Genetic Variability and Distribution of Mating Type Alleles in Field Populations of *Leptosphaeria maculans* from France

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Leptosphaeria maculans is the most ubiquitous fungal pathogen of *Brassica* crops and causes the devastating stem canker disease of oilseed rape worldwide. We used minisatellite markers to determine the genetic structure of *L. maculans* in four field populations from France. Isolates were collected at three different spatial scales (leaf, 2-m² field plot, and field) enabling the evaluation of spatial distribution of the mating type alleles and of genetic variability within and among field populations. Within each field population, no gametic disequilibrium between the minisatellite loci was detected and the mating type alleles were present at equal frequencies. Both sexual and asexual reproduction occur in the field, but the genetic structure of these populations is consistent with annual cycles of randomly mating sexual reproduction. All *L. maculans* field populations had a high level of gene diversity ($H = 0.68$ to 0.75) and genotypic diversity. Within each field population, the number of genotypes often was very close to the number of isolates. Analysis of molecular variance indicated that >99.5% of the total genetic variability was distributed at a small spatial scale, i.e., within 2-m² field plots. Population differentiation among the four field populations was low ($G_{ST} < 0.02$), suggesting a high degree of gene exchange between these populations. The high gene flow evidenced here in French populations of *L. maculans* suggests a rapid countrywide diffusion of novel virulence alleles whenever novel resistance sources are used.

Stem canker of crucifers, also termed blackleg disease, is caused by *Leptosphaeria maculans* (Desm.) Ces. & de Not. (anamorph *Phoma lingam* Tode ex Fr.), a heterothallic haploid dothideomycete fungus. Stem canker of crucifers is the most damaging disease of oilseed *Brassica* crops, including oilseed rape (OSR; winter cultivars of *Brassica napus*) and canola (spring cultivars of *B. napus* and *B. rapa*) (39). This disease is widespread in Europe, Canada, and Australia and causes average OSR yield losses of 5 to 20% in France due to lodging of the crop (31).

The epidemiology of the stem canker disease has been extensively investigated (19, 39) since the first descriptions of the causal fungus by Tode in 1751 and Desmazières in 1849 (34). In Europe, OSR is grown as a winter crop and *L. maculans* produces pseudothecia in the autumn following sexual outcrosses on infected debris from the previous growing season. Airborne ascospores discharged from these pseudothecia infect leaves of the recently emerged crop, resulting in the development of leaf lesions (phoma leaf spots) that contain the asexual fruiting bodies, pycnidia. Asexual pycnidiospores can cause secondary infections, which are thought to be important in Australia but rare in Canada and Europe, where the disease is thought to be monocyclic (39). From the initial necrotrophic phase when leaf lesions occur, the fungus enters a symptomless biotrophic phase when, during winter and early spring, the fungus grows systemically in intercellular spaces down the petiole toward the crown at the stem base. During late spring and early summer in Europe, *L. maculans* again becomes necro-

trophic and causes necrosis of the crown tissues that may result in the plant lodging prior to harvest (20, 21).

Strategies for stem canker disease management include cultural practices such as crop rotation, isolation of the crops from infected stubble of the previous growing season crops, and stubble management. Some control is achieved through the use of fungicides but, at present, disease control relies mainly on the use of disease-resistant cultivars. There are two types of disease resistance in *B. napus*: polygenic quantitative resistance (29, 30) or specific resistance, which involves major resistance gene(s) (19, 39). Nine avirulence genes (*AvrLm* genes), namely, *AvrLm1* to *AvrLm9*, have been identified in *L. maculans* (2, 6–8), and the corresponding nine major resistance genes (*Rlm1* to *Rlm9*) have been described in *Brassica* sp. (13), as expected in a host-pathogen system with gene-for-gene interactions (17). The *Rlm* genes effectively control the disease as long as the corresponding avirulent allele (*AvrLm*) dominates the pathogen population (3, 9, 31, 35). However, large-scale cropping of cultivars with a single effective *Rlm* gene results in the rapid breakdown of major gene resistance within a few years after commercial release of the cultivars due to the adaptation of the *L. maculans* field populations. Such breakdown of resistance was documented in France for *Rlm1* (35) and in Australia for the breakdown of the “Surpass 400” resistance (23).

Our knowledge of genetic variation in *L. maculans* populations and the role of the evolutionary processes such as mutation, migration, genetic drift, selection, and/or recombination in these rapid shifts in the field population is still very limited. Previous studies of genetic variation in *L. maculans* populations from Canada and Australia found high levels of genetic variation in field populations of *L. maculans* (10, 11, 24), which is consistent with the hypothesis that sexual reproduction is an

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important part of the life cycle of *L. maculans*. However, reports of the distribution of genetic variation among and within field populations have been inconsistent (10, 11, 24). To date, there have been no studies of the genetic structure of *L. maculans* field populations in France and other countries of Western Europe. In these regions, previous work has focused mainly on field population race structure and has used virulence markers to assess genetic diversity in the fungus (5, 9, 22, 35).

Our objective here was to assess genetic variation in French populations of *L. maculans* with minisatellite loci (15), mating type, and virulence markers (8). We tested the hypotheses that the field populations were randomly mating, that populations from different fields were genetically separable, and that epidemics are initiated by ascospores. The present study provides the first estimates of genetic structure of field populations of *L. maculans* from France and suggests that genetic structure of populations from Western Europe is similar to that in Australia.

MATERIALS AND METHODS

Sampling of *L. maculans* field populations. A total of 401 isolates were obtained from pycnidiospores oozing from individual pycnidia of leaf lesions. The resulting cultures were subcultured as hyphal tips for further purification (38). All fungal cultures were maintained on V8 juice agar, and conidia were produced on V8 juice medium as previously described (2). Isolates of *L. maculans* were collected from four OSR fields in France as soon as the first leaf lesions were observed in Le Rheu (western France), Oucques (central France), and Grignon (near Paris) in autumn 2000 and in Versailles (near Paris) in autumn 2003. In each field, cultivar Drakkar, a cultivar that lacks the nine known resistance alleles (*Rlm* genes), was grown, and samples were taken from four arbitrarily selected plots (2 by 1 m). From each 2-m² plot, 25 naturally infected plants were arbitrarily selected, and one leaf with phoma leaf lesions was collected from each plant. One lesion per leaf was arbitrarily selected, and the causal organism was isolated. In addition, isolations were made from each of 50 leaf lesions occurring on one leaf from Oucques (autumn 2000) and from every lesion occurring on five leaves (43 total lesions) collected at Versailles in December 2003.

Pathogenicity tests. Races of the *L. maculans* isolates were determined by inoculating the 401 isolates onto a *B. napus* differential set, comprising fixed lines or commercial cultivars with as few *Rlm* alleles as possible (8). Cotyledons of the fully susceptible cv. Westar (no known *Rlm* allele), the cvs. Columbus (*Rlm1* and *Rlm3*), Bristol (*Rlm2* and *Rlm9*), 22-1-1 (*Rlm3*), Jet Neuf (*Rlm4*), 23-2-1 (*Rlm7*), Falcon-MX (*Rlm4* and *Rlm6*), and Samourai-MX (*Rlm1* and *Rlm6*) or Dar-mor-MX (*Rlm6*) were inoculated according to established protocols (2, 7). At 14 to 27 days after inoculation, symptoms were scored from 10 to 12 plants by using the IMASCO rating scale comprising six infection classes (IC1 to IC6), where IC1 to IC3 corresponded to avirulent isolates (*avrLm*) and IC4 to IC6 corresponded to virulent isolates (*avrLm*) (7).

Mating type determination. A multiplex PCR, developed to rapidly characterize the mating type of *L. maculans* isolates (12), was used to determine the distribution of the *Mat1-1* and *Mat1-2* alleles within the 401 field isolates collected. The distribution of mating type alleles was analyzed with a χ^2 test.

Minisatellite analyses. We evaluated six polymorphic minisatellite loci (*MinLm1* to *MinLm6*), all on different chromosomes in *L. maculans* (4, 15). New specific primers based on the published sequences (accession numbers AJ621802 and AJ621805, respectively) were designed for *MinLm3* and *MinLm5* by using Oligo v.5.0 software (Molecular Biology Insights, Inc., Cascade, Iowa). Primers *MinLm3*-ULG (5'-GGCTCGGTCGGTTAGTTA-3') and *MinLm3*-LLG (5'-AATGATGTACAGGACGGGATT-3') or primers *MinLm5*-ULG (5'-GCCGCCGCCGCTTACC-3') and *MinLm5*-LLG (5'-GAGCTCTGCGCCACAGTG-3') hybridized to the flanks of the minisatellite locus and allow amplification of the repeated motifs only. *MinLm2*, *MinLm4*, and *MinLm6* were amplified by using primers that had been previously described (15).

For PCR, genomic DNA was extracted from pycnidiospores by using the DNeasy 96 plant kit (QIAGEN S.A., Courtaboeuf, France) as previously described (4). PCR amplification was done in a total volume of 15 μ l containing 0.2 μ M of each deoxynucleotide triphosphate, 1.2 μ M of each primer, 0.6 U of *Taq* DNA polymerase (Qiagen, Illkirch, France), 1.5 μ l of a 10 $\times$ reaction buffer supplied with the enzyme, and 10 to 30 ng of genomic DNA. PCR amplifications

were done in an Eppendorf Mastercycler EP Gradient thermocycler (Eppendorf, Le Pecq, France), with 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 80 s, with a final extension at 72°C for 5 min. The cycling conditions were the same for the amplification of all minisatellite loci. The PCR products were separated by electrophoresis on a 2.5% SeaKem LE agarose gel (FMC, Rockland, Maine) and visualized by staining with ethidium bromide and UV illumination. Allele sizes were determined by using Quantity One 1-D Analysis software (Bio-Rad, Marne-la-Coquette, France) and comparing the bands for the alleles with those of the 1-kb ladder (Invitrogen, Cergy Pontoise, France) and with PCR products obtained after amplification of alleles of known size for each minisatellite. Each allele identified at the six minisatellite loci was previously sequenced from at least two isolates to determine the exact allele size and to confirm that the size polymorphism observed after electrophoresis was due to variability in the number of tandemly repeated core motifs (4, 15). Sequencing of the same allele amplified from at least three different individuals also confirmed that sequences of the same size are homologous and size homoplasy did not occur (4, 15). The data were scored as the number of repeat units for each minisatellite.

Statistical analysis. For statistical analyses, the 401 isolates were divided into a "leaf-scale group" comprising 50 isolates collected from one leaf in Oucques and 43 isolates collected from five leaves in Versailles and a "field-scale group" comprising 308 isolates. The field scale group was structured into four field populations, each of which was further subdivided into four subpopulations corresponding to four 2-m² plots.

Genetic diversity, which was studied separately for each field population and for all fields sampled, was estimated from genotype and gene diversity. Genotypes of isolates in both the field- and leaf-scale groups were determined by combining information from the alleles at minisatellite loci and at the mating type locus. Genotype diversity (*G*) was then estimated as the percentage of unique genotypes obtained in each sample unit, in each field population, and in the overall sample units. A discriminatory index (*DI*), based on Simpson's index of diversity, was used to estimate the discriminatory power of the six minisatellites. *DI* measures the probability that two individuals randomly selected from a small sample will have the same haplotype. *DI* is determined by the number and relative frequencies of the different haplotypes and is calculated as follows:

$$D = \frac{1}{N(N-1)} \sum_{j=1}^k n_j(n_j-1)$$

where *N* is the total number of isolates, *k* is the total number of haplotypes, and *n_j* is the number of isolates sharing the *j*th multilocus haplotype (14).

POPGENE version 1.31 software (40) was used to compute allele frequency, Nei's gene diversity (*h*) (27) for each population and Nei's measures of genetic identity (*I*) between populations over all loci (28). The mean gene diversity (*H*) was the mean of *h* over loci within each population.

Gametic linkage disequilibria were computed by using GENEPOP version 3.3 software (32) for each pair of loci within each of the four field populations and in the overall field samples. GENEPOP performs the Fisher exact test using a Markov chain to test for gametic disequilibrium between two loci. The null hypothesis *H*₀ was: "genotypes at one locus are independent from genotypes at the other locus." The significance of departure from linkage equilibrium was assessed by using Bonferroni adjusted *P* values (33). Linkage disequilibrium also was estimated by pairwise comparison of haplotypes using the index of multilocus gametic disequilibrium $\bar{r}_d$ (1), which tests to what extent individuals that are identical (or different) at one locus are more likely to be identical (or different) at the other loci. To determine whether an observation deviates significantly from the null hypothesis of random mating (no linkage disequilibrium among loci), the observed value was compared to the results of 500 randomized data sets. The program MULTILOCUS version 1.3 (1) was used for both estimation and randomization.

Heterogeneity of allele frequencies among field populations was investigated for each locus with contingency tables analysis and the Fisher exact test as implemented in the GENEPOP program. The null hypothesis tested was *H*₀: "allele frequencies are homogeneous across populations." Genetic structure was analyzed with Nei's coefficient of population differentiation (*G*_{ST}), which indicates the proportion of the total genetic variation attributable to population differentiation. Analyses of molecular variance (AMOVA) were computed with Arlequin version 2.000 software (37) and used to estimate how genetic variability is partitioned among fields, among plots within fields, and within plots. Φ statistics associated with these components of variance (Φ_{CT} , Φ_{SC} , and Φ_{ST} , respectively) were estimated and their significance tested by using a nonparametric permutation approach (16).

TABLE 1. Allelic frequencies and gene diversities at two avirulence loci of *Leptosphaeria maculans* in French field populations

Locus ^b	Allele frequency ^a in population at:				<i>P</i> ^c
	Grignon (<i>n</i> = 82)	Le Rheu (<i>n</i> = 81)	Oucques (<i>n</i> = 82)	Versailles (<i>n</i> = 63)	
<i>AvrLm1</i>	0.05	0.40	0.22	0.03	<0.05
<i>h</i>	0.09	0.48	0.34	0.06	
<i>AvrLm4</i>	0.10	0.07	0.11	0.06	0.77
<i>h</i>	0.18	0.14	0.20	0.12	
<i>H</i>	0.13	0.31	0.27	0.09	

^a Frequency of avirulence allele; *n*, number of isolates analyzed.^b *h*, gene diversity per locus; *H*, gene diversity averaged over both loci.^c Probability for the Fisher exact test for frequency heterogeneity across the populations.

RESULTS

Diversity of virulence. Six avirulence loci (*AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm6*, and *AvrLm7*) were characterized in four field-scale populations. All isolates were avirulent at the *AvrLm6* and *AvrLm7* loci and were virulent at the *AvrLm2* and *AvrLm3* loci. Isolates were polymorphic only at the *AvrLm1* and *AvrLm4* loci where virulent isolates (*avrLm1* and *avrLm4* isolates, respectively) were more frequent. The frequency of *avrLm1* varied significantly by population, ranging from 0.60 in the Le Rheu population to 0.97 in the Versailles population, whereas *avrLm4* frequencies were close to 0.90 at all locations (Table 1).

Minisatellite variation. Five of the six minisatellite loci tested in the present study were polymorphic at a 5% level in the populations analyzed (Table 2). The data from the *MinLm4* marker were not evaluated further because one of the three alleles at this locus was found in >95% of the isolates (Table 2). The remaining five minisatellite loci—*MinLm1*, *MinLm2*, *MinLm3*, *MinLm5*, and *MinLm6*—all had a high level of allelic variation in the four field populations studied with 7, 16, 12, 6, and 7 alleles, respectively (Table 3). Based on the discriminatory index *DI*, the probability that two isolates randomly selected from the 401 isolates would have the same combination of alleles over these five loci was 8.5×10^{-4} (Table 4).

Genetic diversity in field populations. Overall, 349 different multilocus genotypes were obtained with the mating type and five minisatellite loci among the 401 analyzed isolates (Table 4). The mean number of isolates per genotype was low (1.15) and 309 isolates (78%) had unique genotypes. A few putatively clonal genotypes were distributed within and among the four populations of *L. maculans*. These isolates are unlikely to be true clones since the addition of the two polymorphic virulence loci, *AvrLm1* and *AvrLm4*, enabled us to distinguish 12 additional genotypes among the 92 isolates with “clonal” genotypes (data not shown). The most common genotype identified with the mating type locus and the five minisatellite loci was shared by five isolates, of which four originated from the four field plots sampled in Le Rheu. One genotype, shared by three isolates, was found in three different populations, and 17 genotypes occurring twice were found in two populations. The genotype distribution exhibited no variation between the different geographic scales. The genotypic diversity reached its

TABLE 2. Frequencies of the most common minisatellite alleles, genic differentiation, and gene diversity^d for minisatellite loci across four field populations of *Leptosphaeria maculans*

Locus	Allele ^a	Allele frequency in population at:				<i>P</i> ^b
		Grignon	Le Rheu	Oucques	Versailles	
<i>MinLm1</i>	2×	0.20	0.25	0.24	0.16	<0.05
	5×	0.72	0.52	0.61	0.70	
	<i>h</i>	0.44	0.65	0.57	0.48	
<i>MinLm2</i>	6×	0.23	0.20	0.07	0.25	0.07
	7×	0.09	0.12	0.15	0.18	
	9×	0.21	0.21	0.20	0.16	
	<i>h</i>	0.86	0.86	0.88	0.86	
<i>MinLm3</i>	3×	0.56	0.35	0.40	0.57	0.12
	9×	0.13	0.21	0.16	0.10	
	15×	0.04	0.17	0.12	0.11	
	<i>h</i>	0.65	0.78	0.78	0.64	
<i>MinLm4</i>	3×	0.99	0.96	0.96	0.98	0.86
	<i>h</i>	ND ^c	ND	ND	ND	
<i>MinLm5</i>	4×	0.52	0.59	0.45	0.61	0.59
	5×	0.33	0.28	0.38	0.32	
	<i>h</i>	0.61	0.56	0.64	0.51	
<i>MinLm6</i>	4×	0.43	0.51	0.39	0.40	0.18
	5×	0.31	0.37	0.37	0.35	
	<i>h</i>	0.69	0.60	0.69	0.70	
	<i>H</i>	0.68	0.71	0.75	0.71	

^a 2×, 5×, etc., alleles are scored as the number of repeat units; *h*, gene diversity per locus; *H*, gene diversity averaged over all four loci (*MinLm2*, *MinLm3*, *MinLm5*, and *MinLm6*).^b Probability for the Fisher exact test for frequency heterogeneity across the populations.^c ND, not determined.^d Gene diversity was computed only for polymorphic loci at a 5% level.

maximum value within 2-m² field plots or at the leaf scale (Table 4). Even at the smallest spatial scale, i.e., the leaf scale, the number of genotypes often was very close to the number of individuals collected (Table 4). Only 2 of 50 isolates collected from the same leaf had the same genotype. These two isolates originated from lesions in close proximity to each other, and we think that they originate from a single strain that either had caused two separate lesions or was sampled twice.

The Grignon and Oucques populations contained the most

TABLE 3. Number of alleles at the six minisatellite loci in field populations of *Leptosphaeria maculans*

Locus	No. of alleles ^a			
	Grignon	Le Rheu	Oucques	Versailles
<i>MinLm1</i>	5	7 (1)	6	6
<i>MinLm2</i>	13 (1)	12	15 (1)	12
<i>MinLm3</i>	12 (2)	9	11 (3)	9
<i>MinLm4</i>	3*	3*	3*	3*
<i>MinLm5</i>	5	6	5	4
<i>MinLm6</i>	7	6	6	6
Total	45	43	46	40

^a The number of low-frequency alleles present in only one field population is indicated in parentheses. *, not polymorphic at a 5% level.

TABLE 4. Genotype diversity at three spatial scales in field populations of *Leptosphaeria maculans* in France

Scale group and geographic origin	Subpopulation	<i>n</i> ^c	No. of leaves	No. of genotypes ^a	<i>G</i> ^b
Field scale Grignon	P1	20	20	20	100
	P2	24	24	23	96
	P3	18	18	17	94
	P4	20	20	20	100
Le Rheu	P1	22	22	22	100
	P2	22	22	22	100
	P3	18	18	18	100
	P4	19	19	19	100
Oucques	P1	18	18	17	94
	P2	21	21	20	95
	P3	21	21	21	100
	P4	22	22	21	95
Versailles	M5	13	13	13	100
	M6	17	17	17	100
	M7	17	17	15	88
	M9	16	16	16	100
Leaf scale Oucques	FI	50	1	49	98
	Versailles	FI	8	1	100
Versailles	FII	10	1	10	100
	FIII	7	1	7	100
	FIV	10	1	10	100
	FV	8	1	8	100

^a Genotypes are based on multilocus haplotypes determined by combining information from the alleles at *MinLm1*, *MinLm2*, *MinLm3*, *MinLm5*, *MinLm6*, and *Mat1*.

^b Genotypic diversity is expressed as the percentage of unique genotypes.

^c *n*, number of isolates analyzed.

alleles over the six loci (Table 3). These two populations also had alleles at the *MinLm2* and *MinLm3* loci that occurred at low frequency and that were not present in the other field populations (Table 3). Two or three alleles at *MinLm2*, *MinLm3*, *MinLm5*, and *MinLm6* were more frequent and were present at similar frequencies in all of the analyzed populations (Table 2). *MinLm1* is physically close to the *AvrLm1* avirulence gene, and the allele frequencies at these two loci were significantly different across the four populations (Tables 1 and 2). *MinLm1* was excluded from analysis of gene diversity and distribution of genetic diversity because this locus was in linkage disequilibrium with *AvrLm1* (4, 18). Nei's measures of gene diversity averaged over the four loci *MinLm2*, *MinLm3*, *MinLm5*, and *MinLm6* were similar among the four field populations and ranged from 0.68 in the Grignon population to 0.75 in the Oucques population (Table 2).

Mating type distribution and linkage disequilibria in field populations. PCR products corresponding to one of the two mating type idiomorphs could be amplified from all 401 isolates. Both mating types were present in the populations at all of the tested spatial scales, including different lesions on the same leaf, different plots within a field, and different fields within the country. No significant departures from the 1:1 ratio between the two mating types were observed at the field scale

TABLE 5. Distribution of the mating type alleles of *Leptosphaeria maculans* in field populations in France

Scale group and geographic origin	Subpopulation(s)	<i>n</i> ^b	No. of alleles for mating type ^a :	
			<i>Mat1-1</i>	<i>Mat1-2</i>
Field scale Grignon	P1-P4	82	49	33
	Le Rheu	81	34	47
	Oucques	82	48	34
	Versailles	63	34	29
Leaf scale Oucques	FI	50	22	28
	Versailles	FI	8	6
Versailles	FII	10	5	5
	FIII	7	4	3
	FIV	10	4	6
	FV	8	1	7
	FI-FV	43	16	27

^a Whenever sample sizes were large enough to perform a χ^2 test, mating type frequencies were not significantly different at $P = 0.05$.

^b *n*, number of isolates analyzed.

(Table 5). Linkage disequilibria were assessed with *MinLm1*, *MinLm2*, *MinLm3*, *MinLm5*, and *MinLm6* and using two different approaches. Within field populations, only two pairwise tests of linkage disequilibria were significant in the Le Rheu and Versailles populations, but none of them were significant after Bonferroni's corrections for multiple comparisons were made. When the complete field-scale data set was analyzed as a single group, none of the pairwise comparisons were significant. The multilocus linkage disequilibrium values ($\bar{r}_d$) obtained for the four field populations were close to zero and not significant ($P > 0.05$). No significant deviation from random association of alleles was detected within the total population ($\bar{r}_d < 0.01$, $P = 0.40$), suggesting that recombination regularly generates new genotypes at the field level. Linkage disequilibria between the minisatellite loci and the polymorphic avirulence loci (*AvrLm1* and *AvrLm4*) also were assessed in field populations. Overall, only one pairwise test of linkage disequilibria, which corresponded to the *MinLm1* and *AvrLm1* loci, was significant after Bonferroni's corrections. These two loci were in significant linkage disequilibria only within the Le Rheu and Oucques populations.

Distribution of genetic diversity within and among populations. Distribution of genetic diversity was estimated using

TABLE 6. Pairwise comparisons of population differentiation (G_{ST}) and Nei's unbiased measure of gene identity (I) in *Leptosphaeria maculans* field populations from France

Population	G_{ST} (I) values for population:		
	Le Rheu	Oucques	Versailles
Grignon	0.011 (0.95)	0.008 (0.96)	0.004 (0.98)
Le Rheu		0.010 (0.95)	0.011 (0.95)
Oucques			0.011 (0.95)

TABLE 7. Analysis of molecular variance of the multilocus haplotypes for four *Leptosphaeria maculans* populations isolated from OSR fields in France

Source of variation	df	Variance components	% of variation	P^a	Φ statistics
Among field populations	3	0.00053	0.11	0.062	$\Phi_{CT} = 0.001$
Among field plots, but within field populations	12	0.00165	0.33	0.017	$\Phi_{SC} = 0.003$
Within field plots	292	0.49864	99.56	0.001	$\Phi_{ST} = 0.004$

^a Probability of having values larger than those observed based on 1,000 randomizations of the treatments.

MinLm2, *MinLm3*, *MinLm5*, and *MinLm6*. Pairwise estimates of Nei's coefficient of population differentiation (G_{ST}) were relatively low and ranged from 0.004 to 0.011 (Table 6). These estimates are consistent with the hypothesis that there is no genetic differentiation between these French field populations. Similar results were obtained from the Fisher exact test for heterogeneity of allele frequencies among field populations (Table 2) and from an AMOVA performed with the four field populations as a whole ($\Phi_{ST} = 0.0007$, $P = 0.14$; data not shown). The results of the AMOVA, which partitions the genetic variation hierarchically from field plot level to an among-field level, revealed that most of the allelic variability (>99.5%) was found in the 2-m² field plots and that the genetic variation among field populations accounted for only 0.11% of the total variability (Table 7). Nei's measure of genetic identity was high for each pairwise comparison of field populations (Table 6). The highest estimate of genetic identity was obtained from a pairwise comparison of the Versailles and Grignon populations, which also were the geographically closest locations (Table 6).

DISCUSSION

The four minisatellite loci examined had a large number of alleles at each locus, which enabled a high level of discrimination between field isolates. Most of the isolates could be distinguished by using this set of minisatellite loci, and adding one or two more polymorphic minisatellite loci (currently unavailable) to this set probably would differentiate all of the isolates except those that are true clones. The most common genotype among the 401 isolates was shared by only five isolates and corresponds to the combination of the most frequent alleles at each locus. Furthermore, these five isolates were polymorphic at the virulence loci and correspond to at least two genotypes (data not shown), suggesting that some of these isolates are genetically distinct individuals and that they do not represent a widely distributed clone.

The 1:1 distribution of mating type alleles we observed also has been observed in field populations of *L. maculans* in Australia (10) and is expected in randomly mating populations (26). Milgroom (26) proposed several tests for random mating in natural populations of fungal pathogens. Among these, the frequent occurrence of fungal sexual structures, a high level of genotypic diversity, and gametic linkage equilibrium, all provide evidence that is consistent with sexual reproduction. In *L. maculans*, the frequent occurrence of pseudothecia has

been documented in many instances (19, 39). The present study further supports the importance of sexual reproduction by showing that minisatellite loci were in linkage equilibrium and that genotypic diversity was very high even at small spatial scales in field populations of *L. maculans* in France.

Linkage disequilibrium was detected between the *MinLm1* and *AvrLm1* loci, which are physically close and genetically linked. The genetic distance between these loci ranges from 0 to >30 cM depending on the *L. maculans* genetic map considered (L. Gout, unpublished data). The suppression of recombination observed in some genetic maps may be related to non-homologous sequences present in this region in the avirulent and virulent parental isolates used (L. Gout, unpublished data). The nonhomologous genome organization of the *AvrLm1* region in avirulent and virulent isolates has been conserved in field populations from Western Europe based on the lack of apparent recombination between markers spanning this region (5). In the Le Rheu and Oucques populations, the presence of avirulent and virulent isolates could have reduced recombination near *AvrLm1* and have led to the linkage disequilibrium between *MinLm1* and *AvrLm1* via "hitchhiking" to *AvrLm1*, which would be selected for when cultivars carrying *Rlm1* were planted. *MinLm1* and *AvrLm1* were in linkage equilibrium in the Grignon and Versailles populations, but >95% of the isolates in these field populations were virulent. The lack of disequilibrium in this genomic region in these populations could result because the recombination rate in the region is not the same when populations are mainly virulent as it is when both avirulent and virulent isolates coexist in the population.

L. maculans has a mixed reproductive system (39). Ascospores are thought to be the major source of inoculum for the annual epidemics. Our results are consistent with this hypothesis and similar to those of studies of field populations from Canada (24) or Australia (10). Pycnidia are produced throughout the growing season, but the role of the asexual pycnidiospores in the epidemiology of the disease remains unclear, and secondary infections via pycnidiospores are thought to be rare in Western Europe (39). Our collections were mainly made at a time corresponding to the initiation of epidemics, and the variation we observed is consistent with the hypothesis that ascospores are the primary inoculum. Additional samples collected throughout the growing season are needed to determine the role of asexual spores in the epidemiology of *L. maculans* in France.

Most of the total genetic variability in these populations of *L. maculans* (>99.5%) was distributed at a small spatial scale, i.e., within 2-m² field plots. These populations also had a very high level of gene ($0.68 \leq H \leq 0.75$) and genotypic ($88\% \leq G \leq 100\%$) diversity even over small spatial scales. The four field populations, despite being collected in two different years and from sites more than 500 km apart, may be coevolving parts of a large panmictic population. Ascospores are airborne, can potentially be dispersed over distances of several kilometers, can survive up to 6 weeks (19, 39), and could enable this genetic homogenization.

In contrast to the minisatellite loci, the level of diversity at the virulence loci was low, with only two of the six characterized avirulence loci (*AvrLm1* and *AvrLm4*) being polymorphic. Polymorphism was similar across the four field populations for *AvrLm4* but not for *AvrLm1*. The two corresponding resistance

genes, *Rlm1* and *Rlm4*, have been widely deployed in France, with cultivars carrying *Rlm4* in use since the 1970s (36) and cultivars carrying *Rlm1* in use since the early 1990s (36). Thus, subpopulations of *L. maculans* may be differentiated based on selectable markers, e.g., the *AvrLm1* locus, as a result of spatial heterogeneity in the use of *Rlm1* cultivars (35). This genic differentiation of subpopulations may be transitory, and the genetic exchange occurring in French populations of *L. maculans* should homogenize the frequencies of these virulence alleles. Increased use of cultivars carrying *Rlm* gene(s) across all OSR growing regions could give the same result, but this scenario is less likely since the commercial success of cultivars with a given *Rlm* gene should be inversely proportional to the frequency of the corresponding virulence allele in the pathogen populations. Indeed, the frequencies of the *avrLm4* virulence allele are very similar across all of the field populations we studied, and all of the isolates characterized in the present study carried the *avrLm2* virulence allele. The corresponding resistance gene, *Rlm2*, was used in France prior to *Rlm4* and is still present in many cultivars (36).

A low level of differentiation between field populations of *L. maculans* also was observed in Australia, where the high level of genetic variability revealed by amplified fragment length polymorphism analyses was distributed mainly within field populations (10). In contrast, AMOVA done on RAPD [random(ly) amplified polymorphic DNA] data attributed only 55% of the total variability to differences within field populations in Canada. Simultaneous analysis of field populations from these continents together with some European field populations is now needed (i) to evaluate the genetic structure of worldwide populations, (ii) to identify potential subpopulations, and (iii) to estimate the level of intercontinental genetic exchange. The amount of intercontinental genetic exchange is of particular interest since gene flow, due to natural long distance dispersal of propagules or linked to human activities, may lead to genetic exchange between populations located on different continents. Thus, the durability of a resistance gene on one continent could be altered by the durability of the same resistance gene on another. Resistance gene durability is important because only a few *Rlm* genes are known (13) and maintaining the effectiveness of each resistance gene is critical for the sustainable control of stem canker. The minisatellite markers we used in the present study were useful in determining the amount of genetic variability within and between field populations in France and may be useful for a global population study.

Our study is the first to evaluate the genetic variability of *L. maculans* field populations in France. Based on the level of gametic linkage equilibria observed and the frequency-dependent selection of mating type alleles, we showed that sexual recombination is an important part of this fungus's life cycle and occurs regularly in *L. maculans* field populations in France. Indeed, we showed that ascospores could be the primary source of inoculum and could be responsible for the leaf-spotting phase of the disease in both autumn and winter. High levels of gene flow also were inferred from the low levels of population differentiation. McDonald and Linde (25) recently proposed a framework to predict the evolutionary potential of pathogen populations based on analysis of their genetic structure. According to their model, pathogens such as *L.*

maculans with high gene flow and mixed reproduction system have moderate to high evolutionary potential. However, the recent breakdown of the *Rlm1* resistance occurred in a time frame (only 3 years) that is similar to that predicted for pathogens with high evolutionary potential, which pose the greatest "risk" of breaking down novel specific resistance genes. Fungal pathogens such as rusts or mildews assigned to this highest risk category (25) often have mixed reproductive systems and high levels of migration (genotype flow) through long-distance dispersal. Thus, the rapid breakdown of *Rlm1* now questions the level of migration among *L. maculans* field populations.

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