002) proposed that sexual recombination promotes a pathogen's ability to evolve quickly in response to control methods such as deployment of resistant wheat varieties or fungicides. In contrast to the splash-dispersed conidia, the air-dispersed ascospores also have the potential to rapidly disseminate new mutations that encode virulence or fungicide resistance over a large area.

Several earlier studies noted a lack of structuring in populations of *M. graminicola* at regional and global scales attributed to long distance gene flow (Linde et al. 2002; McDonald et al. 1999). But some recent studies reported a significant degree of differentiation, consistent with restricted gene flow, among regional populations in France (based on SSR markers, El Chartouni et al. 2011) and Iran (based on AFLP markers, Abrinbana et al. 2010). These contrasting results were explained by geographical barriers and local adaptation of the fungus. Highly polymorphic loci, in particularly microsatellites, are one of the most efficient tools for studying moderately structured populations (Balloux and Lugon-Moulin 2002). Our analyses using microsatellite markers showed a lack of genetic differentiation among Tunisian sampling sites, consistent with a high degree of gene flow across the wheat growing regions.

No gene flow out of Tunisia was detected, whereas most populations included in the analysis contributed to gene flow into Tunisia. A global RFLP study of *M. graminicola* populations sampled from different regions on the same continent and from different continents also suggested that substantial gene flow had occurred across long distances (Zhan et al. 2003). Regional gene flow can be explained by dispersal of wind-transported ascospores as suggested by Fraaije et al. (2005). This mechanism could explain the lack of population subdivision observed in Tunisia. Gene flow between continents could result from man-mediated dispersal of infected host material such as seeds or straw and stubble used for animal feed or bedding. Unidirectional gene flow could also be explained by the dominant wind directions in the Mediterranean Sea (Bakun and Agostini 2001) which could carry ascospores over long distances.

The question whether *M. graminicola* comprises two host-adapted ecotypes specialized on durum and bread wheat, respectively, is still debated. Kema et al. (1996) provided statistical evidence for host adaptation from cross-inoculation experiments. This finding was supported by a genetic study from Zhan et al. (2004), showing specific RFLP haplotypes for isolates collected from durum wheat. However, both studies included only a limited number of isolates collected from durum wheat (11and 62, respectively. In contrast, our study compared microsatellite data from 161 isolates collected from durum wheat and 57 isolates collected from bread wheat. We did not find evidence for population substructure due to host specialization. Similarly, no evidence for host specialization in *M. graminicola* resulted from a recent coalescence analysis based on mitochondrial sequence data (Torriani et al. 2011).

In conclusion, mutations associated with resistance to fungicides are presently at low frequencies in Tunisian *M. graminicola* populations. However, based on the observations made in Europe (eg Fraaije et al. 2005; Brunner et al. 2008), we believe that resistant populations could emerge rapidly if fungicides are used more intensively. Tunisian populations of *M. graminicola* are characterized by sexual reproduction and high gene flow, two important parameters for defining pathogens with high evolutionary potential (McDonald and Linde 2002). Thus, control strategies against Septoria blotch on wheat should be optimized to reduce the spread of resistant isolates. For example, wheat stubble should be considered an important source of ascospores and the use of azole fungicides should be carefully regulated to avoid a rapid increase in the frequency of strains carrying resistant *CYP51* alleles.

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