Multilocus Sequence Typing of Strains of Bacterial Spot of Lettuce Collected in the United States

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ABSTRACT

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Studies on genetic diversity and recombination in bacterial pathogens are providing a better understanding of the mechanisms shaping bacterial diversity, which can affect disease control. *Xanthomonas campestris* pv. *vitians*, causal agent of bacterial leaf spot of lettuce, is a threat to the worldwide lettuce industry. We examined the genetic variation within a sample of 83 strains from California, Florida, and Ohio using multilocus sequence typing of six housekeeping genes, totaling 2.7 kb. Additionally, polymorphism in two virulence-related genes, *hrpB2* and a putative glycosyl

hydrolase, were examined. Based on housekeeping genes, we found three genetic groups of strains that were all able to induce the disease. These included strains collected from weeds and irrigation water that had haplotypes identical to strains from diseased lettuce. High linkage disequilibrium across the sequenced loci indicates that the pathogen is predominantly clonal but recombination has contributed to the observed sequence variation. Although there was significant genetic variation in *X. campestris* pv. *vitians* within and among sampled states, identical haplotypes were observed across all three states. This finding suggests that seedborne inoculum may contribute to the diversity of *X. campestris* pv. *vitians* in the United States. Knowledge of the genetic structure of the pathogen may be used for developing resistant lettuce varieties.

First reported in South Carolina in 1916 (Brown 1918), *Xanthomonas campestris* pv. *vitians*, causal agent of bacterial leaf spot of lettuce, has recently become a potential threat to the lettuce industry in many parts of the world, including North America (Burkholder 1954; Pernezny et al. 1995; Toussaint 1999), Africa (Yigit 2011), Asia (Sahin 2000; Myung et al. 2010), and Europe (Sahin 2000; Zoina and Volpe 1994). Control of this disease with bactericides is difficult; thus, there is a need to identify host resistance (Bull et al. 2015; Wang et al. 2015). When lettuce cultivars and lines were screened for resistance to three single strains of *X. campestris* pv. *vitians*, disease incidence and severity showed a lettuce genotype by *X. campestris* pv. *vitians* strain interaction (Wang et al. 2015). This result suggests that genetic variation in *X. campestris* pv. *vitians* should be considered when developing management strategies based on host resistance.

The causal agent of the bacterial leaf spot of lettuce pathogen has created some confusion. Analysis of a large number of genetically diverse *Xanthomonas* strains from many plant species in 1995 reclassified the *X. campestris* pv. *vitians* pathotype strain LMG 937 (NCPB 976 = ATCC 19320) as *X. axonopodis* pv. *vitians*, while another bacterial spot of lettuce strain, LMG 938, was placed in *X. hortorum* (Vauterin et al. 1995). Subsequent studies showed that strain LMG 937 was genetically different from pathogenic strains collected from diseased lettuce, including LMG 938, and was not pathogenic on lettuce but weakly pathogenic on tomato and

pepper (Barak et al. 2001; Sahin et al. 2003). Although the strains associated with lettuce were reclassified, the pathogenic strains continue to be referred to as *X. campestris* pv. *vitians*.

Investigation of the diversity of *X. campestris* pv. *vitians* strains isolated from lettuce in Ohio further distinguished two phenotypic and genetic groups similar to LMG 938 (Sahin et al. 2003). The grouping was based on pathogenicity tests, monoclonal antibodies, fatty acid methyl ester analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and BIOLOG profiles. Group A strains were reported to produce local and systemic symptoms on lettuce leaves and blight on stems, whereas group B strains caused necrotic spots on leaves (Sahin et al. 2003). No sequence variation was found in the 16S–23S ribosomal DNA spacer regions of representative strains. Another study also found that the spacer regions of strains from different geographical regions were similar and that there was no genetic evidence for different groupings of *Xanthomonas* spp. associated with bacterial leaf spot of lettuce, based on repetitive element polymerase chain reaction (rep-PCR) and whole-genome genetic fingerprinting (Barak and Gilbertson 2003).

Multilocus sequence typing (MLST) was proposed in 1998 as an approach to identify clones within populations of pathogenic microbes (Maiden et al. 1998). It consists of the sequencing of multiple housekeeping genes whose evolution is either neutral or selectively constrained (Sarkar and Guttman 2004). The variants at each locus are given different allele numbers and each strain is defined by its allelic profile across the sequenced loci, known as the sequence type (ST) (Feil et al. 2003; Maiden 2006; Scally et al. 2005). Multilocus sequence analysis (MLSA) uses the same data set of housekeeping gene sequences but differs in that the analysis uses nucleotide sequences rather than simply the ST. Since 1998, MLSA has been extensively used to study bacterial population structure and evolution (Cooper and Feil 2004; Didelot and Falush 2007; Yan

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et al. 2008). MLST schemes have been developed for *Pseudomonas syringae* (Bull et al. 2011; Hwang et al. 2005; Morris et al. 2008), *Ralstonia solanacearum* (Castillo and Greenberg 2007), *Xylella* spp. (Scaally et al. 2005), and xanthomonads (Almeida et al. 2010; Young et al. 2008), among others.

Bacteria reproduce clonally but their genomes can be modified through the exchange of gene fragments. Horizontal gene transfer has affected the evolution of *Xanthomonas* genomes (Lima et al. 2008; Lin et al. 1999). Horizontal gene transfer has acted on genes involved in pathogenicity as well as housekeeping genes (Mhedbi-Hajri et al. 2013). MLSA has been useful in the estimation of relative contributions of mutation and recombination to the evolution of bacterial plant pathogens (Goss et al. 2005; Sarkar and Guttman 2004; Smith 1999).

Variation has or has not been observed in population samples of *X. campestris* pv. *vitians* depending on the sample and the electrophoretic and phenotypic markers used (Barak and Gilbertson 2003; Sahin et al. 2003). MLST has not previously been applied to study diversity in *X. campestris* pv. *vitians* and there is a need to study this pathogen using a multilocus approach to unambiguously assign ST and discriminate among differentiated groups of strains. We examined the population structure of *X. campestris* pv. *vitians* in the United States, as represented by strains from California, Florida, and Ohio, using MLST and MLSA. Our objectives were to determine the level of genetic variation in *X. campestris* pv. *vitians* within states and genetic differentiation among states, examine the support from MLSA analysis of housekeeping genes for two genetic groups consistent with the previous A/B grouping described by Sahin et al. (2003), and estimate the role of recombination in generating variation in *X. campestris* pv. *vitians*. Based on previous population studies (Barak and Gilbertson 2003; Sahin et al. 2003) and plant resistance screening in Florida (Wang et al. 2015), we hypothesized that *X. campestris* pv. *vitians* would exhibit little genetic variation in California but two differentiated genetic groups in Ohio and possibly Florida.

MATERIALS AND METHODS

Bacterial strains. The *X. campestris* pv. *vitians* strains, including their sources, year of isolation, and geographical origin,

used in this study are listed in Table 1. The strains primarily came from three sources: diseased lettuce (representing many cultivars), epiphytic populations on symptomless weeds, and irrigation water. Florida samples from 2010 to 2014 were collected from three commercial farms in the Everglades Agricultural Area (EAA). Strains from weeds and irrigation water were collected as part of an ecological study of bacterial leaf spot of lettuce (Fayette 2014). Strains were identified as *X. campestris* pv. *vitians* based on amplification of an *X. campestris* pv. *vitians*-specific fragment using the B162 primer pair (Barak et al. 2001) or the F-glyco and R-glyco primer pair (see below), and the sequence of *hrpB* (Obradovic et al. 2004). For long-term storage, bacterial strains were stored in sterile 15% aqueous glycerol solution at -80°C . Working cultures were kept on nutrient agar slants.

Pathogenicity test. Strains were grown on nutrient agar and incubated at 28°C . Each strain collected in the Florida EAA was tested for pathogenicity in crisphead ‘Belle Glade’ and romaine ‘Okechobee’ lettuce cultivars. Plants were grown in a greenhouse in 10-cm pots containing Fafard 2 Mix, a mixture of Canadian sphagnum peat, perlite, and vermiculite (Conrad Fafard, Inc., Agawan, MA). Plants were grown for 4 to 5 weeks. They were inoculated with a hand sprayer until run-off with a bacterial suspension adjusted to 1×10^8 CFU ml^{-1} (absorbance at 600 nm = 0.3) obtained from 24-h culture. The positive control consisted of plants inoculated with known pathogenic *X. campestris* pv. *vitians* strain L7 (Pernezny et al. 1995), whereas plants inoculated with tap water constituted the negative control. Plants were bagged and placed in a growth room adjusted to 21°C (Robinson et al. 2006). After 48 h, bags were removed and plants were returned to the greenhouse for the remainder of each experiment. Fourteen days after inoculation, plants were evaluated for the presence or absence of disease symptoms.

Amplification and sequencing of genes. Six primer pairs (*lacF*, *gyrB*, *gltA*, *gap-I*, *fusA*, and *lepA*) designed for MLST of *Xanthomonas* spp. (Almeida et al. 2010) were used in this study. Two additional gene fragments were also sequenced. These were the type III secretion system gene *hrpB2* (Obradovic et al. 2004), and a putative glycosyl hydrolase gene, designated *JF1* here, which was originally identified as a random amplified polymorphic DNA product found in

TABLE 1. Strains of *Xanthomonas campestris* pv. *vitians* used in this study

Strain designation	Source ^a	Origin	Year ^b	Reference
LMG 938	Lettuce	Zimbabwe	1965	Vauterin et al. 1995
L41, L43, L44	Lettuce	Florida	1993	Pernezny et al. 1995
B55	Lettuce	California	1994	Sahin et al. 2003
700A, 701A, 701B, 701C	Lettuce	Ohio	1995	Sahin et al. 2003
906, 917, 919, 921, 923, 924	Lettuce	Ohio	1996	Sahin et al. 2003
B57	Lettuce	California	1996	Sahin et al. 2003
B59	Lettuce	California	1997	Sahin et al. 2003
L1, L2, L3, L7, L11, L13, X251, X252, X253	Lettuce	Florida	1998	Pernezny et al. 1995
9805, 9812, Sal, Xav 98-37	Lettuce	California	1998	Barak et al. 2001
<i>X. campestris</i> pv. <i>vitians</i> 5/01	Lettuce	California	2001	J. Barak
BS2861	Lettuce	California	2010	S. Koike
JF150, JF152, JF154	Lettuce	Florida	2010	This study
JF52, JF58, JF70, JF72, JF74, JF88, JF90, JF116, JF156, JF158, JF160, JF168, JF180, JF182, JF184	Lettuce	Florida	2011	This study
JF170, JF172, JF174, JF176, JF178, JF178, JF186, JF188, JF196, JF200, JF202, JF204, JF206, JF208, JF210, JF214	Lettuce	Florida	2012	This study
JF162, JF164, JF166, JF190, JF192, JF198	Lettuce	Florida ^c	2012	This study
JF390, JF398, JF400, JF402	Lettuce	Florida	2014	This study
W3, W5, W8,	Weeds	Florida	2011	This study
W13, W14, W15	Weeds	Florida	2012	This study
H01, H02, H03, H04,	Water	Florida	2012	This study
W17, W18, W21, W28	Weeds	Florida	2013	This study

^a Isolation host. Weeds = strains from weeds isolated from the following species: *Lactuca seriola* (W3), *Sonchus oleraceus* (W5, W15, and W28), *Solanum americanum* (W17, W18), *Chenopodium album* (W8), *Amaranthus spinosus* (W13), *Eclipta prostrata* (W14), and *Portulaca oleracea* (W21). Water = irrigation water.

^b Year isolated.

^c Strains were collected from lettuce in a Florida supermarket.

X. campestris pv. *vitians* strains (Barak et al. 2001). We amplified and sequenced *JF1* using the PCR primers designed by Barak et al. (2001), and used the sequence to design new internal primers for sequence analysis: F-glyco 5'-CTGCTGATTGCGACGGACAATGAGG-3' and R-glyco 5'-CGCGATATCGATTGCGAAGGCTTGG-3'.

Genomic DNA was extracted using the Qiagen DNeasy Plant mini kit or NucleoSpin Tissue kit (Macherey-Nagel, Deer Park, NY). DNA template was added to PCR reagents of 1.25 U of *Taq* DNA polymerase (Qiagen, Macherey-Nagel) in 1× *Taq* Buffer, 0.2 mM dNTP, the primers, and water, for a final reaction volume of 25 µl. Amount of primers used varied among primer pairs (Almeida et al. 2010). For *hrpB* and *JF1*, the primer concentration was 0.5 mM. After a hot start at 95°C for 30 s followed by the denaturation for 5 min at 95°C, the amplification profile was 30 cycles of 95°C for 45 s, 58°C for 30 s, and 72°C for 30 s, which was followed by an extension time of 5 min at 72°C. Amplified DNA fragments were inspected by gel electrophoresis in a 1.0% agarose gel in 0.5 Tris-borate EDTA buffer. ExoSAP-It (Amersham Biosciences, Piscataway, NJ) was used to prepare the PCR products for sequencing following the manufacturer's instructions. The products were sequenced in both directions at the Interdisciplinary Center for Biotechnology Research, University of Florida. Reads were assembled and edited using Phred and Phrap implemented in CodonCode Aligner (CodonCode Corporation, Dedham, MA). Contigs were aligned using Clustal W and trimmed to the desired length. Sequences representing each haplotype were deposited in the National Center for Biotechnology Information GenBank under accessions KU095848 to KU095849 and KU136320 to KU136335. Sequences for the concatenated MLST loci and for the *JF1* locus for all strains are provided as Supplementary Files S1 and S2.

Population genetic analysis. Statistics summarizing nucleotide diversity and number of polymorphic sites were estimated for each gene. MLST uses housekeeping genes representative of the core genome of the species. Housekeeping genes are generally expected to be under purifying selection. Tajima's D statistic is based on differences between two estimators of nucleotide polymorphism, Washington's θ (θ_w) and nucleotide diversity (θ_π). If Tajima's D is significantly different from zero, the null hypothesis of neutral evolution is rejected. A significantly negative D can be an indication of a selective sweep or population growth, whereas a positive D can result from diversifying selection. Fu and Li's tests are particularly useful for the detection of rare alleles or young mutations, genetic hitchhiking, and population expansion (Fu 1996; Kreitman 2000). MLST sequences were tested for departure from neutral evolution using Tajima's D (Tajima 1989), Fu and Li' D* and Fu and Li' F* (Fu and Li 1993). The tests of neutrality were conducted in DnaSP (Librado and Rozas 2009). Assignments of alleles to ST were made through the MLST nonredundant database program (<http://pubmlst.org>). To test for linkage disequilibrium among loci, the index of association was estimated in LIAN 3.7 (Haubold and Hudson 2000, Smith et al. 1993).

Population structure. Genetic variation between and within populations was evaluated by analysis of molecular variance

(AMOVA) carried out using the software Arlequin 3.5 (Excoffier et al. 2005). The software Sequence Matrix (Vaidya et al. 2011) was used to concatenate the MLST fragments. The strains were divided into three groups: Florida ($n = 57$), Ohio ($n = 13$), and California ($n = 12$). The fixation index (F_{ST}), a measure of population differentiation due to genetic structure, was also estimated.

Phylogenetic analysis. The phylogenetic relationship among strains was estimated by maximum-likelihood phylogenetic analysis. jModeltest was used to determine an appropriate nucleotide substitution model for the concatenated sequences by evaluating 88 substitution models using maximum-likelihood with nearest neighbor interchange (NNI) tree searching. The best model was selected using the corrected Akaike information criterion. The maximum-likelihood phylogeny was inferred using PhyML with the TPM2uf substitution model and NNI tree searching, as implemented in Geneious 6.1.6 (Biomatters Ltd., Auckland, New Zealand). Branch support was assessed with 500 bootstrap samples.

Coalescent analysis. The clonal genealogy of the strains was estimated in ClonalFrame, which uses a coalescent-based population model to infer the evolutionary history of bacterial populations that are predominantly clonal (Didelot and Falush 2007). The Markov Chain Monte Carlo (MCMC) simulation was run with the following parameters: 100,000 MCMC iterations after a burn-in period of 100,000 iterations. The thinning interval was 100, which provided independent successive samplings. Two independent simulations were carried out to check the convergence of parameters. The statistic ρ/θ is the ratio of rates of recombination relative to mutation, and r/m is a measure of the impact of recombination (r) relative to mutation (m). Parameters were also estimated using ClonalFrameML using $\kappa = 2.454$ and 1,000 pseudoreplicates for bootstrapping (Didelot and Wilson 2015).

Recombination. The software packages RDP4 (Martin et al. 2010) and DnaSP were also used to detect recombination events. To estimate the mutation rate and recombination rate for each locus in RDP4, the default values for a recombination rate plot were chosen. The settings were starting $\rho = 30$, block penalty = 10, minor allele frequency cutoff = 0.05, average gene conversion tract length = 1,000, MCMC updates 100,000, and MCMC burn-in 100,000. For

TABLE 3. Results of neutrality tests^a

Gene	Tajima's D	Fu and Li's D*	Fu and Li's F*
<i>fusA</i>	-0.51	0.504	0.235
<i>gap-1</i>	3.896**	1.381	2.663**
<i>gltA</i>	-1.216	0.700	0.135
<i>gyrB</i>	-1.774	-4.577**	-4.267**
<i>lacF</i>	-1.882**	-3.098**	-3.182**
<i>lepA</i>	-1.048	-1.982	-1.982
Concatenated MLST loci	-0.008	-2.677	-1.898
<i>JF1</i>	1.383	0.525	0.899

^a MLST = multilocus sequence typing. Asterisks (**) indicate statistical significance: $P < 0.01$ for Tajima's D and $P < 0.02$ for Fu and Li's statistics.

TABLE 2. Polymorphism statistics for all sequenced loci

Gene	Number of sequences	Length	Number of haplotypes	Haplotype diversity	θ_π ^a	θ_w ^b
<i>fusA</i>	83	591	2	0.115	0.0002	0.0003
<i>gap-1</i>	83	444	3	0.637	0.0112	0.0045
<i>gltA</i>	83	501	2	0.048	0.0002	0.0007
<i>gyrB</i>	83	411	3	0.304	0.0020	0.0058
<i>lacF</i>	83	408	3	0.048	0.0003	0.0025
<i>lepA</i>	83	390	2	0.049	0.0001	0.0005
Concatenated MLST loci ^c	83	2745	9	0.677	0.002	0.002
<i>JF1</i>	62	409	2	0.444	0.0005	0.0005
<i>hrpB</i>	48	420	1	...	...	...

^a Nucleotide diversity per site using Jukes and Cantor correction factor.

^b Watterson's θ per site.

^c MLST = multilocus sequence typing.

analysis in DnaSP, the genomic state and chromosomal location were selected as haploid and prokaryotic, respectively. The minimum number of recombination events (the four-gamete test) (Hudson 1987) was estimated for each MLST locus and the MLST concatenated sequence.

RESULTS

Pathogenicity. The *X. campestris* pv. *vitians* strains tested for pathogenicity were recovered from various sources, including

leaves with visible symptoms of bacterial leaf spot of lettuce, epiphytic populations on weeds from commercial lettuce farms in the EAA, and irrigation water. Typical bacterial leaf spot symptoms are characterized by dark-brown to dark lesions surrounding by yellow halos on lettuce leaves. All of the Florida strains from 2010 to 2104 produced symptoms similar to those induced by the *X. campestris* pv. *vitians* strain L7. No symptoms were observed in plants that were inoculated with tap water. Strains from earlier Florida, Ohio, and California collections (Table 1) were previously characterized (Barak and Gilbertson 2003; Pernezny et al. 1995;

Fig. 1. Maximum-likelihood phylogeny of *Xanthomonas campestris* pv. *vitians* strains from California, Florida, and Ohio based on multilocus sequence analysis. Strains previously assigned to two genetic groups by Sahin et al. (2003) are shown in bold, with A/B grouping in parentheses. Bootstrap support values are shown above branches. Recombination was inferred on branches indicated with asterisks. Branch lengths are in substitutions per site.

Sahin et al. 2003) and include strains representing both groups proposed by Sahin et al. (2003).

Polymorphism. In all, 83 strains of *X. campestris* pv. *vitians*, including the reference strain LMG 938, were sequenced at six loci for MLSA. The length of the six concatenated loci was 2,745 bp.

These sequences resulted in nine multilocus ST among the 83 strains. Individual MLST loci produced two or three haplotypes. For *hrpB* and *JF1*, 48 and 62 strains were sequenced. The *hrpB* locus produced a single haplotype. *JF1* produced two haplotypes based on single-nucleotide polymorphisms. The θ_{π} was relatively low for

Fig. 2. Consensus clonal genealogy of *Xanthomonas campestris* pv. *vitians* strains using multilocus sequence analysis genes. Branches shown were present in at least 50% of the simulated coalescent genealogies, and branches present in 80% of genealogies are in bold. The scale indicates time from the present in coalescent units, which is a function of population size and generation time. The collection location and year are shown for strains together with the sequence type of the glycosyl hydrolase gene, *JF1*. The *JF1* sequence was not obtained for all strains.

each locus (Table 2) and much higher for *gap-1* (0.01) than for the other loci (0 to 0.002), while θ_w was high for *gap-1* relative to the other loci but θ_w for *gyrB* was slightly higher.

The neutrality tests did not reject neutral evolution of the housekeeping genes *fusA*, *gltA*, and *lepA* and the glycosyl hydrolase gene *JF1*. Only *lacF* produced significantly negative values for all three neutrality tests, consistent with purifying selection. In contrast, *gap-1* had significantly positive values of Tajima's D and Fu and Li's F^* (Table 3). The statistics were not estimated for *hrpB* because there was no polymorphism among 48 strains.

Genealogy. The maximum-likelihood phylogeny and clonal genealogy of the MLST loci both supported three groups of strains (Figs. 1 and 2). Strains from California, Ohio, and Florida grouped together in the trees and, in some cases, had identical sequences (Fig. 1). The Sahin et al. (2003) group A strains were all found in one clade but the group B strains were found in all three major clades (Fig. 1). The *JF1* locus was not included in the MLST trees; instead, the ST is given in Figure 2 and shows that haplotypes 1 and 2 are found in all three clades.

Recombination. Based on the four-gamete test, a recombination event was detected in the concatenated MLST dataset but not within loci, potentially indicating recombination among loci. The index of association is a commonly used test of recombination in bacteria and measures the degree of linkage among loci. The standardized index of association (I_{SA}) (Haubold et al. 1998) for the MLST sample was $I_{SA} = 0.11$. The statistic I_{SA} is equal to zero when the population is under free recombination. The observed variance ($V_D = 30.46$) was significantly greater than the expected variance under linkage equilibrium ($V_e = 6.38$) based on 1,000 trials ($P < 0.001$). The null hypothesis of free recombination among loci was rejected, indicating linkage disequilibrium among loci.

One of the approaches used to examine recombination rates is the estimation of the population recombination rate (ρ) relative to the population mutation rates (θ), or ρ/θ . The program RDP4 was used to estimate ρ and θ (Table 4). The value of ρ/θ was <1 for *gap-1*, *gltA*, *gyrB*, and *lacF*, averaging 0.0772. Values could not be estimated for the other MLST loci or for *JF1*. For the concatenated MLST data set, $\rho/\theta = 0.0061$.

The ρ/θ rate was also estimated for the concatenated MLST data set using the coalescent models implemented in ClonalFrame and ClonalFrameML. Significant recombination was found using ClonalFrame ($R = 0.868$, 95% confidence interval [0.240 to 1.745]). However, the ρ/θ rate could not be reliably estimated. ClonalFrameML estimated the rate of recombination to have been half the rate of mutation (Table 5). These estimates of recombination are more than an order of magnitude higher than those obtained from RDP4. We calculated the relative impact of recombination to mutation to be 3.2 ($r/m = R/\theta \times v \times \delta$), indicating that recombination is responsible for three times as many substitutions as mutation. Recombination events were inferred for strains JF166 and 924, and the branch leading to at least one of the major multilocus ST by both models (Fig. 1). The recombinant sequences were located in *lacF*, *gyrB*, and *gap-1*.

Population structure. We conducted an AMOVA analysis on 57 strains from Florida, 10 strains from Ohio, and 9 from California. The variation between the Florida sample and the combined

other strains was significant at 20% of the total genetic variation (Table 6). The F_{ST} of 0.20 suggests genetic structure but limited variation among groups relative to within groups.

DISCUSSION

Understanding the diversity of *X. campestris* pv. *vitians* is important for breeding programs that aim to develop resistant lettuce varieties because of the variation in disease severity that is observed among *X. campestris* pv. *vitians* strains (Bull et al. 2015; Sahin et al. 2003; Wang et al. 2015). MLST has been valuable as an unambiguous, highly reproducible tool to assess bacterial diversity and discriminate among different evolutionary lineages that may also differ in phenotype. MLST has not previously been used to examine the diversity of *X. campestris* pv. *vitians*. MLSA revealed the presence of genetically distinct groups of strains, as previously reported by Sahin et al. (2003). Our analysis supported three groups of strains but there was not a clear relationship between our supported groups and the groups of Sahin and colleagues. Strains from the A group identified by Sahin et al. (2003) were represented by a single multilocus ST but the B group strains produced multiple ST. Florida strains were present in each of the *X. campestris* pv. *vitians* genetic clusters. Some of the MLST loci showed little or no variation among strains; therefore, our analysis was largely dependent on variation observed in *gap-1* and *gyrB*. This is consistent with the report of Barak and Gilbertson (2003) that *X. campestris* pv. *vitians* strains constitute a relatively homogenous group, based on pathogenicity, fatty acid profiles, rep-PCR, and internal transcribed spacer sequences.

MLST analyses are based on housekeeping genes, which are expected to be evolutionarily conserved. Housekeeping genes are core genes, which tend to be characterized by clonal descent rather than lateral gene transfer. Core genes can be used to define species boundaries (Goss et al. 2005; Young et al. 2008), and their evolution is usually either neutral or selectively constrained. In contrast, the flexible genome evolves largely via horizontal genetic exchange (Sarkar and Guttman 2004). The glycosyl hydrolase gene (*JF1*) may be part of the flexible genome but we did not detect departure from neutrality. However, we rejected neutral evolution for some of the housekeeping genes. The MLST genes that had statistically significant values of Tajima's D or Fu and Li's statistics also were the genes that are likely to have undergone recombination. Therefore, the departure from neutrality may be due to the allele frequencies that resulted from rare recombination events. We found only a single haplotype for *hrpB*, suggesting the possibility of strong purifying selection on this gene,

TABLE 5. ClonalFrameML parameter estimates for the concatenated multilocus sequence typing loci

Parameters ^a	Estimate	95% confidence interval
R/θ	0.50	0.18–1.00
δ	425	214–1,175
v	0.015	0.009–0.023

^a R/θ is the ratio of recombination to mutation, δ is the average length of a recombination event, and v is the per-site probability of recombination.

TABLE 4. Estimates of recombination and mutation rates

Genes	Rm ^a	ρ	θ	ρ/θ ^b
<i>gap-1</i>	0	0.0400	6.6	0.0066
<i>gltA</i>	0	0.0350	2.0	0.0180
<i>gyrB</i>	0	0.0340	8.0	0.0043
<i>lacF</i>	0	0.9000	3.3	0.2800
Concatenated loci	1	0.0700	11.4	0.0061

^a Minimum recombination events estimated with DnaSP.

^b Estimates of ρ and θ were obtained in RDP4; θ is per locus. Parameters could not be estimated for *fusA*, *lepA*, *JF1*, and *hrpB*.

TABLE 6. Analysis of molecular variance comparing samples from Florida, Ohio, and California^a

Source of variation	df	Sum of squares	Variance components	Variation (%)	F_{ST}
Among states	2	44.0	0.65*	20.08	0.20
Within states	148	384.7	2.60*	79.92	...
Total	150	428.7	3.25	...	...

^a Strains were grouped according to their origin: 57 strains from Florida, 10 from Ohio, and 9 from California. An asterisk (*) indicates that variance components were statistically significant.

which codes for a component of the type III secretion system and is an essential gene for pathogenicity.

The MLSA approach can provide an estimate of the relative contributions of mutation and recombination to bacterial diversification, because MLST loci are core genes found in all strains and they undergo homologous recombination. The ratio of recombination to mutation is commonly averaged across MLST loci (Castillo and Greenberg 2007; Scally et al. 2005). Using this approach, *P. syringae* and *Xylella fastidiosa* were shown to evolve mainly via mutation (Sarkar and Guttman 2004; Scally et al. 2005). However, recombination was reported to be greater than mutation in bacterial diversification in other plant pathogens, including *P. viridiflava* (Goss et al. 2005) and *Xanthomonas axonopodis* pv. *vesicatoria* (Basim et al. 1999). For *X. campestris* pv. *vitians*, we found that mutation occurred at a greater rate than recombination; however, the impact of recombination was greater than the impact of mutation in generating the polymorphism observed in the dataset. We also observed evidence of recombination in comparison to the glycosyl hydrolase gene. These results suggest that recombination is infrequent but has contributed to the genetic variation in *X. campestris* pv. *vitians*. Based on the clonal genealogy, the most frequently isolated ST, including the type strain, is characterized by a recombination event affecting *gap-1*. Using a similar analysis for a collection of *X. axonopodis* strains, it was reported that mutation occurs as frequently as recombination, and the impact of recombination was at least three times greater than mutation (Mhedbi-Hajri et al. 2013). Whole-genome sequences for *X. campestris* pv. *vitians* would provide greater understanding of the impact of recombination on both housekeeping and virulence-related genes.

We found strains with identical ST in Florida, California, and Ohio. In addition, strain LMG 938 collected in 1965 had the same ST as strains found more recently in Florida and California. As bacterial leaf spot of lettuce has recently become more important in Florida (Pernezny et al. 1995), California (Barak and Gilbertson 2003), and Ohio (Sahin et al. 2003), inoculum appears to be moving among production regions. For bacterial spot of tomato and pepper caused by *Xanthomonas* spp., it has been suggested that the introduction of pepper and tomato seed may serve as sources of races that eventually become endemic in a given production area (Pohronezny et al. 1992). Lettuce in Florida is directly seeded and commercial lettuce seed are mainly produced in California. Infested seed can be produced from diseased lettuce plants (Barak et al. 2002; Sahin and Miller 1997) but *X. campestris* pv. *vitians* has not been detected from commercial seed lots (Barak et al. 2001). Based on these MLSA results, two strains from different clades, L7 and JF196 (collected in 1998 and 2012, respectively), were used to screen 30 lettuce genotypes, including crisphead, romaine, butterhead, leaf, and latin types. Strain JF196 was found, overall, to be slightly less virulent than L7 (Wang et al. 2015). Additional tests are required to determine whether strains exhibiting genotypes identical to JF196 and L7 have similar levels of virulence. Continued efforts to identify resistant germplasm may lead to a reduction of inoculum available for seed infestation on resistant cultivars (Bull et al. 2015).

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