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Genetic Variability in *Puccinia striiformis* f. sp. *tritici* Populations Sampled on a Local Scale during Natural Epidemics

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This study investigated genetic polymorphism on a local scale in *Puccinia striiformis* f. sp. *tritici* populations during natural epidemics, in four fields located in northern France and sampled in 1998 or 1999. Two hundred and forty-seven isolates were analyzed for their amplified fragment length polymorphism (AFLP) pattern through four primer combinations, and 194 of them were also tested for their virulence factors. Only one or two pathotypes were found in each field, and all isolates had virulence v17, matching the recently introduced *Yr17* resistance gene. Polymorphism on a field scale was low. Although 67 loci were polymorphic, 77% of the isolates had the same AFLP pattern, all other patterns being rare or unique. Analyses of the genetic distance between AFLP patterns based on the Jaccard index allowed us to define 12 groups, but a bootstrap analysis showed that all isolates could be assigned to a single clonal lineage. This leads us to conclude that *P. striiformis* f. sp. *tritici* populations are clonal on a field scale in northern France.

Puccinia striiformis Westend. f. sp. *tritici* is a fungal pathogen responsible for stripe rust, one of the most widespread wheat diseases in the world, which causes yield losses of up to 40% (9, 28). This biotrophic fungus reproduces asexually with no known alternate host (32) and spreads by means of airborne dicaryotic uredospores. In France, natural epidemics occur usually in the northwest and occasionally in the south and are especially damaging when springs are cool and rainy (6, 7, 38). In most cases, the use of specific resistance genes as a disease control method has been unsuccessful because the pathogen population evolves rapidly and is able to overcome a newly introduced resistance gene within only a few years (1, 32, 36). Other methods have been investigated, such as pathotype-nonspecific resistance or cultivar mixtures (37), but more detailed data about the genetic structure of natural populations are needed to understand and predict their efficacy. Our capacity to develop durable and efficient control methods against crop diseases is largely based on the knowledge of the pathogen population structure and its potential for adaptation to new cultivars (3, 4, 8, 15, 22). So far, European stripe rust populations have been studied on a very large scale (10, 11, 13), with the objective to investigate long-distance migration and population exchanges between countries (United Kingdom and Denmark, and, to a lesser extent, France). A more complete understanding of the genetic structure of those pathogen populations necessitates work on different geographical and spatial scales, from the largest to the smallest (field or plant scale, or even a single lesion) (2, 23). In epidemics, stripe rust spreads easily from field to field, but the strongest disease intensification occurs on the field scale, when the pathogen develops on homogeneous host populations. On this spatial scale, the competition among pathogen genotypes for available host tissue is intense and largely determines the population shifts between the beginning and the end of the epidemics (34, 39). The within-field genetic structure of pathogen populations then has major consequences on the effectiveness of disease control methods. It has been shown for instance (14) that the efficacy of host resistance management strategies like cultivar mixtures or multilines is largely influenced by the local diversity of the pathogen.

When molecular polymorphism is found in stripe rust, it generally occurs at a low level and in most cases shows no relationship with pathotypes. Newton et al. (25) investigated genetic variation among several pathotypes of *P. striiformis* f. sp. *tritici* with cytoplasmic (double-stranded RNA) and nuclear (isozyme) markers. All isolates of *P. striiformis* examined gave identical phenotypes for double-stranded RNA and for 19 isozyme markers, irrespective of their race or geographical origin. Chen et al. (5) studied a sample of 115 isolates from the United States and identified six pathotypes and five random amplified polymorphic DNA (RAPD) groups, with a low correlation coefficient found between pathotypes and RAPD groups. Although five RAPD groups were identified, the genetic diversity level appeared low. Other results are contradictory. Shan et al. (29) found a relatively high polymorphism using restriction fragment length polymorphism (RFLP) probes; studying stripe rust populations on a regional scale, they distinguished 97 RFLP types out of 160 isolates from China, screening 26 putative genetic loci.

Amplified fragment length polymorphism (AFLP) analysis has proved suitable to study populations with a low genetic

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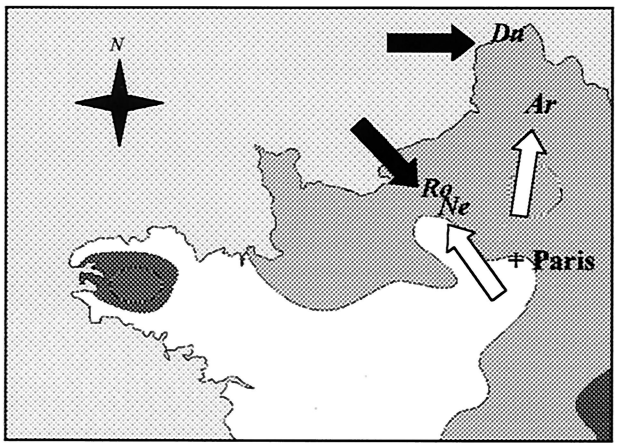

FIG. 1. Location of fields in northwestern France, where natural populations of *P. striiformis* f. sp. *tritici* were sampled in 1998 (fields Ro and Du [black arrows]) and 1999 (fields Ne and Ar [white arrows]). North is indicated in the upper-left corner. Three areas are shaded with respect to mean summer rainfall: 120 to 160 mm (white), 160 to 200 mm (light grey), and 160 to 240 mm (grey).

diversity (26, 35) because it provides a large number of reproducible markers. In addition, it is difficult to obtain large amounts of *P. striiformis* f. sp. *tritici* spores, and DNA amplification necessitates very small amounts of biological material. This method was first used for plants and bacteria (17), and significant results have now been obtained for various fungi (21, 33). In Western Europe, cooperative efforts have been undertaken in order to characterize *P. striiformis* f. sp. *tritici* with AFLP markers. Hovmøller et al. (10), using AFLP markers, showed that stripe rust develops clonal populations in Northern Europe, with a capacity for long-distance migration. In 1997 and 1998 in Denmark, Justesen et al. (13) identified two pathotypes and three AFLP phenotypes. The two most frequent AFLP phenotypes often occurred within the same field. Naestoft et al. (24) described latent period and lesion growth for nine *P. striiformis* f. sp. *tritici* isolates representing four AFLP groups, which were previously defined by Hovmøller and Justesen.

The objectives of the present study were to characterize genetic polymorphism on a field scale for natural populations, in order to estimate the genetic diversity of local populations during an epidemic season. Two hundred and forty-seven isolates were multiplied and tested with four AFLP primer combinations, and their AFLP patterns were described with 67 loci. The distribution of AFLP patterns within fields was analyzed at the beginning and at the end of the epidemics. An unweighted pair-group method using arithmetic averages (UPGMA) analysis of AFLP patterns was used to draw conclusions about genetic diversity on a field scale. Virulence patterns were also determined.

MATERIALS AND METHODS

Sampling. Uredospores were sampled in wheat fields during natural epidemics in the northwest of France (Fig. 1). In 1998, two 0.5-ha fields located near Rouen (field Ro) and Dunkerque (field Du) and in 1999, two others of 0.25 ha, located near Le Neubourg (field Ne) and Arras (field Ar), were sown with the wheat cultivar Victo, which has no identified resistance gene to *P. striiformis* f. sp. *tritici* pathotypes but is resistant to leaf rust (*Puccinia triticina*) and powdery mildew (*Blumeria graminis* f. sp. *tritici*). Fields Ro and Ne were located in the west, and fields Du and Ar were in the north. In 1998 and in 1999, fields were separated by 170 to 220 km. No fungicide was applied to the fields.

In 1998, spores were sampled once in May, from the fields Ro and Du, at the end of epidemics. Ten sampling points about 5 m apart were defined along a transect, and 20 sporulating leaves were collected at each point. In 1999, spores were sampled twice, at the beginning and at the end of epidemics. At the beginning, several foci of about 1 m^2 in which plants were severely diseased were observed. Ten sampling points were defined along two parallel transects in the field, and, in addition, five (field Ne) or six (field Ar) foci were also sampled. At two of these foci (Ne_3 and Ar_3), 11 isolates were sampled. At the other foci, one to three isolates only were sampled. At the end of epidemics, the whole fields were heavily diseased, and 15 to 20 sporulating leaves were collected in each field.

For transportation, sporulating leaves were kept in plastic bags at 4°C. In the laboratory, leaves were taped on plates, sporulating side up, and placed in a plastic bag with 100% humidity at 8°C. After 12 h, spores were collected, desiccated, and stored in liquid nitrogen.

Isolation and multiplication of the fungus. Spores were taken out of liquid nitrogen, heat shocked (40°C, 5 min), and used to inoculate 7-day-old seedlings of cultivar Victo. Twelve seedlings were sown in 7-cm-wide square pots, in a row along one of the pot edges, and protected from airborne contamination in isolated compartments. When seedlings were 1-cm high, growth was reduced by adding 15 ml of maleic hydrazide acid (0.25 $g \cdot liter^{-1}$) per pot. When 7 days old, 5 to 12 pots of seedlings were placed horizontally in a settling tower, 50 cm wide and 1 m high. In order to obtain very low lesion density on the leaves, *P. striiformis* f. sp. *tritici* spores were diluted 25 times in lycopodium spores, and 5 mg of the mixture was projected in the settling tower. The inoculated seedlings were placed in a dew chamber at 8°C for 16 h and then in a climate chamber (day: 17 h at 15°C; night: 7 h at 10°C) in individual booths. After 10 days, latent lesions could be observed for each successful infection. One leaf per pot, bearing a single lesion, was then chosen, and the remaining leaves were removed. Pots were then replaced in individual booths within the climate chamber. After 7 days, isolated lesions were sporulating, and spores were collected in 2.2-ml microtubes before multiplication. For each isolate, 20 cultivar Victo seedlings were grown in a 7-cm-wide plastic square pot, protected from airborne spores and treated with 20 ml of maleic hydrazide acid (0.25 $g \cdot liter^{-1}$) when 1 cm high. Collected spores were mixed with talc and applied manually along each leaf with a cotton swab. Inoculated plants were then incubated in a dew chamber at 8°C for 16 h and then in a climate chamber (day: 16 h, 350 $\mu E \cdot m^{-2} \cdot s^{-1}$, 17°C; night: 8 h, 14°C). During the whole multiplication procedure, plants were kept separated from each other in individual booths. Eighteen days after inoculation, spores were collected, dried in a desiccator at 4°C for 3 days, and stored in microtubes at −80°C. For each isolate, two independent multiplication procedures were carried out, from which collected spores were stored apart.

For the whole experiment, 247 individual specimens were isolated and multiplied (Table 1). For fields Ro and Du, 29 isolates were obtained per field. For fields Ne and Ar, at least three isolates were multiplied for each of the 10 sampling points at the beginning of epidemics, and at least 30 isolates were also multiplied at the end of epidemics. For the same fields, 1 to 11 isolates were multiplied per focus. Ninety-seven and 92 isolates, respectively, were obtained from fields Ne and Ar.

Virulence tests. Virulence spectra of the isolates were determined using the European and world sets of 15 differential cultivars (12) and three additional cultivars with specific resistance genes: Clement (*Yr9*), VPM1 (*Yr17*), and Kalyansona (*Yr2*) (6, 7, 18). This differential set made it possible to detect the virulences matching resistance genes *Yr1*, *Yr2*, *Yr3*, *Yr4*, *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr10*, *Yr17*, SD, SP, CV, and SU. Twelve milligrams of uredospores of each isolate was mixed with talc (spore/talc ratio, 1:3 by weight) and sprayed at the two-leaf-stage onto six seedlings of each variety. After inoculation, seedlings were placed in a dark climatic room for 24 h at 8°C and 100% relative humidity and then transferred into a greenhouse at 20°C for 10 days. Daylight was supplemented to 16 h with high-pressure sodium vapor lamps. Plants were scored individually on a scale from 1 to 9, 15 and 17 days after inoculation, based on the presence of necrosis and chlorosis and the intensity of sporulation (19). Virulence reactions were defined as infection types 7 to 9. Pathotypes were named according to binary codes generated from the infection types (virulent or avirulent) on the differential cultivars (first code from the world subset, and second code, preceded by the letter E, from the European subset).

DNA extraction. DNA was extracted from uredospores using a cetyltrimethylammonium bromide protocol with one chloroform step. For each isolate, 15 mg of spores was placed in a 2.2-ml microtube with seven autoclaved 3-mm-wide glass beads and 100 μl of extraction buffer (0.1 M Tris-HCl [pH 8], 140 mM

TABLE 1. *P. striiformis* f. sp. *tritici* isolates sampled in four fields relative to the number and type of sampling points per field and to the epidemic state when sampling was done

Yr	Field	Epidemic state	Sampling point		Isolates		Pathotype[b]	AFLP pattern(s)
			Type[a]	No.	No./point	Total no.		
1998	Ro	End	Field	10	1–6	29	169E136V17	0
							233E137V17	0
1998	Du	End	Field	10	1–5	29	233E137V17	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10
1999	Ne	Beginning	Focus Ne_3	1	11	11	233E127V17	0, 20, 21, 22
			Foci	4	1–3	10	233E137V17	0, 11, 16, 18, 19, 23, 24
			Field	10	3–5	45	233E137V17	0, 11, 12, 13, 14, 15, 16, 17
		End	Field	2	14–17	31	233E137V17	0, 12, 14, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34
1999	Ar	Beginning	Focus Ar_3	1	11	11	169E136V17	0
							233E137V17	0, 35, 36
			Foci	5	1–3	8	169E136V17	0
							233E137V17	0, 18
			Field	10	3–5	40	169E136V17	0, 18
							233E137V17	0, 18, 28, 31, 35
		End	Field	1	33	33	233E137V17	0, 18, 35, 37, 38, 39

[a] For fields Ne and Ar, at the beginning of the epidemic, foci were sampled separately.
[b] Pathotypes are named according to a double binary code generated from infection types on two subsets of differential varieties.

D-sorbitol, 30 mM *N*-lauroyl-sarcosine, 0.8% cetyltrimethylammonium bromide, 0.8 M NaCl, 20 mM EDTA, 0.1% polyvinylpyrrolidone), and vortexed for 2 min 45 s. At this step, grinding efficiency was assessed under a microscope. Four hundred microliters of extraction buffer was then added with 5 μl of proteinase K (10 mg · ml^{-1}), and tubes were incubated 1 h 30 min at 65°C. The mixture was then extracted with 500 μl of chloroform, gently inverted for 5 min, and centrifuged for 20 min at 17,000 × *g* at 4°C. The supernatant was transferred to a new tube, 500 μl of cold isopropanol was added, and the mix was gently inverted. After 40 min of incubation at −20°C, the solution was centrifuged for 20 min at 17,000 × *g* at 4°C. The pellet was washed with 150 μl of 70% ethanol, dried in a vacuum, and dissolved in 40 μl of TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA). The solution was then treated with 1 μl of RNase at 10 mg · ml^{-1} at 37°C for 1 h. The DNA was quantified on agarose gel. An average of 25 ng · μl^{-1} was obtained, i.e., 1.0 μg from 15 mg of uredospores.

AFLP protocol, electrophoresis, and gel staining. Genomic DNA samples (100 ng) were digested simultaneously with *Eco*RI and *Mse*I (5 U each; Life Technologies) at 37°C in 40 μl of RL buffer (final concentration: 10 mM Tris-H acetate [pH 7.5], 10 mM Mg acetate, 50 mM K acetate, 5 mM dithiothreitol, bovine serum albumin [50 ng · μl^{-1}]). After 1 h, 10 μl of RL buffer was added with 5 pmol of *Eco*RI adapter, 50 pmol of *Mse*I adapter, 1 pmol of ATP, and 1 U of ligase (Euromedex), and the whole mix (50 μl) was incubated 3 h more at 37°C. The resulting DNA solution was then diluted four times with T 0.1E buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) and stored at 4°C.

The first amplifications, with no selective nucleotides, were carried out in volumes of 20 μl of PCR buffer (10 mM Tris at pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM concentrations of each deoxynucleoside triphosphate) containing 30 ng of each primer (Table 2), 5 μl of diluted DNA, and 0.4 U of *Taq* DNA polymerase (Life Technologies). This preamplification was carried out in a thermal cycler (Hybaid) programmed for 30 cycles at 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min. The amplification products were then diluted 50 times in T 0.1E buffer. The selective amplifications were performed using four selected combinations of primers with two selective nucleotides (Table 2) (*Eco*RI+AC-*Mse*I+AC, *Eco*RI+GC-*Mse*I+AA, *Eco*RI+A-*Mse*I+AG, *Eco*RI+GT-*Mse*I+GT). They were carried out in volumes of 20 μl of PCR buffer containing 25 ng of *Eco*RI + 2 primer, 30 ng of *Mse*I + 2 primer, 5 μl of diluted preamplified DNA, and 0.4 U of *Taq* DNA polymerase (Life Technologies). The program ran for 13 cycles at 94°C for 30 s, 65°C for 30 s with a 0.7°C decrement per cycle, and 72°C for 1 min, and then 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min.

Final amplification products were separated on a 5.2% polyacrylamide denaturing gel (38 by 50 cm; Power Pac 3000; Sequigen Bio-Rad) for 1 h 40 min at a constant power of 90 W. One of the glasses was treated with γ-methacryloxypropyltrimethoxysilane and the other was treated with Sigmacote (Sigma) in order that, after the run, the gel could be kept fixed on the first glass for staining. Amplicons were revealed with a silver nitrate stain, after successive treatments with 10% acetic acid, silver nitrate solution (Prolabo, 99.8% purity [R. P. Normapur], 3 g · $liter^{-1}$), and sodium carbonate (Prolabo, 99.8% purity, 60 g · $liter^{-1}$).

Data analysis. For each primer combination, all bands were manually recorded as present or absent, and their lengths were calculated. Only clearly repeatable markers between 100 and 650 bp were used for analysis. For each of the 247 isolates, the AFLP pattern was described as binary strings coded by the presence (1) or absence (0) of each polymorphic marker. Subpatterns were attributed to each primer combination, and whole AFLP patterns were therefore described as an $a_i\ b_j\ c_k\ d_l$ assembly. Exact tests for population differentiation were conducted by the Raymond and Rousset procedure (27). Relationships between AFLP patterns were studied using an UPGMA procedure based on Jaccard's index (30). Five hundred replications were generated from the original set of data using Scilab (version 2.6; INRIA/Scilab Group) for randomization and calculation. Resulting distance matrices were used for a bootstrap test with Felsenstein's Phylip software (version 3.5c; University of Washington), and a consensus tree was determined.

RESULTS

Sampling design. In France, in 1998 the stripe rust epidemic was late, and fields Ro and Du were sampled at the end of the epidemics. In 1999, the epidemic began early in spring and ran from the north towards the southern and western regions. According to the progress of the epidemic, field Ar, being

TABLE 2. Nonselective AFLP primers and selective AFLP primers with two selective nucleotides

Primer denomination in the text	Primer sequence
*Eco*RI+0	5′-GTAGACTGCGTACCAATTC-3′
*Eco*RI+AC	5′-GACTGCGTACCAATTCAC-3′
*Eco*RI+AG	5′-GACTGCGTACCAATTCAG-3′
*Eco*RI+GC	5′-GACTGCGTACCAATTCGC-3′
*Eco*RI+GT	5′-GACTGCGTACCAATTCGT-3′
*Mse*I+0	5′-GACGATGAGTCCTGAGTAA-3′
*Mse*I+AA	5′-GATGAGTCCTGAGTAAAA-3′
*Mse*I+AC	5′-GATGAGTCCTGAGTAAAC-3′
*Mse*I+AG	5′-GATGAGTCCTGAGTAAAG-3′
*Mse*I+GT	5′-GATGAGTCCTGAGTAAGT-3′

more northern than field Ne, showed heavier infection than field Ne at a similar sampling date. When possible, we chose to sample at the beginning and at the end of the epidemic.

No other leaf disease, except septoria leaf blotch (*Mycosphaerella graminicola*), was present in the sampled plots. The septoria epidemic was delayed in time relative to stripe rust. Leaves were first infected by stripe rust and eventually by septoria, depending on the local climatic conditions. Therefore, in all locations, the amount of leaves presenting a large number of stripe rust lesions was not limiting, and the development of other pathogens did not interfere with stripe rust epidemics.

Diversity of virulence. One hundred and ninety-four isolates were tested for diversity of virulence. All isolates had virulence v17, matching the recently introduced *Yr17* resistance gene. Pathotypes 169E136V17 (avirulent types 4, 6, 7, 8, 10, CV, SP, SU; virulent types 1, 2, 3, 9, 17, SD) and 233E137V17 (avirulent types 6, 7, 8, 10, CV, SP; virulent types 1, 2, 3, 4, 9, 17, SD, SU) represented, respectively, 62 and 38% of the population in field Ro in 1998 and 12 and 88% of the population in field Ar in 1999. Only pathotype 233E137V17 was present in field Du in 1998 and in field Ne in 1999 (Table 1).

AFLP polymorphism. The AFLP protocol was carried out a first time for the 247 isolates. One hundred and eighty of them had a clear identical and reproducible AFLP pattern (pattern 0), and 67 had different patterns. These 67 isolates were subjected to a second AFLP protocol after an independent enzymatic restriction. Out of these 67 replications, 7 isolates were identified as belonging to pattern 0, and 60 showed a reproducible different pattern. A third AFLP protocol was carried out for these 60 isolates and for 10 isolates identified as belonging to pattern 0, after a new DNA extraction from a second independent spore sample. Two of these isolates appeared to have been contaminated (the first spore sample showed original extra-bands, whereas the second spore sample showed a clear pattern 0), and we only took into account the second spore sample. Finally, 189 isolates out of the 247 tested presented pattern 0.

On average, 82.5 bands per primer combination could be identified between 100 and 650 bp, and 20.3% of them were polymorphic markers. For the *Eco*RI+AC-*Mse*I+AC combination, 88 bands were observed, and 18 were polymorphic (20.5%). This ratio was 15 polymorphic bands out of 80 bands for *Eco*RI+GC-*Mse*I+AA (18.8%), 17 out of 99 for *Eco*RI+AG-*Mse*I+AG (17.2%), and 17 out of 63 for *Eco*RI+GT-*Mse*I+GT (27.0%). Considering the four chosen combinations, 67 polymorphic markers were detected among the 247 isolates tested (Tables 3 to 6).

Subpatterns were attributed to each primer combination, and then whole AFLP patterns were described as an $a_i\ b_j\ c_k\ d_l$ combination. There were 14 a_i subpatterns for the *Eco*RI+AC-*Mse*I+AC combination, 19 b_j subpatterns for *Eco*RI+GC-*Mse*I+AA, 12 c_k subpatterns for *Eco*RI+AG-*Mse*I+AG, and 16 d_l subpatterns for *Eco*RI+GT-*Mse*I+GT (Tables 3 to 6). Among all possible $a_i\ b_j\ c_k\ d_l$ combinations, a total of 40 patterns were observed, with one major pattern (pattern 0), nine rare patterns and 30 patterns found for only one isolate each (Table 7). Pattern 0 represented 78% (189 isolates) of the whole population; pattern 18 represented 3.2% of the population; patterns 12, 16, 28, and 35 each represented 1.2% of the population; patterns 8, 11, 14, and 31 each represented 0.8% each of the population; and the others were unique (accounting for 0.4% of the population each).

TABLE 3. Description of molecular markers of AFLP subpatterns for *Eco*RI+AC-*Mse*I+AC primer combination

Molecular marker size (bp)	Presence of[a]:													
	a_0	a_1	a_2	a_3	a_4	a_5	a_6	a_7	a_8	a_9	a_{10}	a_{11}	a_{12}	a_{13}
130	0	0	0	0	0	0	0	0	0	0	0	1	0	0
135	0	0	0	0	0	0	0	1	1	0	0	0	0	0
169	0	0	0	0	0	0	0	0	0	1	0	0	0	0
187	0	0	0	0	0	0	1	0	0	0	0	0	0	0
210	0	0	0	0	0	0	1	0	0	1	0	0	0	0
245	0	1	1	0	0	0	0	0	0	0	0	0	0	0
251	0	0	0	0	0	0	0	0	0	0	1	0	0	0
260	0	0	0	0	0	0	1	0	0	0	0	0	0	0
286	0	0	0	0	0	0	0	1	0	0	0	0	0	1
292	1	1	0	1	1	1	1	0	1	1	1	1	1	1
367	1	1	1	1	0	1	1	1	1	1	1	1	1	1
393	0	0	1	0	1	0	0	0	0	0	0	0	0	0
445	0	0	0	1	0	0	0	0	0	0	0	0	1	0
463	1	1	1	1	0	1	1	1	1	1	1	1	1	1
469	1	1	1	1	0	0	1	1	1	1	1	1	1	1
532	0	0	0	0	0	0	1	0	0	0	0	0	0	0
575	0	1	1	0	0	0	0	0	0	0	0	0	0	0
650	0	0	0	1	0	0	0	0	0	0	0	0	0	0

[a] 0, absent; 1, present.

A test was designed to evaluate the sensitivity of the AFLP protocol. Genomic DNAs from two isolates exhibiting different AFLP patterns were mixed in various proportions, and the AFLP protocol was then carried out with these mixtures. We used DNA from the major pattern 0 to dilute DNA from pattern 20 (which had specific additive bands), and we determined the minimal concentration of pattern 20 in the mixture that allowed the observation of these specific bands. Test dilutions were 1:2, 1:3, 1:5, 1:10, and 1:20. For each dilution, we noted the presence or absence of the following markers: +321, +267, and +251 for the *Eco*RI+GC-*Mse*I+AA combination and +560, +476, +464, +428, and +365 for the *Eco*RI+GT-*Mse*I+GT combination. Band intensity after staining seemed

TABLE 4. Description of molecular markers of AFLP subpatterns for *Eco*RI+GC-*Mse*I+AA primer combination

Molecular marker size (bp)	Presence of[a]:																		
	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9	b_{10}	b_{11}	b_{12}	b_{13}	b_{14}	b_{15}	b_{16}	b_{17}	b_{18}
147	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
205	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0
240	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
243	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
251	0	1	1	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0
267	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
321	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1
342	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
370	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
374	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1
380	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1
480	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
509	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
545	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0
595	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] See Table 3, footnote *a*.

TABLE 5. Description of molecular markers of AFLP subpatterns for *Eco*RI+AG-*Mse*I+AG primer combination

Molecular marker size (bp)	Presence of[a]:											
	c_0	c_1	c_2	c_3	c_4	c_5	c_6	c_7	c_8	c_9	c_{10}	c_{11}
136	0	1	1	0	0	0	0	0	1	0	0	0
137	0	1	1	0	0	0	0	0	1	0	0	0
149	0	0	0	0	0	0	0	0	1	0	0	0
200	0	0	0	1	0	0	0	0	0	0	0	0
214	0	0	0	0	0	0	1	0	0	1	0	0
218	0	0	0	0	0	0	1	0	0	0	0	0
247	0	0	0	0	0	0	1	1	0	0	0	1
250	0	0	0	0	0	0	0	0	0	1	0	0
280	0	1	1	0	0	0	0	0	1	0	0	1
282	0	1	1	0	0	0	0	0	0	0	0	0
325	0	0	0	0	0	0	0	0	0	0	1	0
404	1	1	0	1	1	1	1	1	0	1	1	1
457	1	1	1	1	1	0	1	1	1	1	1	1
459	1	1	1	1	1	0	1	1	1	1	1	1
470	0	0	1	0	1	0	0	0	0	0	1	0
473	0	0	0	0	1	0	0	0	0	0	1	0
542	0	1	1	0	0	0	0	0	0	0	0	0

[a] See Table 3, footnote *a*.

to be proportionally dependent on DNA concentration but also dependent on the nature of the individual marker. Specific bands of pattern 20 were never observed for 1:20 dilutions, and only rarely for 1:10 dilutions. Markers +321 and +267 for the *Eco*RI+GC-*Mse*I+AA combination were observed for 1:2, 1:3, and 1:5 dilutions. They were no longer present for more-diluted DNA. Marker +251 for the same combination was observed for 1:2, 1:3, and 1:5 dilutions and could hardly be observed for the 1:10 dilution. Marker +560 for the *Eco*RI+GT-*Mse*I+GT combination was clearly present for the 1:2, 1:3, 1:5, and 1:10 dilutions. On the contrary, for the same combination, markers +476, +464, and +428 were only present in the 1:2 dilution, and marker +365 appeared in 1:2 and 1:3 dilutions.

TABLE 6. Description of molecular markers of AFLP subpatterns for *Eco*RI+GT-*Mse*I+GT primer combination

Molecular marker size (bp)	Presence of[a]:															
	d_0	d_1	d_2	d_3	d_4	d_5	d_6	d_7	d_8	d_9	d_{10}	d_{11}	d_{12}	d_{13}	d_{14}	d_{15}
123	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
194	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
214	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
261	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
263	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0
310	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
315	0	0	1	0	1	1	1	0	0	0	1	0	0	0	0	0
337	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
343	0	0	1	0	0	0	1	0	1	0	0	1	1	1	1	1
365	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
374	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0
410	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
428	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
464	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
476	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0
560	0	1	0	1	0	0	0	0	0	1	0	1	0	0	0	1
580	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	1

[a] See Table 3, footnote *a*.

TABLE 7. AFLP patterns described as subpattern assemblies and number of isolates found in each pattern

Pattern	Subpattern assembly	No. of isolates
0	$a_0\ b_0\ c_0\ d_0$	189
1	$a_1\ b_1\ c_1\ d_1$	1
2	$a_0\ b_0\ c_0\ d_2$	1
3	$a_2\ b_2\ c_2\ d_3$	1
4	$a_3\ b_3\ c_0\ d_4$	1
5	$a_0\ b_0\ c_0\ d_4$	1
6	$a_4\ b_4\ c_0\ d_0$	1
7	$a_3\ b_5\ c_0\ d_4$	1
8	$a_5\ b_6\ c_0\ d_0$	2
9	$a_6\ b_0\ c_3\ d_0$	1
10	$a_5\ b_0\ c_0\ d_5$	1
11	$a_0\ b_7\ c_0\ d_0$	2
12	$a_0\ b_8\ c_4\ d_0$	3
13	$a_0\ b_9\ c_5\ d_0$	1
14	$a_0\ b_0\ c_4\ d_0$	2
15	$a_0\ b_{10}\ c_6\ d_6$	1
16	$a_0\ b_9\ c_0\ d_0$	3
17	$a_0\ b_{11}\ c_0\ d_0$	1
18	$a_0\ b_0\ c_0\ d_7$	8
19	$a_0\ b_0\ c_7\ d_8$	1
20	$a_7\ b_{12}\ c_8\ d_9$	1
21	$a_8\ b_8\ c_0\ d_{10}$	1
22	$a_9\ b_{13}\ c_9\ d_{11}$	1
23	$a_{10}\ b_{14}\ c_0\ d_0$	1
24	$a_{10}\ b_0\ c_0\ d_0$	1
25	$a_0\ b_{15}\ c_4\ d_0$	1
26	$a_0\ b_{16}\ c_4\ d_0$	1
27	$a_{11}\ b_0\ c_{10}\ d_{12}$	1
28	$a_0\ b_8\ c_0\ d_0$	3
29	$a_0\ b_0\ c_0\ d_{13}$	1
30	$a_{12}\ b_8\ c_{11}\ d_{14}$	1
31	$a_0\ b_0\ c_7\ d_0$	2
32	$a_0\ b_{17}\ c_0\ d_0$	1
33	$a_0\ b_{18}\ c_4\ d_8$	1
34	$a_0\ b_0\ c_4\ d_7$	1
35	$a_0\ b_0\ c_0\ d_8$	3
36	$a_{13}\ b_0\ c_0\ d_8$	1
37	$a_{12}\ b_0\ c_0\ d_7$	1
38	$a_{13}\ b_0\ c_0\ d_0$	1
39	$a_0\ b_0\ c_0\ d_{15}$	1

Polymorphic loci. Allelic frequencies for all isolates varied from 0.0020 to 0.9251. Nevertheless, as markers were either almost always present, or almost always absent, allelic frequencies were either lower than 0.05 for 85.1% of them or higher than 0.85 for the other 14.9%. There were from 0.0% polymorphic loci in field Ro to 68.6% in field Du, but if rare markers (polymorphic for fewer than 5% of isolates) were not taken into account, fields Ro and Ar appeared monomorphic and fields Du and Ne showed, respectively, only 23.88 and 13.43% polymorphic loci.

Distribution of the genotypic diversity. All 29 isolates of field Ro presented pattern 0. In field Du, 18 out of 29 isolates (62%) presented pattern 0, 2 had pattern 8, and 9 had unique patterns (Table 1). Among the 97 isolates from field Ne, 25 AFLP patterns were present, but 66 isolates were assigned pattern 0 (68%). Patterns 12 and 16 were identified for three isolates each; patterns 11, 14, and 28 were identified for two isolates each; and the other patterns were identified for only one isolate each. For field Ar, among 92 isolates, nine AFLP patterns were present. Pattern 0 accounted for a total of 76

isolates (82.6%); pattern 18 was identified for 7 isolates; pattern 35 was identified for 3 isolates; and patterns 28, 31, and 36 to 39 were identified for only one isolate each.

For fields Ne and Ar, several patterns were sometimes observed at the same sampling point and also inside the same focus (Table 1). For example, in focus Ne_3 of field Ne, the four patterns 0, 20, 21, and 22 were found for 11 isolates sampled. In focus Ar_3 of field Ar, 11 isolates were from three different AFLP patterns.

For fields Ne and Ar, 30 patterns were observed. Pattern 0 was largely predominant, and only three other patterns were common to both fields. For field Ne, 25 different patterns were found, 3 of them being common at the beginning and at the end of the epidemic (Table 1). For field Ar, nine patterns were found, three of them being common to the beginning and the end of the sampling.

Analysis of the similarities between AFLP patterns showed that they were never very different from each other. Indeed, 24 out of 39 were different from pattern 0 by only one to three amplicons. For this reason, we supposed that a clonal lineage approach could help create a better understanding of the genotypic diversity. We used first a cladistic procedure which calculated most-parsimonious trees, and only patterns 1, 3 (field Du, 1998), and 20 (field Ne, 1999) appeared in a separate branch (data not shown). This analysis showed again that all patterns were very similar. We used Jaccard's index to calculate a distance matrix between the different AFLP patterns. A UPGMA analysis defined 12 groups at a 85% similarity level. One of them grouped 20 different patterns, including pattern 0, and accounted for a total of 90.7% of the isolates. Nevertheless, considering that each pattern differed from the other ones by only a few markers, the bootstrap procedure lost almost all information, and all isolates were finally grouped into one lineage. Populations were thus considered to be clonal on the field scale, even when different pathotypes were present.

Field differentiation. In 1998 there was no differentiation between fields Ro and Du ($P = 1.0000$), nor was there a differentiation in 1999 between fields Ne and Ar ($P = 1.0000$), even though in the latter case, four individual loci showed differentiation at the 0.05 level. On the other hand, there was a significant differentiation between year 1998 (fields Ro and Du) and year 1999 (fields Ne and Ar): eight individual loci showed a differentiation at the 0.05 level, with an overall P of 0.0001.

DISCUSSION

Virulence. All isolates were virulent on the recently introduced *Yr17* resistance gene in northern European cultivars. First reports (1) about v17 appeared in 1994 in the United Kingdom and 1997 in Denmark, where *Yr17* had been used extensively. v17 also appeared in 1997 in France and Germany, where *Yr17* cultivars had been grown on a relatively small scale. Bayles et al. (1) also reported that v17 isolates reached a frequency higher than 70% in 1999 in these countries. For each year and field, presence and proportions of pathotypes as defined by virulence genes were consistent with other regional surveys (1, 7). We found that pathotypes 233E137V17 and 169E136V17 represented, respectively, 88.7 and 11.3% of the sampled isolates for years 1998 and 1999 (Table 1). Bayles et al. (1) reported 81 and 5% for the same pathotypes in France in 1999, with the other 14% represented by another pathotype mostly found in the south of France.

AFLP reproducibility. Only clear and reproducible bands ranging from 100 to 650 bp were analyzed. For eight samples, several additional bands of the same average weight were observed, but these bands could not be reproduced with an independent restriction and were therefore attributed to incomplete digestion or problems in ligation or PCR steps. Only two cases of contamination by exogenous DNA, out of the 247 isolates, were found. In these cases, an independent extraction and AFLP protocol carried out from independent spore samples from the same isolate (obtained from different multiplication pots) allowed the identification of clear patterns. Additional bands could be due to hyperparasite development, mixing of spores from different clones, or mixing with any other biological agent. Hyperparasites were sometimes observed in the growth chamber, developing on old sporulating lesions. In that case, the plants and the spores were destroyed. Overall, 58 isolates were clearly identified as different from pattern 0 and distributed among 39 other AFLP patterns.

The sensitivity test gave somehow unexpected results, as AFLP appeared not as sensitive as we first thought. In this experiment, we considered the DNA of pattern 20 to be a contaminant for the DNA sample of pattern 0. Among eight "contaminant" bands, none could be detected at a 1:20 dilution, only two of them could be detected at a 1:10 dilution, three others could be detected at a 1:5 or 1:3 dilution, and three could only be detected at a 1:2 dilution. It therefore seemed that, in the best case, contaminant DNA could only be detected when accounting for at least 1/10 of the total DNA. Another conclusion was that bulk spore samples from direct field sampling, with no strain isolation, could not be used in a preliminary work to infer the presence of isolates different from pattern 0 by additive bands.

Because of *Pst*I sensitivity to methylation, we chose to use *Eco*RI in our AFLP study. Although this choice does not allow direct comparisons with the Hovmøller et al. (10) and Justesen et al. (13) studies on European stripe rust isolates, most AFLP works (21) are conducted with *Eco*RI, and a preliminary work with 16 primer combinations gave good amplification results (data not shown).

Diversity of *P. striiformis* f. sp. *tritici*. Most analyses of genetic diversity of *P. striiformis* f. sp. *tritici* were conducted on a relatively small number of isolates sampled across large geographical areas and generally showed few polymorphisms (5, 10, 29, 31). Studies on such a large scale give insight into the general evolution of the parasite and are of great interest for predicting pandemic dynamics but do not answer more-specific questions about the dynamic of pathogen populations within fields.

We found 67 polymorphic loci with four primer combinations (20.3%), which is consistent with AFLP results for other fungi. For example, Majer et al. (20) used AFLP to study genetic polymorphism in 79 field isolates of *Pyrenopeziza brassicae* sampled in Europe. They screened three primer combinations and found 48 polymorphic markers; i.e., 24.6% of loci were polymorphic. This supports our hypothesis that AFLP combinations are representative and the fact that our results

show a low genotypic diversity for *P. striiformis* f. sp. *tritici* on a field scale.

In another study, Hovmøller et al. (10) used 21 primer combinations for the same pathogen and selected only 28 markers (1.8%). In this study, however, only 42 isolates were sampled over a large spatial scale, from 17 different locations, on 14 different host cultivars and over 5 years, which probably allowed them to select mainly frequent markers, with frequencies ranging from 10 to 90%. Our isolates were sampled in four locations, on the same host cultivar and during 2 years, and the differences between AFLP patterns were due to rare alleles. In our study, we found that field Ro was monomorphic. Most of our AFLP variants were rare, and in particular, isolates from field Ne showed several rare markers. The Ro area, as well as the Du area, are at highest risk for stripe rust epidemics in France. The Ar area is probably directly influenced by the northern population. The Ne area is at an intermediate position and might accumulate different influences. At this point of our knowledge of stripe rust populations in France, however, it is difficult to decide whether this is an explanation for the presence of unusual variants in Ne.

Chen et al. (5) carried out an RAPD analysis on a large scale. Twenty-three isolate collections representative of North American populations were studied, and 50 polymorphic markers were found out of 107 RAPD bands. They identified five RAPD groups, with some minor differences in RAPD patterns within groups. Genetic diversity was assumed to be sufficient to differentiate several RAPD groups within a pathotype, and the correlation between virulence and RAPD patterns was low. In our study, we observed only two virulence patterns and 40 AFLP patterns grouped in a single lineage. Most isolates (88.7%) were from pathotype 233E137V17, the other ones being from pathotype 169E136V17. Two AFLP patterns (0 and 18) had isolates from both pathotypes, but the others were only represented by one to three isolates. These results lead us to the conclusion that our AFLP markers were not related to virulence.

A recent study by Steele et al. (31) using AFLP markers for 14 isolates from Australia and New Zealand, and RAPD analysis for 7 of them, showed no polymorphism on the continent scale, whereas several pathotypes were identified. The authors concluded that this observation was consistent with a stepwise mutation theory of pathotype evolution following a single introduction. These results are similar to ours, though we tested far more isolates and sampled on a field scale.

AFLP pattern distribution. There was no correlation in our study between AFLP pattern and pathotypes. We observed a major pattern (pattern 0) that was the same for the four fields and the two years. A few other rare patterns and several unique patterns were also identified. Pattern 0 had also been found in previous AFLP tests conducted on 10 isolates from a collection representing a 10-year period in France (1987 to 1997), in which it was also associated with different pathotypes (data not shown). It seems therefore that this pattern was present in France during several years and included several pathotypes. The other patterns were rarely found in two different fields, and a given pattern was difficult to record both at the beginning and at the end of the epidemics. Because of the difficulty in sampling such rare patterns, it was not possible to separate sampling bias from genetic drift effects. In field Ne, pattern 0 was always dominant, but three other patterns were present both at the beginning and at the end of the epidemic. For field Ar, three AFLP patterns were present both at the beginning and at the end. These results are partly comparable to those of Justesen et al. (13). Testing 48 isolates of the same pathogen also sampled in 1997 and 1998, but on a larger spatial scale, they found 16 different AFLP patterns. They also noticed, for example, that six isolates sampled in 1998 at Klarup on cultivar Brigadier showed three different AFLP patterns, and that two of these patterns were from the same focus.

In our case, all isolates could be attributed to the same clonal lineage, which suggested that they were derived from one another by minor mutations. We cannot determine whether these mutations occurred before the field contamination or during the epidemic. The first hypothesis suggests that a field population could develop from several independent infection events. The predominance of pattern 0 in the population suggests a better competitive ability of the isolates presenting this phenotype. Intrafocus diversity is more surprising but has also been detected in another study (13). We found up to four different AFLP patterns in the same focus, Ne_3. Several hypotheses could be raised to explain such a result: initiation of primary foci by several independent genotypes, but also secondary mixing of close foci, or mixing of a focus and the epidemic background in the field. A more detailed study of this point would be necessary to draw more-specific conclusions about the genetic structure of foci.

Origin of genetic variation in *P. striiformis* f. sp. *tritici*. Stripe rust is assumed to reproduce only asexually (32), and this assumption has been supported by another AFLP study (10). Winter survival and long-distance migration are decisive factors for stripe rust epidemics to develop (10). It is not known whether stripe rust is able to oversummer in northern France although two different areas are considered to be areas of endemicity (the Caen area and the Calais area). It is also assumed that northern France is largely influenced by the United Kingdom population through migration, as it is known that the migration distance for this parasite's uredospores can be up to 800 km (16). Genetic variation observed in field populations could then derive from a genetically differentiated inoculum, with a major source (pattern 0) and other patterns originating from long-distance migration or local oversummering. We only found two pathotypes in our samples that were also predominant throughout the whole country (7).

An important conclusion of our study is that *P. striiformis* f. sp. *tritici* populations can be considered to be clonal on a field scale under the conditions in northern France. This should be taken into account for the development of management strategies for host resistance (14, 15, 22).

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