Genetic Structure of *Clavibacter michiganensis* subsp. *michiganensis* Populations in Michigan Commercial Tomato Fields

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Abstract

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Clavibacter michiganensis subsp. *michiganensis*, causal agent of bacterial canker of tomato, is distinguished into four fingerprint types (A, B, C, and D) using BOX-polymerase chain reaction (PCR). To characterize the variation within the *C. michiganensis* subsp. *michiganensis* population in Michigan, 718 strains of *C. michiganensis* subsp. *michiganensis* were isolated from infected foliage and fruit collected from 14 and 9 Michigan commercial tomato fields in 1997 and 1998, respectively. The frequency of PCR types detected with BOX-PCR in all strains, and Bayesian cluster analysis, pairwise differentiation index comparisons, and genetic diversity estimates of 96 strains genotyped for six virulence-related genes revealed that *C. michiganensis* subsp.

michiganensis populations in Michigan tomato fields are geographically structured. A multilocus haplotype cladogram was also consistent with geographic stratification in *C. michiganensis* subsp. *michiganensis* populations. Some regions had strains predominantly of only one PCR type or belonging mostly to one genetic cluster, while other regions presented more diversity of occurrence of PCR types and genetic clusters. Results derived from this study provide information about the genetic structure of *C. michiganensis* subsp. *michiganensis* populations in Michigan and genetic diversity of *C. michiganensis* subsp. *michiganensis* inocula, which is key in developing disease management strategies.

Clavibacter michiganensis subsp. *michiganensis* causes bacterial canker of tomato (50), a destructive disease that constitutes a threat to the tomato industry worldwide. *C. michiganensis* subsp. *michiganensis* was first reported in a tomato greenhouse in Michigan in 1909 and it is currently found in most tomato-growing regions (52,54). Although *C. michiganensis* subsp. *michiganensis* can also infect other members of the family Solanaceae, including pepper, tomato is the only crop on which bacterial canker is economically important (40). Processing-tomato growers in Michigan can suffer losses as high as \$300,000 per grower in a single year when conditions are favorable for disease (19). Bacterial canker symptoms include marginal leaf necrosis that may be bordered by chlorosis, fruit lesions (superficial white spots [3 to 6 mm] that develop a necrotic center), plant wilting, stunting, reduced yields, and plant death (19).

The pathogen may be seedborne and overwinters in fields of the north-central United States for up to 2 years, providing a source of inocula during the growing season (15). *C. michiganensis* subsp. *michiganensis* can be transmitted to emerging seedlings (4) because it survives on the surface and in the layers of the seed coat (5,54). *C. michiganensis* subsp. *michiganensis* populations thrive in humid greenhouses and may be spread to uninfected plants via overhead watering (19). In the Midwest, tomato transplants are grown in local greenhouses to later establish production fields. Greenhouse transplants can look healthy but may harbor bacterial populations that can cause severe disease symptoms in the field (19). Epiphytic populations on field plants may spread by water splashing, pesticide spraying, pruning, tying to stakes or trellises, sand blasting, and harvesting (15).

Bacterial canker may be prevented by using certified plant material (23) or applying preventive antibiotic or copper compounds in the greenhouse and the field (60). No commercial varieties with effective resistance to *C. michiganensis* subsp. *michiganensis* have been developed to date (62). Characterizing the population structure and genetic diversity of *C. michiganensis* subsp. *michiganensis* is needed to determine possible inoculum sources and design effective disease management strategies. Repetitive-sequence-based polymerase chain reaction (PCR) with a BOX primer (BOX-PCR) has been used to differentiate *C. michiganensis* subsp. *michiganensis* isolates into four PCR types (A, B, C, and D), which has provided valuable information about the genetic diversity of populations (25,29,35,40). Several studies have tried to find associations between *C. michiganensis* subsp. *michiganensis* genotypes and tomato cultivar, region, or year of isolation using phylogenetic methods but no significant correlation has been detected (8,40). Because genes involved in pathogen virulence have been shown to be under diversifying or positive selection that can obscure evolutionary relationships among individuals, neutrally evolving markers have been preferred for populations and evolutionary studies (2,24,28,33,53). Nonetheless, several studies have recognized the potential usefulness of virulence-related genes to study plant pathogen populations because they can provide information on adaptation processes that can shape population structure (25,39,43). The biological significance of the four PCR types detected using BOX-PCR is unknown but these markers are not strongly related to virulence because virulent as well as avirulent *C. michiganensis* subsp. *michiganensis* strains can have identical PCR types (29).

In this study, we aimed to investigate the genetic structure of *C. michiganensis* subsp. *michiganensis* populations in Michigan commercial tomato fields by using genotype data from BOX-PCR and six virulence-related genes. More specifically, we sought to (i) characterize the genetic variation of strains within and among fields using BOX-PCR fingerprinting, (ii) determine if we could detect population structure using Bayesian cluster analysis in a sample of isolates genotyped for six virulence-related genes and PCR type, and (iii) examine the distribution of genotypes from

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each inferred genetic cluster in the sample of isolates within predefined geographic and PCR type categories.

Materials and Methods

Sample collection. Foliar and fruit samples were collected from 14 and 9 commercial fields from four regions of Michigan in 1997 and 1998, respectively (Supplementary Table 1). Samples were arbitrarily collected according to cultivar or location in a field. One exception was a field in southwest Michigan (SW1/1/97), where individual plants were flagged and sampled along diagonal transects in July, August, and October 1997. Foliar samples consisted of two to three lower leaves exhibiting bacterial canker foliar symptoms. Fruit exhibiting bird's-eye lesions were collected. All samples were placed in plastic bags, transported in ice chests, and stored at 4°C for a maximum of 3 days before processing.

Pathogen isolation. Stems and leaves were chopped with a sterile single-edged razor blade, weighed, and homogenized for 2 min in a Lab-Blender 400 stomacher (Tekmar Co., Cincinnati, OH) in sterile phosphate buffer (0.05 M, pH 7.4; 2 ml/g of plant tissue) amended with Tween-20 detergent (0.02%). The plant extract (1 ml) was mixed with 0.5 ml of 40% glycerol (vol/vol) and the resulting suspension stored at -20°C (9). Each sample was subjected to a 10-fold serial dilution and plated onto nutrient broth yeast extract agar modified by omitting glucose (MNBY) and semiselective media for *C. michiganensis* subsp. *michiganensis* (SCM) (11), both amended with nalidixic acid (0.03 g/liter) and cycloheximide (0.1 g/liter). The plant extract (1 ml) was vortexed with 0.5 ml of 40% glycerol and stored at -20°C. After a pure culture was obtained, each isolate was inoculated into 1 ml of a sterile solution of distilled water (19%), tryptic soy broth (78%), and glycerol (3%). Fruit lesions resembling bird's-eye lesions were probed using sterilized toothpicks directly under the surface of the lesion and the toothpicks were streaked on MNBY. Colonies resembling *C. michiganensis* subsp. *michiganensis* were stored at -20°C in the *C. michiganensis* subsp. *michiganensis* culture collection maintained in the laboratory of Dr. Hausbeck at Michigan State University (MSU) and subjected to rep-PCR within a 2-year period as previously described (9).

Pathogen fingerprinting by rep-PCR. Strains were isolated from approximately 30 individual plant or fruit samples from each field and analyzed using the direct-colony rep-PCR method described by Louws et al. (29), with modifications. Isolates for PCR amplification were grown on either MNBY or SCM agar. Amplification was performed twice in a thermal cycler (Genemate-Technie, Princeton, NJ). Reactions included DNA AmpliTaq polymerase (0.4 µl; Perkin Elmer, Norwalk, CT) and the BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') primer (32) (MSU Macromolecular Structure Facility, East Lansing, MI). PCR products (6 µl) were analyzed by electrophoresis in 1.5% agarose gels in 0.5× Tris-acetate-EDTA (0.4 M Tris-acetate and 1 mM EDTA, pH 8.0) buffer at 4°C and 83 V for 13 h. The gels were stained with ethidium bromide (5 µg/ml) for visualization and compared with a 100-bp ladder (Invitrogen, Carlsbad, CA). Genomic DNA isolated from strain 936 (29) of PCR type A was included as a positive control, and a negative control consisting of the reaction mixture without bacterial cells was included with each run. Gel photographs were analyzed by eye to determine the designation of PCR fingerprint types with the corresponding strains.

Primer design, amplification, and sequencing of genes. A sample of 96 isolates representing different PCR types found in four regions sampled was selected for population structure analysis using two plasmid genes known to be involved in virulence (*celA* and *pat-1*) as well as four chromosomal genes that may be related to virulence (*ABCtransporter*, *mop*, *perforin*, and *phosphataseC*) (Supplementary Table 5). Sequences for the selected six genes with virulence-related annotation were obtained from the *C. michiganensis* subsp. *michiganensis* genome (13) for primer design using the software Primer3 (51) (Supplementary Table 6). Cultures were grown in Luria-Bertani broth (Invitrogen) amended with nalidixic acid (0.03 g/liter) and cycloheximide (0.1 g/liter). Then, 1

µl of cells was transferred to PCR tubes containing 5 µl of sterile water and heated at 95°C for 10 min in a programmable Eppendorf mastercycler ep systems thermal cycler (Eppendorf, Westbury, NY). The PCR mix was directly added to this suspension. Pairs of specific primers for each gene were used for PCR. Direct-colony PCR reactions were performed in a total volume of 25 µl containing, 5 µl of boiled bacterial cells, 5 µl of 5× PCR reaction buffer (Invitrogen), 1.25 µl of 25 mM MgCl₂ (Invitrogen), 0.5 µl of 10 mM dNTP mix (Invitrogen), 1 µl of each 10 µM primer (MSU Macromolecular Structure Facility), 0.7 µl of Platinum Taq DNA polymerase (Invitrogen), and 10.5 µl of sterile water. The PCR reaction was performed in a programmable Eppendorf mastercycler ep systems thermal cycler (Eppendorf) under the following protocol: 3 min of denaturation at 94°C; followed by 45 cycles at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 60 s; with a final extension step of 10 min at 72°C. PCR products were analyzed by electrophoresis in 2% (wt/vol) agarose gel in 0.5× Tris-borate-EDTA buffer (31), stained with ethidium bromide (5 µg/ml) for visualization, and compared with a 100-bp ladder (Invitrogen). Controls with extracted DNA and water instead of cells as a template were included. PCR products were cleaned for direct sequencing using the QIAquick PCR purification kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Cycle sequencing reactions were done directly from the clean PCR products twice with the forward primer in the MSU Research Technology Support Facility (East Lansing, MI) using an ABI PRISM 3100 Genetic Analyzer.

Sequence analysis. Manual editing of base calls and sequence alignments were performed using Lasergene SeqMan Pro (version 8.0; DNASTAR Inc., Madison, WI). Alignments were exported as FASTA files and imported into MacClade (30) to generate NEXUS files required for subsequent analyses. Sequences were compared with sequence data publicly available by using nucleotide BLAST (1) to confirm that sequences corresponded to *C. michiganensis* subsp. *michiganensis* target genes. Sequences from each gene were analyzed individually and concatenated and collapsed into unique haplotypes using DnaSPv5 (27). Haplotypes found for each gene were deposited in GenBank under accession numbers HQ636574 to HQ636603. Base substitutions were classified as phylogenetically informative or uninformative, transitions or transversions, and synonymous or nonsynonymous substitutions using DnaSPv5.

DNA sequence variability, neutrality tests, recombination, and genealogies. Polymorphism, neutrality, and recombination analysis were performed for strains within geographic (northeast, north-central, southeast, and southwest) and PCR type (A, B, C, and D) categories using the program DnaSPv5. Sequence diversity estimates and statistics, including the number of segregating sites (*s*) and haplotypes (*h*), haplotype diversity (*Hd*) (41), Tajima's π (55), average number of pairwise nucleotide differences (*k*) (55), and Watterson's θ (θ_w) per gene (59), were calculated. Sequence variation was tested for deviations from neutrality by using Fu and Li's *D* and *F* and Tajima's *D* (12,56). The recombination parameter (*R*) per gene (20) and minimum number of recombination events (*RM*) (21) were estimated. A neighbor-joining (NJ) tree was generated using MEGA4 (57) with default settings. Node support was assessed by running 1,000 bootstrap replicates. The NJ tree was imported into Dendroscope (22) to generate a radial cladogram of *C. michiganensis* subsp. *michiganensis* concatenated data haplotypes.

Population subdivision analysis. Population subdivision was assessed with the model-based Bayesian cluster algorithm implemented in Structure 2.3X (46). The values for burnin, chain replication, and lambda were set at 200,000, 100,000, and 1, respectively, based on results obtained in preliminary analyses. The optimal number of populations (*K*) was determined by comparing posterior distribution likelihoods among three independent runs of *K* = 1 to *K* = 10 using the established parameters. Data included all genes individually coded as haplotypes, which were analyzed under the admixture model with correlated allele frequencies and without previous population information. Population structure

figures with prior population information, obtained from the defined geographic and PCR type categories and sorted by Q, were generated using the Population Sorting Tool, a graphic editing program created in R (47) to visualize the distribution of clusters in predefined categories (J. J. Morrice, *unpublished data*). Genetic differentiation indexes (F_{ST}) were calculated for data grouped by geographic and PCR type categories using DnaSPv5. Statistical significance was determined for each index by running 5,000 permutations ($\alpha = 0.05$).

Results

In total, 718 *C. michiganensis* subsp. *michiganensis* strains were obtained from sampled tomato fields located in southwest (SW), north-central (NC), northeast (NE), and southeast (SE) Michigan.

The most commonly observed *C. michiganensis* subsp. *michiganensis* strain in SW Michigan was type C (302 of a total of 319 strains), regardless of the number of cultivars present in a field (Supplementary Tables 1 and 2). In 1997, the SW fields containing only 'Mountain Spring' tomato (SW/1/1, SW/1/2, and SW/2/1) or Mountain Spring and Roma-type tomato (SW/3/1) harbored *C. michiganensis* subsp. *michiganensis* strains representing primarily the type C strains (27 to 30 strains/field) and a few type B strains (0 to 3 strains/field). Field SW/4/1/97 contained at least four cultivars; strains were mostly type C strains (20 strains), although type A strains (8 strains) were also present and two plants had a mix of type A and C strains. In 1998, two fields (SW/1/2 and SW/2/3), each containing only Mountain Spring tomato, harbored only type C strains.

Fig. 1. Estimated population structure of 96 *Clavibacter michiganensis* subsp. *michiganensis* strains collected from tomato fields in Michigan. The same isolates are sorted by **A**, region and **B**, polymerase chain reaction (PCR) type. Each strain is represented by a single vertical bar, which is divided into colored segments with lengths representing proportional estimated membership of the isolate in each of the three clusters identified by Bayesian clustering. Colors correspond to clusters as follows: green = cluster 1, light blue = cluster 2, and dark blue = cluster 3. Region coding corresponds to NE, northeast; NC, north-central; SW, southwest; and SE, southeast. PCR type was assigned according to PCR using BOX primer. **C**, Percentage of each PCR type within each region.

C. michiganensis subsp. *michiganensis* strains of type C were also the dominant type in yearly samplings of SW fields. When Mountain Spring tomato was planted in the same fumigated field in consecutive years (SW/1/2/97-98), the C fingerprint type was dominant in 1997 (29 strains) and 1998 (26 strains), with only one type B strain found in 1997 (Supplementary Table 3). The same trend of dominance of C-type *C. michiganensis* subsp. *michiganensis* strains was also observed in monthly samplings of SW fields. When a commercial Mountain Spring tomato field (SW/1/1/97) was sampled three times over the course of a growing season, the *C. michiganensis* subsp. *michiganensis* PCR types remained relatively stable and consisted primarily of type C (26 to 29 strains/sample time) and a few type B strains (0 to 3 strains/sample time) (Supplementary Table 4). Of the 31 plants sampled, 3 had both type B and C strains over the time sequence.

All strains from the only NE farm sampled (NE/1/1) were of type D, regardless of number of tomato cultivars or year of sampling. This farm was planted to 9 and 14 tomato cultivars during 1997 and 1998, respectively. In spring 1998, some fruit produced in a greenhouse on this grower's farm had symptoms of bird's-eye spots from which *C. michiganensis* subsp. *michiganensis* type D strains (GH1f, GH2-4, and GH3-5) were isolated.

The PCR type of *C. michiganensis* subsp. *michiganensis* strains sampled in four fields planted to one to three tomato cultivars in NC presented spatial variation. In 1997, the strains isolated from

fields (NC/1/1 and NC/1/2) were primarily type C (≥ 28), with a few type D strains (≤ 2). Conversely, in 1998, the same grower's fields (NC/1/3 and NC/1/4) contained mostly type D strains (≥ 14) and a few type C strains (≤ 9) (Table 1).

Similar numbers of A, B, and C PCR types were found on multiple cultivars sampled from fields in SE Michigan. The obtained strains presented spatial and temporal variation in PCR type. In field SE/1/2 in 1997, the type A fingerprint was dominant (10 of 13), with one type B and two type C strains detected in low numbers. When the same field was sampled the following year, 12 type A strains and an increased number of type B strains (16 strains) were detected. Two plants in field SE/1/1/97 harbored mixed PCR type strains; one with A and B and another with B and C. Field SE/3/1/97 contained one plant with mixed A and C fingerprint types and one plant with B with C types. In field SE/5/1/98, three plants had mixed A and C strains and five plants had mixed B and C strains.

A set of 96 strains was selected to perform population structure analyses using sequencing data from two plasmid virulence genes and four chromosomal genes that may be related to virulence. Sequence data from the six genes revealed 58 polymorphisms, 17 of which were parsimony informative (Table 2). Most of the nucleotide substitutions were nonsynonymous (35 of 58); both transitions (34 of 58) and transversions (23 of 58) were detected. All genes were polymorphic but *pat-1* was more variable than other genes, as indicated by polymorphism analysis (Table 2) and diversity estimates (Table 1). Nonetheless, Hd estimates suggest that

Fig. 2. Neighbor-joining radial cladogram of *Clavibacter michiganensis* subsp. *michiganensis* concatenated data haplotypes. Colored circles represent predominant membership of concatenated haplotypes in one or two genetic clusters. Colors correspond to clusters as follows: green = cluster 1, light blue = cluster 2, and dark blue = cluster 3. Region coding corresponds to NE, northeast; NC, north-central; SW, southwest; and SE, southeast. Strains belonging to a particular concatenated haplotype are listed in Supplementary Table 5. Bootstrap support higher than 50% is indicated at the nodes.

perforin contained the highest level of DNA polymorphism. Between 2 and 11 haplotypes were detected for all genes (Table 1). Unique haplotypes occurred in *pat-1* (6%), *perforin* (1%), and *phosphataseC* (2%). In general, all genes presented no or little recombination according to R and RM values. Tajima's D indicated a significant deviation from neutrality in *pat-1* and *perforin* but all other genes presented no significant deviations from neutrality (Table 1).

Bayesian cluster analysis detected significant population structure by geography in the set of 96 strains, where some clusters occurred more frequently in some regions than others (Fig. 1A). High likelihoods were observed when the number of clusters was set to three ($K = 3$, $\ln P = -357$; $K = 2$, $\ln P = -433$; $K = 4$, $\ln P = -394$; Fig. 1A and B). Individual ancestry coefficients were highly consistent across replicate runs. The bar plots indicate that some strains are admixed, while others belong mostly to one particular cluster.

When the percentage of strains belonging to a PCR type was calculated within each region, geographic stratification was also apparent (Fig. 1C). Strains from NC and NE were PCR type C and D, respectively, whereas strains from SE and SW presented PCR types A, B, and C. Strains from NC and NE belonged predominantly to cluster one and three, respectively (Fig. 1A). The NC category contained some admixed strains and NE strains presented little admixture. Most strains from SE were predominantly members of cluster two but some strains mostly from cluster three and admixed strains were also found. Strains from SW had the highest diversity of cluster occurrence from all regions, and strains predominantly from clusters one, two, and three were sampled as well as admixed strains. Diversity estimates for strains from NE were higher than for other regions, whereas strains from NC presented the lowest values for diversity indexes (Table 3). Diversity values for strains from SE and SW were high and comparable. F_{ST} estimates indicated high genetic differentiation among strains from all regions but NC strains had higher genetic differentiation values compared with other strains (Table 4). The highest differentiation occurred between strains from NE and NC and the lowest between strains from SE and SW.

When the PCR type categories were used as prior population information in the cluster analysis, some population structure was revealed in the selected 96 strains (Fig. 1B). Strains of PCR type A and D belonged mostly to clusters two and three, respectively. The PCR type A category contained some admixed strains, whereas the type D strains showed little admixture. Strains of PCR type B had predominant membership in clusters two and three, and also presented some admixed strains. The PCR type C category contained similar numbers of strains with predominant membership in clusters one and three, and some admixed strains were also present. Diversity estimates for strains of PCR type A presented the lowest values of all PCR type categories (Table 3). The highest diversity estimates were found in strains of PCR type B, followed by strains of type D. Strains of PCR type C presented moderate values of genetic diversity compared with strains of other PCR types. F_{ST} values detected the highest genetic differentiation between strains of PCR type D and strains of all other types (Table 4). High genetic differentiation was found between strains of PCR types A and C, while moderate differentiation was detected between strains of PCR types B and C. F_{ST} estimates indicated low genetic differentiation between strains of PCR types A and B.

When gene sequencing data was concatenated, 36 multilocus haplotypes were detected and 25% of these were unique. Three of the multilocus haplotypes (numbers 1, 3, and 7) included strains of two different PCR types, for a total of 39 multilocus haplotypes if PCR type is taken into account. The NJ radial cladogram of concatenated haplotypes showed three large clades of strains with a bootstrap support of 66% that were grouped by geography (Fig. 2). When Bayesian clusters and geographic origin of multilocus haplotypes were mapped on the cladogram, it was apparent that cladogram groups were consistent with results from population structure analysis, which detected geographic stratification in *C. michiganensis* subsp. *michiganensis* strains. Two clades contained strains from NE, SE, and SW, while the third clade contained strains from all regions. Smaller clades within larger clades were usually grouped according to genetic cluster membership or geographic origin. Three multilocus haplotypes (C1, C2, and C4) in-

Table 1. Diversity estimates, neutrality tests, and recombination estimates for target plasmid and chromosomal genes in *Clavibacter michiganensis* subsp. *michiganensis*^a

Target DNA	Diversity estimates						Recombination estimates		Neutrality tests		
	s	h	Hd	π	θ_w	k	R	RM	Tajima's D	Fu and Li's D	Fu and Li's F
Plasmid											
<i>celA</i>	2	3	0.608	0.001	0.389	0.71	0	0	1.277	0.688	1.01
<i>pat-1</i>	45	11	0.271	0.006	8.761	2.548	0	0	-2.27*	0.717	-0.578
Chromosome											
<i>ABCtransporter</i>	4	5	0.615	0.001	0.779	1.199	4	1	1.077	0.944	1.16
<i>mop</i>	1	2	0.442	0.001	0.195	0.442	0	0	1.467	0.494	0.916
<i>perforin</i>	2	4	0.663	0.001	0.389	0.926	5	1	2.134*	0.688	1.325
<i>phosphataseC</i>	4	5	0.327	0.001	0.779	0.346	0	0	-1.108	-1.455	-1.58

^a Symbols: s = number of segregating sites, h = number of haplotypes, Hd = haplotype diversity, π = nucleotide diversity, θ_w = Watterson's theta estimator per gene from sequence, k = average number of nucleotide differences, R = recombination parameter, RM = minimum number of recombination events, and * indicates significant at 0.05.

Table 2. Polymorphism types for target plasmid and chromosome genes in *Clavibacter michiganensis* subsp. *michiganensis*

Target DNA	Parsimony informative sites	Synonymous changes	Replacement changes	Transitions	Transversions
Plasmid					
<i>celA</i>	2	1	1	2	0
<i>pat-1</i>	6	17	28	28	17
Subtotal	8	18	29	30	17
Chromosome					
<i>ABCtransporter</i>	4	3	1	1	3
<i>mop</i>	1	0	1	0	1
<i>perforin</i>	2	0	2	2	0
<i>phosphataseC</i>	2	1	2	1	2
Subtotal	9	4	6	4	6
Total	17	22	35	34	23

cluded strains from two different regions. Otherwise, strains that shared a multilocus haplotype were found in the same region of sampling (Fig. 2). Eight multilocus haplotypes (C9, C18, C21, C20, C31, C32, C34, and C35) had membership in two different genetic clusters but the rest belonged mostly to one cluster.

Discussion

Bacterial canker is one of the most serious diseases affecting field and greenhouse-grown tomato. In order to study the population structure of *C. michiganensis* subsp. *michiganensis* populations in Michigan tomato fields, all strains obtained were genotyped using BOX-PCR, and a sample of 96 strains was selected for sequencing of six virulence-related genes. The frequency of PCR types detected with BOX-PCR for all strains and Bayesian cluster analysis, pairwise F_{ST} comparisons, and genetic diversity estimates for 96 strains genotyped for six virulence-related genes revealed that *C. michiganensis* subsp. *michiganensis* populations in Michigan tomato fields are geographically structured. A multilocus haplotype cladogram was also consistent with geographic stratification in *C. michiganensis* subsp. *michiganensis* populations. Some regions had strains predominantly of only one PCR type or belonging mostly to one genetic cluster, while other regions presented more diversity of occurrence of PCR types and genetic clusters.

The dominant PCR type found in SW Michigan was type C but some type A and B strains were also found using BOX-PCR. Bayesian cluster analysis showed that most strains from SW belonged to cluster three but some strains from clusters one and two were also sampled. Regardless of the dominance of Mountain Spring in this region, SW presented strains from three PCR types (A, B, and C) and all three genetic clusters. In a *C. michiganensis* subsp. *michiganensis* collection spanning 1981 to 1994, type C strains (11 strains) detected in SW fields were present at a higher

incidence than type A (5 strains) and B (3 strains) strains, in agreement with our findings (29). Predominance of type C strains and strains from cluster three could be due to overwintering of inocula (6). In one SW field (SW/1/2) that was sampled in 1997 and 1998, symptom severity varied between years. In 1997, this field had young, newly established seedlings that were exhibiting severe wilting and eventually died, probably due to high numbers of *C. michiganensis* subsp. *michiganensis* on the seedlings at the time of transplanting (19). However, in 1998, disease symptoms in that same field were minor, consisting of marginal leaf necrosis on a few plants. Although overwintered host debris was not readily observed and plant beds were fumigated, the soil between the plant beds was not, leaving a possible source of inocula from infected plant debris. *C. michiganensis* subsp. *michiganensis* numbers on the transplants used to establish the field in 1998 could have been below the threshold required for more severe symptoms to occur during that year. Also, the fumigation efforts could have successfully reduced populations within the plant bed, leaving only inocula present from between plant beds.

Seedlings used to establish field SW/1/2 during 1997 and 1998 could have been contaminated with genetically similar strains, resulting in an increased sampling of type C strains. When recommendations to control bacterial canker on tomato are considered, a major focus has been on the source of inocula. Prior to 1990, Michigan growers routinely utilized transplants that had been grown in southern outdoor seedbeds. Infected transplants were recognized as a source of inocula after a severe bacterial canker outbreak in the Midwestern United States and Canada that was traced back to infected transplants from Georgia (14). Although Michigan growers have transitioned to local greenhouse-grown tomato transplants to avoid bacterial canker outbreaks, the disease has not been eliminated. The warm and humid environment of the

Table 3. Number of strains of *Clavibacter michiganensis* subsp. *michiganensis* and diversity estimates with strains grouped in geography and polymerase chain reaction (PCR) type categories^a

Category ^b	N ^c	Hd		π		θ_w		K	
		P	C	P	C	P	C	P	C
Region									
NC	18	0	0.186	0	0	0	0.218	0	0.186
NE	17	0.415	0.224	0.01	0	5.621	0.148	4.066	0.224
SE	29	0.206	0.338	0.003	0.001	4.47	0.497	1.211	0.615
SW	32	0.311	0.336	0.002	0.004	1.49	0.435	0.865	0.488
PCR type									
A	16	0.234	0.169	0.001	0.001	0.452	0.527	0.242	0.369
B	19	0.366	0.418	0.006	0.002	5.722	0.572	2.416	0.731
C	44	0.289	0.372	0.001	0.001	1.265	0.46	0.45	0.506
D	17	0.415	0.224	0.01	0	5.621	0.148	4.066	0.224

^a Average for plasmid (P) and chromosomal (C) genes. Hd = haplotype diversity, π = nucleotide diversity, θ_w = Watterson's theta estimator per gene from sequence, and K = average number of nucleotide differences.

^b Regions: NC, north-central; NE, northeast; SE, southeast, and SW, southwest. PCR type is designated according to PCR using BOX primer.

^c Number of strains.

Table 4. Genetic differentiation (F_{ST}) estimates for *Clavibacter michiganensis* subsp. *michiganensis* with strains grouped in geography and polymerase chain reaction (PCR) type categories

Category ^b	F_{ST} ^a										
	Region						PCR type				
	NE	SE	SW	B	C	D					
Region											
NC	0.456*	0.593*	0.482*	0.426*	0.256*	0.344*	...	...	...	...	...
NE	...	...	0.333*	0.512*	0.321*	0.381*	...	...	...	...	...
SE	...	...	...	...	0.208*	0.342*	...	...	...	...	...
PCR type											
A	...	...	...	...	...	...	0.023	0.135*	0.294*	0.451*	0.351*
B	...	...	...	...	...	...	...	...	0.171*	0.202*	0.289*
C	...	...	...	...	...	...	...	...	...	...	0.36*

^a Average for plasmid (first column) and chromosomal (second column) genes; * indicates significant at 0.05.

^b Regions: NC, north-central; NE, northeast; and SE, southeast. PCR type is designated according to PCR using BOX primer.

greenhouse in addition to frequent overhead watering creates favorable growing conditions for bacterial populations (19); therefore, infected transplants continue to be a concern as a source of inocula in Michigan.

All samples from NE Michigan analyzed with BOX-PCR were PCR type D, and a sample of isolates genotyped for virulence-related genes were all members of cluster three, even though samples were collected from multiple tomato cultivars (≤ 14). However, NE isolates analyzed with Bayesian clustering presented the highest diversity estimates from a sample of 96 strains from all regions. All NE samples were collected from the tomato grower with the largest production in this region, who produced tomato within a 5-acre plot that was rotated within a 20-acre unfumigated field. Transplants used in the production fields were grown in greenhouses located on the farm but not adjacent to the production field. After the transplants were moved to the field, the grower used the greenhouse to produce full-sized plants and fruit from which *C. michiganensis* subsp. *michiganensis* strains type D were isolated in 1998. The greenhouse-grown plants did not show foliar symptoms but minimal fruit spotting could be observed. A recent study reported that plants are most susceptible to fruit infection at the flowering and small fruit stages of plant development (36). This suggests that the pathogen populations were high enough at the time of flowering to cause minimal fruit spotting, yet not high enough at an early stage of plant growth to cause severe symptoms on the mature plants. Sanitation practices in this greenhouse were lacking. The practice of extended greenhouse production coupled with a lack of field rotation created a situation in which bacterial populations may have survived on buried debris or alternative weed hosts in the field or the greenhouse, thereby providing a reservoir of *C. michiganensis* subsp. *michiganensis* strains type D and from cluster three from 1997 to 1998.

Type D strains were not found in the major fresh-market tomato-growing regions of the SW or SE but they were found on another Michigan farm located in the NC region, approximately 129 km from the NE grower. The grower from the NC region had purchased transplants from the NE grower, although the exact years of purchase are unknown. The NC grower practiced short rotation (< 2 years), did not fumigate the plant beds, and typically grew multiple cultivars (≥ 3). Infected transplants that were grown in greenhouses of the NE grower likely harbored type D strains that were then transmitted to NC fields according to BOX-PCR analyses. Louws et al. (29) identified three type D strains from this farm from samples collected in 1994. The NC grower did not have type D strains exclusively, as did the NE grower, but also had type C strains. A shift in the incidence of the type D strains was observed from 1997 (3%) to 1998 (80%). If type D strains were introduced into NC due to the purchase of infected transplants from the grower in NE, their number may have increased during 1998 compared with 1997 due to the type of cultivars grown in this farm or simply because *C. michiganensis* subsp. *michiganensis* strains had time to spread throughout the field after their introduction.

Host selection is an important factor shaping the structure of plant pathogen populations (10); nonetheless, the effect of cultivars on population stratification of *C. michiganensis* subsp. *michiganensis* has not been elucidated. BOX-PCR analysis did not show strong associations of particular PCR types with tomato cultivar. Likewise, Bayesian cluster analysis did not indicate clear structuring of *C. michiganensis* subsp. *michiganensis* by tomato cultivar (*data not shown*). Type A strains have been described as being most frequently associated with processing cultivars (29). In our study, type A strains were only found in one SW Michigan field (SW/4/1/97) that included a processing cultivar. However, in this field, the type A strains were detected on both the processing cultivar and fresh-market cultivars. Louws et al. (29) described four *C. michiganensis* subsp. *michiganensis* strains obtained in 1994 from a SW Michigan field (SW/4) that contained processing cultivars; a type A strain was detected in this field and was the only type A strain detected in commercial fields in that region in 1994.

Farms growing diverse tomato cultivars (NE and SE) did not yield a higher diversity of PCR types or genetic clusters. However, diversity estimates did indicate higher genetic diversity in a sample of strains from NE and SE (≤ 14 cultivars) than in the sample of strains from NC and SW (≤ 4 cultivars). The diversity of tomato cultivars could have a diversifying effect in *C. michiganensis* subsp. *michiganensis* virulence genes, as has been noted in other pathogens (2,24,28,33,53). Nevertheless, host stratification may be obscured by the detected geographic structure. Hence, future studies that analyze population structure by host nested within geography are needed to understand whether specific genetic clusters are associated to certain tomato cultivars. If associations between PCR type or genetic cluster with virulence or pathogenicity were found, fingerprinting information could be applied to determine what isolates are more virulent or pathogenic to particular tomato cultivars. This type of information may help to explain the presence of dominant fingerprint types and clusters in some of the fields and regions examined in this study. Louws, et al. (29) found a significant number (30%) of avirulent type A strains, whereas type C strains were found to be predominantly virulent. Nonetheless, in our study, 16% of the type C strains were avirulent (*data not shown*). Understanding the diversity of *C. michiganensis* subsp. *michiganensis* is key to select virulent strains to use in host resistance screenings and develop resistant varieties.

Typical cultural practices of the SE region included long rotation (> 3 years), no fumigation, and a diversity of cultivars within and between farms. Field SE/4/1/98, containing 3 type A, 15 type B, and 33 type C strains, had never been planted to tomato before 1998. With the exception of three Florida-grown cultivars, all of the seedlings used to establish this field were grown in the grower's greenhouses that previously had been used only for production of flowering ornamentals and melon transplants. With the elimination of overwintered debris and greenhouse transplants as possible inoculum sources, seed or the Florida-grown transplants are the most likely source of inocula for the disease outbreak in this field. Disease symptoms were severe within some cultivars (wilting and stunting), yet minor within others (marginal necrosis). Seed from cultivars that were already contaminated in the greenhouse would likely exhibit severe symptoms in the field because the pathogen had time to reach threshold levels that result in disease symptoms (19). Spread from infected plants to uninfected plants in the greenhouse or field is suggested by the presence of bird's-eye fruit spotting on all cultivars. Ricker and Reidel (49) determined that secondary spread causes less severe symptoms of marginal necrosis and fruit spotting, and does not lead to yield loss. Nonetheless, fruit spotting can result in severe losses in fresh-market tomato.

Bayesian cluster analysis and F_{ST} values of SE strains again indicated geographic structure in *C. michiganensis* subsp. *michiganensis* populations. Regardless of the high diversity of tomato cultivars planted (≥ 4) and PCR types (A, B, and C) found in this region, most strains belonged predominantly to cluster two. Still, strains from clusters one and three were also sampled, which may explain the lower genetic differentiation between SE and SW, because strains from SW also presented PCR type and cluster occurrence similar to SE. The general geographic stratification observed in our study is probably due to the mode of transmission of *C. michiganensis* subsp. *michiganensis*. *C. michiganensis* subsp. *michiganensis* is not dispersed long-distance by wind, which would likely result in little or no geographic structuring because gene flow would homogenize populations (18). Because *C. michiganensis* subsp. *michiganensis* depends on human activity for long-distance transport (6,14), the observed geographic structure indicated that gene flow between the studied regions is not common but it is possible. Once strains are introduced into a region, *C. michiganensis* subsp. *michiganensis* populations can survive on plant debris, depending on the agricultural practices of each farm, and serve as inocula for the next growing season (6,7). Microbial populations that are spatially structured have been found to diverge quickly, given that gene flow between them is low (38). Establish-

ing the spatial distribution of the three *C. michiganensis* subsp. *michiganensis* clusters detected in this study provides an initial map of population structure of *C. michiganensis* subsp. *michiganensis* in Michigan tomato-growing regions. Nonetheless, migration analysis using neutral markers would be useful in better understanding the historic movement of *C. michiganensis* subsp. *michiganensis* strains in Michigan, as has been done with other plant pathogens (16,17).

When Bayesian cluster data for the sample of 96 *C. michiganensis* subsp. *michiganensis* strains was sorted by PCR type, strains seemed to be somewhat structured. Strains of type A and D were mostly members of cluster two and three, respectively. However, type D strains were all from NE; therefore, PCR type structure is probably an artifact of underlying geographic structure. This confounding effect of geographic stratification on structure by PCR type was also observed in type B strains that belonged mostly to cluster two when sampled in SE and to cluster three when isolated from plants in SW. Strains of PCR type C were somewhat structured because they included all strains with predominant membership in cluster one obtained from NC and SW.

Groups of multilocus haplotypes in the NJ radial cladogram were consistent with results from BOX-PCR and Bayesian clustering, which detected geographic stratification in *C. michiganensis* subsp. *michiganensis* strains. Strains that shared a multilocus haplotype were found in the same region of sampling and belonged mostly to one cluster. Population subdivision was also supported by the positive values for Tajima's D obtained for most genes (42). Other studies using genetic similarity dendrograms of *C. michiganensis* subsp. *michiganensis* populations in Israel did not identify clustering based on tomato cultivar, location, or year of isolation (8). It is desirable to perform population-based analysis such as Bayesian clustering to complement phylogenetic-based analyses commonly used in the study of bacterial populations. Several bacterial pathogens have been found to present recombination or horizontal transfer in their evolutionary histories, which could obscure phylogenetic relationships of strains (3,44,61). The role of horizontal transfer in *C. michiganensis* subsp. *michiganensis* populations is unclear but could be contributing to population structuring due to the limited exchange of plasmids between bacterial strains restricted by geographic barriers (48). Recombination events in *C. michiganensis* subsp. *michiganensis* populations seem to be uncommon but detectable in our recombination estimates. Our observations of closely related multilocus haplotypes suggest that mutation events seem to be more important as a diversifying force in *C. michiganensis* subsp. *michiganensis* populations than recombination, as observed for other clonal organisms (38,42,45).

BOX-PCR has been a useful technique for rapid and inexpensive identification of bacteria, which has yielded important epidemiological information of potential sources of inoculum and genetic variation in *C. michiganensis* subsp. *michiganensis* (29). Nonetheless, information about population divergence can be obtained by using sequencing data, in which polymorphisms are reproducible (58). Sequencing of housekeeping genes has been preferred for bacterial population studies because they are believed to evolve neutrally (34). However, sequencing of virulence-related genes can provide information on the evolution of virulence characteristics in bacterial pathogens and aid in the identification of strains with different virulence levels (42). The high variation detected in *pat-1* using diversity estimates has been observed in genes encoding virulence proteins (26). Significant and negative values for Tajima's D indicate an excess of low-frequency polymorphisms, a population expansion, or purifying selection for *pat-1*. The presence of *pat-1* and *celA* has been found to be conserved in *C. michiganensis* subsp. *michiganensis* populations and is correlated with pathogenicity (25). The loss of plasmids harboring these genes is correlated with a reduction of virulence (37). In addition, *perforin* presented significant and positive values for Tajima's D, indicating low levels of high- and low-frequency polymorphisms, balancing selection or a recent population contraction. Hence, our findings support the role of *pat-1* and *perforin* as viru-

lence determinants. Further studies are required to demonstrate the role of both genes in virulence and functional differences associated with different alleles.

C. michiganensis subsp. *michiganensis* has been previously found in infected transplants, seed, weed hosts, and infected tomato debris that can serve as sources of initial inocula for some fields (6,7,14). Our genetic analyses indicated that *C. michiganensis* subsp. *michiganensis* populations are geographically structured and that gene flow is rare but does occur among Michigan tomato-growing regions included in this study. Nonetheless, studies on *C. michiganensis* subsp. *michiganensis* population structure that include information of pathogen diversity, phylogeny, and the partitioning of variation in space and time are still needed. Studies that compare the populations analyzed in our study with current populations found in Michigan tomato fields would provide valuable information about population stability through time and inoculum sources, which are key to develop disease management strategies. This study suggests that introduction of *C. michiganensis* subsp. *michiganensis* strains into tomato fields and greenhouses due to use of infected seed or transplants remains a concern for disease management; hence, growers are advised to use certified planting material and utilize preventive antibacterial sprays in both the greenhouse and the field (60). Crop rotation and sanitation of trellising stakes and greenhouse production facilities are also recommended to avoid overwintering of inocula.

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