An Assessment of the Genetic Diversity in a Field Population of *Phytophthora nicotianae* with a Changing Race Structure

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ABSTRACT

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One hundred fifty-three isolates of *Phytophthora nicotianae* that were collected over a 4-year period from a single field were subjected to amplified fragment length polymorphism (AFLP) analysis to investigate the effect of different types of resistance in tobacco (*Nicotiana tabacum*) on genetic diversity in the pathogen population. No race 1 isolates were detected in the field prior to initiating the study, but the race was present in multiple plots by the end of the 4-year period. There were 102 race 0 isolates and 51 race 1 isolates characterized. Seventy-six of the 153 isolates had a unique AFLP profile, whereas the remaining 77 isolates were represented by 27 AFLP profiles shared by at least two isolates. Isolates of both races were found in both the unique and shared AFLP profile groups. Twenty-three of the AFLP profiles were detected in multiple years, indicating a clonal component to the pathogen population. Race 1 isolates that were detected over multiple years were always obtained from the same plot. No race 1 profile was found in more than one plot, confirming the hypothesis that the multiple occurrences of the race throughout the field were the result of independent events and not pathogen spread. Three identical race 0 AFLP profiles occurred in noncontiguous plots, and in each case, the plots contained the same partially resistant variety. Cluster analysis provided a high level of bootstrap support for 41 isolates in 19 clusters that grouped primarily by race and rotation treatment. Estimates of genetic diversity ranged from 0.365 to 0.831 and varied depending on tobacco cultivar planted and race. When averaged over all treatments, diversity in race 1 isolates was lower than in race 0 isolates at the end of each season. Deployment of single-gene resistance initially decreased genetic diversity of the population, but the diversity increased each year, indicating the pathogen was adapting to the host genotypes deployed in the field.

Black shank, caused by the soilborne pathogen *Phytophthora nicotianae*, is an economically important disease of cultivated tobacco (*Nicotiana tabacum*) throughout the southeastern United States and most production areas of the world (11,31). Infection of roots, stems, and leaves can occur at any stage of plant growth, resulting in root necrosis, wilting, chlorosis, stem lesions, and stunting of plants. The disease is managed using an integrated approach including cultural and chemical practices along with the use of multiple sources of resistance (11,31,34). Cultivated *N. tabacum* and the wild species *N. plumbaginifolia* and *N. longiflora* have been used as sources of resistance, with

both partial and complete resistance incorporated into available cultivars. Partial resistance is effective against all races of *P. nicotianae* and ranges from a low to a high level (32).

Single-gene resistance introgressed from both wild species confers complete resistance to race 0 but no resistance to race 1 of *P. nicotianae* (2,6,8,33). Race 0 has been the predominant race in the flue-cured tobacco areas of North Carolina since the pathogen was introduced in 1931 (31). Although race 1 was observed in breeding nurseries in the 1960s, it was not observed widely in production areas until cultivars containing the *Php* gene, from the species *N. plumbaginifolia*, were widely deployed in the 1990s (10,31). The rapid emergence of race 1 prompted studies on ways to preserve the effectiveness of the *Php* gene. A 4-year cultivar rotation study was conducted to investigate the short- and long-term effects of *Php* gene deployment on the race structure of the tobacco black shank pathogen (32). In that study, two partially resistant cultivars (K 326 and K

346) and one completely resistant cultivar (NC 71) were cropped continuously or in various rotations of complete and partial resistance. In a field where no race 1 was initially detected, race 1 was detected after a single growing season of deploying the *Php* gene and continued to increase in prevalence over space and time during the study. At the end of the 4-year study, race 1 was recovered from all plots throughout the field that had the cultivar NC 71 in the rotation. The occurrence of race 1 in non-contiguous plots led the authors to speculate that the increase in race 1 was due to multiple independent selections of race 1 from the race 0 population (32).

In this study, amplified fragment length polymorphism (AFLP)-based genetic markers were used to characterize the genetic diversity within a subset of the population of *P. nicotianae* obtained from various rotation treatments during the 4-year rotation study. Analyses of AFLP data were conducted to monitor changes in the genetic diversity of race 0 and race 1 over the course of the study and to determine if there was an association of AFLP profile with race.

MATERIALS AND METHODS

Isolates. A subsample of 153 isolates of *P. nicotianae* was selected from all isolates obtained in the rotation study (32). All isolates were collected from soil assays taken from a single tobacco field in Duplin County, NC between 2000 and 2003 in a 4-year cultivar rotation experiment. Nineteen of the 153 isolates were selected from the isolates collected in the spring of 2000 prior to initiating the study to provide a baseline estimate on the genetic diversity of the population. These baseline isolates originated from 14 of the 32 plots from across the field. The remaining 134 isolates used for AFLP analysis were collected each fall after final harvest in the field, beginning in the fall of 2000, year 1, and ending in the fall of 2003, year 4. Briefly, the rotation experiment involved the planting of three flue-cured tobacco cultivars: NC 71 with complete single-gene resistance (C) to race 0; K 326 with a low level of partial resistance (LP) to race 0 and 1; and K 346 with a high level of

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partial resistance (HP) to race 0 and 1. Varieties were planted continuously or alternately over the duration of the 4-year experiment (Table 1). The origin (year collected and rotation treatment) and race of each isolate for which AFLP data were collected are presented in Table 2. Each isolate was identified as *P. nicotianae* based upon morphological characteristics and results using one universal (28) and five different sets of species-specific primers (12,18,22,24,25).

DNA extraction. Total genomic DNA was extracted from lyophilized mycelium of *P. nicotianae* grown in 15 ml of pea broth at 22°C for 7 to 10 days. DNA for AFLP analysis was prepared by extraction with cetyl trimethyl ammonium bromide (CTAB) and phenol/chloroform, followed by precipitation in 95% EtOH and 3 M sodium acetate as previously described by Gonzalez et al. (17).

AFLP analysis. The AFLP protocol was a modification of Vos et al. (35) using the AFLP Core Reagent Kit (Invitrogen) and procedure of Ceresini et al. (7). Approximately 250 ng of genomic DNA was digested overnight at 37°C with *EcoRI* and *MseI*. Double-stranded adaptor sequences were ligated to the digested samples for 6 h with the addition of T4 DNA ligase. The polymerase chain reaction (PCR) pre-amplification step employed primers containing the adaptor sequence, restriction endonuclease recognition sequences, plus one selective nucleotide as provided in the AFLP Pre-Amplification Primer Mix (Invitrogen). Each sample included 2.5 µl of DNA template, 20.0 µl of pre-amplification primer mix, 2.5 µl of enzyme specific PCR buffer, and 0.5 µl of *Taq* DNA polymerase (Qiagen). PCR was performed for 20 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min. The pre-amplification reaction mixture was diluted 10-fold with sterile deionized water and stored at -20°C until needed for the selective amplification step.

Three *EcoRI* (5'-GACTGCGTACCA ATTCAC-3', 5'-GACTGCGTACCAATT CGC-3', and 5'-GACTGCGTACCAATT CGG-3') and two *MseI* (5'-GATGAGTCC TGAGTACA-3' and 5'-GATGAGTCC

TGAGTAACC-3') primers with two selective nucleotides were used for selective amplification of the secondary template. The *EcoRI* primers were 5' labeled with the fluorescent dye carboxyfluorescein (6-FAM) and screened for polymorphisms in all six possible combinations (*EcoRI*-AC and *MseI*-CC, *EcoRI*-AC and *MseI*-CA, *EcoRI*-GC and *MseI*-CC, *EcoRI*-GC and *MseI*-CA, *EcoRI*-GG and *MseI*-CC, and *EcoRI*-GG and *MseI*-CA) on a sample of 24 isolates of *P. nicotianae* collected over multiple years and locations, including Indonesia, Virginia, Kentucky, Connecticut, and multiple counties within North Carolina that represented different races, mating types, and tobacco types. The primer pair *EcoRI*-GG/*MseI*-CC generated easily resolvable and reproducible fragments, and was subsequently used in the selective amplification step for characterizing the 153 isolates used in this study. The *EcoRI* primers were synthesized by MWG Biotech (High Point, NC), and all other primers were synthesized by Applied Biosystems.

Each selective PCR mixture included 10 µl of reaction mixture, 1.5 µl of diluted secondary DNA template obtained from the pre-amplification, 1 µm of *MseI* selective primer, 1 µm of *EcoRI* selective primer, 2.0 mM dNTPs, enzyme-specific buffer, and 0.5 µl *Taq* DNA polymerase (Qiagen). The thermal cycling parameters were an initial denaturation at 94°C for 2 min; 13 cycles of 94°C for 30 s, 65°C (-0.7°C per cycle) for 1 min, and 72°C for 2 min; followed by 23 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 2 min. Completed reactions were stored at 4°C.

Following selective PCR amplification, products were diluted 10-fold with sterile distilled H₂O, and 0.8 µl of the resulting diluted samples was mixed with 0.5 µl of the fluorescent sequencing dye GeneScan ROX-500 containing formamide and an internal molecular marker standard (Applied Biosystems). The mixture was denatured at 90°C for 3 min and placed on ice. One microliter of each reaction mixture was loaded on a 6% denaturing polyacrylamide gel in an ABI model 377 sequencing electrophoresis system (Applied

Biosystems). The running buffer was 1× TBE, and electrophoresis was performed at 60 W for 3.0 h. The AFLP fragments were sized using GeneScan 2.02 (ABIPRISM, Applied Biosystems) software. The 257 polymorphic AFLP fragments were scored with the software Genographer (Montana State University) and coded as present (1) or absent (0) to determine the AFLP profile. The reproducibility of the AFLP analysis was determined by scoring fragments from two independent extractions of each isolate.

Data analysis. Phylogenetic analysis was conducted with the Phylip 3.65 package (13). The program Seqboot was initially used to create 1,000 bootstrapped datasets. Restdist was then used to calculate distance using the modified restriction sites distance method of Nei and Li (29) using 14 as the number of sites. The neighbor-joining program was used to estimate Unweighted Pair-Group Mean Analysis (UPGMA) clustering. Consense was used to determine the Majority Rule dendrogram. Isolates with identical AFLP profiles (clones) were removed for clustering analysis. Genetic diversity was calculated as Weir's gene diversity using $D = 1 - (1/m)\sum p_{iu}^2$, where p is the frequency of the u th allele at the l th locus averaged over m loci (36). Diversity was calculated on both the entire and clone-corrected data sets.

RESULTS

AFLP analysis. The primer pair *EcoRI*-GG and *MseI*-CC provided 302 fragments (loci) that could be scored reliably across the 153 isolates of *P. nicotianae*. These data are available upon request to the authors. Of these loci, 257 (85%) were polymorphic, which produced 84 unique AFLP profiles (Table 2). Nineteen of the unique AFLP profiles were observed from the 19 isolates of race 0 collected in the spring of 2000 and were used to represent the pathogen population present in plots prior to initiating the study (data not shown). None of these original profiles were subsequently recovered in the fall of 2000, 2001, 2002, or 2003.

The remaining 65 AFLP profiles identified varied with host cultivar planted. Experimental plots continuously planted with K 326 (treatment 1, Table 1), a tobacco cultivar with a low level of partial resistance to races 0 and 1, resulted in the identification of 1, 5, 12, and 3 different AFLP profiles in 2000, 2001, 2002, and 2003, respectively, with 8 profiles recovered multiple years (Table 2). Five, six, and one AFLP profiles were identified in 2001, 2002, and 2003, respectively, from experimental plots continuously planted with K 346 (treatment 3, Table 1), a tobacco cultivar with a high level of partial resistance to race 0 and race 1 (Table 2); seven profiles from this treatment were found in multiple years. Continuous planting of the

Table 1. Treatments in the 4-year tobacco cultivar rotation experiment

Treatment ^b	Tobacco cultivar planted, by year ^a			
	2000	2001	2002	2003
1	K 326	K 326	K 326	K 326
2	NC 71	NC 71	NC 71	NC 71
3	K 346	K 346	K 346	K 346
4	K 346	NC 71	K 346	NC 71
5	K 326	NC 71	K 326	NC 71
6	K 346	NC 71	K 346	K 346
7	K 326	NC 71	K 326	K 326
8	NC 71	K 326	NC 71	K 326

^a Tobacco planted in Duplin County, NC with the rotation treatments from 2000 to 2003.

^b Cultivars with varying levels and types of resistance: K 326 = low level of partial resistance to race 0 and race 1, K 346 = high level of partial resistance to race 0 and race 1, and NC 71 = complete (*Ph*) single gene resistance to race 0 and a low level of partial resistance to race 1 (32).

experimental plots with a tobacco cultivar with single gene resistance to race 0 (NC 71, treatment 2, Table 1) resulted in the identification of one AFLP profile in 2000, two in 2001, nine in 2002, and nine in 2003, with seven profiles found multiple years (Table 2). The only rotationally planted treatment where profiles were recovered multiple years was from treatment 5 (Table 1), which was alternately planted with K 326 and NC 71; of 16 profiles identified in this treatment, two AFLP profiles were recovered multiple years.

Diversity. Relative differences among treatments were similar when genetic diversity was calculated with the entire or clone-corrected set of data (*data not shown*). Therefore, estimates of genetic diversity were calculated on the entire data set. The mean diversity in the field population of *P. nicotianae* prior to initiating the experiment was 0.629 ($n = 19$, Table 3). The mean diversity for all isolates characterized in the rotation study for each treatment ranged from 0.349 in treatment 5 planted with K 326, NC 71, K 326, and NC 71, to 0.717 in treatment 4 planted

with K 346, NC 71, K 346, and NC 71 (Table 3).

Diversity values varied across years in all treatments. For example, isolates of *P. nicotianae* from plots planted continuously with the tobacco cultivar K 326 were 0.526 ($n = 5$) in 2001, 0.563 ($n = 21$) in 2002, and 0.70 ($n = 10$) in 2003. The diversity of isolates from plots continuously planted with NC 71 were 0.40 ($n = 4$) in 2001, 0.466 ($n = 13$) in 2002, and 0.55 ($n = 17$) in 2003. Continuous planting of K 346 resulted in diversity values of 0.254 ($n = 6$) in 2001, 0.386 ($n = 11$) in 2002, and 0.383 ($n = 10$) in 2003. The diversity observed from isolates sampled from rotated plots planted with NC 71 in years 2 and 4 and with K 326 in years 1 and 3 was 0.463 ($n = 9$) in 2003. Because of the limited number of isolates (<3) of *P. nicotianae* sampled from experimental plots for treatment 5 in 2001 and 2002 and from treatments 4, 6, 7, and 8 from 2001 to 2003, no estimates of diversity were calculated for these treatments.

When all isolates for each race were combined, estimates for genetic diversity were higher for race 0 isolates than for race 1 isolates in each year of the study (Table 4). Diversity values for race 0 isolates ranged from 0.439 to 0.705 and for race 1 isolates from 0.394 to 0.547 (Table 4). The genetic diversity for race 0 isolates

Table 2. Source code, number, and amplified fragment length polymorphism (AFLP) designation of isolates of *Phytophthora nicotianae* sampled from soil in the 4-year crop rotation study^a

Isolate code ^b	No. of isolates ^c	Years collected ^d	Isolate code ^b	No. of isolates ^c	Years collected ^d
T1-Y0-17A-R0	1		T3-Y1-12B-R0	5	2001, 2002 (2), 2003 (2)
T1-Y1-17A-R0	3	2001, 2002, 2003	T3-Y1-24A-R0	2	2001, 2002
T1-Y1-21A-R0	2	2001, 2002	T3-Y1-24B-R0	4	2001, 2002, 2003 (2)
T1-Y1-21B-R0	3	2001, 2002, 2003	T3-Y1-30A-R0	3	2001, 2003 (2)
T1-Y1-39A-R0	4	2001, 2002 (2), 2003	T3-Y1-7A-R0	3	2001, 2002, 2003
T1-Y1-5A-R0	3	2001, 2002 (2)	T3-Y2-12A-R0	1	
T1-Y2-17A-R0	1		T3-Y2-12C-R0	2	2002, 2003
T1-Y2-17D-R0	1		T3-Y2-24C-R0	1	
T1-Y2-17E-R0	1		T3-Y2-30A-R0	1	
T1-Y2-17F-R0	1		T3-Y2-30B-R0	2	2002, 2003
T1-Y2-21C-R0	1		T3-Y2-7A-R0	1	
T1-Y2-21D-R0	1		T3-Y3-42A-R0	2	2003 (2)
T1-Y2-21E-R0	1		T4-Y0-32A-R0	1	
T1-Y2-36A-R0	2	2002, 2003	T4-Y2-44A-R0	1	
T1-Y2-36D-R0	1		T4-Y3-26A-R1	1	
T1-Y2-39B-R0	3	2002 (2), 2003	T4-Y3-26B-R0	1	
T1-Y2-39C-R0	1		T4-Y3-26C-R0	1	
T1-Y2-5B-R0	3	2002 (2), 2003	T4-Y3-9A-R1	1	
T1-Y3-39A-R0	1		T4-Y3-9B-R0	1	
T1-Y3-39C-R0	1		T5-Y0-20A-R0	1	
T1-Y3-5A-R0	2	2003 (2)	T5-Y0-2A-R0	1	
T2-Y0-43A-R0 ^e	4	2000, 2001, 2002, 2003	T5-Y2-34A-R1	1	
T2-Y1-6A-R1	5	2001 (2), 2002, 2003 (2)	T5-Y2-34B-R1	5	2002, 2003 (4)
T2-Y1-6B-R0	1		T5-Y2-34C-R1	1	
T2-Y2-18A-R1	3	2002 (2), 2003	T5-Y3-20A-R1	1	
T2-Y2-22A-R1	1		T5-Y3-34A-R1	1	
T2-Y2-22B-R1	2	2002 (2)	T5-Y3-34B-R1	2	2003 (2)
T2-Y2-28A-R1	2	2002, 2003	T5-Y3-34C-R1	2	2002, 2003
T2-Y2-43A-R1	1		T5-Y3-34E-R1	1	
T2-Y2-43C-R1	2	2002, 2003	T6-Y0-15A-R0	1	
T2-Y2-43D-R1	1		T6-Y2-45A-R0	1	
T2-Y2-6B-R1	1		T6-Y3-15A-R1	1	
T2-Y2-6C-R1	2	2002, 2003	T7-Y2-29A-R1	1	
T2-Y3-22B-R1	1		T7-Y2-41A-R0	1	
T2-Y3-28B-R0	1		T7-Y3-25A-R0	1	
T2-Y3-43A-R1	1		T7-Y3-29B-R0	1	
T2-Y3-43B-R0	1		T7-Y3-29C-R0	1	
T2-Y3-6A-R1	1		T8-Y0-40A-R1	1	
T2-Y3-6C-R1	1		T8-Y2-40C-R1	1	
T2-Y3-6E-R1	1		T8-Y3-11B-R0	1	
T2-Y3-61-R1	1		T8-Y3-35A-R0	1	
T2-Y3-6P-R1	1		T8-Y3-40A-R1	1	

^a Baseline isolates collected prior to year 1 are not shown.

^b Isolate code indicates treatment (T), year (Y, 2000-2003), experimental plot, isolate (alpha-numeric), and race (R) of *P. nicotianae*. AFLP profiles are named for the isolate in which they first occurred.

^c Number of isolates with that AFLP profile.

^d Years collected are the years in which that AFLP profile was detected. For profiles that were detected multiple times in a year, number of occurrences is given in parentheses.

^e Isolate from 2000 was race 0; isolates having this AFLP profile in subsequent years were race 1.

Table 3. Estimates of genetic diversity for isolates sampled from a field population of *Phytophthora nicotianae* from each treatment

Treatment	Genetic diversity ^a
1	0.595 ($n = 37$)
2	0.519 ($n = 34$)
3	0.365 ($n = 27$)
4	0.717 ($n = 7$)
5	0.349 ($n = 16$)
6	0.575 ($n = 3$)
7	0.612 ($n = 5$)
8	0.623 ($n = 5$)

^a Baseline estimate of genetic diversity prior to establishing treatments was 0.629 ($n = 19$). Genetic diversity was calculated using $D = 1 - (1/m)\sum p_{lu}^2$, where p is the frequency of the u th allele at the l th locus averaged over m loci (36). Treatments are given in Table 1. Numbers in parentheses indicate number of samples for each treatment.

Table 4. Estimates of genetic diversity of race 0 and race 1 sampled from a field population of *Phytophthora nicotianae* in experimental plots cropped with tobacco cultivars with different levels of resistance from 2000 to 2003

Year	Genetic diversity ^a	
	Race 0	Race 1
2000	0.564 ($n = 6$)	N/A ($n = 1$)
2001	0.439 ($n = 11$)	0.394 ($n = 3$)
2002	0.539 ($n = 35$)	0.414 ($n = 19$)
2003	0.705 ($n = 31$)	0.547 ($n = 28$)

^a Genetic diversity was calculated using $D = 1 - (1/m)\sum p_{lu}^2$, where p is the frequency of the u th allele at the l th locus averaged over m loci (36).

was 0.564 ($n = 6$) in 2000, 0.439 ($n = 11$) in 2001, 0.539 ($n = 35$) in 2002, and 0.709 ($n = 31$) in 2003. Diversity of race 1 was not calculated at the end of 2000, as only one isolate of race 1 was recovered at the end of the growing season; however, diversity increased from 0.394 ($n = 3$) in 2001

to 0.414 ($n = 19$) in 2002, and to 0.547 ($n = 28$) in 2003.

UPGMA clustering. Isolates with identical AFLP profiles were removed for clustering analysis, leaving 84 isolates with unique profiles. Forty-one of the isolates formed 19 clusters of two to four isolates each with a

high level of bootstrap support (Fig. 1). Isolates clustered primarily by race and treatment. All clusters contained isolates of a single race, except for three instances. Isolates from clusters that contained both races were recovered from the same plot in two cases and from an adjacent plot in an-

Fig. 1. Majority-Rule Unweighted Pair-Group Mean Analysis (UPGMA) bootstrap consensus dendrogram of *Phytophthora nicotianae* isolates analyzed in this study. Bootstrap values less than 50% were collapsed, and values greater than 50% are shown below the corresponding node. Arrows indicate separation of dendrogram. Abbreviations are given in Table 2.

other case. Thirty-one isolates representing all treatments were unresolved.

DISCUSSION

The AFLP technique can reveal polymorphisms in a genome and was used in this study to identify differences among closely related isolates to elucidate small changes within a field population over time in response to selection pressures imposed by host resistance. The primers used in this study produced a large number of reproducible and unambiguous fragments and were developed based on screening multiple sets of primers on a diverse subsample of 24 isolates of *P. nicotianae* representing different races and mating types. This subset of isolates was collected from different host tobacco types across diverse geographic locations including Indonesia, Kentucky, Virginia, Connecticut, and multiple counties in North Carolina. AFLP-based genetic markers have been used extensively to assess the genetic diversity and structure of populations of species of *Phytophthora* (1,3,5,20,21,23,27). However, except for the molecular characterization of natural hybrids of *P. nicotianae* and *P. cactorum* (4), these genetic markers have not been used previously to examine a large sample of isolates from a single-field population of *P. nicotianae*.

Previous studies have examined the genetic diversity in *P. nicotianae* with isozyme and restriction fragment length polymorphism (RFLP) analyses of nuclear and mitochondrial DNA (14,15,26,30). In these studies, a small sample of isolates of *P. nicotianae* collected from a range of hosts other than tobacco was examined. Colas et al. (9) were able to distinguish a genetically distinct group of isolates causing black shank from other isolates of *P. nicotianae* in a sample from tobacco ($n = 57$) and other hosts ($n = 16$) by examining the association of virulence with RFLP-based genetic markers. However, no estimates of genetic diversity within tobacco-specific isolates or races of *P. nicotianae* were conducted.

The field site in Duplin County provided a unique opportunity to study the spatial and temporal dynamics of a field population of *P. nicotianae* with a changing race structure. The subset of race 0 isolates recovered from the plots in the spring of 2000 all had unique AFLP profiles. At the end of year 1, fall 2000, additional AFLP profiles were recovered among race 0 isolates, and the one isolate of race 1 recovered from an NC 71 plot also had a unique AFLP profile. In subsequent years, numerous isolates of races 0 and 1 were recovered from plots, and additional AFLP profiles were recovered each year. These results confirmed the hypothesis that race 1 was developing in the field as independent events, since the profiles of isolates from noncontiguous plots were always

unique. There was only one example of race 0 and race 1 isolates having identical profiles. Three of the race 1 isolates recovered from an NC 71 plot in 2001, 2002, and 2003 had identical AFLP profiles to a race 0 isolate from the same plot in 2000. This result also indicates that the bands scored in this study did not correspond to the region associated with race of an isolate.

There were numerous examples of an AFLP profile being collected in multiple years. In each case, these profiles were from the same plot, indicating that the pathogen is surviving within this small plot and was not being moved to contiguous plots. These observations support previous reports on the effects of host resistance on movement of the pathogen (31). There were only three cases where identical AFLP profiles were obtained from noncontiguous plots. Interestingly, in each case, these noncontiguous plots were planted with the same variety with partial resistance. There have been no reports of host specialization to partial resistance in this pathosystem, but this observation may suggest that the pathogen adapts to partial resistance as well as complete resistance.

The repeated recovery of isolates with identical AFLP profiles across years suggests that there is a clonal component to the population structure of *P. nicotianae* and the survival of these numerous clones (clones limited to small areas of the field) may account for disease variability observed across a field. The moderate level of recovery of isolates with a unique AFLP profile (84 profiles from the 153 isolates, 55%) is interesting and provides some evidence for a mixed population structure for *P. nicotianae*, i.e., not entirely clonal. Although beyond the scope of this study, further investigation with a larger sample is needed to better determine the population processes (mutation, recombination, and selection) and aspects of reproductive biology (asexual versus sexual) that contribute to a mixed population structure for this pathosystem.

The small sample of isolates of *P. nicotianae* included in this study from the rotation plots limited the ability to compare yearly estimates of genetic diversity for each rotation treatment. However, sample size was taken into account in the diversity calculation to allow for comparisons between sets of isolates with differing sample sizes. The data suggest that there was a decrease in genetic diversity in the experimental plots in 2001 (second year of the study), followed by a gradual increase in genetic diversity in subsequent years (2002 and 2003). A comparison of the genetic diversity from plots continuously planted with a tobacco cultivar indicated that isolates sampled from plots planted continuously with K 346 had the lowest genetic diversity over the course of the study. In this study, we also calculated genotypic

diversity as recommended by Grunwald et al. (19), and the results for isolates from the continuously planted treatments were similar. However, the rotationally planted treatments did not have sufficient numbers to utilize this method.

Race and treatment were confounded in this study; treatments with continuous planting of partial resistance led to maintenance of race 0 (continuous planting of either K 326 or K 346, treatments 1 and 3, respectively), and continuous planting with complete resistance led to development and maintenance of race 1 (continuous planting of NC 71, treatment 2). The clustering of isolates from multiple plots of the same treatment provides support for the presence of a treatment signature in the AFLP profiles. Likewise, clusters consisting of isolates of the same race that were obtained from the same treatment indicate the close association of treatment and race. However, the 19 clusters consisting of isolates from multiple treatments were largely based on race, with only three of these being of mixed race. These data suggest that the clustering is primarily a result of race, and that race 1 could also be maintained on a variety with low partial resistance.

The AFLP profiles within a race often were very similar and differed by only two to four fragments. However, no unique fragments were identified that had a one-to-one correspondence with race. Similar results were observed from an RFLP analysis of *P. sojae* with 48 isolates representing 25 physiological races of the pathogen (16). In that study, one group of isolates, representing seven races, had nearly identical RFLP profiles to race 1 and probably arose clonally by mutation. All other races seem to have arisen from rare outcrosses among four progenitor lines. In this study, the evolutionary relatedness of isolates of race 0 and race 1 could not be determined, but should be the focus of future studies.

This study illustrates the dynamic nature of a field population of a soilborne pathogen undergoing severe selection pressure due to the introduction of complete resistance. In the case of the black shank pathogen, the race structure of the population can adapt relatively quickly in response to the deployment of this type of resistance. A comparable level of genetic diversity was observed both within and between races of *P. nicotianae*, but the genetic composition and diversity of the population changed in response to the deployment of complete resistance. Current recommendations to use an integrated approach to disease management will continue to be necessary. By rotating types of resistance, isolates of race 0 and race 1 can be altered and potentially managed over time (32), regardless of their inherent genetic diversity in field populations of *P. nicotianae*. The biological and ecological

characteristics (e.g., mating type, zoospore production, virulence) of isolates of race 1 and race 0 belonging to different AFLP profiles are currently being studied, and further study is needed with additional co-dominant genetic markers.

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