

Research Papers

Evolution of *Mycosphaerella graminicola* at the wheat leaf and field levels

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Summary. The aim of this study was to compare *Mycosphaerella graminicola* populations at the field and lesion levels. The evolution of *M. graminicola* populations from a single field in the “Morbihan” county (France), between 2005 and 2006, was first investigated for 37 strains using molecular fingerprinting by microsatellite markers (ST1A4, ST1E3, ST1E7 and ST1D7) and SSCP analysis of partial actin and β -tubulin encoding sequences. Similar gene diversity was observed in the 2005 and 2006 populations, with no common clones between the two years. This indicates frequent sexual recombination by the fungus. When considering each marker independently and comparing marker genetic variability for the two populations, differences in the genetic variability were detected in 2006 population compared to the 2005 population. ST1A4, ST1D7 and the partial sequence of actin presented a decrease in genetic variability of the 2006 strains, while for ST1E3, ST1E7 and the partial sequence of β -tubulin showed an increase, revealing the importance of the chosen markers. In addition, 29 strains collected in 2006 from three distinct lesions on the same wheat leaf in the “Nord” county were also investigated for genetic diversity. MAT1-1 and MAT1-2 were found in the same lesion offering opportunities for sexual contact.

Key words: diversity, microsatellite markers, SSCP, local populations, lesions.

Introduction

In agroecosystems submitted to conventional control strategies against pathogens, growing of resistant cultivars and spraying with fungicides are frequently used. A good knowledge of the genetic structure of pathogens is therefore necessary, in order to develop the most durable disease management strategies, as the amount of genetic variation present within a population indicates how rapidly a pathogen can evolve. Zhan *et al.* (2003) revealed that

host dynamics, chemical applications and cultural practices affect *Mycosphaerella graminicola* allele extinction and re-colonization in a wheat field. A study of *M. graminicola* genetic variation in a time course and in a wheat leaf may be used to predict how long control measures such as fungicides and resistant cultivars are likely to be effective (Linde *et al.*, 2002; Razavi and Hughes, 2004a).

In Europe and more specifically in France, *M. graminicola* (Fuckel) Schroeter (anamorph *Zymoseptoria tritici* (Desm.) Quaedvlieg & Crous [Quaedvlieg *et al.*, 2011]) is one of the most devastating wheat pathogens. At the field level, the genetic structure of the fungus has been intensively investigated through continents at different scales (Zhan *et al.*, 2003; Banke *et al.*, 2004; Razavi and Hughes, 2004b; El Chartouni

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et al., 2011), and 94% of the total gene diversity was reported to be distributed within individual fields (Linde *et al.*, 2002). In a study of Iranian populations collected from seven distinct provinces, Komijani *et al.* (2010) found that 77% of genetic variability occurred within locations while little variability (23%) occurred among locations. *Mycosphaerella graminicola* has apparently a limited level of clonal dispersal and low levels of clonality even at spatial scales of a few square meters (Linde *et al.*, 2002). In addition, it has been hypothesized that the center of origin for the pathogen is the Middle East, and that human activities have contributed to its global dispersion as a result of the expansion of wheat cultivation (Banke and McDonald, 2005).

At the interaction site level, a histopathological study of wheat leaves infected with a single isolate of *M. graminicola* indicated that multiple penetrations of individual stomata are possible (Kema *et al.*, 1996). In addition, McDonald and Martinez (1990) and Boeger *et al.*, (1993) showed that co-infection of a single stoma by several isolates is common. As *M. graminicola* is a heterothallic fungus, the occurrence of multiple genotypes in the same lesion may allow isolates of opposite mating types to occur together and to reproduce sexually. Multiple genotypes in the same lesion can increase competition and, thus, select for higher aggressiveness/virulence (Kema *et al.*, 1996). In their study of genetic diversity in lesions, Linde *et al.* (2002) showed that the number of genotypes per lesion was generally low for most of the analyzed lesions, with two to six genotypes occurring within individual lesions. Moreover, they revealed that high diversity among lesions may indicate that ascospore infection plays an important role, and that frequent sexual reproduction will ensure frequent recombination and increased evolutionary adaptability.

In a previous study, we investigated the genetic diversity of a French population of *M. graminicola*, demonstrating that the population was highly polymorphic with 84% of unique haplotypes confirming the active sexual state of the fungus in France (El Chartouni *et al.* 2011). The even distribution of mating type idiomorphs confirmed the probability of sexual recombination leading to new strains of the pathogen every year (Siah *et al.*, 2010). Genetic study of a French population revealed that the population was relatively structured but diverse, and is potentially moving towards equilibrium between different locations (El Chartouni *et al.* 2011). In the present study,

we are giving preliminary answers to the following questions: How do clonality and reproduction evolve during time in the same wheat field? How does clonality affect the distribution of strains on a wheat leaf? The aims of the study were, therefore: (1) to compare the evolution of a local population on a period of 2 years, (2) to assess asexual reproduction by measuring the diversity of populations found in different lesions on a wheat leaf, and (3) to evaluate diversity in a single wheat lesion.

Materials and methods

Isolate sampling, strain isolation, culture and DNA extraction

For the study of population evolution in one naturally inoculated wheat field over 2 years, 38 *M. graminicola* isolates were collected in 2005 and 2006 from a field in the "Morbihan" county (number 56), France. The 2005 population consisted of 25 isolates and the 2006 population consisted of 13 isolates. In addition, 29 isolates were collected from three distinct lesions (a, b and c) on one wheat leaf, from a field in the "Nord" county (number 59), during 2006. The 29 isolates were distributed as follow, ten strains were isolated from each of lesion "a" and lesion "b", and nine strains from lesion "c". Strain isolation took place as described by Siah *et al.* (2010). The infected leaves were incubated in Petri dishes on wet filter paper overnight following the method of Kema and Annone (1991). High relative humidity is required to induce oozing of cirrhi containing spores from pycnidia of *M. graminicola*. The cirrhus from a single pycnidium was collected under stereo-microscope observation using a sterile needle, and spores were suspended in sterile water and then plated on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) in Petri dishes. After incubation for 4 days at 18°C with a 12/12h light/dark cycle (1.10³ lux), the plates were screened visually for the presence of characteristic microcolonies of *M. graminicola* and *Phaeosphaeria nodorum*, but only microcolonies of *M. graminicola* were developed. One colony per Petri dish was then randomly selected and transferred onto a new PDA plate. The cultures were then incubated at 18°C under continuous exposure to fluorescent light. The *M. graminicola* strains obtained were then conserved at -80°C for further analysis.

The resulting monospore strains were each grown on YESA medium (Yeast extract, 10 g L⁻¹; sac-

charose, 10 g L⁻¹, agar 7 g L⁻¹, chloramphenicol, 200 mg L⁻¹; ampicilin 100 mg L⁻¹) in a Petri dish kept at room temperature in constant dark, and then grown for 1 week in a YES liquid medium (yeast extract 10 g L⁻¹, saccharose 10 g L⁻¹) at 18°C under continuous light in an agitator as described previously (El Chartouni *et al.*, 2011). Pycnidiospores were then collected and the genomic DNA was extracted from approximately 100 mg using the QUIAGEN® DNeasy Plant Mini Kit (250).

DNA amplification for mating type, microsatellite and PCR-SSCP analysis

Mating types of the strains were determined by multiplex PCR amplification of partial sequences from the mating type idiomorphs (Waalwijk *et al.*, 2002). The multiplex PCR amplification was performed as described by Siah *et al.* (2010). Four pairs of primers corresponding to single locus microsatellites ST1A4, ST1E3, ST1E7, and ST1D7 (Owen *et al.*, 1998), and two pairs of primers corresponding to partial β -tubulin and actin sequences (Banke *et al.*, 2004) that were to be studied by means of PCR-SSCP were synthesized by Invitrogen® (California, CA, USA) in order to study the genetic variability within the pathogen population. Microsatellite PCR amplifications were carried out as described by Razavi and Hughes (2004b). Electrophoresis of PCR products was performed on 6% urea-polyacrylamide gels at a constant 10 W power for 90 min. After electrophoresis, microsatellite and PCR-SSCP gels were silver stained according to Bassam *et al.* (1991). The molecular mass markers were the pGEM® DNA markers (Promega, Madison, WI, USA). Many isolates presented microsatellite profiles with more than one amplified fragment. These fragments corresponded to tandem repeats in the genome that possessed different molecular weights. The PCR-SSCP amplification was performed as described by El Chartouni *et al.* (2011). For PCR-SSCP, each isolate presented two major bands corresponding to the two single stranded conformations of the PCR products.

Data analyses

To avoid bias due from repeated sampling of the same clone within the assayed *M. graminicola* populations, only one representative from each clone was included in statistical analyses of mating types.

Frequencies of the two mating types across the sampled locations as well as across host wheat cultivars were examined using the χ^2 test at significant level $P=0.05$. False rejection of the null hypothesis for possible Type I error in χ^2 analyses was tested using the Bonferroni procedure (Weir, 1997). This statistical correction assumes that the probability of incorrectly rejecting a null hypothesis in each test conducted at $P=0.05$ is 0.05.

When scoring gel profiles, null alleles were considered to be representative of a single class as previously determined by Razavi and Hughes (2004b). A preliminary dendrogram obtained by cluster analysis realised after multiple correspondence analysis (XLSTAT version 2007) revealed 38 different haplotypes for 2005-2006 field populations and 20 different haplotypes for the 2006 "lesion" populations represented by one strain or more. Isolates with identical DNA fingerprints were considered as clones, likely to be potential products of asexual reproduction.

The clonal fraction was calculated as 1– (number of different haplotypes/total number of isolates) (Zhan *et al.*, 2003). The PopGene software (Version 1.32; 2000, Yeh *et al.*, Molecular Biology and Biotechnology Centre, University of Alberta, Canada, URL <http://www.ualberta.ca/~fyeh/index.htm>) was run on clone corrected allele frequency data in order to avoid errors due to over-representation. Gene diversity was studied for the 2005-2006 populations and within and among the 2006 lesion populations. The following genetic parameters were calculated: Nei's gene diversity (Nei, 1973), average number of alleles, and gametic disequilibrium (GD). A hierarchical ascendant classification (HAC) was calculated for the period of 2005 and 2006 to study the spatial distribution of the isolates. An HAC is the progressive aggregation of individuals based on their similarity or dissimilarity index. An Euclidian distance was calculated between the strains. The algorithm starts with n singletons (a singleton is a strain). At each stage of measure, classes were formed by aggregation of the two closest elements to the previous partition. A χ^2 test was calculated to study the distributions of alleles.

Mating type analyses

A total of 67 *M. graminicola* isolates were screened in this study: 38 isolates came from the same field and were collected in 2005 and 2006, whereas 29 iso-

lates were obtained from the one wheat leaf. The 67 isolates were assayed for mating type frequencies using multiplex PCR amplification of parts of mating type encoding sequences. No *Phaeosphaeria nodorum* isolates were identified from the sampled wheat leaves during the isolation procedure, both in 2005 and 2006. All *M. graminicola* isolates generated single amplicons of the expected size, corresponding to either *mat1-1* or *mat1-2* amplicons, except for one isolate where the mating type could not be determined. After taking into account clone correction, a subset of 58 isolates was included in mating type frequency and polymorphism analyses.

Both idiomorphs were found within each of the two field populations. Of the 38 isolates, 16 (42.1%) were MAT1-1 and 22 (57.0%) were MAT1-2. A χ^2 test applied to examine deviation from the null hypothesis of 1:1 ratio revealed non-significant differences between mating type frequencies. Isolates from lesions "a" and "c" on the single leaf were exclusively MAT1-1, while isolates from lesion "b" were of the two mating types, MAT1-1 (40%) and MAT1-2 (50%), with one isolates for which mating type was not determined.

Isolates collected from one field shared similar genetic characteristics.

Twenty-five isolates were collected in 2005 and, after clone correction, 24 distinct haplotypes were found, with an average observed number of alleles of 2.89. A maximum of 5.53 was scored for the partial sequence of actin and a minimum of 1.28 for the ST1A4 marker. In 2006, 13 isolates were collected and no clones were found, revealing that the 2006 population included 13 distinct haplotypes. The average observed number of alleles was 2.82, with a maximum of 4.57 for the partial sequence of β -tubulin and a minimum of 1.17 for the ST1A4 marker (Table 1). No common haplotypes were found between the two populations.

The Nei's gene diversity index had an average value of 0.55 for 2005 and 0.58 for 2006. The greatest values for gene diversity in 2005 and 2006 were 0.82 (Actin) and 0.78 (β -tubulin) respectively. The least values for 2005 (0.22) and 2006 (0.14) were scored for the ST1A4 marker (Table 1).

In 2005, the tests of gametic disequilibrium showed that 31 tests out of 313 were significant at $P < 0.05$, and in 2006, ten tests out of 217 were significant

at $P < 0.05$ (Table 2) illustrating the high frequency of occurrence of sexual reproduction in 2005-06. HAC (Figure 1) showed that the 2005-2006 populations were distributed into four distinct classes. In the first class, all strains except one (T0313) were 2006 strains. In the second class, two out of 13 strains were 2006 strains. In the 3rd class, the distribution between 2005 and 2006 collections was even. In the 4th class all isolates originated from the 2005 collection.

The single leaf populations from county 59 shared common haplotypes and showed similar genetic diversity

The population isolated from three distinct lesions on one wheat leaf consisted of 29 isolates, from which 29 monospore strains were collected and analyzed. Of these, 20 corresponded to distinct haplotypes according to their identification using the six molecular markers. The remaining nine strains were of four small distinct groups containing clones.

Overall, and after clone correction, 69% (20 strains) of the population were analyzed since 31% (nine strains) were members of one clonal population. One clone population was common between lesions "b" and "c" as revealed by the comparison of molecular profiles and the study of their distribution.

Discussion

In a previous study based on a single year (2005) population, El Chartouni *et al.* (2011) revealed that a French population of *M. graminicola* exhibited a high level of genetic diversity, although it was relatively structured. This confirmed that the fungus undergoes frequent sexual reproduction. The aims of the present study were: to examine for the first time the genetic evolution of a French population of *M. graminicola* collected from the same field in a county, over two years (growing seasons), and to assess asexual reproduction by evaluating the genetic diversity of lesion populations isolated from a single wheat leaf.

Mycosphaerella graminicola diversity in a given field depends both on the year of sampling and the considered marker

At the Morbihan field level, we noticed that the population's gene diversity was similar in 2005 (0.55)

Table 1. Description of two French populations of *Mycosphaerella graminicola* from county 56 (Morbihan) for the six molecular markers collected in the years 2005 and 2006 and before clone correction.

Locus	Number of strains		Observed number of alleles		Gene diversity ^a	
	2005	2006	2005	2006	2005	2006
ST1A4	25	13	1.28	1.17	0.22	0.14
ST1E3	25	13	2.24	2.68	0.55	0.63
ST1E7	25	13	1.57	2.25	0.36	0.56
ST1D7	25	13	4.14	2.68	0.76	0.63
ACT	25	13	5.53	3.6	0.82	0.72
TUB	25	13	2.55	4.57	0.61	0.78
Mean	25	13	2.89	2.82	0.55	0.58

^aNei (1973).

Table 2. Observed gametic disequilibrium revealed for *Mycosphaerella graminicola* populations collected in 2005 (below the diagonal) and 2006 (above the diagonal).

	ST1A4	ST1E3	ST1E7	ST1D7	ACT	TUB
ST1A4	*	0/8	0/6	0/8	0/10	0/10
ST1E3	0/16	*	4/12	0/16	1/20	0/20
ST1E7	0/8	2/8	*	0/12	1/15	1/15
ST1D7	1/20	2/20	2/10	*	1/20	1/20
ACT	4/36	1/36	2/18	6/45	*	1/25
TUB	0/16	3/16	2/8	2/20	4/36	*

Thirty one tests out of 313 presented gametic disequilibrium ($P < 0.05$) for the 2005 population while ten tests out of 217 presented gametic disequilibrium ($P < 0.05$) for the 2006 population.

and 2006 (0.58), even though the number of collected isolates in 2006 was almost half of that collected in 2005. These high diversity levels may result from repeated sexual cycles. These genotype diversity values were close to the European values (Linde *et al.*, 2002; Zhan *et al.*, 2003; Zhan and McDonald, 2004), but were less than that obtained in a French population of *M. graminicola* collected from the main wheat growing areas in France, where a gene diversity of 0.71 was found after clone correction (El Chartouni *et al.*, 2011). We presume that the climatic conditions in 2005 were particular, and because the values obtained in 2006 were closer to the European one, the importance of evaluating population diversity over

several years is emphasized. Average observed numbers of alleles and gene diversity values at the locus level revealed differences. We found two markers (ST1D7 and Actin) presented in 2006 an average observed number of alleles and gene diversity values that were close to those found for the 2005 populations. More important is the finding that clones are not conserved over time, and the alleles are shared over time with new recombination. However, the ST1A4 marker tended to present lesser values in 2006 compared to 2005; while ST1E3, ST1E7 and β -tubulin revealed greater values in 2006 than in 2005. These results indicate that some markers presented higher allele diversity in 2006 compared to 2005, and sug-

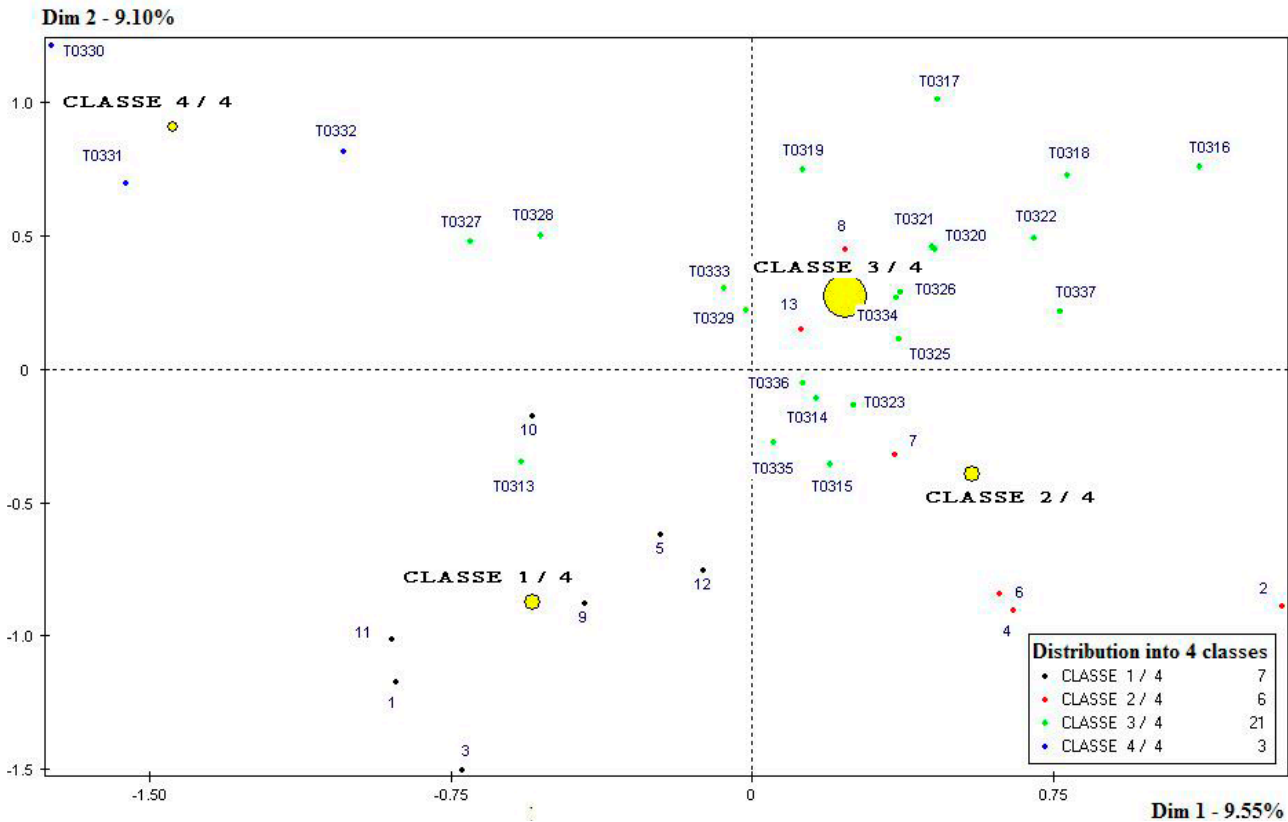


Figure 1. HAC of the 2005-2006 populations of *Mycosphaerella graminicola* for the county 56 (Morbihan). T0313 to T0337 are the strains collected in 2005. The 2006 strains are represented by numbers 1 to 13. Strains are distributed in four classes. The centre of each class is defined by a circle.

gest that these markers are good tools to indicate frequent sexual recombination.

Sexual reproduction generated high diversity levels in a given year and from one year to another

Concerning gametic disequilibrium, the 2005 population showed that 31 tests out of 313 (9.9%) were statistically significantly different. On the other hand, in 2006, ten tests out of 217 (4.6%) were significant. Therefore, these data show that the 2005 population had greater gametic disequilibrium than the 2006 population, with more frequent sexual reproduction. Both populations mostly showed random associations among alleles, consistent with high levels of recombination in the 2006 collection. The HAC analysis of the 2005-2006 populations showed that they were in four classes, with entirely new strains in

2006, since no common clones were found between 2005 and 2006. The new 2006 strains may be the result of sexual reproduction that occurred during the season. Migrant pycnidiospores could also explain the diversity observed in 2006 strains, although this has still to be tested. New clones could also originate from wild grasses or volunteer wheat plants near the sampling sites.

Are lesions formed after sexual reproduction and dissemination of pycnidiospores?

The study of genetic diversity at the leaf level allowed assessment of asexual reproduction. The percentage of unique haplotypes within the three lesions was about 69%, confirming that a single lesion often represents more than one infection event. Those infections can originate both from pycnidi-

ospores and ascospores. Therefore, single lesions can be composed of different genotypes that are likely to compete with each other, offering opportunities for sexual contact, as revealed by the high number of strains isolated at the wheat leaf level.

All three lesions revealed different amounts of gene diversity (data not shown), each of them containing multiple genotypes (Linde *et al.*, 2002). In the heterothallic fungus *M. graminicola* the occurrence of two mating types in the same lesion allows sexual reproduction and therefore leads to potential adaptation of the fungus (Linde *et al.*, 2002).

Exploitative competition exists at the lesion level

The three studied lesions contained different haplotypes, which were in competition for nutritive resource. This has been described as exploitative competition, since one individual had depleted a limited resource without restricting another individual's access to the remaining resource (Lockwood, 1992). Intraspecific exploitative competition is clearly a function of density and survival of fungi. A wheat leaf may be infected by numerous clones but only few will survive when the leaf dies (Lobban and Harrison, 1994). Linde *et al.* (2002) reported that exploitative competition may result from genotypes competing for available, uncolonized, host tissue, with intermingled hyphae and production of pycnidia whenever sufficient nutrients have been sequestered and space is available. Although vegetative incompatibility has not yet been identified in *M. graminicola*, the possibility that vegetative incompatibility affected these growth patterns cannot be excluded (Linde *et al.*, 2002).

Conclusions

Although our sampled populations may be considered as small, and may have led to biased results because of the limited sample sizes, B-Rao (2001) revealed that a sample size of 20 diploid isolates is sufficient to detect all alleles when the number of expected alleles is equal or less than five. In our case, the maximum number of observed alleles (5.53) was for the 2005 isolates analyzed with the partial sequence of actin. On the other hand, in their study of genetic diversity at the lesion level, Linde *et al.* (2002) studied one to 12 strains per lesion per wheat leaf and compared the obtained results. In our case nine

to 10 strains per lesion were collected from three distinct lesions on one wheat leaf.

To have a better knowledge of the diversity of these populations, the use of a greater number of microsatellite markers could be proposed. In addition, the study of mutations at the *CYP51* gene using the SSCP technique may reveal the evolution of resistance and aggressiveness of the fungus towards the fungicides frequently used for disease control. This investigation must be performed not only at the wheat leaf level but also on populations coming from the one field and collected in different years and at different time points over a year.

Acknowledgments

We thank FSOV (Fond de Soutien à l'Obtention Végétale) for funding this project.

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Accepted for publication: December 24, 2011