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Absence of isolation by distance patterns at the regional scale in the fungal plant pathogen *Leptosphaeria maculans*

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Abstract

Outcomes of host-pathogen coevolution are influenced by migration rates of the interacting species. Reduced gene flow with increasing spatial distance between populations leads to spatial genetic structure, as predicted by the isolation by distance (IBD) model. In wind-dispersed plant-pathogenic fungi, a significant spatial genetic structure is theoretically expected if local spore dispersal is more frequent than long-distance dispersal, but this remains to be documented by empirical data. For 29 populations of the oilseed rape fungus *Leptosphaeria maculans* sampled from two French regions, genetic structure was determined using eight minisatellite markers. Gene diversity (H = 0.62–0.70) and haplotypic richness (R = 0.96–1) were high in all populations. No linkage disequilibrium was detected between loci, suggesting the prevalence of panmictic sexual reproduction. Analysis of molecular variance showed that >97 % of genetic diversity was observed within populations. Genetic differentiation was low among populations (F_{st} < 0.05). Although direct methods previously revealed short-distance dispersal for *L. maculans*, our findings of no correlation between genetic and geographic distances among populations illustrate that the IBD model does not account for dispersal of the fungus at the spatial scale we examined. These results indicate high gene flow among French populations of *L. maculans*, suggesting high dispersal rates and/or large effective population sizes, two characteristics giving the pathogen high evolutionary potential against the deployment of resistant oilseed rape cultivars.

Keywords: Gene flow, Indirect measures, Minisatellites, Spatial genetic structure, Spore dispersal

Highlights

- We investigate the spatial genetic structure of *Leptosphaeria maculans* in France.
- We test the relevance of the isolation by distance model to describe spore dispersal.
- We found no correlation between genetic and geographic distances among populations.
- Results indicate high gene flow among populations.
- High dispersal rates and large effective population sizes characterize *L. maculans*.

Introduction

Dispersal has an important influence on patterns of local adaptation of plant pathogens to their host (Kaltz & Shykoff 1998), especially in agricultural ecosystems where migration of virulent pathogen genotypes is favoured compared to immobile, cultivated resistant host genotypes. These unbalanced migration rates between host plants and pathogens eventually lead to a reduced efficacy of plant resistance (Gandon *et al.* 1996). Durable disease control, therefore, requires the deployment of resistant varieties in a spatial arrangement that limits disease transmission (Aubertot *et al.* 2006). The spatial scale of relevance for such an arrangement should match the scale of gene flow between pathogen populations. For wind-dispersed, plant-pathogenic fungi, spore dispersal usually occurs both at long distances over hundreds of kilometres (low-frequency, atmospheric dispersal) and at short distances within a few hundred metres (high-frequency, local dispersal) (Aylor & Irwin 1999). Locally, the number of deposited spores decreases with increasing distance from the inoculum source (Gregory 1973). Spore trapping experiments, however, do not always yield consistent estimates at increasing distances from the inoculum source, thereby underestimating rare events of long-distance dispersal (McCartney *et al.* 2006).

When direct measures fail, the scale of spore dispersal can be inferred from indirect genetic measures. If local dispersal is predominant, the resulting pattern of neutral genetic differentiation between populations is expected to follow the 'isolation by distance' (IBD) model (Wright, 1943, Slatkin, 1987; Rousset 1997). Assuming that neutral genetic structure is primarily due to genetic drift and is counterbalanced by gene dispersal (in a two-dimensional environment), and that it reflects the dispersal capacities of the studied species, a linear correlation is expected between the logarithm of the geographical distance and

estimated between pairs of populations (F_{st} being the index of genetic differentiation among populations; Wright 1943). The detection of an IBD pattern requires a precise framework, assuming mutation–drift equilibrium, and considering spatial distances ranging between σ and 20σ, where σ^2 is the second moment of parent-offspring distance dispersal (Rousset 1997). Estimations through this method have been shown to be robust to the mutational process of microsatellite loci (*i.e.* allele size homoplasy), and to spatial and temporal heterogeneities of demographic parameters (*i.e.* variation in dispersal and density) (Leblois *et al.* 2003, 2004). Furthermore, analysis conducted at a local scale allows reducing the influence of selection pressure on genetic differentiation (Rousset 2001). For several tropical tree species, indirect gene dispersal estimates were consistent with direct estimates of seed and pollen dispersal (Hardy *et al.* 2006).

Among wind-dispersed fungi, IBD patterns have been tested at inter-continental scales, at which the spatial genetic structure could either reflect past, stochastic, and rare long-distance colonisation events (Linde *et al.* 2002; Rivas *et al.* 2004; Zaffarano *et al.* 2006), or the presence of physical barriers to gene flow (mountains, seas, and deserts) (Hayden *et al.* 2007). At broad spatial scales, the effects of selection, mutation, and demographic variation on genetic differentiation are not negligible; thus, translating measures of genetic differentiation such as F_{st} and related indices into a quantitative evaluation of spore dispersal when transformed as a direct measure of the product of effective population size and number of successful migrants per generation, could often lead to misinterpretation (see for example Whitlock & McCauley 1999). One way to test for the relevance of the IBD model to plant-pathogenic fungi when σ is not known is to compare spatial scales. For instance, in populations of the poplar rust fungus *Melampsora larici-populina*, Barrès *et al.* (2008) detected IBD patterns within Europe, but population genetic structures from Iceland and Canada were shown to result from rare long-distance dispersal events.

Leptosphaeria maculans (Desm.) Ces. & de Not. (anamorph *Phoma* lingam Tode ex Fr.), is a heterothallic, haploid fungus causing Phoma stem canker, a disease responsible of severe yield losses of *Brassica*, especially oilseed rape (*Brassicanapus*) world-wide (Fitt *et al.* 2006). In French oilseed rape crops, the life-cycle of the fungus matches the host presence, which is from Sep. to Jun. A single, sexual reproduction cycle per year occurs during summer on infected stubble (Fitt *et al.* 2006). While the resulting ascospores were once thought to travel on distances up to 10 km (McGee 1977), direct measures recently suggested that most ascospores were indeed concentrated within a few hundred metres from the previous year's crop (Salam et al., 2001, Marcroft et al., 2004). From previous indirect measures, contradictory conclusions were drawn on the dispersal abilities of *L. maculans*. At the inter-continental scale, Amplified Fragment Length Polymorphism (AFLP) analysis differentiated Australian, North American, and European *L. maculans* populations (Purwantara *et al.* 2000). In Australia, AFLP and Restriction Fragment Length Polymorphism markers did not reveal a genetic east–west differentiation among populations (Barrins *et al.* 2004), a differentiation which was subsequently revealed by microsatellite and minisatellite markers (Hayden *et al.* 2007), and explained as a result of the separation of these two regions by an arid desert. In Canada, Random Amplified Polymorphic DNA markers differentiated two populations sampled from 20 km distant fields (Mahuku *et al.* 1997). In contrast, a study conducted in France, including four minisatellite markers used in the present study, showed low genetic differentiation among *L. maculans* populations (Gout *et al.* 2006). Findings of Gout *et al.* (2006) are based on only four populations with a scatter distribution across France. This sampling was not designed to infer spore dispersal parameters through indirect genetic measures, but rather allowed the examination of levels and distribution of within-population genetic diversity. The low measures of genetic differentiation reported by Gout *et al.* (2006), who estimated that >99.5 % of genetic diversity measured at four minisatellite markers was present within two square metres field plots, do not allow the assessment of the minor variation in genetic differentiation within a local spatial scale, which could possibly reflect the evolution of *L. maculans* populations under an IBD model.

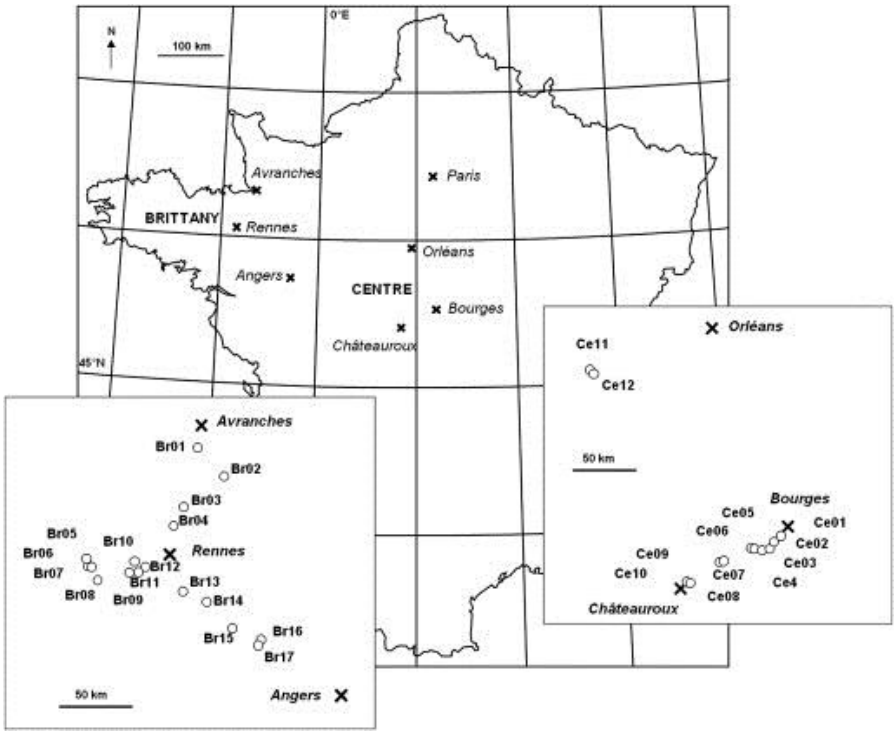
The aim of the present study was to test the relevance of the IBD model to a windborne plant-pathogenic fungus. Indeed, it remains to be proven that IBD patterns can be detected in agricultural ecosystems characterized by pathogen populations with a high multiplication rate, a large population size alternating with recurrent yearly bottlenecks, or with a recent range expansion. Based on *a priori* knowledge of the organism's dispersal abilities, the genetic structure of *L. maculans* was investigated at a spatial scale where gene flow was expected to occur. Direct measures of ascospore dispersal distances suggest that *L. maculans* populations separated by geographic distances ranging from several hundred metres to 10 km evolve under IBD (Salam et al., 2001, Marcroft et al., 2004). We developed four new minisatellite markers and genotyped 693 isolates at eight loci. We sampled 29 field populations in two regions of France, covering distances ranging from a few hundred metres to one hundred kilometres in each region. Our objective was to test for the presence of IBD pattern in the genetic structure of *L. maculans* populations. Also, we tested whether the detection of IBD pattern was not erased by a recent range expansion of the pathogen. To this aim, we compared pathogen genetic structure in Region Brittany, where the cultivation of *B. napus* – and thus the presence of its *L. maculans* pathogen – is less than 25-y-old, with that of Region Centre, where oilseed rape has been intensively cultivated for more than 60 y.

Materials and methods

Sample collection and DNA extraction

In autumn 2004 and 2005, 693 isolates were collected from 29 commercial oilseed rape fields in two regions [western France (Region Brittany, $n = 17$) and central France (Region Centre, $n = 12$) (Fig 1)]. In 2007, 66 405 ha of oilseed rape were grown in the Department Cher (Region Centre), compared to only 12 950 ha in the Department Ille-et-Vilaine (Region Brittany) (Prolea 2008). Pair-wise distances between field populations ranged from 0.44 km to 364 km (from 0.44 km to 99.7 km and from 1.32 km to 114.5 km in Region Brittany and in Region Centre, respectively). In each field, one infected leaf displaying typical phoma leaf spots was collected from each of 23–25 plants. Monospore mycelial cultures were increased on V8 juice agar at 20 °C for 21 d (Chèvre *et al.* 2008), then scraped with a sterile scalpel. DNA was extracted from lyophilized mycelia following the standard protocol supplied in the Nucleospin® Plant II kit (Macherey-Nagel, Düren, Germany).

Fig 1. *Map of France showing the location of the 29 French field populations sampled (○) in Regions Brittany and Centre. The main towns in the sampling areas are marked with a × on the map.*



Minisatellite identification

A systematic search approach described by Eckert *et al.* (2005) was undertaken in order to identify minisatellite loci in *Leptosphaeria* maculans. BAC-end sequence data, from the v23.1.3 genomic DNA *Hind*III BAC library (Attard *et al.* 2002), were screened for regions with tandem repeat using TANDEM REPEAT FINDER (Benson 1999). Primers were designed using Oligo6 (MedProbe, Norway). Genomic DNA of 56 isolates was extracted, comprising 50 *L. maculans* 'brassicae', one *L. maculans* 'lepidii' (IBCN84), as well as one *Leptosphaeria biglobosa* 'brassicae' (IBCN93), 'thlaspii' (IBCN65),'canadensis' (IBCN82), 'australensis' (IBCN91), and 'erysimii' (IBCN83). These isolates were obtained from existing collections maintained at INRA Versailles, and previously described (Gall *et al.* 1994; Balesdent et al., 2001, Mendes-Pereira et al., 2003). In order to analyse the genetic control of minisatellite segregation, progeny from *in vitro* cross a.2 × H5 (Balesdent *et al.* 2001) was used. TANDEM REPEAT FINDER highlighted 203 out of 2550 BAC-end sequences analysed as sequences with putative tandem repeats, for which primer pairs specific to flanking regions of tandem repeats were designed. These primer pairs were first tested on genomic DNA from a set of 7 *L. maculans* 'brassicae' isolates originating from diverse geographical locations. This first screen resulted in 23 primer pairs generating polymorphic products (2–4 variants of alleles). Then, these 23 primer pairs were tested on 47 *L. maculans* isolates of diverse geographical origin. Moreover, 12 of them displayed polymorphism between a.2 and H5 parental isolates. Meiotic segregation of minisatellite markers confirmed that these 12 new markers were single-locus sequences. Among these 12 markers, four were chosen according both to their polymorphism, and to their specificity to *L. maculans*.

Minisatellite amplification

Multilocus genotypes were characterized with minisatellite loci *MinLm2*, *MinLm4*, *MinLm5*, *MinLm6* (Eckert *et al.* 2005), and *MinLm8*, *MinLm9*, *MinLm632*, *MinLm1377* for which primers were designed (Table 1). Forward primers were fluorescently-labelled. PCR reactions were performed in a 20-µl final volume containing 10–30 ng of genomic DNA, 1× PCR buffer, 200 µM of each dNTPs, 1.5 mM $MgCl_2$, each primer at 0.5 µM, 0.25 U GoTaq® DNA Polymerase (Promega, WI, USA). Amplifications were carried out in a G-STORM GS4 thermocycler (GRI Ltd., Braintree, UK) using one cycle of 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, annealing temperature for 30 s (Table 1), 72 °C for 1 min, and 5 min of final extension at 72 °C. Allele assignments were performed on an ABI PRISM® 3130xl sequencer using manufacturer's instructions with the GENEMAPPER 3.7 software (Applied Biosystems).

Table 1. *Minisatellite markers and PCR primers used to characterise molecular diversity in populations of* Leptosphaeria maculans *sampled in two regions of France.*

Minisatellite	Accession number[b]	Minisatellite consensus (5′–3′)	Consensus length (bp)	Primer name	Primer sequence (5′-3′)	Annealing temperature (°C)
MinLm2[a]	AJ294758	GAGACATGGGAAATAGAGGAGGAA	24	*MinLm2F* *MinLm2R*	AAGAAGGTGGGTGGATTGGTAAG GCCCTCTTTTCCTCGTTGC	60
MinLm4[a]	AJ621803	ACAACTTCAATCCGCCTGCTGATTATCCCTATACATATCAGACCAAGCAAG	51	*MinLm4F* *MinLm4R*	ACCAGGTGGAGTTGATAACAT TCCTGCGAATCCCATTAG	53
MinLm5[a]	AJ621804	ACCGCCCAGCCACTCA	16	*MinLm5-ULG* *MinLm5-LLG*	GCCGCCCGCCGCCTTACC GAGCTCCTGCGCCACAGTG	60
MinLm6[a]	AJ621805	GGCAGGGCTGGCCATGGGGA	21	*MinLm6F* *MinLm6R*	GGAAGGAACACACGGTGAC AATTGAATGATTTGCGACACA	53
MinLm8	HQ637176	TGTATATACAGTACAAAGACACACACA	27	*MinLm8F* *MinLm8R*	ATTTGCTGGCGGTGTAGGTA TGTTTGTACATGTGGTAAGTAAAGCA	53
MinLm9	HQ604861	GTGTGTGTGTGCGTGTGT	18	*MinLm9F* *MinLm9R*	GCATTTGTTGGCTGGCATTGGA TGCGTTGGCGTGTGACATGACT	60
MinLm632	HQ604859	CTCTGTCTTTCTCTTTCTCTCTCTCTGT	28	*MinLm632F* *MinLm632R*	TCAGACGCTGCTGGCCTGTGTG AGTGAGCCGACGAGACGCGAGAG	64
MinLm1377	HQ604860	TTGTCGGATGTCCCCTGTCCTGTCTTGTGCTGTGTC	36	*MinLm1377F* *MinLm1377R*	CGTCCAGTCGTGCCTGCCTTTG GGTGCATGTCTTGGCGGACCATT	64

[a] Eckert *et al.* 2005.
[b] GenBank database accession number.

Data analysis

Multilocus haplotypes were constructed for each isolate. All isolates collected within the same field were considered as one population. We determined the number of identical multilocus haplotypes within and across populations, using GENCLONE 2.0 (Arnaud-Haond & Belkhir 2007). The same program was used to estimate the probability that identical multilocus haplotypes were the result of a distinct sexual reproductive event (P_{sex}), rather than clonal spread, based on the number of loci and allelic frequencies. For each locus within each population, mean number of alleles (N_{all}), Nei's gene diversity corrected for sample size (h) (Nei 1987) and allele frequencies were estimated using SPAGEDI 1.2 (Hardy & Vekemans 2002). At a given locus, Nei's gene diversity (h) is defined as the probability that two sampled alleles are different. Mean gene diversity (H) was calculated as the mean of h across loci within each population. In each population, haplotypic richness (R) was estimated as $R = (G - 1)/ (N - 1)$, where G is the number of unique haplotypes and N is the number of isolates (Dorken & Eckert 2001).

For each pair of loci within each population, gametic linkage disequilibria were computed using GENEPOP 4.0 (Raymond & Rousset 1995). The index of multilocus linkage disequilibrium r_d (Agapow & Burt 2001) was computed for each population. The significance of r_d, which is expected to equal zero if there is no linkage disequilibrium, was tested by comparing the observed variance with the distribution of the variance expected under the null hypothesis of random mating, as determined from 1000 randomizations of the haplotype data implemented in MULTILOCUS 1.3 (Agapow & Burt 2001).

For each locus and across all populations, Nei's estimator of genetic differentiation (G_{ST}) was calculated with FSTAT 2.9.3 (Goudet 1995). G_{ST} indicates the proportion of the total genetic variation attributable to population differentiation. Values close to zero indicate little differentiation while values close to unity indicate nearly complete differentiation. The null hypothesis of no genetic differentiation between field populations was also tested using FSTAT to estimate θ (Weir & Cockerham 1984), an unbiased estimator of the parameter F_{st}. Significance levels were determined after Bonferroni corrections based on the adjusted P-value following 8000 permutations. Genetic differentiation was estimated with a hierarchical analysis of molecular variance (AMOVA) using ARLEQUIN 3.1 (Excoffier *et al.* 2005). AMOVA was used to determine the proportion of genetic variation partitioned among the two regions, among fields within a region, or among individuals within a field. The number of permutations to test for significance was set at 5000.

IBD between populations was tested using the method described by Rousset (1997), under which a correlation is expected between the logarithm of the geographical distance and the genetic distance $\theta/ (1-\theta)$, assuming gene flow in a two-dimensional environment. Data analysis involves the regression of $F_{ST} / (1-F_{ST})$ estimates for each pair of subpopulations. Pair-wise geographical distances among field populations were calculated without correcting for the Earth's curvature because of the limited spatial distance among fields. Significance was tested using a Mantel test (1000 permutations) implemented in GENEPOP.

A test to detect evidence of recent demographic fluctuations was performed with BOTTLENECK (Piry *et al.* 1999). For each population and for each locus, we estimated gene diversity as expected under the assumption of mutation–drift equilibrium (expected gene diversity), from the number of alleles and sample size, and compared it to gene diversity calculated directly from the allele frequencies (H_{obs}, observed gene diversity). In a population that has undergone a bottleneck, gene diversity is higher than expected at equilibrium, due to a high-frequency of rare alleles (Cornuet & Luikart 1996). Gene diversity was estimated under three mutation models: the infinite allele model (IAM), the stepwise mutation model (SMM), and the two-phase model (TPM) (Rienzo *et al.* 1994). In the IAM, each mutation creates a novel allele at constant rate. In the SMM, each mutation creates a novel allele by adding or deleting a single repeated unit of the minisatellite. TPM is an offshoot of SMM developed to account for a proportion of larger mutation events (addition or deletion of several units). We tested these three mutation models because patterns of mutation for minisatellites are not perfectly known and can vary from one locus to another. The proportion of alleles attributed to SMM under TPM was 70 %, with a variance of 30 (default parameters). Estimations were based on 1000 replications. The Wilcoxon sign-rank test (Luikart *et al.* 1997) was performed to determine if the allele frequency distribution deviates significantly from that expected under mutation-drift equilibrium.

Results

Genetic diversity within populations

Across all 29 populations, the number of alleles at each locus ranged from 3 (*MinLm4*) to 21 (*MinLm632*), and only one locus (*MinLm4*) presented one allele occurring at a frequency greater than 95 %. Average gene diversity across populations ranged from 0.05 (*MinLm4*) to 0.87 (*MinLm2*) (Table 2). Mean number of alleles per locus (N_{all}) was 5.88 (±0.45, standard deviation), ranging from 4.88 to 7 in the Ce11 (Region Centre) and Br14 (Region Brittany) field populations, respectively. Mean gene diversity *H* across all loci was high (0.66 ± 0.02) and homogeneous (Table 3). Among all 693 isolates analysed, 687 multilocus haplotypes were obtained. Isolates sharing identical multilocus haplotypes were recovered from the same population (Br06, Ce08, and Br16) and likely resulted from clonal spread, as the probability that these repeated multilocus haplotypes originated from distinct sexual reproduction events was low ($P_{sex} < 0.005$). In contrast, isolates sharing identical multilocus haplotypes were identified from different populations in the same region (Br16-Br06, Br04-Br13) or from different populations in different regions (Ce10-Br15) and likely resulted from distinct sexual reproduction events ($P_{sex} > 0.096$). Numbers of haplotypes equalled numbers of isolates in 26 field populations (Table 3), and haplotypic richness (*R*) was high in all field populations (0.96–1).

Table 2. *Frequency of the most common (frequency greater than 0.05) minisatellite alleles, gene diversity (*h*), number of alleles at the eight minisatellite loci, and Nei's estimator of genetic differentiation (G_{ST}) per locus across the 29 field populations of* Leptosphaeria maculans.

Minisatellite	Allele[a]	Allele frequency	*h* (gene diversity)	Number of alleles	G_{ST}[b]
MinLm2	6X	0.23	0.87	20	0.001
	7X	0.15			
	8X	0.07			
	9X	0.19			
MinLm4	3X	0.97	0.05	3	−0.008
MinLm5	7X	0.55	0.58	6	−0.01
	8X	0.34			
MinLm6	3X	0.11	0.65	8	0.009
	4X	0.44			
	5X	0.37			
	6X	0.06			
MinLm8	5X	0.13	0.77	14	0.008
	6X	0.26			
	7X	0.36			
	8X	0.14			
MinLm9	3X	0.14	0.85	17	−0.001
	5X	0.13			
	6X	0.16			
	7X	0.05			
	9X	0.26			
	10X	0.07			
	11X	0.11			
MinLm632	4X	0.15	0.77	21	0.008
	7X	0.41			
	8X	0.10			
	18X	0.15			
MinLm1377	4X	0.11	0.84	13	0.003
	5X	0.20			
	6X	0.28			
	7X	0.14			
	8X	0.08			

[a] Alleles are scored as the number of repeat units.
[b] Nei's estimator of genetic differentiation.

Table 3. *Field population code, number of analysed isolates (*N*), number of observed haplotypes (*G*), haplotypic richness (*R*), mean number of alleles over the eight loci (*N_{all}*), mean gene diversity over the eight loci (*H*) and index of multilocus linkage disequilibrium (*r_d*).*

Population[a]	N	G	R	N_{all}	H	r_d
Br01	24	24	1	6.38	0.69	−0.019
Br02	23	23	1	5.75	0.66	−0.037
Br03	24	24	1	5.88	0.64	0.008
Br04	24	24	1	5.88	0.64	−0.004
Br05	24	24	1	5.63	0.65	−0.004
Br06	24	23	0.96	6.00	0.66	0.041
Br07	24	24	1	6.25	0.70	−0.019
Br08	23	23	1	5.25	0.65	−0.018
Br09	23	23	1	5.75	0.66	0.009
Br10	24	24	1	5.88	0.65	−0.002
Br11	25	25	1	5.88	0.66	−0.011
Br12	24	24	1	5.00	0.62	0.003
Br13	24	24	1	6.00	0.65	<0.001
Br14	24	24	1	7.00	0.67	0.001
Br15	24	24	1	5.88	0.66	−0.014
Br16	24	23	0.96	5.63	0.65	0.028
Br17	24	24	1	5.50	0.67	0.008
Ce01	24	24	1	6.13	0.69	0.013
Ce02	23	23	1	6.63	0.68	−0.015
Ce03	24	24	1	5.63	0.64	0.016
Ce04	24	24	1	6.63	0.67	0.038
Ce05	24	24	1	6.00	0.64	−0.020
Ce06	24	24	1	6.13	0.69	−0.008
Ce07	24	24	1	6.13	0.66	0.018
Ce08	24	23	0.96	5.63	0.62	0.010
Ce09	24	24	1	5.50	0.64	−0.008
Ce10	24	24	1	5.88	0.65	−0.009
Ce11	24	24	1	4.88	0.62	0.031
Ce12	24	24	1	5.75	0.65	0.004

[a] Field population from region Brittany (Br01–Br17) and from region Centre (Ce01–Ce12), geographically localized in Fig 1.

Linkage disequilibrium

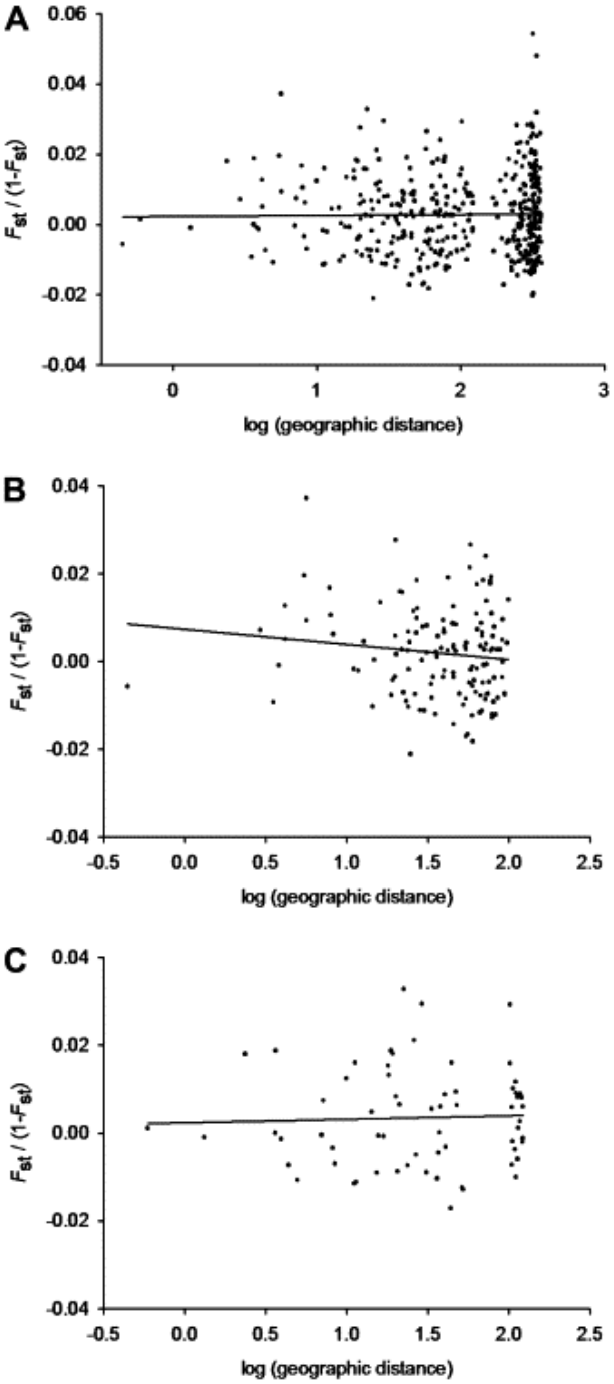
Seventeen populations showed random association of all alleles at all loci. Linkage disequilibrium among alleles was not significant in 801 of 812 pair-wise comparisons (98.6 %). The index of multilocus linkage disequilibrium r_d was low ($|r_d| < 0.041$) and not significant, and the null hypothesis of random mating was not rejected for all field populations (Table 3). When the complete data set was analysed as a single population, none of the pair-wise comparisons showed evidence of significant linkage disequilibrium.

Genetic diversity among populations

Hierarchical AMOVA revealed that ca. 97 % of the genetic variability was distributed within populations, compared to only 2.56 % among populations within a region, and 0.06 % among regions. None of these three factors contributed significantly to total molecular variance ($P > 0.05$). Similarly, none of the pair-wise comparisons revealed significant population differentiation among populations ($\theta = -0.02$ to 0.05; $P > 0.05$), with an average F_{st} value of 0.003 (±0.004). F_{st} maximal value (0.05) was reached for the comparison between field populations Ce08 and Br10, two field populations from the two distinct regions sampled, separated by one of the farthest distance in the overall sampling (318 km; Fig 1). Nonetheless, there was no significant correlation between logarithmic geographic distance and genetic distance F_{ST} / (1-F_{ST}), neither among all populations, nor within regions. Among all populations, the maximum distance between two populations was 364.2 km; the linear regression between logarithmic geographic distance and pair-wise genetic distance was $y = 1.13 \times 10^{-4}x + 0.0015$ ($r^2 = 1.7 \times 10^{-4}$; $P = 0.35$) (Fig 2A). Among populations from Region Brittany, the maximum distance between two populations was 99.6 km; the linear regression between logarithmic

geographic distance and pairwise F_{ST} / (1-F_{ST}) was $y = -1.5 \times 10^{-3}x + 0.018$ ($r^2 = 0.016$; $P = 0.88$) (Fig 2B). Among populations from Region Centre, the maximum distance between two populations was 122.2 km; the regression is $y = 3.4 \times 10^{-4}x + 2.53 \times 10^{-5}$ ($r^2 = 1.5 \times 10^{-3}$; $P = 0.38$) (Fig 2C).

Fig 2. *Genetic differentiation among Leptosphaeria maculans populations. Multilocus estimates (θ) of genetic differentiation* F_{st} *expressed as* F_{ST} */ (1-*F_{ST}*) are plotted against logarithm of geographic distance (km) for (A) each pair of* L. maculans *field populations (maximum distance between two populations is 364.2 km; the regression is* $y = 1.13 \times 10^{-4}x + 0.0015$; $r^2 = 1.7 \times 10^{-4}$; $P = 0.35$*), (B) each pair of* L. maculans *field populations from region Brittany (maximum distance between two populations is 99.6 km; the regression is* $y = -1.5 \times 10^{-3}x + 0.018$; $r^2 = 0.016$; $P = 0.88$*), and (C) each pair of* L. maculans *field populations from region Centre (maximum distance between two populations is 122.2 km; the regression is* $y = 3.4 \times 10^{-4}x + 2.53 \times 10^{-5}$; $r^2 = 1.5 \times 10^{-3}$; $P = 0.38$*).* F_{st} *was estimated according to Weir & Cockerham (1984).*



Effective population size fluctuations

Comparisons between observed and expected gene diversity revealed significant gene diversity excess for 20 of 29 populations, under TPM (Wilcoxon sign-rank test, $P < 0.05$; Table 4) and IAM ($P < 0.05$). Conversely, under SMM, none of the populations displayed significant gene diversity excess ($P > 0.05$). For the three models, no differences were found between regions.

Table 4. *Probability for an excess in observed gene diversity (H_{obs}) compared to that expected under mutation-drift equilibrium, in each field population, tested with Wilcoxon sign-rank test, under three models: the IAM, the TPM, with 70 % of alleles attributed to SMM and the SMM.* P-*values after 1000 simulation replicates are presented.*

Population	Wilcoxon sign-rank test		
	Probability for H_{obs} excess (P-values)		
	IAM	TPM	SMM
Br01	0.014	0.098	0.629
Br02	0.010	0.037	0.371
Br03	0.037	0.230	0.844
Br04	0.010	0.156	0.320
Br05	0.004	0.008	0.234
Br06	0.004	0.012	0.406
Br07	0.004	0.014	0.156
Br08	0.010	0.098	0.527
Br09	0.010	0.037	0.422
Br10	0.004	0.004	0.289
Br11	0.010	0.098	0.629
Br12	0.004	0.004	0.148
Br13	0.020	0.037	0.770
Br14	0.004	0.020	0.594
Br15	0.010	0.037	0.578
Br16	0.004	0.008	0.148
Br17	0.006	0.014	0.191
Ce01	0.004	0.027	0.098
Ce02	0.014	0.273	0.680
Ce03	0.008	0.039	0.406
Ce04	0.014	0.098	0.770
Ce05	0.004	0.039	0.594
Ce06	0.014	0.098	0.527
Ce07	0.010	0.027	0.473
Ce08	0.039	0.234	0.711
Ce09	0.004	0.020	0.406
Ce10	0.004	0.020	0.766
Ce11	0.004	0.008	0.289
Ce12	0.004	0.008	0.148

Discussion

In characterizing the genetic structure of *Leptosphaeria maculans* populations in France, we intended to demonstrate that, in the case of this wind-dispersed phytopathogenic fungus, the scale of spore dispersal could be obtained from the theoretical migration model IBD. Despite the fact that our sampling scheme was based on *a priori* knowledge of spore dispersal, however, we did not detect a significant IBD pattern among populations. We will discuss how deviations from model assumptions occurring in populations, including departure from genetic equilibrium or gene flow higher than previously assumed, are likely to explain the inapplicability of the IBD model at the spatial scale studied.

The detection of IBD pattern strongly depends on the spatial scale studied, as the studied area should match the spatial scale at which gene dispersal occurs (Rousset 1997) and sampling at larger distances leads to an overestimation of demographic parameters (Leblois *et al.* 2003). Our sampling scheme was designed assuming that migration was due to ascospore dispersal over a few hundred metres, distances estimated from

spore (Salam *et al.* 2001) and disease (Marcroft *et al.* 2004) gradients. As both methods are known to be inaccurate at large distances, *i.e.* over several kilometres (McCartney *et al.* 2006), it is possible that our sampling scale was too small, and that we underestimated ascospore dispersal. Our findings suggest that ascospore dispersal occurs at distances greater than several hundred metres. Undetected by direct methods, widespread spore dispersal at continental scale by atmospheric turbulence could occur for this fungus. Such long-distance dispersal events are known to be the main cause of the world-wide spread of plant diseases (Brown & Hovmøller 2002). But assuming that these events have a low-frequency compared to local dispersal, based on reduced fungal spore viability under atmospheric conditions (Rotem *et al.* 1985), an IBD pattern should be detectable at the studied regional spatial scales.

Alternatively, it is possible that human transport of infected seeds is responsible for long-distance dispersal of *L. maculans* (Hall *et al.* 1996). Long-distance dispersal *via* human transport and, thus, high migration rates that reduce genetic differentiation between populations (Slatkin 1987), were suggested to explain the world-wide spread of other plant pathogens, such as the banana black leaf streak fungus *Mycosphaerella fijiensis* (Rivas *et al.* 2004), the wheat leaf blotch fungus *Mycosphaerella graminicola* (Linde *et al.* 2002), and the poplar rust fungus *Melampsora larici-populina* (Barrès *et al.* 2008). Nonetheless, long distance human-mediated transport of infected plant material would have to be regular and intense to erase population differentiation, which is locally driven by genetic drift in the absence of selection. Further investigations of infected seed material are necessary to elucidate its effect on the spread of *L. maculans*.

The absence of demographic equilibrium in the studied populations may have prevented the detection of IBD patterns, though empirical data showed that demographic estimates through the method used were robust to population density changes that occur within just a few generations (Leblois *et al.* 2004). In Region Brittany, oilseed rape acreage increased from 0 ha in 1983 to ca. 50 000 ha in 2007 (Agreste 2008), yet a matching increase in effective population sizes was not detected in this region. As the test implemented in the BOTTLENECK software only allows the detection of recent size fluctuations, pathogen spread in Brittany, which possibly dates back from about 25 generations ago assuming one sexual cycle per season, may be too ancient for detection. In Region Centre, where oilseed rape has been intensively grown for more than 60 y, we might expect that coevolving *L. maculans* populations have reached demographic equilibrium. Nonetheless, annual, recurrent bottlenecks could hinder the detection of IBD patterns. For annual crop pathogens, this decrease in effective population size is expected when host plant material available for pathogen development dramatically decreases between growing seasons (Burdon 1993). However, the high levels of within-population genetic diversity revealed in this study, concordant with previous studies (Mahuku et al., 1997, Barrins et al., 2004, Gout et al., 2006, Hayden et al., 2007), do not support recent or recurrent bottlenecks. Furthermore, with SMM, considered the most conservative mutation model in the test conducted (*i.e.* the null hypothesis of no population size fluctuation is rejected only when a strong bottleneck signal is detected; Cornuet & Luikart 1996), none of the populations showed significant recent changes in size. Multiple sampling dates and years are necessary to characterize the genetic diversity of *L. maculans* populations and to further examine their local and temporal demography.

The absence of both bottleneck signatures and significant spatial genetic structure among French populations of *L. maculans* suggest that these populations do not undergo strong loss of genetic diversity through genetic drift during the saprophytic stage on oilseed rape stubble. Instead, high proportion of genetic diversity distributed within-population (>97 %) suggests that the examined populations have large effective population sizes. Large effective population sizes reduce genetic differentiation among populations (Criscione *et al.* 2005) and hence limit the chance to detect IBD patterns. The substantial quantities of stubble that is not buried, but is instead left on the soil surface (Lydia Bousset, personal observation), can explain the maintenance of large effective population size in *L. maculans*.

The absence of linkage disequilibrium and the uniform distribution of mating types in all populations (data not shown) confirm the significance of sexual reproduction in the life-cycle of *L. maculans*, and are consistent with previous research identifying ascospores, and not conidia (*i.e.* spores resulting from asexual multiplication), as the main source of inoculum to initiate epidemics (Fitt *et al.* 2006). Nonetheless, three repeated haplotypes were detected in the same field and were unlikely to originate from distinct sexual reproduction events. These findings provide indirect evidence for the spread of isolates through splash-dispersal of conidia in field conditions, hence supporting the involvement of conidia in the epidemiology of

phoma stem canker on oilseed rape (Travadon *et al.* 2007). In contrast, similar associations of alleles in repeated haplotypes detected in distant fields were likely the result of random mating. Overall, genetic diversity indices estimated in all field populations are in agreement with the hypothesis that, in France, populations of *L. maculans* operate as one panmictic population.

As discussed in this study, the absence of IBD patterns in a wind-dispersed, plant-pathogenic fungus may not be only due to dispersal of spores or infected plants across large geographic distances. Instead, a focus on the inter-generation evolution of local genetic diversity may reveal the contribution of large effective population sizes and the absence of demographic equilibrium as factors that mask IBD patterns. Buffering the effects of genetic drift, the effective population size has a large influence on overall levels of genetic diversity (Criscione *et al.* 2005). For plant-pathogenic fungi not experiencing strong effects of genetic drift during their life-cycle, either because of constant host availability (*i.e.* perennial hosts), or because of their ability to survive saprophytically between host growing seasons, or due to their ability to infect multiple hosts, and thus to survive independently of one single host persistence, high levels of within-population genetic diversity and low levels of among-population differentiation are expected. In these cases the IBD model does not seem appropriate to infer dispersal distances.

Large effective population sizes, high dispersal rates and random mating confer a high evolutionary potential to *L. maculans* (McDonald & Linde 2002), and such characteristics are consistent with its rapid adaptation to major resistance genes (Sprague *et al.* 2006). Since effective population sizes affect local adaptive potential to evolve in response to host resistance, it is necessary to reduce effective population size in *L. maculans* populations. Accordingly, management practices between growing seasons, especially burial of the oilseed rape stubble from which the pathogen fruits, is recommended (Aubertot *et al.* 2006). Disease control strategies aiming at either the prevention of virulent spore dispersal from resistant crops to new resistant crops through spatial cultivar deployment, or at the reduction of sexual reproduction *via* stubble management, are essential to preserve the efficacy of the few available specific resistance genes against *L. maculans* in *B. napus*.

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