The Dynamics of Mefenoxam Insensitivity in a Recombining Population of *Phytophthora capsici* Characterized with Amplified Fragment Length Polymorphism Markers

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ABSTRACT

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Recent findings from Michigan suggest that recombination may play a role in the survival and evolution of sensitivity to the fungicide mefenoxam in populations of *Phytophthora capsici* on cucurbit hosts. In 1998, 63 mefenoxam insensitive isolates were recovered from a squash field in which mefenoxam had been applied. Additional isolates were recovered from untreated squash fields planted at this location in 1999 (200 isolates) and the spring of 2000 (34 isolates). Isolates from 1998 and 1999 were characterized using fluorescent amplified fragment length polymorphism (AFLP) markers and all isolates were screened for com-

patibility type and mefenoxam sensitivity. In 1998 and 1999, 92 and 71% of the isolates, respectively, had unique multilocus AFLP genotypes with no identical isolates recovered between years. Seventy-two identical AFLP markers were clearly resolved in both the 1998 and 1999 sample sets, and fixation indices for the 37 polymorphic AFLP loci indicate little differentiation between years. There was no decrease in the frequency of resistant isolates during the 2 years without mefenoxam selection. We conclude that oospores play a key role in overwintering and that the frequency of mefenoxam insensitivity may not decrease in an agriculturally significant time period (2 years) once mefenoxam selection pressure is removed.

Additional keywords: fungicide resistance, genetic diversity, population genetics.

Crown, root, and fruit rot caused by *Phytophthora capsici* is increasing in Michigan cucurbit production fields, and unfested land suitable for rotation is becoming increasingly scarce, especially in areas undergoing rapid urban development. The phenylamide fungicide (PAF) mefenoxam is a systemic fungicide that appears to be acting at the level of DNA translation, and is fungistatic to fully sensitive isolates of *P. capsici* (2,13). Although mefenoxam has been considered by some growers to be helpful, mefenoxam insensitive isolates were reported on bell peppers in North Carolina and New Jersey by Parra and Ristaino in 1998 (18) and have since been recovered from 10 of 11 farms sampled in Michigan (13), as well as, in Georgia (15) and southern Italy (19). Mefenoxam insensitivity in Michigan *P. capsici* isolates is inherited as a single gene exhibiting incomplete dominance (13), which is consistent with the reports for a variety of other oomycetous organisms (2). Investigations with *P. infestans* indicate that insensitivity may be conferred by genes at different chromosomal positions (5), suggesting that the basis of insensitivity in different populations may not be identical. Sexual recombination, in particular, has the potential to impact management strategies that employ PAFs because the fully insensitive (two copies of the insensitivity allele) phenotype may be directly generated. *P. capsici* is heterothallic and the sexual stage is initiated when isolates of opposite compatibility type, designated A1 and A2, come into close association to form thick-walled oospores (4). The asexual stage includes the production of caducous sporangia born on long pedicels, which may release motile zoospores if free water is present. Asexual spores are thought to be responsible for the polycyclic nature of disease development (20).

PAF resistance in the genus *Phytophthora* and, in particular, the *P. infestans*–potato pathosystem, is well documented (2,4,9). Until recently, the population structure of *P. infestans* appeared to be largely clonal outside of *P. infestans* putative center of origin (6). The recent detection of both *P. infestans* compatibility types along with increased genotypic diversity in some potato growing regions indicates that the sexual stage is likely active and may significantly impact control strategies that have proved useful in the past (3,8). When PAF resistance in European *P. infestans* populations increased significantly in the early 1980s, the efficacy of the PAF metalaxyl was only regained after the product was not made available to growers for a period of time (2). This strategy apparently allowed the resistant populations to decline or become extinct and depends on ephemeral populations or, in the case of resident populations, upon a significant cost for resistance outside of selection pressure. A recent study of sensitive versus PAF resistant *P. nicotianae* isolates from citrus suggests negligible fitness costs for PAF resistance and reports that 2 years without PAF use did not reduce the proportion of resistant isolates in groves (21). Kadish and Cohen report that PAF-resistant *P. infestans* isolates in Israel were more aggressive in colonizing tuber tissue than sensitive isolates (12).

Novel techniques have been developed recently that allow characterization of DNA-level polymorphism in organisms for which little is known about the genome. An example is the amplified fragment length polymorphism (AFLP) technique introduced by Vos et al. in 1995 (23). This technique relies on restriction enzyme fragmentation of genomic DNA with the concomitant ligation of synthetic adaptors to the DNA fragment ends. Stringent polymerase chain reaction (PCR) amplification using adaptor-complementary primers with additional selective nucleotides allow for the amplification of fragment subsets. DNA fragment subsets are termed fingerprints and may be resolved with a range of techniques (1). AFLP markers have been used on a variety of organ-

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isms (14,22) and the procedure generates a large number of reproducible markers (1,22). The limitation that these markers are generally scored as dominant markers (e.g., either present or absent) for diploid organisms requires the use of relatively large sample sets (11,25).

Our null hypotheses are that sexual recombination has a significant impact on the population structure of *P. capsici* in Michigan and that mefenoxam insensitivity may not decrease in the time frame of a typical 2-year rotation outside of mefenoxam selection pressure.

MATERIALS AND METHODS

Field plot. Research was conducted on a commercial farm in southwest Michigan, with a history (>11 years) of *P. capsici* on bell peppers and squash and intensive use of PAF. The 4.05-ha field sampled had previously been cropped to soybeans and corn with no known record of *P. capsici* susceptible crops (e.g., tomatoes, peppers, or cucurbits) prior to 1997. During 1997 and 1998, yellow squash and zucchini grown in this field became diseased with *Phytophthora* crown, root, and fruit rot and the grower applied mefenoxam as part of a disease management strategy (Novartis, Greensboro, NC). In 1998, all isolates recovered were either intermediately or fully insensitive to mefenoxam. Both A1

TABLE 1. Fixation indices (F_{ST}) for 37 amplified fragment length polymorphism loci from unique *Phytophthora capsici* isolates collected from a single Michigan cucurbit field during 1998 ($N = 57$) and 1999 ($N = 141$)

Fragment ^a	1998 f(aa) ^b	1999 f(aa)	F_{ST} ^c
45	0.02	0.06	0.018
54	0.29	0.29	0.000
64	0.82	0.55	0.048
104	0.11	0.06	0.007
106	0.11	0.04	0.025
110	0.41	0.36	0.002
130	0.41	0.30	0.009
146	0.47	0.24	0.038
149	0.12	0.27	0.029
154	0.39	0.31	0.004
156	0.53	0.83	0.054
172	0.56	0.33	0.034
189	0.16	0.56	0.121
192	0.16	0.37	0.044
193	0.35	0.20	0.022
211	0.47	0.15	0.088
241	0.48	0.32	0.018
256	0.04	0.01	0.022
258	0.43	0.49	0.002
261	0.55	0.54	0.000
270	0.57	0.41	0.015
282	0.35	0.40	0.002
285	0.51	0.73	0.030
314	0.51	0.34	0.019
320	0.41	0.51	0.006
333	0.16	0.20	0.002
346	0.36	0.33	0.001
361	0.33	0.49	0.017
383	0.21	0.15	0.005
418	0.40	0.34	0.002
431	0.34	0.32	0.001
438	0.67	0.45	0.028
454	0.65	0.49	0.015
492	0.29	0.40	0.009
504	0.51	0.47	0.001
511	0.38	0.28	0.007
548	0.78	0.78	0.000

^a EcoRI-AC/MseI-CA selectively amplified fragment size in base pairs.

^b Observed frequency of the absent state where "a" represents the absence of a fragment.

^c F_{ST} calculated from estimated allele frequencies. According to Wright's qualitative guidelines, values from 0 to 0.05 indicate little genetic differentiation and values from 0.05 to 0.15 indicate moderate genetic differentiation.

and A2 compatibility types were present, and oospores were detected in diseased fruit. In 1999 and 2000, yellow squash was established in a 1,124-m² experimental plot in this field, and mefenoxam was not applied. Diseased plants and fruit were sampled on 20 August 1998 (63 isolates from entire field), June through August 1999 (200 isolates from experimental plot), and 13 July 2000 (34 isolates from experimental plot). All isolates were recovered from single diseased plants or fruit.

Isolate collection and maintenance. 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 Bacto agar) plates. Procedures for obtaining single zoospore isolates were as previously described (13). Single zoospore cultures were maintained on 30 ppm of rifampicin and 100 ppm of ampicillin (RA)-UCV8 plates and transferred bimonthly. Long-term storage consisted of a single 7-mm plug of expanding mycelium from each single zoospore culture being placed in a 1.5-ml microfuge tube with one sterilized hemp seed and 1 ml of sterile distilled water, incubated for 2 to 3 weeks at 23 to 25°C, and stored at 15°C long term.

Phenotypic characterization. Isolates were screened for compatibility type as previously described (13). Mefenoxam sensitivity was characterized according to the in vitro screening technique described by Lamour and Hausbeck (LH technique) for *P. capsici* isolates in Michigan (13). Isolates were scored as sensitive (S) if growth on UC-V8 agar amended with 100 ppm of mefenoxam was less than 30% compared with a control, as intermediately sensitive (IS) if between 30 and 90%, and fully insensitive (I) if greater than 90% compared with the unamended control. These mefenoxam sensitivity categories are based on a trimodal distribution of 523 field isolates of *P. capsici*. Clear modal distributions were only attained when screening was conducted with a single high rate of mefenoxam-amended (100 ppm) media (K. Lamour, unpublished data). These putative mefenoxam sensitivity categories were tested by in vitro crosses (I × S, IS × IS, IS × S, and S × S), and chi-square analysis confirmed that the observed progeny numbers were not significantly different than expected for Mendelian inheritance of an incompletely dominant trait (13).

The LH technique differs from a commonly used method described by Goodwin, Sujkowski, and Fry (GSF technique) (9) for *P. infestans* which uses two levels of amended media (5 and 100 ppm) to differentiate the three mefenoxam sensitivity phenotypes and which has been used to characterize *P. capsici* isolates (15,18,19). Unfortunately, analysis of our in vitro crosses and field isolates by the GSF technique did not resolve a clear modal distribution (K. Lamour, unpublished data). Assignment of Michigan *P. capsici* isolates to the S category was the same whether using the LH or GSF technique. The only difference was that some *P. capsici* isolates from Michigan rated as fully insensitive by the GSF technique were rated as intermediately sensitive by the LH technique.

DNA extraction and AFLP fingerprinting. A technique for avoiding bacterial contamination prior to growing isolates for DNA extraction was implemented using a modified Van Teigham cell (4). The uppermost portion of a 7-mm plug of mycelium was placed onto the surface of RA-WA plates (30 ppm of rifampicin, 100 ppm of ampicillin, 1,000 ml of distilled water, and 16 g of Bacto 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 media. 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 × 15 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 (Dneasy; Qiagen Inc., Valencia, CA) according to the manufacturers directions. DNA was quantified (Nucleic Acid QuickSticks; Clontech, Palo Alto, CA) according to the manufacturers directions and approximately 100 ng of DNA was subjected to a restriction/ligation reaction, preselective amplification, and selective amplifications using the PCR core mix, adaptor sequences, core primer sequences, and fluorescent-labeled primers available in an AFLP microbial fingerprinting kit (Perkin-Elmer Applied Biosystems, Foster City, CA, henceforth referred to as PE/ABI) and performed exactly as described in protocol part 402977 Rev A (23). 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 (13). 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 carboxy-fluorescein (FAM), carboxytetramethylrhodamine (TAMRA), or carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) fluorescent dyes. The fluorescent dyes are excited by laser radiation and visualized by their characteristic absorption-emission frequencies. Only the fragments containing an *EcoRI* restriction site are resolved.

Products from three reactions labeled with different colored dyes and a carboxy-X-rhodamine (ROX) size standard were loaded into each lane on a denaturing polyacrylamide gel and the fragments resolved in a DNA sequencer (ABI Prism 377). 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 or absence profiles were scored.

A single isolate, OP97, was subjected to the aforementioned protocol using three primer pair combinations that were chosen as optimal on three separate occasions, approximately 3 months apart, to test for reproducibility of AFLP profiles.

Clone detection and cluster analysis. AFLP fragments were considered polymorphic if the most common allele was present in less than 95% of the isolates from a given sample set and scored for presence (1) or absence (0) (10). AFLP fragments present in more than 95% of the isolates from a given sample set were considered monomorphic. Analysis of the resulting binary data matrix was performed using NTSYS-pc version 2.02k (Exeter Software, Setauket, NY). Unweighted pair group method with arithmetic averages cluster analysis was performed on the matrix of similarity coefficients calculated from all possible pairwise comparisons of individuals within and among the 1998 and 1999 populations and a tree generated. Isolates showing complete homology at all loci were considered to be clones and except for a single representative isolate were excluded from frequency calculations.

Allele frequency and fixation indices. Allele frequencies for AFLP markers were estimated utilizing the expected relationship between gene and genotype frequencies in a randomly mating population (i.e., Hardy-Weinberg proportions). The frequency of the recessive (absent) allele (q) was calculated from the observed number of recessive homozygote individuals (X) in a sample of n individuals by the formula for dominant markers described by Jorde et al. (11):

$$\hat{q} = \sqrt{x + \frac{1-x}{4n}}$$

where $x = X/n$ is the observed proportion of individuals that do not display the dominant (present) marker phenotype. In order to test whether the composite genetic profiles from 1998 and 1999 were consistent with a single randomly mating population, the fixation index was calculated for each AFLP loci from the variance in allele frequencies according to the following formula: $F_{ST} = [(p_1 - p_2)^2/4]/(\text{average } p \times \text{average } q)$, where p is the allele frequency for the present state with p_1 and p_2 indicating the two sample populations, and q is the allele frequency for the absent state (10). Fixation indices for individual loci were interpreted according to the qualitative guidelines suggested by Wright (24), where the range 0 to 0.05 indicates little genetic differentiation, range 0.05 to 0.15 indicates moderate genetic differentiation, and greater than 0.25 indicates great genetic differentiation (10).

RESULTS

AFLP band characterization. Evaluation of the 16 *EcoRI* + 2-*MseI* + 2 selective primer pair combinations indicated that *EcoRI* + AC-*MseI* + CA gave the most clearly resolved fragment profile and was used to amplify genomic DNA from all isolates in both the 1998 and 1999 sample sets. This primer combination resulted in 72 clearly resolved fragments of which 37 (51%) fragments were polymorphic in both 1998 and 1999 (Table 1). All 72 fragments were present in both 1998 and 1999 and no novel fragments were detected between years. The following 35 fragments (size in base pairs) were monomorphic in both the 1998 and 1999 sample sets: 41, 43, 47, 49, 58, 66, 70, 82, 85, 114, 118, 123, 133, 135, 140, 159, 174, 235, 247, 249, 272, 278, 295, 298, 300, 341, 351, 355, 367, 402, 474, 488, 502, 519, and 527. AFLP profiles for isolate OP97, generated from separate DNA extractions on three separate occasions over a 1-year period, resulted in identical banding patterns with the only difference being minor changes 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.

Phenotypic, genotypic, and gene diversity. No isolates sensitive to mefenoxam were recovered in 1998 or 2000, and single A1 sensitive and A2 sensitive isolates were recovered in 1999 (Table 2). In 1998, 18% of the isolates were intermediately sensitive and 82% were insensitive, in 1999, 2% were sensitive, 28% were intermediately sensitive and 70% were insensitive, and in 2000, 15% of the isolates were intermediately sensitive and 85% were insensitive to mefenoxam (Table 2).

In 1998, 57 of the 63 isolates recovered, and 141 of the 200 isolates recovered in 1999 were unique based on multilocus AFLP profiles. No identical multilocus genotypes were recovered between 1998 and 1999. Five isolates (two A2/I, two A2/IS, and

TABLE 2. Phenotypic diversity of *Phytophthora capsici* isolates recovered from the same cucurbit field in 1998, 1999, and 2000

Year ^a	No. of isolates ^b	Compatibility type and mefenoxam sensitivity ^c					
		A1/S	A1/IS	A1/I	A2/S	A2/IS	A2/I
1998	57	—	4	31	—	6	16
1999	141	1 (2)	17 (20)	57 (53)	1 (1)	23 (18)	42 (47)
2000	34	—	2	8	—	3	21

^a Mefenoxam was applied in 1998 but not in 1999 or 2000.

^b Sample sets from 1998 and 1999 consist of unique multilocus genotypes as determined with amplified fragment length polymorphism fingerprinting. The 2000 sample set was recovered at the beginning of the growing season and was not fingerprinted.

^c S = sensitive, IS = intermediately sensitive, and I = insensitive as determined by in vitro screening on 100 ppm of mefenoxam-amended agar. Numbers in parentheses indicate the expected number of isolates when mefenoxam insensitivity is assumed to be controlled by a single incompletely dominant gene in Hardy-Weinberg equilibrium unlinked to compatibility type.

one A1/I) of *P. capsici* collected in 1998 had one clonal representative. Fourteen isolates collected in 1999 had between two and four clones (Table 3). A single A1 compatibility type insensitive isolate had 40 clones recovered over the course of the 1999 season and comprised 3% of the early, 15% of the mid-, and 43% of the late sampling intervals (Table 3). The 1999 sampling intervals (early, mid, and late) are based on the dates of sampling and are not intended to reflect stages of plant growth or the epidemiology of *P. capsici*. Cluster analysis of AFLP fingerprint variation indicated no significant clustering of isolates between 1998 and 1999.

The majority (98%) of the 37 polymorphic AFLP fragments showed little genetic differentiation ($F_{ST} < 0.05$) between 1998 and 1999 according to Wrights qualitative criterion (Table 1) (24).

DISCUSSION

P. capsici causes significant damage to cucurbit hosts in Michigan each year. In an effort to prevent or control epidemics, many growers have used either metalaxyl or the newer, but similarly acting compound, mefenoxam as a part of their disease management strategy. This study was initiated in an effort to address the concerns of growers who have high levels of mefenoxam insensitivity.

Phenotypic data (mefenoxam sensitivity and compatibility type) from a 1998 survey suggested that insensitivity to mefenoxam was common and that some level of recombination is occurring in the field (13), but without the application of additional polymorphic markers our ability to assess population structure was severely restricted. AFLP analysis proved to be a powerful tool for resolving the population dynamics of *P. capsici*. A single selective primer combination, *EcoRI*-AC-*MseI*-CA, generated 72 bands of which 37 were polymorphic in our 1998 and 1999 sample sets. AFLP fingerprinting, in conjunction with temporal sampling, provided a useful characterization of *P. capsici* from one season to the next and allowed us to track asexual disease development over the course of a single season.

Our data suggests that sexual recombination significantly impacts the structure of this *P. capsici* population. The finding that 198 of the 262 isolates recovered between 1998 and 1999 had unique multilocus AFLP genotypes is consistent with the high level of genotypic diversity expected in an outcrossing population

(7,16,17). Although clonal reproduction occurred in 1998 and 1999, no identical genotypes were recovered between years, suggesting that oospores are important for overwintering. The finding that 35 of the 37 polymorphic fragments exhibited very little differentiation (i.e., change in allele frequency) based on the estimated fixation indices between 1998 and 1999 is consistent with the expectations for a recombining population large enough to avoid dramatic changes due to genetic drift.

In 1999 and 2000, sensitive and intermediately sensitive isolates (42 of 175) did not increase in a manner suggesting selection in favor of mefenoxam sensitivity outside of mefenoxam selection pressure. The fact that 14 of the 15 isolates with clonal reproduction in 1999 were fully insensitive may be another indication that mefenoxam insensitivity does not have significant costs outside of mefenoxam selection pressure. If we assume that there is only a single mefenoxam insensitivity gene in this population unlinked to compatibility type, designated *I*, and that this population is effectively free from the effects of migration and genetic drift, some interesting speculations can be made. For instance, in 1999, if the mefenoxam sensitivity phenotypes are assumed to represent genotypes (e.g., a fully insensitive isolate has two copies of the *I* allele) then the frequency of *I* can be estimated and the observed number of unique isolates that fall into each of the six mefenoxam sensitivity/compatibility type categories can be compared with the expectations under Hardy-Weinberg equilibrium. In 1999, the estimated frequency of *I* was 0.84, and chi-square analysis, using the data in Table 2, indicates that the observed numbers do not differ from those expected under Hardy-Weinberg equilibria at $P = 0.50$ ($\chi^2 = 3.09$, $df = 4$). Although this is not a particularly powerful test due to the large number of assumptions (10), it does lend support to the hypothesis that this population meets the criterion for panmixia.

Our results do not allow us to reject the null hypothesis that sexual recombination significantly impacts the structure of this population. It appears that sexual recombination plays a significant role in maintaining genotypic and gene diversity while concomitantly producing overwintering inoculum. Our data also suggest that sexual recombination may serve as a potent force for integrating a beneficial allele based on the finding that there were a total of 133 unique multilocus genotypes fully insensitive to mefenoxam between 1998 and 1999. An interesting question that can only be answered by following a fully sensitive population as it shifts to insensitivity is how much genetic diversity is lost, if any, during the PAF selection process? The question of how long mefenoxam resistance will remain in a population of *P. capsici* when selection pressure is removed can only be answered in a tentative way. It appears that in this population, insensitivity will not decrease within the time frame of a typical 2-year rotation and, once resistance to mefenoxam is established, the future usefulness of this fungicide may be extremely limited.

Comparison of the population structure reported at this single location is currently being compared with other locations in Michigan and the United States and should provide useful insight into the amount of genetic diversity in sensitive versus insensitive populations as well as the contribution of migration to *P. capsici* population structure.

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TABLE 3. Clone contribution of 15 *Phytophthora capsici* isolates to the total number of isolates collected in 1999 ($N = 200$)

Isolate	No. of clones ^a	CT/MS ^b	No. of clones in early, mid, and late season ^c		
			6/22 – 7/16 $N = 60$	7/20 – 8/3 $N = 80$	8/5 – 8/18 $N = 60$
JP571	2	A1/I	2	–	–
JP583	2	A1/I	2	–	–
JP944	3	A1/I	2	1	–
JP999	3	A1/I	2	1	–
JP1007	2	A1/I	1	1	–
JP1042	2	A2/I	1	1	–
JP1096	2	A1/I	–	1	1
JP1102	2	A2/I	–	2	–
JP1215	3	A2/I	3	–	–
JP1342	2	A2/IS	–	2	–
JP1369	2	A1/I	1	1	–
JP1384	4	A2/I	3	1	–
JP1512	2	A1/I	1	–	1
JP1555	3	A1/I	–	–	3
JP1632	40	A1/I	2	12	26

^a Total number of isolates with identical multilocus amplified fragment length polymorphism profiles.

^b CT = compatibility type and MS = mefenoxam sensitivity where S = sensitive, IS = intermediately sensitive, and I = insensitive as determined by in vitro screening on 100 ppm of mefenoxam-amended agar.

^c Sample intervals based on sampling dates only.

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