Investigating the Spatiotemporal Genetic Structure of *Phytophthora capsici* in Michigan

K. H. Lamour and M. K. Hausbeck

Department of Plant Pathology, Michigan State University, East Lansing 48824.
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ABSTRACT

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Phytophthora capsici isolates were recovered from pepper and cucurbit hosts at seven locations in Michigan from 1998 to 2000. Isolates were characterized for compatibility type (CT), mefenoxam sensitivity (MS), and amplified fragment length polymorphism (AFLP) marker profiles. In total, 94 AFLP bands were resolved. Individual populations were highly variable. Within populations, 39 to 49% of the AFLP bands were polymorphic and estimated heterozygosities ranged from 0.16 to 0.19. Of the 646 isolates fingerprinted, 70% (454) had unique AFLP

profiles. No clones were recovered between years or locations. Pairwise F statistics (Φ_{ST}) between populations from different locations ranged from 0.18 to 0.40. A tree based on unweighted pair-group method with arithmetic average cluster analysis indicates discrete clusters based on location. Isolates from the same location showed no clustering based on the year of sampling. Analysis of molecular variance partitioned variability among (40%) and within populations (60%). The overall estimated Φ_{ST} was 0.34 (SD = 0.03). A1/A2 CT ratios were $\approx$ 1:1, and MS frequencies were similar between years for the two locations sampled over time. These data suggest that *P. capsici* persists in discrete outcrossing populations and that gene flow among locations in Michigan is infrequent.

Phytophthora capsici Leonian causes significant damage to a variety of plant hosts worldwide, and in the United States, it seriously impacts the production of cucurbits, tomatoes, and peppers (9,14,20). In Michigan, the life history of *P. capsici* is divided between an active growth phase in the presence of susceptible host tissue and a state of dormancy over the winter. Overwintering survival is thought to be accomplished by thick-walled oospores that are produced during sexual reproduction (9,10). *P. capsici* is heterothallic, and completion of the sexual stage requires both A1 and A2 compatibility types (CT). Sexual reproduction is mediated by extracellular hormonal signals, and there is the potential for both self and cross-fertilization (8). Oospores generally require a dormancy period prior to germination. Germinating oospores produce coenocytic mycelium, which can directly infect or differentiate into caducous sporangia under suitable conditions. Sporangia can be dislodged and cause infection directly, or, in the presence of free water, release 20 to 40 motile zoospores. Polycyclic asexual spread of *P. capsici* between and down rows has been clearly documented in the pepper/*P. capsici* pathosystem (21).

Ristaino and Johnston recently summarized management strategies useful for disease control (20). The primary strategy is to manage soil water dynamics by providing the best possible drainage for the host plant's rhizosphere and the field, in general. Growers are advised to rotate fields to nonsusceptible hosts, and when appropriate to apply fungicides.

The phenylamide fungicide mefenoxam is fungistatic to sensitive isolates of *P. capsici* (16), but, as has occurred with many oomycetes, insensitivity has developed in field populations (9,17,18). Research with *P. capsici* isolates from Michigan indicates that insensitivity is controlled by an incompletely dominant

gene of major effect (9), which is consistent with the findings for other oomycetes (3).

In Michigan, fruit, stem, and root rots caused by *P. capsici* on susceptible hosts have increased in recent years, and growers employing available management strategies have experienced significant losses. Over the last 4 years, an investigation of *P. capsici* populations in Michigan commercial vegetable production fields has been conducted (9,10). The initial phase of this study was based on the distribution and frequency of CT and mefenoxam sensitivity (MS) phenotypes within fields. In 1998, an approximate 1:1 ratio of A1/A2 isolates was discovered in the majority of fields sampled, and oospores were detected in diseased cucurbit fruit on four separate farms. All six CT/MS phenotypes were recovered as oospore progeny from a single diseased cucumber fruit as well as from a single diseased cucumber field (9). These initial findings suggested that the sexual stage occurs in populations of *P. capsici* in Michigan and, based on the MS findings, that sexual recombination may play an important role in generating the fully insensitive MS phenotype.

The ability to assess population structure by only CT and MS is limited by the fact that only six phenotypic combinations are resolvable and is further limited because some populations appear to have only sensitive or insensitive isolates (9). Amplified fragment length polymorphism (AFLP) markers are increasingly used as a tool to investigate population genetic structure in a wide variety of living organisms including plants (22,24), animals (19), insects (4), and microorganisms (11). A molecular map of the *P. infestans* genome was constructed based on AFLP and restriction fragment length polymorphism markers and corroborates the finding of other researchers that AFLP markers span the genome (23). The recent characterization of a single mefenoxam-insensitive population of *P. capsici* with AFLP markers over a 2-year period revealed that genotypic and genic diversity were high, that clonal reproduction (the recovery of identical multilocus genotypes from different locations within a field) was significant within a single season but that members of the same clonal lineage were not detected between years, that AFLP marker frequencies did not

change significantly between years, and that the frequency of mefenoxam insensitivity did not appear to decrease in the absence of mefenoxam use (10).

In this paper, we report on the genetic structure of *P. capsici* populations from fields located in different regions of Michigan. It was our goal to consider dispersal between locations and the impact of outcrossing on natural populations. We also report on the frequency of self-fertilized versus outcrossed progeny in a sexual cross between isolates from different geographical locations and the inheritance of AFLP markers in this cross. Portions of the information in this paper have been reported previously (9,10).

MATERIALS AND METHODS

Isolate collection and maintenance. Pepper, cucumber, pumpkin, tomato, and squash plant tissue (root, crown, and fruit) with typical signs and symptoms of infection by *P. capsici* were collected from six farms in four different regions of Michigan between 1998 and 2000. Sampling was conducted using field-specific grids with grid quadrants varying from 40 m² to 12 km², depending on the size of the field. Sample sets are labeled according to the following notational approach: location (SW = southwest, SC = south central, C = central, and NW = northwest) followed by a farm designation (1,2,...n) with a hyphen separating a field designation (A,B,...n) and the year sampling was conducted (98, 99, and 00). Diseased plant tissue (between 4 to 20 per quadrant) was collected from quadrants in an arbitrary fashion. Isolation from diseased plant material was made onto BARP (25 ppm of benomyl, 100 ppm of ampicillin, 30 ppm of rifampicin, and 100 ppm of pentachloronitrobenzene)-amended UCV8 (840 ml of distilled water, 163 ml of unclarified V8 juice, 3 g of CaCO₃, and 16 g of agar) plates. Procedures for obtaining single zoospore isolates were as previously described (9). Single zoospore cultures were maintained on RA (30 ppm of rifampicin and 100 ppm of ampicillin)-UCV8 plates and transferred bimonthly. For long-term storage, a 7-mm plug of expanding mycelium from each culture was placed in a 1.5-ml microfuge tube with one sterilized hemp

seed and 1 ml of sterile distilled water (SDW). Isolates were then incubated for 2 to 3 weeks at 23 to 25°C before being stored at 15°C.

CT and MS determination. Agar plugs from the edge of an expanding single zoospore colony were placed at the center of UCV8 plates approximately 2 cm from ATCC isolate 15427 (A1 CT) and ATCC 15399 (A2 CT) and incubated in the dark at 23 to 25°C for 3 to 6 days. Following incubation, CT was determined. Thereafter, all CT determinations were crossed with field isolates OP97 (A1) and SP98 (A2).

Agar plugs from the edge of actively expanding single zoospore colonies were placed at the center of UCV8 plates (100 × 15 mm) amended with 0 or 100 ppm of mefenoxam (Ridomil Gold EC, Novartis, Greensboro, NC; 48% active ingredient, suspended in SDW; added to UCV8 cooled to 49°C). Inoculated plates were incubated at 23 to 25°C for 3 days, and colony diameters were measured. Percent growth of an isolate on amended media was calculated by subtracting the inoculation plug diameter (7 mm) from the diameter of each colony and dividing the average diameter of the colony on amended plates by the average diameter of the colony on unamended control plates. All tests were conducted at least two times. An isolate was scored as sensitive (S) if growth at 100 ppm was <30% of the control, intermediately sensitive (IS) if growth was between 30 and 90% of the control, and insensitive (I) if growth was >90% of the control (9).

DNA extraction and AFLP fingerprinting. Bacterial contamination was avoided by using a modified Van Teigham cell (5). The uppermost portion of a 7-mm plug of mycelium was placed on the surface of RA-WA plates (30 ppm of rifampicin, 100 ppm of ampicillin, 1,000 ml of distilled water; and 16 g of agar) and an autoclaved cap from a 1.5-ml microfuge tube was placed over the plug, which forced the isolate to grow through the amended medium. Isolates were incubated in the dark for 2 to 3 days before two 7-mm plugs were transferred to approximately 15 ml of RA-UCV8 broth in petri dishes (100 × 5 mm) and incubated in the dark for 3 days at 23 to 25°C. Mycelial mats were washed with distilled water and dried briefly under vacuum before being frozen to -20°C and lyophilized.

Lyophilized mats were ground with a sterile mortar and pestle. Whole genomic DNA from approximately 50 mg of ground mycelium was extracted with a plant mini kit (Qiagen Dneasy; Qiagen Inc., Valencia, CA) according to the manufacturer's directions or using a cetyltrimethylammonium bromide (CTAB) procedure in conjunction with an automated DNA extractor. DNA was

TABLE 1. Inheritance of 17 amplified fragment length polymorphism (AFLP) markers, compatibility type (CT), and mefenoxam sensitivity (MS) in 107 progeny of a cross between *Phytophthora capsici* isolates OP97 (A1/IS) and SFF3 (A2/S)

Marker ^a	Progeny ratio ^b	χ^2 ^c	P ^d
E+AC/M+CA-66	47:60	1.58	0.20
E+AC/M+CA-97	51:56	0.23	0.70
E+AC/M+CA-146	53:54	0.01	0.90
E+AC/M+CA-149	60:47	1.58	0.20
E+AC/M+CA-156	64:43	4.12	0.04
E+AC/M+CA-159	56:51	0.23	0.70
E+AC/M+CA-244	46:61	2.10	0.17
E+AC/M+CA-258	52:55	0.08	0.80
E+AC/M+CA-270	53:54	0.01	0.98
E+AC/M+CA-282	56:51	0.23	0.70
E+AC/M+CA-290	62:45	2.70	0.13
E+AC/M+CA-328	55:52	0.08	0.80
E+AC/M+CA-351	61:46	2.10	0.15
E+AC/M+CA-398	55:52	0.08	0.80
E+AC/M+CA-431	55:52	0.08	0.80
E+AC/M+CA-435	57:50	0.46	0.90
E+AC/M+CA-444	49:58	0.76	0.85
CT	53:54	0.01	0.98
MS	47:60	1.58	0.20

^a AFLP marker labels indicate the restriction enzymes (E = *Eco*RI, M = *Mse*I), the two selective nucleotides, and the size of the DNA fragment in base pairs.

^b Presence/absence ratios for AFLP markers, A1/A2 for CT, and sensitive (S)/intermediately sensitive (IS) for MS as determined by in vitro screening.

^c χ^2 value for testing 1:1 segregation (1 df).

^d Probability of the observed ratio occurring by chance under the null hypothesis of 1:1 segregation.

TABLE 2. Estimates of genetic diversity within populations of *Phytophthora capsici* in Michigan

Population ^a	No. of isolates ^b	No. of AFLP bands	No. and % polymorphic bands ^c	Estimated average heterozygosity
SW1-A98	57	72	37 (39)	0.16
SW1-A99	141	72	37 (39)	0.16
SW1-B99	35	69	38 (40)	0.16
SW1-B00	24	69	38 (40)	0.16
SC1-A98	50	68	42 (45)	0.17
SC2-B99	45	71	43 (46)	0.17
C1-A00	48	77	41 (44)	0.17
NW1-A99	37	80	44 (47)	0.19
NW2-B98	24	73	46 (49)	0.18

^a First two capital letters indicate location in Michigan with S = south, W = west, C = central, and N = north, the number following the location designator indicates the farm, the capital letter following the hyphen is a field designator, and the numbers following the field designator indicate year (e.g., 00 = 2000).

^b Total number of isolates with unique multilocus amplified fragment length polymorphism (AFLP) profiles.

^c Percent polymorphic bands determined by dividing the number of polymorphic bands by the total number of bands recovered in Michigan (N = 94).

quantified by Nucleic Acid QuickSticks (Clontech, Palo Alto, CA) according to the manufacturer's directions or on 1.5% agarose gels. Approximately 100 ng of DNA was subjected to a restriction/ligation reaction, preselective amplification, and selective amplifications using the polymerase chain reaction (PCR) core mix, adaptor sequences, core primer sequences, and fluorescence-labeled primers provided by an AFLP microbial fingerprinting kit (Perkin-Elmer Applied Biosystems [PE/ABI], Foster City, CA) and performed exactly as described in the AFLP microbial fingerprinting protocol part 402977 Rev A (PE/ABI) (25). All PCR reactions were performed with a minicycler (MJ Research Inc., Waltham, MA) in 0.2-ml tubes according to the cycling parameters outlined in the microbial fingerprinting protocol.

An initial optimization set of reactions was performed with preselective products from *P. capsici* isolate OP97, which was isolated from a cucumber fruit in 1997 (9). Selective amplifications with the selective primers *EcoRI*-AA, AC, AG, and AT were performed in all 16 combinations with the *MseI*-CA, CC, CG, and CT selective primers. *EcoRI*-selective primers available from PE/ABI were labeled at the 5' end with either carboxyfluorescein (FAM), carboxytetramethylrhodamine (TAMRA), or carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) fluorescent dyes. The fluorescent dyes were excited by laser radiation and visualized by their characteristic absorption-emission frequencies. Only the fragments containing an *EcoRI* restriction site were resolved.

Selective amplification AFLP products and a carboxy-X-rhodamine size standard were loaded into each lane on a denaturing polyacrylamide gel and the fragments resolved in a DNA sequencer (Prism 377; ABI). Results were prepared for analysis in the form of electropherograms using GeneScan Analysis software (PE/ABI). AFLP fragments were scored manually as present = 1 or absent = 0 using Genotyper (PE/ABI). Only DNA bands that consistently exhibited unambiguous presence/absence profiles were scored.

In order to assess the reproducibility of AFLP profiles, a single isolate, OP97, was subjected to the aforementioned protocol using three optimal primer pair combinations on three separate occasions approximately 3 months apart.

No prior sequencing or cloning of fragments is needed to utilize this marker system and it is highly reproducible between labs (1). AFLP markers are generally scored as present or absent (e.g., dominant markers), and the confidence with which population level inferences can be made is greatly increased by sample sets that are approximately twice the size used for codominant markers (7,12,28).

Marker inheritance. Oospore progeny ($N = 107$) resulting from a cross between isolate OP97 (A1/IS) $\times$ SFF3 (A2/S) were subjected to AFLP analysis as described previously. Protocols for the generation, germination, and phenotypic characterization of the F1 oospores from this cross have been reported previously (9). The inheritance of AFLP bands present in one parent and absent in the other were analyzed by chi-square analysis to compare observed numbers to those expected under simple Mendelian inheritance (23). Bands present in a single parent and inherited in all the progeny were assumed to be present in two copies in the parent. Bands present in both parents, or present in two copies in one parent and absent in the other, are not reported on in this study. Individual oospore isolates were evaluated to determine if they were the products of self-fertilization or outcrossing between the parent isolates.

Clone detection. AFLP fragments for each field isolate were scored for presence or absence, and the binary data matrix was converted to a similarity matrix using a simple matching coefficient of resemblance with the program NTSYS-pc version 2.02k (Exeter Software, Setauket, NY). Unweighted pair group method with arithmetic averages (UPGMA) cluster analysis was performed on the similarity matrix and a tree was generated. Isolates showing complete homology at all loci were considered members of the same clonal lineage and, except for a single representative

isolate (referred to as a clone), were excluded from population genetic analysis (13).

Population genetic analysis. Sample sets collected from single fields during a single year were considered a population. Populations were assumed to be in Hardy-Weinberg equilibrium, and each AFLP locus was assumed to be diallelic and selectively neutral. The program tools for population genetic analysis (TFPGA) (M. P. Miller, Northern Arizona University, Flagstaff) was used to assess genetic diversity within each population on the basis of estimated average heterozygosity (15) and the proportion of polymorphic loci at the 95% level (6), and to calculate pairwise and overall fixation indices (F statistics) according to the methods of Weir and Cockerham (26). Confidence intervals for F statistics at the 95% confidence level were generated by bootstrapping using 1,000 iterations.

The fixation index, as described by Wright, equals the reduction in heterozygosity expected with random mating at any one level of a population hierarchy relative to another more inclusive level of the hierarchy (27). Weir and Cockerham's approach to estimating fixation indices attempts to correct for the effects of sampling a limited number of organisms from a limited number of populations and is reported as Φ_{ST} instead of F_{ST} (26). Theoretically, the fixation index has a minimum of 0 (no loss of heterozygosity between the populations compared) and a maximum of 1 (indicating fixation for alternative alleles in different populations or a total loss of heterozygosity), but, as discussed by Hartl and Clark (6), the observed maximum is usually much less than 1. Wright (27) has suggested the following qualitative guidelines for the interpretation of fixation indices: the range 0 to 0.05 indicates little genetic differentiation, 0.05 to 0.15 indicates moderate genetic differentiation, 0.15 to 0.25 indicates great genetic differentiation, and values above 0.25 indicate very great genetic differentiation.

Using the program NTSYS-pc, the combined 0/1 data matrix for isolates from all populations was used to construct a genetic similarity matrix of all possible pairwise comparisons of individuals within and among populations using Jaccard's similarity coefficient: $GS(ij) = a/(a + b + c)$. $GS(ij)$ is the measure of genetic similarity between individuals i and j , where a is the number of polymorphic bands shared by i and j , b is the number of bands present in i and absent in j , and c is the number of bands present in j but absent in i . Trees were constructed using UPGMA cluster analysis to provide a graphic representation of the relationships among isolates. A cophenetic correlation coefficient was computed to assess the goodness of fit of the tree to the similarity matrix.

TABLE 3. Clonal component of genotypic diversity within populations of *Phytophthora capsici* from Michigan

Population ^a	Total no. of isolates	Unique AFLP genotypes (%) ^b	No. of clonal lineages	Minimum:maximum no. of isolates per clonal lineage
SW1-A98	63	57 (90)	5	2:2
SW1-A99	200	141 (71)	15	2:40
SW1-B99	71	34 (48)	12	2:9
SW1-B00	36	24 (67)	5	2:8
SC1-A98	57	50 (88)	5	2:3
SC2-B99	56	45 (80)	5	2:5
C1-A00	51	48 (94)	3	2:2
NW1-A99	88	37 (42)	12	2:12
NW2-B98	24	18 (75)	3	2:3
Total	646	454 (70)	65	...

^a First two capital letters indicate location in Michigan with S = south, W = west, C = central, and N = north, the number following the location designator indicates the farm, the capital letter following the hyphen is a field designator, and the numbers following the field designator indicate year (e.g., 00 = 2000).

^b Percentages calculated by dividing the number of unique amplified fragment length polymorphism (AFLP) genotypes by the total number of isolates recovered.

Genetic structure was also examined by analysis of molecular variance (AMOVA) using the ARLEQUIN software package (L. Excoffier, University of Geneva). The AMOVA analysis was used to partition the variance in banding patterns within and among populations from the same geographical site over consecutive years, between sites on the same farm separated by approximately 1 km, and between all the locations sampled in Michigan. Significance values were assigned to variance components based on a set of null distributions generated by a permutation process, which randomly assigned individuals to populations and drew 1,000 independent samples. In order to clearly summarize the spatial aspect of genetic differentiation, regression was used to fit an appropriate model to the plot of pairwise Φ_{ST} values and geographical distances.

RESULTS

AFLP band characterization and marker inheritance. Evaluation of 16 *EcoRI* + 2/*MseI* + 2 selective primer pair combinations indicated that *EcoRI* + AC/*MseI* + CA (EAC/MCA) provided the most clearly resolved fragment profile, and this primer pair was used to analyze DNA from the isolates in this investigation. AFLP profiles for isolate OP97, generated from separate DNA extractions on three separate occasions over a 1-year period, were identical, with only minor differences in the intensity of the electropherogram signal. Occasionally, individual reactions resulted in poorly resolved fingerprint profiles (e.g., low intensity of signal) and were repeated until signals were deemed optimal. The EAC/MCA primer combination resulted in 94 clearly resolved fragments between 40 and 550 bps when considering all the isolates recovered from Michigan.

AFLP analysis of oospore progeny from cross OP97 × SFF3 revealed that all 107 progeny had a combination of bands that were present in only a single parent, indicating that each was a product of outcrossing and not self-fertilization. A comparison of the observed to the expected ratios (1:1) for 17 bands, which were present in only one parent, indicated that only one band segregated in a manner significantly different than expected ($P = 0.05$) (Table 1). Chi-square analysis also indicated that the observed ratios of A1/A2 CT and S/IS MS were not significantly different than expected under Mendelian inheritance (Table 1).

Gene and genotypic diversity. Each isolate was scored for the presence or absence of all 94 AFLP bands. The number of AFLP bands present in each population ranged from 68 to 80, with an average of 72; the number of polymorphic bands ranged from 39 to 49, with an average of 43; and the estimated average heterozygosity ranged from 0.16 to 0.19, with an average of 0.17 (Table 2). These measurements fall within the range described for a wide range of obligately outcrossing diploid plant species. Seventeen (18%) AFLP loci were fixed for the present state (every isolate analyzed had these AFLP markers) in all populations; 12 (13%) were polymorphic in all populations, and 65 (69%) were fixed for presence or absence in some populations and polymorphic in others. The high proportion of AFLP markers fixed among the populations gives a strong indication that significant genetic differentiation exists.

Of the 646 isolates analyzed, 70% had unique multilocus AFLP profiles (Table 3). This suggests that inoculum originating from oospores plays a surprisingly large role in contributing to epidemic development. The number of clonal lineages detected from single locations in Michigan varied from 3 to 15, and the number of isolates within any single clonal lineage ranged from 2 to 40

TABLE 4. Pairwise F statistics (Φ_{ST})^a (below diagonal) and geographical distances (in kilometers, above diagonal) among *Phytophthora capsici* populations in Michigan

Populations ^b	SW1-A98	SW1-A99	SW1-B99	SW1-B00	SC1-A98	SC2-B99	C1-A00	NW1-A99	NW2-B98
SW1-A98	...	0	1	1	165	169	150	180	185
SW1-A99	0.04	...	1	1	165	169	150	180	185
SW1-B99	0.18	0.25	...	0	166	170	150	180	185
SW1-B00	0.25	0.24	0.03	...	166	170	150	180	185
SC1-A98	0.36	0.37	0.29	0.29	...	8	135	260	265
SC2-B99	0.33	0.35	0.32	0.33	0.28	...	130	255	260
C1-A00	0.36	0.37	0.33	0.32	0.38	0.40	...	140	145
NW1-A99	0.32	0.34	0.30	0.30	0.32	0.32	0.38	...	5
NW2-B98	0.36	0.37	0.31	0.32	0.33	0.33	0.33	0.27	...

^a Estimated fixation index calculated according to the methods of Weir and Cockerham.
^b First two capital letters indicate location in Michigan with S = south, W = west, C = central, and N = north, the number following the location designator indicates the farm, the capital letter following the hyphen is a field designator, and the numbers following the field designator indicate year (e.g., 00 = 2000).

TABLE 5. Results of nested analysis of molecular variance (AMOVA) for *Phytophthora capsici* isolates based on 94 amplified fragment length polymorphism markers

Source of variation ^a	Degrees of freedom	Sum of squares	Variance component	Percent variation	P^b
SW1-A (1998-1999)					
Among populations	1	39.658	0.396	5.05	<0.0001
Within populations	197	1,461.559	7.457	94.95	
SW1-B (1999-2000)					
Among populations	1	6.678	0.016	0.27	0.0029
Within populations	57	312.399	6.248	99.73	
SW1-A vs. SW1-B					
Among populations	1	234.790	2.762	27.34	<0.0001
Within populations	255	1,820.294	7.340	72.66	
All locations					
Among populations	6	1,169.295	4.814	39.67	<0.0001
Within populations	273	1,984.345	7.322	60.33	

^a First two capital letters indicate location in Michigan with S = south, W = west, C = central, and N = north, the number following the location designator indicates the farm, and the capital letter following the hyphen is a field designator. Variance is partitioned between 1998 and 1999 at SW1-A, between 1999 and 2000 at SW1-B, between combined sample sets from SW1-A and SW1-B, and within and between sample sets from seven locations in Michigan. AMOVA analysis for all locations includes sample sets from a single year for locations SW1-A and SW1-B.
^b P = the probability of obtaining a more extreme variance component estimate by chance alone based on 1,000 sampling realizations.

(Table 3). In all cases, isolates with identical multilocus AFLP profiles had identical CT and fell into the same MS category. No clones were recovered among separate locations, and cluster analysis indicated that isolates from the same location grouped discretely. The percentage of genotypically unique isolates recovered at locations ranged between 42 and 94% (Table 3). This wide variation may be due to when the samples were collected. Sample sets collected at SW1-A over the course of the 1999 growing season exhibited significantly less genotypic diversity at the end of the season due to the spread of clonal lineages (10). This is expected for an organism with polycyclic disease develop-

ment and suggests that samples collected early in a *P. capsici* epidemic may provide a better estimate of genic diversity than samples collected at the height of an epidemic.

Temporal dynamics. *F* statistics (Φ_{ST}) comparing populations of *P. capsici* recovered from field SW1-A over 1998 and 1999, and field SW1-B over 1999 and 2000 were 0.04 and 0.03, respectively. These values indicate that very little genetic differentiation or loss of heterozygosity occurred between years at either location (Table 4). At both locations, the number and identity of AFLP bands resolved remained identical over time, with 72 total bands recovered from populations at SW1-A and 69 bands recovered

Fig. 1. Unweighted pair-group method with arithmetic average cluster analysis of *Phytophthora capsici* isolates from location SW1-B over 1999 and 2000 ($N = 58$) and SW1-A in 1998 ($N = 57$) based on the Jaccard similarity coefficient using 94 amplified fragment length polymorphism markers. Nodes contain isolates exclusively from single locations. Location identifiers precede the inclusive node and are indicated by region (S = south, N = north, W = west, and C = central) and a farm identifier (1,2,... n) prior to the hyphen with a field indicator (A, B,... n) and the year of sampling (e.g., 00 = 2000) following.

TABLE 6. Location, year, hosts, compatibility type, and mefenoxam sensitivity of genetically unique *Phytophthora capsici* isolates collected in Michigan between 1998 and 2000

Population ^a	Hosts ^b	No. of isolates ^c	Compatibility type/mefenoxam sensitivity ^d					
			A1/S	A1/IS	A1/I	A2/S	A2/IS	A2/I
SW1-A98	S, PK	57	...	4 (0.07)	31 (0.54)	...	6 (0.11)	16 (0.28)
SW1-A99	S	141	1 (0.01)	17 (0.12)	57 (0.40)	1 (0.01)	23 (0.16)	42 (0.30)
SW1-B99	S	34	14 (0.41)	4 (0.12)	...	11 (0.32)	4 (0.12)	1 (0.03)
SW1-B00	S	24	7 (0.29)	5 (0.21)	...	5 (0.21)	5 (0.21)	2 (0.08)
SC1-A98	C	50	10 (0.20)	17 (0.34)	2 (0.04)	10 (0.20)	11 (0.22)	...
SC2-B99	C	45	...	6 (0.13)	22 (0.49)	...	2 (0.04)	15 (0.33)
C1-A00	P	48	20 (0.42)	...	...	28 (0.58)	...	...
NW1-A99	S, C	37	25 (0.68)	...	...	12 (0.32)	...	...
NW2-B98	P	18	10 (0.56)	...	...	7 (0.39)	1 (0.05)	...
Total		454	87 (0.19)	53 (0.12)	112 (0.25)	74 (0.16)	52 (0.11)	76 (0.17)

^a First two capital letters indicate location in Michigan with S = south, W = west, C = central, and N = north, the number following the location designator indicates the farm, the capital letter following the hyphen is a field designator, and the numbers following the field designator indicate year (e.g., 00 = 2000).

^b S = squash, C = cucumber, PK = pumpkin, and P = pepper.

^c Total number of isolates with unique multilocus amplified fragment length polymorphism profiles.

^d Mefenoxam sensitivity determined by in vitro screening on 100 ppm of mefenoxam-amended media with S = <30% growth of control (GC), IS = between 30 and 90% GC and I = >90% GC. Observed numbers are followed by proportion of total sample size in parenthesis.

from populations at SW1-B (Table 2). The number and identity of bands polymorphic at the 95% level (37 for SW1-A and 38 for SW1-B) and the estimated average heterozygosity (0.16 for both locations) also remained constant over time (Table 2). AMOVA analysis of SW1-A and SW1-B over time partitioned 5% of the total variability between years for SW1-A and <1% of the total variability between years at SW1-B (Table 5). Significant clonal reproduction was detected at both field sites within a given year, but no members of the same clonal lineage were detected among locations or years (Table 3). Thus, even though individual genotypes did not appear to survive the winter, the data suggest that there was enough outcrossing and survival of the resulting recombinant progeny at both these locations to maintain genic diversity.

Cluster analysis showed that isolates from SW1-A and SW1-B branched from location-specific nodes (branch points on the tree). If there was migration between the locations, then isolates from

SW1-A and SW1-B would be expected to be intermixed in the cluster analysis. On the other hand, there was no clustering within either of the location-specific clusters based on year (Fig. 1). The ratio of A1/A2 CT approximated a 1:1 ratio at each location (Table 6). The percentage of isolates falling into the six MS/CT categories remained relatively similar between years at each location, with a breakdown of 0 and 1% A1/S, 7 and 12% A1/IS, 54 and 40% A1/I, 0 and 1% A2/S, 11 and 16% A2/IS, and 28 and 30% A2/I for location SW1-A in 1998 and 1999, respectively (Table 6). The percentage of isolates in each of the six categories for SW1-B was 41 and 29% A1/S, 12 and 21% A1/IS, 0 and 0% A1/I, 32 and 21% A2/S, 12 and 21% A2/IS, and 3 and 8% A2/I between 1999 and 2000, respectively (Table 6).

Genetic structure. Pairwise Φ_{ST} values ranged from 0.18 to 0.40 when comparing populations from different locations (Table 4). According to Wright's criterion, this means that populations

Fig. 2. Unweighted pair-group method with arithmetic average cluster analysis of 255 *Phytophthora capsici* isolates from six locations in Michigan based on the Jaccard similarity coefficient using 94 amplified fragment length polymorphism markers. Nodes contain isolates exclusively from single locations. Location identifiers precede the inclusive node and are indicated by region (S = south, N = north, W = west, and C = central) and a farm identifier (1,2,...n) prior to the hyphen with a field indicator (A, B,...n) and the year of sampling (e.g., 00 = 2000) following.

were greatly differentiated, even when located as close as 1 km apart. The overall Φ_{ST} value when analyzing isolates from all seven locations combined was 0.34 (SD = 0.03), which indicates that approximately 34% of the total genetic variation was present among locations. An AMOVA analysis of sample sets from all locations corroborated this finding and attributed 40% of the genetic variation among populations and 60% within populations (Table 5). Cluster analysis was also in agreement with the overall fixation index and revealed that populations from different geographical locations branched from specific nodes (Figs. 1 and 2), with population-specific clusters being between 63 and 75% similar. Genetic similarities between individuals within each of the clusters showed similar patterns with individuals ranging between 75 to 95% for SW1-A (1998 and 1999), 77 to 94% for SW1-B (1999 and 2000), 75 to 94% for SC1-A98, 69 to 92% for SC2-B99, 76 to 95% for C1-A00, 71 to 97% for NW1-A99, and 72 to 93% similar for NW2-B98 (Figs. 1 and 2). The cophenetic correlation coefficient for the overall tree (Fig. 2) was 0.84, indicating that the tree provided a good fit to the data matrix. The results of fitting a linear model to describe the relationship between pairwise Φ_{ST} and pairwise geographical distances indicated a significant relationship ($r^2 = 42.67$; $P < 0.01$) (Fig. 3). Although this analysis should be interpreted with caution due to the unbalanced nature of the sample (28 observations between 130 to 265 km and only 6 observations between 1 to 8 km), it suggests that the genetic differentiation among locations only becomes more substantial with increasing distance.

DISCUSSION

In Michigan, producers of tomatoes, peppers, and cucurbits have experienced increasing losses to *P. capsici* during the last 10 to 15 years. Land suitable for vegetable production is limited in some areas and the length of crop rotation is restricted. A minimum of 3-years rotation to nonsusceptible hosts is a standard recommendation (20). The efficacy of rotation in a disease management program depends on the ability of *P. capsici* to survive and move among locations. Determining the survival period of naturally produced *P. capsici* propagules is difficult (2) because inoculum may be present in a small, often undetectable amount. Although significant local spread via water has been demonstrated (21), there is little information concerning the movement of *P. capsici* among geographically separated locations. We report on isolates from seven geographically separated locations as part of an ongoing investigation aimed at determining how *P. capsici* survives and characterizing the dynamics of dispersal. Segregation analysis of 17 of the AFLP markers used in this study suggests they are generally inherited as diallelic Mendelian characters and therefore are useful for estimating population genetic measures with *P. capsici*.

Earlier studies suggest that outcrossing is an important component of the life history of *P. capsici* and that recombination has a significant impact on the genetic structure of populations (9,10). The data reported here support these previous conclusions, but suggest that outcrossing occurs on a local scale. This is best illustrated by the grouping of isolates into location-specific clusters. Gene flow among locations serves as a powerful evolutionary force to reduce genetic differentiation (6), and the distinct grouping of isolates based on location is typical for populations that are reproductively isolated. It is unlikely that incompatibility among the isolates from different locations is responsible because the progeny from the interregional cross (OP97 $\times$ SFF3) were all hybrid and previous crosses between isolates from separate populations suggested similar results (9). The estimated pairwise fixation indices and AMOVA analysis quantified the differences among the populations and indicated that >25% of the observed genetic variation was unique to single locations. Hartl and Clark state that migration of a small number of individuals (e.g., one to two) per

generation is generally sufficient to keep fixation indices at 0.10 or less (6). The observed pairwise fixation indices among the populations presented here suggest that movement among locations was rare. Although polycyclic disease development appears to play an important role in disease development within a single growing season, there were no members of the same clonal lineage recovered among the seven locations or among years at SW1-A or SW1-B.

The finding that movement among locations appears to be rare suggests that the efficacy of rotation may depend more on the long-term survival of *P. capsici* than on movement among locations. The fields at farm SW1 provided a unique opportunity to investigate survival and spread. Both SW1-A and SW1-B had *P. capsici* epidemics in 1999, and the only difference among the two was previous rotation patterns. SW1-A was continuously cropped to squash from 1997 to 1999. Location SW1-B was the site of a severe *P. capsici* epidemic on squash in 1994 that was followed by a soybean and corn rotation until squash was planted again in 1999. The locations are irrigated from separate wells, do not share drainage water, and plant tissue is not knowingly moved among the sites. These fields are of particular interest because they differed significantly in the proportion of mefenoxam insensitive isolates collected in 1999. Only 2 of the 141 genetically unique isolates collected from SW1-A were sensitive to mefenoxam, whereas 24 of the 35 unique isolates recovered from SW1-B were sensitive to mefenoxam. This suggests very little, if any, movement of isolates from SW1-A to SW1-B. The patterns of diversity at the DNA level clearly separate the isolates into two discrete populations and effectively rule out gene flow in 1999. The genic stability of *P. capsici* at SW1-A from 1998 to 1999, and at SW1-B from 1999 to 2000, suggests that movement into these sites was rare. In light of these findings, a reasonable explanation for the epidemic at SW1-B in 1999 is that oospores formed during the 1994 epidemic remained dormant over five winters and provided the initial inoculum. There are reports of oospores surviving extended periods for other *Phytophthora* spp. (5), and continued tracking of the *P. capsici* populations at the locations presented here should help us decipher the relative contributions of reintroduction and survival.

The finding that population differentiation increased with distance, considering the magnitude of genetic differentiation at even the closest sites, is consistent with rare founding events originating from nearby locations or from a similar source population. For example, farms SC1 and SC2 are not connected by waterways, nor do they share equipment, but both produce cucumbers for the pickling industry and utilize the same processing station.

Fig. 3. Association between the degree of genetic subdivision (pairwise Φ_{ST}) and pairwise geographical distances among *Phytophthora capsici* populations at seven locations in Michigan ($r^2 = 42.6$; $P < 0.01$).

When a semitrailer of cucumber fruit is delivered to the processing station the weight of oversized, undersized, or diseased cucumbers (culls) is estimated and the trailer is reloaded with a corresponding weight of culls sorted from previous deliveries. Traditionally, these culls are spread onto fields with a manure spreader. A single cucumber cull infected with A1 and A2 CT may contain thousands of oospores (K. H. Lamour and M. K. Hausbeck, *unpublished data*) and it is possible that transfer of infected culls may have contributed to the dissemination of *P. capsici* in Michigan. All of the locations sampled in this study had a history of *P. capsici* epidemics and investigation of a newly infested field should provide insight into the nature of founding events.

In summary, it appears that *P. capsici* persists in Michigan fields as reproductively isolated outcrossing populations in which the sexual stage is effectively linked to long-term survival. Thus, even though single genotypes have the potential to increase significantly within a single season, genic diversity is maintained over time and new gene combinations are constantly generated. Comparison of future sample sets to the baseline data presented here should provide an opportunity to further clarify the contributions of movement among locations and survival to the population structure of *P. capsici* in Michigan.

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