Mycologia, 103(4), 2011, pp. 764–774. DOI: 10.3852/10-184

Genetic diversity and population structure in French populations of *Mycosphaerella graminicola*

Lea El Chartouni
Benoît Tisserant

Université Lille-Nord de France, Unité de Chimie Environnementale et Interactions sur le Vivant (UCEIV), Université du Littoral Côte d'Opale, B.P. 699, F-62228 Calais cedex, France. GIS PhyNoPi: Groupement d'Intérêt Scientifique Phytopathologie Nord-Picardie

Ali Siah

Université Lille-Nord de France, Biotechnologie des Microorganismes, ISA de Lille, 48 Boulevard Vauban, F-59046 Lille, France. GIS PhyNoPi: Groupement d'Intérêt Scientifique Phytopathologie Nord-Picardie

Florent Duyme

Université Lille-Nord de France, Laboratoire Statistiques et Informatique, ISA de Lille, 48 Boulevard Vauban, F-59046 Lille, France

Jean-Baptiste Leducq

Université Laval, Institut de Biologie Intégrative et des Systèmes, 1030 Avenue de la Médecine, Québec (Québec) G1V0A6, Canada

Caroline Deweer

Université Lille-Nord de France, Biotechnologie des Microorganismes, ISA de Lille, 48 Boulevard Vauban, F-59046 Lille, France. GIS PhyNoPi: Groupement d'Intérêt Scientifique Phytopathologie Nord-Picardie

Céline Fichter- Roisin
Jean Sanssené

Institut Polytechnique Lasalle-Beauvais, Rue Pierre Waguet, B.P. 30313, F-60026, Beauvais cedex, France. GIS PhyNoPi: Groupement d'Intérêt Scientifique Phytopathologie Nord-Picardie

Roger Durand

Université Lille-Nord de France, Unité de Chimie Environnementale et Interactions sur le vivant, Université du Littoral Côte d'Opale, B.P. 699, F-62228 Calais cedex, France. GIS PhyNoPi: Groupement d'Intérêt Scientifique Phytopathologie Nord-Picardie

Patrice Halama

Université Lille-Nord de France, Biotechnologie des Microorganismes, ISA de Lille, 48 Boulevard Vauban, F-59046 Lille, France. GIS PhyNoPi: Groupement d'Intérêt Scientifique Phytopathologie Nord-Picardie

Philippe Reignault[1]

Université Lille-Nord de France, Unité de Chimie Environnementale et Interactions sur le vivant, Université du Littoral Côte d'Opale, B.P. 699, F-62228 Calais cedex, France. GIS PhyNoPi: Groupement d'Intérêt Scientifique Phytopathologie Nord-Picardie

Submitted 9 Jun 2010; accepted for publication 22 Dec 2010.
[1] Corresponding author. E-mail: Philippe.Reignault@univ-littoral.fr

Abstract: *Mycosphaerella graminicola* populations were examined in France with microsatellite markers and PCR-SSCP analysis of partial actin and β-tubulin encoding sequences. A total of 363 isolates was sampled in 2005 from 17 provinces, and genotypes from corresponding strains were characterized. Unique haplotypes comprised 84% of the population, and gene diversity was high nationwide (0.70) and locally. A moderate genetic differentiation (G_{ST} = 0.18) was found and indicated that in France the *M. graminicola* population was more structured than in other previously studied European countries. Bayesian structure analysis identified three genetic clusters distributed among the 17 provinces. Our results highlighted the potential for the adaptation of the fungus to local conditions, leading to genetic clusters among the French population of the fungus as well as genotype flow between regional clusters.

Key words: gene diversity, microsatellite markers, sexual reproduction, SSCP

INTRODUCTION

Mycosphaerella graminicola (Fuckel) J. Schröt in Cohn (anamorph *Septoria tritici* Roberge in Desmaz.) is an ascomycete fungus responsible for Septoria tritici blotch and causes major yield losses in winter wheat (*Triticum aestivum* L.) production, especially in temperate regions with high relative humidity. Asexual reproduction of the fungus leads to the formation of pycnidia and conidiospores involved in short distance dissemination. On the other hand pseudothecia, the sexual fruiting bodies, are formed throughout the year (Hunter et al. 1999) and produce airborne ascospores capable of traveling large distances. Several infection cycles occur during a single year (Zhan et al. 1998, 2003), and the disease progress is the consequence of repeated infections by asexual pycnidiospores (Owen et al. 1998). *M. graminicola* ascospores produced in autumn contribute to the first phase of epidemics as a source of inoculum for the following spring. *M. graminicola* first was reported as the sexual state of *Septoria tritici* in New Zealand more than 30 y ago (Sanderson 1972). Halama (1996) described for the first time the

occurrence of the sexual stage in France during autumn 1994 in the north of the country.

Several *M. graminicola* populations have been studied in both the old and new worlds (Schneider et al. 2001; Linde et al. 2002; Razavi and Hughes 2004a, 2004b; Jürgens et al. 2006), and it has been shown that the center of origin of the fungus was in the Fertile Crescent (Linde et al. 2002). The same authors found a high gene diversity at small geographical scales and showed that gene flow occurred at a high frequency between nearby regions and continents. Continuous sexual reproduction is thought to generate a wide variety of *M. graminicola* strains because clones are rarely found beyond 1 m^2 (Banke et al. 2004). Razavi and Hughes (2004a, b) correlated this high genetic variability to null clonality in a Canadian population, in which every sampled isolate corresponded to a unique genotype. Banke et al. (2004) suggested that long distance clone dissemination and propagation were unlikely because no clone was shared among field populations. Moreover continuous sexual reproduction might give *M. graminicola* the advantage of defeating cultivar disease resistance and might increase its ability to adapt rapidly to environmental changes (Zhan and McDonald 2004). Finally agronomic techniques by which crops are being produced, movement of infected seeds (Banke and McDonald 2005) and use of different cultivars influence the population structure (Zhan and McDonald 2004).

These studies of genetic structure and epidemiology in *M. graminicola* populations have been performed with a wide range of molecular markers. For instance populations have been characterized by means of microsatellite markers (Owen et al. 1998, Razavi and Hughes 2004a, Banke and McDonald 2005, Goodwin et al. 2007). Other works have used RAPD (Razavi and Hughes 2004b), ITS sequences (Goodwin et al. 2001) and RFLP markers (Linde et al. 2002, Zhan et al. 2003, Zhan and McDonald 2004, Jürgens et al. 2006).

In France bread wheat is produced mainly in the northern part of the country, in which climatic conditions differ between the east (pre-temperate climate) and the west (temperate oceanic). In the south of France, where wheat production is less compared to the north, a strong Mediterranean climate influence is found. The northern region is more severely affected by Septoria tritici blotch, and yield losses there were reported to represent around 40% of the total French production (Oste et al. 2000). In addition Zhang et al. (2007) reported that in France a yield loss of 28.8% was due to *M. graminicola* in combination with other diseases, yellow rust, brown rust and powdery mildew. However no data are available to date on the distribution and the genetic structure of *M. graminicola* in France.

This work presents the first description of a French population of *M. graminicola*. This population was sampled from 17 provinces throughout France. Our objectives were to (i) determine the genetic diversity of *M. graminicola* countrywide and locally with microsatellite analysis and single-stranded conformation polymorphism (PCR-SSCP) analysis of the partial actin and β-tubulin sequence, (ii) estimate the importance of sexual reproduction in these populations and (iii) describe the structure of this population and estimate gene flow among locations.

MATERIALS AND METHODS

Isolate sampling. Strain isolation, culture and DNA extraction.—Three hundred sixty-three isolates of *M. graminicola* were collected randomly from 17 provinces, mainly in northern France (FIG. 1). These locations were untreated trap plots of susceptible cultivars, infected by natural inoculum. All isolates were collected Apr–May 2005, and they were isolated from eight wheat cultivars (Charger, Orvantis, Caphorn, Apache, Soissons, Robigus, Calisto and Hammac) just before they produced ears. One to five cultivars were sampled per location, 1–5 flag leaves were collected per cultivar and 1–3 isolates were collected per leaf. Infected leaves were incubated in Petri dishes on wet filter paper overnight following the method of Kema and Annone (1991). On each wheat leaf five lesions were chosen and a single pycnidium was isolated from each lesion. To obtain a monospore colony a cirrus was collected, diluted in sterile water and plated on Petri dishes containing potato dextrose agar (PDA). After 4 d at 18 C and continuous day light (1.10^3 lux) one colony was selected randomly.

The resulting monospore strains were grown on YESA (10 g yeast extract L^{-1}, 10 g sucrose L^{-1}, 7 g agar L^{-1}, 200 mg chloramphenicol L^{-1} and 100 mg ampicilin L^{-1}) medium in Petri dishes kept at room temperature in constant dark and grown a week in YES liquid medium (10 g yeast extract L^{-1}, 10 g sucrose L^{-1}) at 18 C under continuous light in an agitator. The spores were collected and genomic DNA was extracted from approximately 100 mg with the QIAGEN® DNeasy Plant Mini Kit (QIAGEN, Mississauga, Ontario, Canada).

DNA amplification for microsatellite and PCR-SSCP analysis.—Preliminary studies let us estimate the suitability of different molecular markers. Four pairs of primers corresponding to microsatellites ST1A4, ST1E3, ST1E7 and ST1D7 (Owen et al. 1998) and two pairs of primers corresponding to β-tubulin and actin partial sequences (Banke et al. 2004) that were to be studied with SSCP were synthesized by Invitrogen (Carlsbad, California). Microsatellite PCR amplification was carried out as described by Razavi and Hughes (2004a). Electrophoresis of PCR products was performed on a 6% urea-polyacrylamide gel at a constant 10 W for 90 min. The molecular mass markers

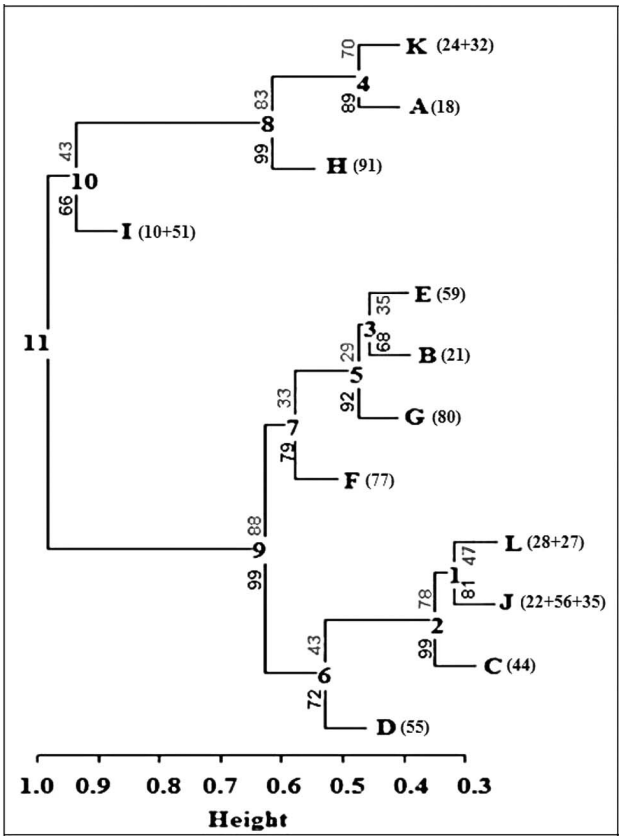


FIG. 1. UPGMA phenogram based on Nei's genetic distance (1978) between the *Mycosphaerella graminicola* populations from 12 French locations using microsatellite loci and SSCP analyzed genes actin and β-tubulin as revealed by bootstrap analysis, with the corresponding bootstraps values (gray) and the approximately unbiased *P* value (black). Corresponding French provinces are in parentheses.

were the pGEM® DNA markers (Promega, Madison, Wisconsin).

PCR-SSCP amplification involved 2 min at 96 C for initial denaturation, followed by 30 amplification cycles involving a denaturation step at 96 C for 1 min, an annealing step at 59 C for 60 s and an elongation step at 72 C for 1 min. A final extension step at 72 C took 10 min. Each 20 µL reaction mixture contained 2 µL PCR buffer (750 mM Tris-HCl pH 8.8, 200 mM $[NH_4]_2SO_4$, 0.1 mL Tween 20), 2 µL $MgCl_2$ (25mM), 0.2 mM of each dNTP, 30 pmol forward primer, 30 pmol reverse primer, 1 unit Taq DNA polymerase (GoldStar™, Eurogenetech, Liege, Belgium) and 11.5 µL distilled water. Preliminary optimization of PCR with different annealing temperatures for each pair of primers showed that the optimum temperature was 60 C for β-tubulin and 54 C for actin. All 363 strains were screened with the optimized annealing temperature for each pair of primers. After amplification an equal volume of SSCP denaturation buffer (95 mL formamide [v/v], 10 mM NaOH, 250 mg bromophenol blue, and 250 mg xylene cyanol) was added to each reaction tube. After denaturation at 95 C for 4 min PCR reaction tubes were cooled in wet ice and 3 µL were used for electrophoresis. Electrophoresis was conducted 17 h at a constant 4 W. Non-denaturing polyacrylamide gel (MDE 2× concentrate, FMC Bioproducts; Rockland, Maine) was poured according to the manufacturer's instructions. After electrophoresis microsatellite and PCR-SSCP gels were stained silver according to Bassam et al. (1991). For SSCP each isolate presented two major bands corresponding to the two single-stranded conformations of the PCR products. In addition verification gels also were performed to check the distinct observed profiles and to eliminate any risk of human error.

Data analysis.—When scoring gel profiles null alleles were considered to be representative of the same class. Except for Bayesian structure analysis, provinces in which fewer than 10 isolates were sampled were grouped into a single location with others according to their geographic proximity (FIG. 1). A preliminary dendrogram was obtained by cluster analysis realized after multiple correspondence analyses (XLSTAT 2007, Addinsoft-Pro, Paris, France). By compiling both microsatellite markers and PCR-SSCP profiles we identified 305 haplotypes corresponding to one or more strains because several strains can share the same haplotypes (data not shown). The detection of repeated multilocus genotypes can provide strong evidence for the presence of some clonal reproduction (Halkett et al. 2005).

The clonal fraction was calculated as 1− (number of different haplotypes/total number of isolates) (Zhan et al. 2003). Popgene software (1.32; Yeh et al. 2000) was run both before and after clone correction of the allele frequency data to estimate possible over representation. These genetic parameters were calculated: average number of alleles, Nei's gene diversity (Nei 1973), gametic disequilibrium (Ia) (Brown et al. 1980), population differentiation (G_{ST}) (Nei 1973), gene flow (Slatkin 1987) and Nei's genetic distance (Nei 1978). The χ^2 test was applied to determine differences in allele frequencies.

Gene diversity was calculated for the entire country and within each location. G_{ST} is defined as the proportion of genetic diversity that resides among populations (Culley et al. 2002). N*m* is a theoretical number estimating both the effective rate of migrant per generation and the effective size of populations (Slatkin 1987). Nei's genetic distance (1978) was used to draw an UPGMA phenogram among *M. graminicola* populations from different locations followed by a bootstrap analysis. Gametic disequilibrium was measured with the method of Brown et al. (1980) to detect the departure to random mating. A lack of statistically significant ($P \leq 0.05$) disequilibrium indicated that the population uses a random mating sexual reproduction.

Population composition was inferred for *M. graminicola* isolates and for the 17 initial provinces with Structure 2.3.3. (Pritchard et al. 2000). It consisted of Bayesian inference cluster analysis of clone-corrected haplotypes and let us identify clusters based on maximizing Hardy-Weinberg equilibrium. The admixed model with the correlated alleles frequencies option was implemented with burn-in period and 1 000 000 Markov chain Monte Carlo (MCMC) iterations without taking into account the prior information of

TABLE I. Population parameters of the total French population for the six markers before (1) and after (2) clone correction

	Number of strains		Average observed number of alleles		Gene diversity[a]		G_{ST}	χ^{2b}
Locus	1	2	1	2	1	2	2	2
ST1A4	363	305	3.16	3.38	0.68	0.70	0.36	236.71
ST1E3	363	305	3.79	3.89	0.74	0.74	0.13	87.38
ST1E7	363	305	1.62	1.66	0.38	0.40	0.16	611.02
ST1D7	363	305	4.82	4.81	0.79	0.79	0.18	11.70
ACT	363	305	8.48	9.05	0.88	0.89	0.12	469.84
TUB	363	305	3.55	3.71	0.72	0.73	0.14	847.18
Mean	363	305	4.23	4.42	0.70	0.71	0.18	

[a] Nei 1973.
[b] $P \leq 0.01$.

locations. Analyses were carried out by allowing the relative contribution of each cluster (parameter α) to vary among populations (Falush et al. 2003). Each dataset of 20 runs was used to determine the variation of the posterior probability Ln *P(D)* of each, assuming the true number of populations or clusters (K) was 1–15. The estimation of the most likely *K* was based on Δ*K* values calculated from Ln *P(D)*, as described in Evanno et al. (2005). In addition the relationship among individuals was calculated with multidimensional scaling (MDS) implemented in XLSTAT 2010; MDS was used to detect genetic divergence among populations.

RESULTS

Genotype and gene diversity, gametic disequilibrium within the entire population.—The French population studied in this work consisted of 363 isolates from which 363 monospore strains were obtained and analyzed. Clone correction was performed in each province individually, and the number of clone-corrected genotypes is therefore not equal to the number of distinct genotypes. Before clone correction was performed within these strains the average number of alleles analyzed was 1.62–8.48. The global Nei's gene diversity index mean value was 0.70. Within the total population 264 strains corresponded to single unique haplotypes according to their molecular profiles based on six molecular markers. The 99 remaining strains formed small groups with strains from different geographical origins and comprised 41 distinct haplotypes. For example in province 59 71 strains were sampled, of which 50 strains presented unique haplotypes and the remaining 21 isolates were distributed among six genotypes (one genotype consisted of two clones, two genotypes consisted of three clones, two genotypes of four clones and a genotype of five clones). After clone correction only 56 haplotypes were studied in this province.

After clone correction a total of 305 unique haplotypes (84%) were analyzed for the six loci; we summarized the average observed number of alleles (TABLE I). Tests for gametic disequilibrium were performed for all loci corresponding to the four studied microsatellite markers and the actin and β-tubulin partial sequences analyzed by SSCP. Only 136 of 1264 (11%) tests for gametic disequilibrium were significant.

Distribution of diversity and gametic disequilibrium within locations.—The clonal fraction was calculated for each population (TABLE II). Location A had no clonal fraction while the clonal fraction reached its greatest value in Location B. Location A also presented the lowest value for Nei's gene diversity index (0.45) whereas location E had the largest (0.68). Gametic disequilibrium values ranged between 1.10^{-3} (location B) and 3.10^{-2} (location E) for $P \leq 0.05$. The difference in the percentages of polymorphic loci in locations A, B and H (TABLE II) is due to the ST1E7 locus, which was monomorphic in these locations (data not shown).

Population differentiation and gene flow.—The subdivision (G_{ST}) within the French population was greatest (0.36) for the ST1A4 locus, whereas the smallest G_{ST} was scored for the actin gene (0.12). Considering all the tested molecular markers together, the average G_{ST} for the clone corrected data (305 strains) was 0.18 (TABLE I). For the location by location comparison G_{ST} ranged between 0.04 (location L vs. location J) and 0.19 (location A vs. location D), and the N*m* values varied between 2.10 (location A versus location D) and 11.52 (location L vs. location J) (TABLE III).

Global and partial UPGMA phenograms for genetic distances between locations.—A comparison of Nei's genetic identity and genetic distance among the

TABLE II. Comparison of the French *Mycosphaerella graminicola* population after clone correction among the 12 distinct locations

Location	Strains		Clonal fraction	Average observed number of alleles	Gene diversity[a]	No. of poly Loci[b]	% poly Loci	Ia[c]
	No.of strains	Clone correction						
A	10	10	0	2.60	0.45	5	83.3%	6.10^{-3}
B	18	11	0.39	2.32	0.48	5	83.3%	1.10^{-3}
C	35	26	0.26	2.72	0.60	6	100%	16.10^{-3}
D	16	14	0.12	2.43	0.54	6	100%	6.10^{-3}
E	71	56	0.21	3.73	0.68	6	100%	3.10^{-2}
F	11	8	0.27	2.73	0.56	6	100%	5.10^{-3}
G	30	27	0.1	2.64	0.53	6	100%	11.10^{-3}
H	35	32	0.09	2.48	0.52	5	83.3%	6.10^{-3}
I	45	43	0.05	3.51	0.61	6	100%	36.10^{-3}
J	31	27	0.13	3.13	0.58	6	100%	18.10^{-3}
K	21	18	0.14	3.41	0.63	6	100%	17.10^{-3}
L	40	33	0.18	3.13	0.65	6	100%	25.10^{-3}

[a] Nei (1973).

[b] Number of polymorphic loci for the six studied markers.

[c] Gametic disequilibrium for tests that showed significant disequilibrium ($P \leq 0.05$) between individual alleles at 2 microsatellite loci and/or SSCP genes.

sampled locations revealed genetic affinities among some of these locations (FIG. 1). UPGMA analysis identified two main groups, one consisting of subpopulations I, A, H and K and a second group subdivided in two subgroups respectively comprising populations B, E, F, G and C, D, J, L.

Genetic structure of the populations sampled in the different provinces.—With Bayesian cluster analysis implemented in Structure 2.3. (Pritchard et al. 2000) we found higher mean posterior probability values Ln*P(D)* and lower standard deviations (SD) estimated over 20 runs for *K* values assumed between 1 and 3. We observed a substantial decrease in mean and increase in SD of Ln *P(D)* for *K* between 3 and 8 (FIG. 2a). The method developed by Evanno et al. (2005) to infer the most likely number of clusters gave a maximum ΔK value for $K = 3$ (FIG. 2b), also corresponding to the higher posterior probability. For $K = 2$ a slightly higher SD in *LnP(D)* (SD = 29.82) indicated slight variations in individual assignations among 20 runs, whereas all runs obtained for $K = 3$ (SD = 11.2) gave the same assignation pattern. For *K* values greater than 12 the observed decrease in Ln*P(D)* SD and subsequent increase of ΔK (FIG. 2b) was a result of progressive mitigation of structure signal, that is all individuals tended to be assigned with a 1/K probability to each cluster (data not shown). We thus retained $K = 3$ as the most likely number of genetic clusters among sampled populations.

TABLE III. Population differentiation (G_{ST}) (above diagonal) and gene flow (N*m*) (below diagonal) among *M. graminicola* populations from 12 French locations

Location	A	B	C	D	E	F	G	H	I	J	K	L
A	***	0.16	0.15	0.19	0.12	0.15	0.18	0.18	0.17	0.14	0.10	0.12
B	2.64	***	0.09	0.12	0.09	0.12	0.10	0.13	0.14	0.09	0.11	0.09
C	2.75	5.20	***	0.07	0.07	0.10	0.07	0.12	0.09	0.05	0.12	0.05
D	2.10	3.48	7.06	***	0.11	0.11	0.12	0.19	0.13	0.05	0.15	0.05
E	3.56	5.22	7.11	4.23	***	0.06	0.06	0.09	0.08	0.07	0.08	0.06
F	2.86	3.53	4.48	4.04	7.84	***	0.10	0.16	0.14	0.07	0.08	0.09
G	2.33	4.42	7.03	3.76	7.87	4.59	***	0.15	0.14	0.07	0.14	0.09
H	2.33	3.20	3.54	2.17	4.81	2.58	2.76	***	0.14	0.15	0.09	0.11
I	2.48	2.99	5.08	3.26	5.97	2.96	3.18	3.07	***	0.10	0.12	0.10
J	3.17	4.84	10.08	10.45	6.69	6.66	6.14	2.85	4.32	***	0.11	0.04
K	4.49	3.89	3.70	2.88	5.98	5.60	3.15	5.04	3.73	4.15	***	0.10
L	3.62	4.74	8.93	9.89	7.24	4.87	5.08	3.88	4.68	11.52	4.42	***

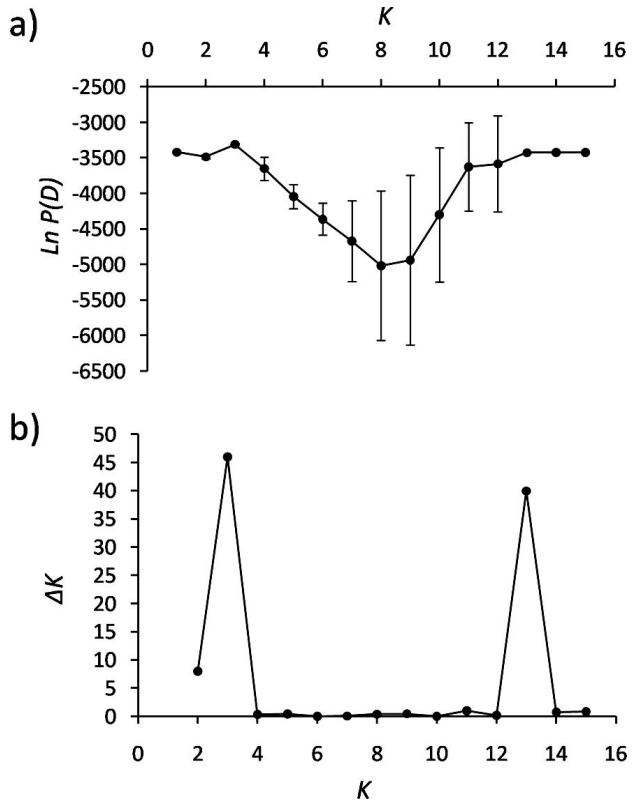


FIG. 2. Detection of the most likely number of clusters from Bayesian inference cluster analysis (Structure 2.3.3.) carried on in 17 populations of *M. graminicola*. a. Mean and standard deviation of posterior probability Ln*P(D)* calculated among 20 runs, function of the number of clusters *K* (range 1–15). Values were obtained after burn in and 1 000 000 MCMC iterations. b. Statistic Δ*K* calculated from Ln*P(D)* according to the method described in Evanno et al. (2005), function of the number of clusters *K* (range 1–15).

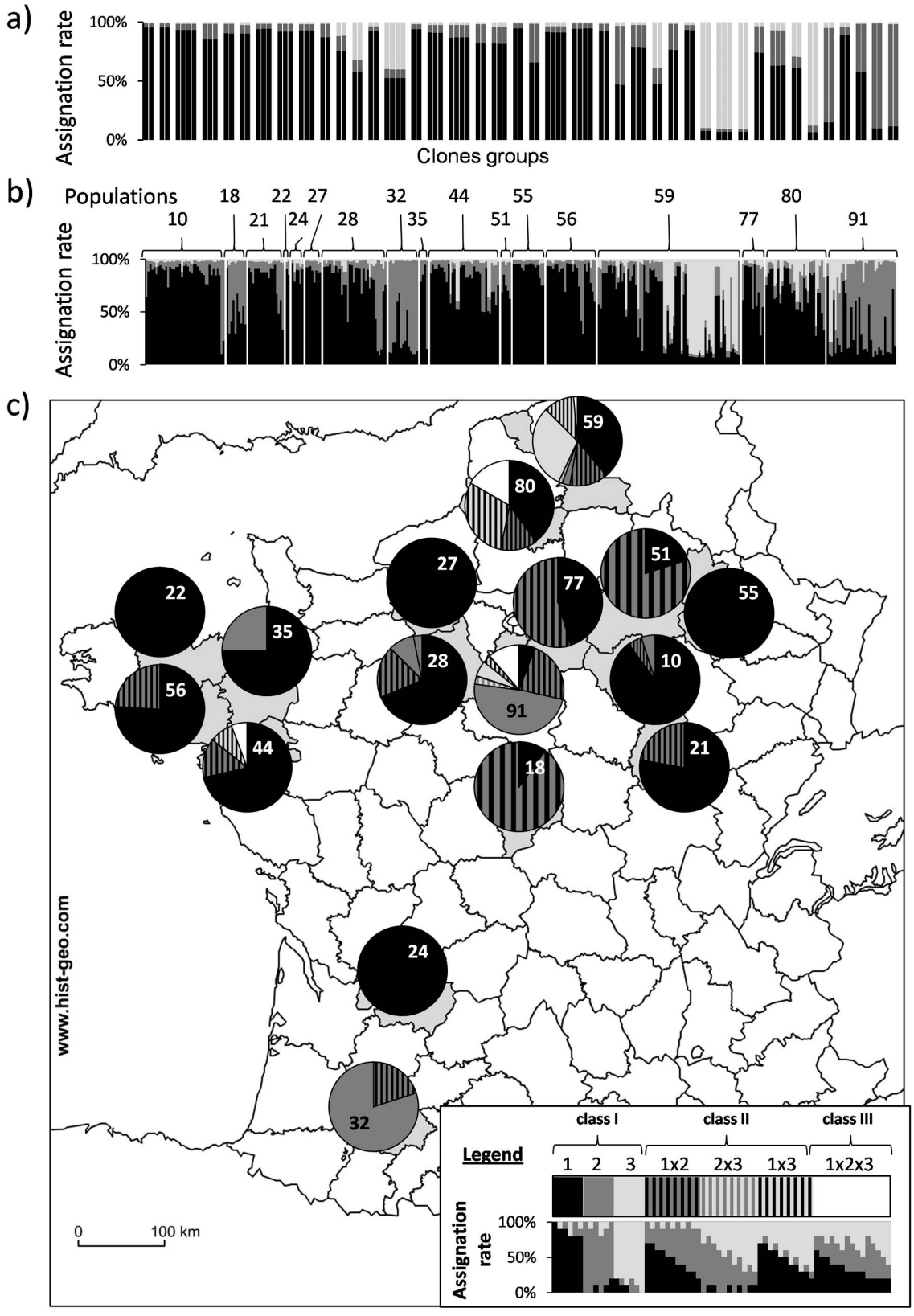
We observed no variation in assignation proportions among individuals sharing the same genotype (FIG. 3a). Because individual assignations showed frequent admixtures of three clusters (FIG. 3b) we defined three classes of individuals according to their degree of admixture (cf. FIG. 3c). An individual belonged to class I when one cluster represented at least 70% of its individual assignation. A large proportion of individuals were assigned to a single cluster (77%) (FIG. 3b) and thus belonged to this class. Class II grouped individuals that showed admixtures of two clusters, with the third cluster only represented in low proportions (below 15%) in individual assignment. Class II represented 22% of our sampling. The third class (III) represented only 1% of our sampling (1%) and corresponded to remaining isolates showing admixtures of three clusters.

Strict clustering based on geographical origin was not found with Structure; each provincial population contained haplotypes with membership in three clusters in proportions varying with populations (FIG. 3c). However among the three Cluster 1 represented 65% of all assignations and was widely represented by class I individuals among all locations, except in populations 32 and 91, where Cluster 2 was predominant. Cluster 3 was represented only by half the individuals of population 59 and some in population 91. A significant proportion of population 80 also showed class II individuals partially assigned to Cluster 3. Clusters 2 and 3 thus represented restricted geographic distributions in respectively southern (32) and northern (59 and 80) populations, as compared to Cluster 1, which was widely distributed. Only population 91, located in the center of our sampling area, showed class I individuals assigned to each cluster. Moreover MDS analysis that was performed to detect genetic divergence among populations confirmed that genotypes belong to clusters that do not significantly correlate with sampling locations (data not shown).

DISCUSSION

The French population of M. graminicola reveals high diversity of its nuclear genome.—In previous studies similar numbers of molecular neutral markers were used for *M. graminicola* population characterizations. For instance nine RFLP markers were used by Zhan et al. (1998), nine microsatellites by Razavi et al. (2004a) and seven RFLPs by Jürgens et al. (2006). In populations of diploid organisms with alleles assumed to be equally frequent about 150 individuals and 40 disclosed alleles had 90% chance of detecting all alleles at corresponding loci (B-Rao 2001). With four microsatellite markers and two partial sequences of genes assessed by SSCP we identified more than 40 alleles in a population of haploid organisms consisting of 363 strains. Moreover because the allele distribution was not equal for most of the loci we examined more than 70 diploid individuals and 40 disclosed alleles are sufficient to get 0.99 probability of finding alleles (B-Rao 2001).

Our results revealed a high degree of genomic diversity in the French population of *M. graminicola*. Low clonality is correlated to high Nei's gene diversity for the total population and is illustrated by the weak differences in the values observed before (0.70) and after (0.71) clone correction. In terms of gene diversity for the microsatellite loci, polymorphisms between alleles might be due either to a few nucleotides or to the number of repeats. Indeed Owen et al. (1998) and Razavi and Hughes (2004a) reported results similar to ours for 12 British isolates and a Canadian population of *M. graminicola*; they interpreted these polymorphisms as arising from


a)
Assignation rate
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Clones groups
b)
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mutation during DNA replication. The failure of amplification with some primer pairs leading to null alleles is thought to be related to a mutation within the primer binding sites of the genome (Owen et al. 1998). With XLSTAT we found no relationship among the cultivars from which sampling was performed and the genetic distribution of *M. graminicola* French populations (data not shown).

The high number of fingerprints revealed by PCR-SSCP for the actin and β-tubulin amplified sequences compared to microsatellites show that they possess a high potential for identification of polymorphisms in *M. graminicola* populations. This result is in agreement with the comparisons of these sequences performed by Banke et al. (2004) in 14 globally distributed populations of this fungus. In our work the actin partial sequence presented the highest number of PCR-SSCP profiles. Moreover this high level of polymorphism has been observed in other ascomycetes such as *Saccharomyces cerevisiae*; the actin gene showed a higher variability compared to the D1/D2 region of the large subunit gene (LSU) of the ribosomal gene complex (Daniel and Meyer 2003). The same authors concluded that the actin gene was a good tool for detecting differentiation within closely related taxa. In our case the actin gene revealed a high haplotype resolution with a Nei's gene diversity index of 0.71. The actin gene therefore was found to be useful in detecting intraspecific variations.

The average of total gene diversity (0.70) agrees with results obtained by Schneider et al. (2001), Linde et al. (2002), Zhan et al. (2003) and Zhan and McDonald (2004). In comparison gene diversity for *Phaeosphaeria nodorum* was reported to range between 0.19 and 0.90 with an average of 0.60 for 693 isolates collected from all continents (Stukenbrock et al. 2006). This indicates that the two *Septoria* species share common features, *M. graminicola* having probably emerged as the major *Septoria* disease because of the erosion of cultivar resistance (Bayles 1991).

Sexual reproduction contributes to M. graminicola *diversity.*—Gametic disequilibrium displayed low values; only 136 tests out of 1264 were significant ($P \leq 0.05$) nationwide. Lower values were scored locally, suggesting the departure from random mating in this French population, probably leading to strains with new genetic combinations every year. Sexual recombination, which has a great impact on the number of genetically distinct individuals in a population, increases gene diversity through recombination and therefore might explain these low values of gametic disequilibrium. The percentage of unique haplotypes (84%) was close to the result obtained by Linde et al. (2002) for a Switzerland population (89–98%) studied with RFLP markers. Moreover the clonal fractions calculated for every population revealed that asexual reproduction occurs in these populations. These rates of clonal fraction might fluctuate owing to periodic events of sexual reproduction (Halkett et al. 2005), and the absence of clonal fraction in location A might be due to sampling size.

The French population of M. graminicola *is more structured than previously studied European populations.*—If frequent sexual reproduction occurs, as linkage equilibrium between populations would indicate, one would expect a lack of genetic structure within populations because any genetic differentiation should be erased after few sexual generations. However a relatively high genetic differentiation index ($G_{ST} = 0.18$) was observed when considering the 12 locations or the 17 provinces. One could speculate that local French populations of *M. graminicola* might be adapted to a particular climate or combination of agricultural practices and climate conditions. For Culley et al. (2002) a low genetic

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FIG. 3. a. Distribution of individual assignations estimated for $K = 3$ clusters, from Bayesian inference cluster analysis (Structure 2.3.3.) carried on in 17 populations of *M. graminicola*. Only groups of 2–4 individuals showing a same genotype are represented and grouped together by genotype. Assignation rates for each individual (y-axis) were calculated by averaging values obtained from two runs. Each individual is represented by a vertical bar with various proportions of cluster 1 (black), cluster 2 (dark gray) and cluster 3 (gray). b. Distribution of individual assignations estimated for $K = 3$ clusters, from Bayesian inference cluster analysis (Structure 2.3.3.) carried on in 17 populations of *M. graminicola*. Assignation rates for each individual (y-axis) were calculated by averaging values obtained from two runs. Each individual is represented by a vertical bar with various proportions of cluster 1 (black), cluster 2 (dark gray) and cluster 3 (gray). c) Geographical distribution of three genetic clusters inferred by Bayesian inference cluster analysis (Structure 2.3.3.) carried on in 17 populations of *M. graminicola*. Each population is represented by a pie diagram showing the proportion of individuals according to their assignation to three clusters (1 = black, 2 = dark gray, 3 = gray). Proportions of class I individuals (assigned to one major cluster) are represented by solid colors, class II individuals (assigned to two major clusters) are represented in hatched colors and class III (other individuals) in white. Classes were defined from proportions presented in the b section, as described in the example of the legend (bottom).

differentiation corresponds to a $G_{ST} < 0.05$ and a high genetic differentiation corresponds to a $G_{ST} > 0.25$. In our case G_{ST} is higher than the global mean ($G_{ST} = 0.05$) obtained by Linde et al. (2002) and was 0.05–0.25. Moreover the French population was collected more recently than the populations studied in Linde et al. (2002). The global G_{ST} value of 0.18 reveals that the French populations studied here were more structured compared to those studied by Schneider et al. (2001) and Zhan et al. (2003), suggesting more restricted gene flow among these populations. This interpretation is supported by the low N*m* values we found among spatially close populations (e.g. 2.8 among contiguous provinces 77 and 91). In agreement with our results a comparison of two field populations, one in Kansas and the second in California, revealed a G_{ST} of 0.21 and an N*m* of 1.8 (Kabbage 2007). The author stated that a migration or a flow of 1–2 individuals per generation might be low enough to keep the two populations diverging significantly but also could be an indication that populations of *M. graminicola* at these two sites might be moving gradually toward equilibrium between genetic drift and gene flow. Abrinbana et al. (2010) found a G_{ST} of 0.38 with an N*m* of 0.78 and noted the considerable contrast with other regional (Schneidar et al. 2001) and global (Linde et al. 2002, Zhan et al. 2003) scales. In addition, concerning high gene flow values found between geographically separated populations (e.g. 9.89 among locations D and L), we assumed that infected grains might have contributed to such a gene flow (Consolo et al. 2009).

French populations were collected in 2005 from sites with different climatic conditions and agricultural practices. Such G_{ST} values detected among *M. graminicola* French populations might have resulted from random genetic drift as well as from adaptations to local conditions, as previously observed in an Iranian population of *M. graminicola* for which the authors found a population structure in *M. graminicola* contrasting to that of most other countries studied (Abrinbana et al. 2010). Moreover Jürgens et al. (2006) reported that climate in Argentina in the 1998 growing season did not favor sexual reproduction of *M. graminicola* populations whereas during the 2000 growing season conditions might have favored the sexual cycle. In the 2005 growing season climate conditions might have favored a sexual cycle in France, resulting in the observed genetic diversity. We therefore suggest that our markers might be not neutral with respect to environmental conditions. This hypothesis could be investigated in the future, but the main question remains the durability of such parameters values later and the effect of distinct environmental conditions on *M. graminicola* population evolution. The investigation of population structure and diversity over several years might provide a clear answer to this question.

The French population of M. graminicola *contains distinct genetic groups.*—Combining the use of two different molecular markers classes let us draw a precise picture of *M. graminicola* gene and genetic diversities in France in 2005. In a preliminary step we obtained a dendrogram drawn with XLSTAT revealing that when using the data obtained for the four microsatellite markers only the number of haplotypes was smaller than that obtained when considering PCR-SSCP markers only (data not shown). However UPGMA showed that location D in the east was associated with locations from the west (C, J and L). This genetic proximity of location D with western locations was confirmed later by the Bayesian structure analysis.

Based on Structure analysis, the French populations of *M. graminicola* comprise three genetic clusters. We found that in northern provinces 65% of assignations corresponded to Cluster 1; we therefore assumed that the *M. graminicola* populations might not present a Hardy-Weinberg equilibrium (e.g. the allele numbers as well as genotype numbers are not multinomially distributed and an island model is followed, at least by the part of the population corresponding to this cluster). However a G_{ST} of 0.18 indicates that gene flow can occur between distant provinces and would homogenize the populations. Populations assigned to Cluster 2 were sampled almost exclusively in provinces 32 (south) and 91 (center), while those of Cluster 3 were collected in provinces 59, 80 (north) and 91 again. Moreover province 91, which is approximately in the center of our sampling area, presented class I individuals assigned to each cluster. Based on this observation, each individual would have inherited some proportion of its ancestry from each population revealing the presence of admixture (Falush et al. 2003).

In conclusion we studied for the first time populations of *M. graminicola* in France with two distinct molecular techniques that helped us in revealing the diversity, structure and mode of reproduction and dissemination of the fungus to assess the genetic diversity and the structure of fungal strain obtained from isolates collected from 17 provinces/12 locations of French wheat-growing areas.

We revealed a high gene diversity of the fungus consistent with active sexual reproduction in France. This result agreed with previous studies concerning

populations from other areas (Zhan et al. 1998, 2003). The equal frequency of the two mating types in France (Siah et al. 2010) is consistent with sexual recombination (Zhan et al. 2003), leading to new strains of *M. graminicola* and a high potential for adaptation. The development of *M. graminicola* resistance to fungicides and its ability to overcome cultivar resistance must be measured. An investigation of the fungus diversity and structure with new additional microsatellite markers (Goodwin et al. 2007) and wider sampling on more locations and spanning several years also has to be examined for a better understanding of the fungus evolution and distribution in France. Particular attention must focus on testing whether the consistence of the cryptic structure observed in this work would be confirmed later.

ACKNOWLEDGMENTS

The authors thank Groupement National Interprofessionnel des Grains et des Semences (GNIS) and the Regional Council of Nord-Pas-de-Calais for financial support. This research also was supported by grants from the Regional Council of Nord-Pas-de-Calais.

LITERATURE CITED

Abrinbana M, Mozafiri J, Shams-bakhsh M, Mehrabi R. 2010. Genetic structure of *Mycosphaerella graminicola* populations in Iran. Plant Pathol 59:829–838, doi:10.1111/j.1365-3059.2010.02309.x

Banke S, Peschon A, McDonald BA. 2004. Phylogenetic analysis of globally distributed *Mycosphaerella graminicola* based on three DNA sequence loci. Fungal Genet Biol 41:226–238, doi:10.1016/j.fgb.2003.09.006

———, McDonald BA. 2005. Migration patterns among global populations of the pathogenic fungus *Mycosphaerella graminicola*. Mol Ecol 14:1881–1896, doi:10.1111/j.1365-294X.2005.02536.x

Bassam BJ, Gaetano-Anolles G, Gresshoff PM. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 196:80–83, doi:10.1016/0003-2697(91)90120-I

Bayles RA. 1991. Varietal resistance as a factor contributing to the increase importance of *Septoria tritici* Rob. & Desm. In the UK wheat crop. Plant Variety Seeds 4:177–183.

B-Rao C. 2001. Sample size considerations in genetic polymorphisms studies. Human Heredity 52(4):191–200, doi:10.1159/000053376

Brown AHD, Feldman MW, Nevo E. 1980. Multilocus structure of natural populations of *Hordeum spontaneum*. Genetics 96:523–536.

Consolo VF, Albani CM, Bern CM, Salerno GL, Cordo CA. 2009. A conventional PCR technique to detect Septoria tritici in wheat seeds. Aust Plant Pathol 38(3):222–227, doi:10.1071/AP08099

Culley TM, Wallace LE, Gengler-Nowak KM, Crawford DJ. 2002. A comparison of two methods for calculating G_{ST}, a genetic measure of population differentiation. Am J Bot 89(9):460–463, doi:10.3732/ajb.89.3.460

Daniel HM, Meyer W. 2003. Evaluation of ribosomal RNA and actin gene sequences for the identification of ascomycetous yeasts. Int J Food Microbiol 86:61–78, doi:10.1016/S0168-1605(03)00248-4

Evanno G, Regnaut S, Goude J. 2005. Detecting the number of clusters of individuals using the software Structure: a simulation study. Mol Ecol 14:2611–2620, doi:10.1111/j.1365-294X.2005.02553.x

Falush D, Stephens M, Pritchard JK. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587.

Goodwin SB, Dunkle LD, Zismann VL. 2001. Phylogenetic analyses of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91:648–658, doi:10.1094/PHYTO.2001.91.7.648

———, van der Lee TAJ, Cavaletto JR, te Lintel Hekkert B, Crane CF, Kema GHJ. 2007. Identification and genetic mapping of highly polymorphic microsatellite loci from an EST database of the Septoria tritici blotch pathogen *Mycosphaerella graminicola*. Fungal Genet Biol 44:398–414, doi:10.1016/j.fgb.2006.09.004

Halama P. 1996. The occurrence of *Mycosphaerella graminicola*, teleomorph of *Septoria tritici* in France. Plant Pathol 45:135–138, doi:10.1046/j.1365-3059.1996.d01-103.x

Halkett F, Simon JC, Balloux F. 2005. Tackling the population genetics of clonal and partially clonal organisms. Trends Ecol Evol 20(4):196–201.

Hunter T, Cohen RR, Royle DJ. 1999. The teleomorph stage, *Mycosphaerella graminicola*, in epidemics of Septoria tritici leaf blotch on winter wheat in UK. Plant Pathol 48:51–57, doi:10.1046/j.1365-3059.1999.00310.x

Jürgens T, Linde CC, McDonald BA. 2006. Genetic structure of *Mycosphaerella graminicola* populations from Iran, Argentina and Australia. Eur J Plant Pathol 115:223–233, doi:10.1007/s10658-006-9000-0

Kabbage M. 2007. Limited gene flow between natural populations of *Mycosphaerella graminicola* from single fields in Kansas and California. In: Amplified fragment polymorphism in *Mycosphaerella graminicola*. Manhattan: Kansas State Univ. p 23–47.

Kema GHJ, Annone JG. 1991. In vitro production of pycnidia by *Septoria tritici*. Nether J Plant Pathol 97:65–72, doi:10.1007/BF01974270

Linde CC, Zhan J, McDonald BA. 2002. Population structure of *Mycosphaerella graminicola*: from lesions to continents. Phytopathology 92:946–955, doi:10.1094/PHYTO.2002.92.9.946

Nei M. 1973. Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA 70:3321–3323, doi:10.1073/pnas.70.12.3321

———. 1978. Estimation of average heterozygosity and

genetic distance from a small number of individuals. Genetics 89:583–590.

Oste B, Huguerot G, Freydier M, Henaff GL, Gatellet J, Pillon O, Feurprier B, Vergnaud A. 2000. Bilan sanitaire de la campagne 1998/1999. Phytoma 523: 12–14.

Owen PG, Pei M, Karp A, Royle DJ, Edwards KJ. 1998. Isolation and characterization of microsatellite loci in the wheat pathogen *Mycosphaerella graminicola*. Mol Ecol 7:1611–1612, doi:10.1046/j.1365-294X.1998.00499.x

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Razavi M, Hughes GR. 2004a. Microsatellite markers provide evidence for sexual reproduction of *Mycosphaerella graminicola* in Saskatechewan. Genome 47: 789–794, doi:10.1139/g04-036

———, ———. 2004b. Molecular variability of *Mycosphaerella graminicola* as detected by RAPD markers. J Phytopathol 152:543–548, doi:10.1111/j.1439-0434.2004.00893.x

Sanderson FR. 1972. A *Mycosphaerella* species as the ascogenous state of *Septoria tritici* Rob. ex Desm. NZ J Bot 10:707–709.

Schneider F, Koch G, Jung C, Verreet JA. 2001. Genotypic diversity of the wheat leaf blotch pathogen *Mycosphaerella graminicola* (anamorph) *Septoria tritici* in Germany. Eur J Plant Pathol 107:285–290, doi:10.1023/A:1011256504146

Siah A, Tisserant B, El Chartouni L, Duyme F, Deweer C, Roisin-Fichter C, Sanssené J, Durand R, Reignault Ph, Halama P. 2010. Mating type idiomorphs from a French population of the wheat pathogen *Mycosphaerella graminicola*: widespread equal distribution and low but distinct level of molecular polymorphism. Fungal Biol (In press).

Slatkin M. 1987. Gene flow and the geographic structure of natural populations. Science 236:787–792, doi:10.1126/science.3576198

Stukenbrock EH, Banke S, McDonald BA. 2006. Global migration patterns in the fungal wheat pathogen *Phaeosphaeria nodorum*. Mol Ecol 25:2895–2904, doi:10.1111/j.1365-294X.2006.02986.x

Yeh FC, Yang R, Boyle T. 2000. Popgene 1.32. The user-friendly software for population genetic analysis. Molecular Biology and Biotechnology Center, Univ Alberta, and CIFOR, Canada. http://www.ualberta.ca/~fyeh/index.htm

Zhan J, McDonald BA. 2004. The interaction among evolutionary forces in the pathogenic fungus *Mycosphaerella graminicola*. Fungal Genet Biol 41:590–599, doi:10.1016/j.fgb.2004.01.006

———, Mundt CC, McDonald BA. 1998. Measuring immigration and sexual reproduction in field populations of *Mycospaherella graminicola*. Phytopathology 88: 1330–1337, doi:10.1094/PHYTO.1998.88.12.1330

———, Pettway RE, McDonald BA. 2003. The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination and gene flow. Fungal Genet Biol 38:286–297, doi:10.1016/S1087-1845(02)00538-8

Zhang XY, Loyce C, Meynard JM, Monod H. 2007. Modeling the effect of cultivar resistance on yield losses of winter wheat in natural multiple disease conditions. Eur J Agron 26:384–393, doi:10.1016/j.eja.2006.12.004