Diversity of *Xanthomonas campestris* Isolates from Symptomatic Crucifers in New York State

H. W. Lange, M. A. Tancos, M. O. Carlson, and C. D. Smart

Plant Pathology and Plant-Microbe Biology Section, School of Integrative Plant Science, Cornell University, Geneva, NY 14456.
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ABSTRACT

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To assess the diversity of *Xanthomonas campestris* spp. infecting crucifers in New York, 154 isolates were collected over 10 years across the state. The goal was to determine if isolates of the pathogen were overwintering in New York and serving as primary inoculum in subsequent years, or if novel isolates were entering the state each year. Pure cultures of isolates were characterized using multilocus sequence analysis (MLSA), a greenhouse pathogenicity assay, repetitive element-polymerase chain reaction (Rep-PCR) using the BOX-A1R primer, and enzyme-linked immunosorbent assay. The MLSA scheme proved to be more efficient than Rep-PCR for a large sample

population and for comparison with global isolates. *X. campestris* isolated from crucifers in New York comprised of *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani*, with *X. campestris* pv. *raphani* being predominately isolated from transplants. Evidence for unique haplotypes persisting on the same farm for several years due to improper seedbed rotations was documented in addition to novel haplotypes being spread throughout states through infected transplants and seed. Rep-PCR confirmed the high diversity of *X. campestris* and was used to generate 15 unique fingerprint patterns from isolates collected in the first 5 years. A worldwide comparison of isolates suggests that the *X. campestris* pv. *campestris* population appears to be very homogenous with dominant haplotypes persisting for extended periods and being globally disseminated.

Xanthomonas campestris infections of cruciferous crops continue to threaten the economic viability of growers worldwide (Williams 1980). *X. campestris* is a seed-disseminated Gram-negative phytopathogenic bacterium that is a continual threat in tropical and subtropical areas where crucifers are planted year round, and it is an annual threat in temperate climates when temperatures are between 22 and 28°C with high humidity (Cook et al. 1952a). Wet conditions are conducive for epiphytic survival of the bacterium as well as the secondary spread between plants (Carisse et al. 1998).

X. campestris has been reclassified into six pathovars in order to incorporate distinctions in host infection, colonization, and symptom development among cruciferous plants, which currently include the following: *X. campestris* pv. *campestris*, *X. campestris* pv. *aberrans*, *X. campestris* pv. *armoraciae*, *X. campestris* pv. *barbareae*, *X. campestris* pv. *incanae*, and *X. campestris* pv. *raphani* (Vauterin et al. 1995). Much debate continues about *X. campestris* pv. *armoraciae* and *X. campestris* pv. *raphani* and whether they are synonymous or distinct pathovars (Alvarez et al. 1994; Bogdanove et al. 2011; Vicente et al. 2006). In the current study we will adopt the definition by Vicente et al. (2006) and define *X. campestris* isolates that cause nonvascular leaf spots on *Brassica* spp. as *X. campestris* pv. *raphani*.

In New York, black rot (*X. campestris* pv. *campestris*) occurs annually, with severe outbreaks causing economic impact when the growing season is warm and accompanied by wind-driven rains. Field maintenance practices such as cultivation, spray applications, and irrigation are all means of pathogen spread. Bacteria can readily

enter the plant through wounds, stomata (cotyledon stage), and hydathodes (Staub and Williams 1972). During cool/moist nights, plants create droplets of guttation fluid on the hydathodes which are drawn back into the vascular system as the day warms; thereby providing an ideal entry route for bacteria (Curtis 1943). Guttation fluid contains minerals, carbohydrates, and amino acids, and has a pH of ~7.0, making it a model medium for *X. campestris* pv. *campestris* growth (Slusarenko et al. 2000). Once inside the leaf, the bacteria multiply rapidly secreting an extracellular polysaccharide, xanthan, which can plug the xylem vessels and cause tissue damage (Beattie 2011; Sutton and Williams 1970a). The typical *X. campestris* pv. *campestris* yellow V-shaped lesions that originate at the leaf edge and extend inward are caused by bacteria that enter through the hydathodes and move through the xylem causing a blockage of water and nutrients, resulting in chlorosis and blackening of veins (Sutton and Williams 1970b).

X. campestris pv. *campestris* infection can also develop on seedlings from bacteria on and/or within the crucifer seed (Cook et al. 1952a,b; Ryan et al. 2011; Schaad et al. 1980; Starr 1981). The presence of bacteria may not decrease the germination rate but can serve as an inoculum source (Walcott 2003). *X. campestris* pv. *campestris* has been found to survive in the xylem of the plant for months until conditions are favorable for growth (Cook et al. 1952a). In addition, plant debris and cruciferous weeds in and around the field have also been implicated as inoculum sources for black rot infections (Alvarez and Lou 1985; Schaad and Dianese 1981; Schaad and White 1974). In a study of cruciferous weeds in California, Ignatov et al. (2007) concluded that bacterial populations isolated from weeds had different genetic properties than those associated with seed infections in cultivated *Brassica* crops (Ignatov et al. 2007).

A second pathovar, *X. campestris* pv. *raphani*, was first isolated from radish and turnip and causes leaf spots rather than vascular infections in *Brassica* spp. (White 1930). The leaf spots are usually more numerous near large leaf veins and can cause total leaf collapse when severe, but do not result in a systemic infection

Corresponding author: C. D. Smart; E-mail address: cds14@cornell.edu

First and second author authors contributed equally to this work.

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(Alvarez et al. 1994; Vicente et al. 2001). Unlike *X. campestris* pv. *campestris*, pathovar *raphani* will enter the stomata of true leaves (Cook et al. 1952a; Hugouvieux et al. 1998; Shaw and Kado 1988). Both pathovars are often found on the same plant in natural field infections, which further complicates diagnosis (Chen et al. 1994).

The objectives of this paper were to (i) understand the role of introduced and overwintering isolates in annual black rot outbreaks in New York, (ii) identify likely inoculum sources, (iii) characterize the diversity among pathogenic *X. campestris* pathovars, and (iv) determine the relative importance of vascular versus leaf spotting isolates in the population. We hypothesize that if overwintering isolates are the primary source of inoculum in New York, those haplotypes should persist for at least 2 years. If novel haplotypes are appearing every year, it would suggest that isolates are arriving from external sources (i.e., seed or transplants) and overwintering isolates are not the primary source of inoculum. Here we report the results of pathogenicity and fingerprinting analyses used to differentiate isolates collected over 10 years throughout New York State.

MATERIALS AND METHODS

Isolate collection. Bacteria were isolated from symptomatic cabbage (*Brassica oleracea*), broccoli (*B. oleracea* var. *botrytis*), Brussels sprouts (*B. oleracea* var. *gemmifera*), kale (*B. oleracea* var. *viridis*), and cauliflower (*B. oleracea* var. *botrytis*) leaves that were collected from greenhouses, seed beds, fields, and winter storage facilities from 2004 to 2013. A total of 154 *X. campestris* isolates were obtained from the Eastern United States with the majority being collected from New York State ($n = 140$), and the remaining isolates from Pennsylvania ($n = 1$), Michigan ($n = 6$), Georgia ($n = 6$), and Florida ($n = 1$). Since 91% of the isolates are from New York, the *X. campestris* isolates will be referred to as the New York population.

Tissue was excised from symptomatic leaves, surface sterilized with 0.6% NaOCl for 3 min, ground with sterile water using a mortar and pestle, plated onto King's B medium (King et al. 1954), and grown at 28°C for 24 to 48 h. Cultures that morphologically appeared to be *Xanthomonas* and did not fluoresce on King's B medium were cultured on YDC (Wilson et al. 1967) medium to check for yellow mucoid growth (typical of *Xanthomonas* species) and also cultured on *Xanthomonas* semiselective SM medium (Chun and Alvarez 1983).

Multilocus sequence analysis (MLSA) and sequence analysis of *X. campestris*. Individual *X. campestris* colony forming units (CFUs) were grown in nutrient broth at 27°C with shaking at 160 rpm for 18 h. Genomic DNA was subsequently extracted using the MasterPure DNA purification kit (Epicentre, Madison, WI) according to the manufacturer's instructions. All DNA was quantified with a Nanodrop ND-1000 (Wilmington, DE). MLSA for *X. campestris* was conducted using the previously described primers that amplify seven housekeeping genes—*atpD* (ATP synthase β chain), *dnaK* (heat shock protein 70), *efp* (elongation factor P), *glnA* (glutamine synthetase), *gyrB* (gyrase subunit β), *rpoD* (RNA polymerase sigma-70 factor), and *tpiA* (triosephosphate isomerase)—and a single transmembrane protein—*fyuA* (TonB-dependent receptor) (Fargier et al. 2011). The eight loci were analyzed for each isolate using total extracted DNA and gene-specific polymerase chain reaction (PCR). Amplification was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using EmeraldAmp GT PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan). Extracted total DNA was used at 50 to 100 ng per reaction with 10 μ M each of forward and reverse primers. PCR was performed in 25 μ l reactions following the previously described protocol (Fargier et al. 2011). PCR products were purified with an ExoSAP (Exonuclease I-Shrimp Alkaline Phosphatase) protocol (New England Biolabs, Ipswich, MA), and the cleaned PCR amplicons were sequenced in both directions on a 3730XL DNA Analyzer (Applied Biosystems) at the Cornell University Life Sciences Core Laboratories Center with the aforementioned PCR amplification primer sets (Fargier et al. 2011).

Raw sequences for each of the eight genes in the MLSA scheme for all 154 isolates were viewed, manually edited according to peak quality, and aligned with Muscle in MEGA 6.06 (Tamura et al. 2013) on an individual gene basis or concatenated. Consensus sequences were used to generate maximum-likelihood (ML) trees based on the Tamura-Nei model (Tamura and Nei 1993) of MEGA 6.06 using all sites with bootstrapping repetitions of 1,000. Sequences from *X. campestris* pv. *campestris* ATCC33913, *X. campestris* pv. *raphani* 756C, and *X. campestris* pv. *vesicatoria* 85-10 were used as references and an outgroup, respectively (Bogdanove et al. 2011; da Silva et al. 2002; Thieme et al. 2005). Discriminatory power was calculated using a web-based discriminatory power calculator (http://insilico.ehu.es/mini_tools/discriminatory_power/index.php). Haplotypes were considered unique if the sequence differed by a single nucleotide (Tancos et al. 2015; Wicker et al. 2012). Additionally, the concatenated sequences (eight loci) from 15 previously published *X. campestris* pv. *campestris* and three *X. campestris* pv. *raphani* isolates, each representing unique haplotypes ($n = 18$), were compared with the shortened aligned sequences (4,486 nucleotides) of the New York haplotypes ($n = 64$) (Fargier et al. 2011). New York haplotypes sequences had to be shortened in order to allow for concatenated sequences from both studies to be compared since different sequencing primers were used in each study.

To provide orthologous support for identified phylogenetic groupings, genetic differentiation among haplotypes was assessed with discriminant analysis of principal components (DAPC) using the *ade4* package in R (Jombart 2008). This method maximizes variation between predefined groups, and minimizes within group variance in data transformed by principal component analysis (Jombart et al. 2010). Genotypes were converted to dummy coding ($A = 1, G = 2, C = 3, T = 4, - = 5$) and variant sites were subsequently converted into a *genind* object in R (Jombart 2008). Groups were defined using the *K*-means clustering method in *ade4* ($k = 3$), as three clusters best described the data. The *a*-score optimization function in *ade4* was used to determine the optimal number of principal components (PCs) to include in the DAPC ($n = 2$) using the New York data set, representing 49.4 and 28.53% of the total variance for New York and total isolates, respectively.

Inoculation test for pathogenicity. Two cultivars of cabbage (*B. oleracea* 'Fresco' and 'Gonzales') susceptible to *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* were grown in the greenhouse with a 16-h photoperiod at 24°C. Cabbage seedlings were inoculated at the three true-leaf stage. Each field isolate was grown on YDC medium for 24 to 48 h at 28°C. A sterile pin dipped into the bacterial culture was pricked into the outer margin of one leaf eight times and into the petiole and midrib of a second true leaf on the same plant (Vicente et al. 1998). The third leaf of each plant was left uninoculated. Control plants were inoculated with known isolates of *X. campestris* pv. *campestris*, *X. campestris* pv. *raphani*, or a sterile pin. Two plants per isolate were inoculated and symptoms were scored visually at 14 days postinoculation. Plants were scored as having no reaction, a typical black rot symptomatic V-shaped yellow-to-brown lesion along the leaf margin, or a necrotic spot reaction. This test was repeated twice for each isolate.

Genomic fingerprinting using BOX-A1R repetitive element-polymerase chain reaction (Rep-PCR). A fingerprint was generated for each isolate collected between 2004 and 2008 using the BOX-A1R primer following a previously described protocol (Louws et al. 1994) with a PTC-100 Peltier thermal cycler (MJ Research, Waltham, MA). Bacterial cells were grown overnight in nutrient broth, the OD₆₀₀ adjusted to 0.1, and 12 μ l was added to the PCR mix (Versalovic et al. 1994). To streamline Rep-PCR, 50 to 100 ng of genomic DNA isolated with the MasterPure DNA purification kit (Epicentre, Madison, WI) (as described above) was used during 2007 to 2008. The PCR program was 95°C for 7 min, and then 35 cycles of 95°C for 1 min, 53°C for 1 min, 65°C for 8 min followed by a final extension step at 65°C for 8 min and held at 10°C. The PCR amplicons (8 μ l) were loaded onto a 1.5% agarose gel using

0.5× TAE buffer and run at 4°C for 18 h at 67V (de Bruijn et al. 1996).

Enzyme-linked immunosorbent assay (ELISA) antigen test. ELISA analysis was performed using the Agdia (Elkhart, IN) bacterial reagent sets to differentiate *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* (syn. *X. campestris* pv. *armoraciae*) following the manufacturer's protocol. The two isolates with fully sequenced genomes, *X. campestris* pv. *campestris* strain 8004 (NCBI ID 314565) and *X. campestris* pv. *campestris* strain ATCC 33913, were used as positive controls. Isolate Xca 071 was used as the *X. campestris* pv. *raphani* positive control. *Pseudomonas syringae* pv. *tomato* and coating buffer alone were included as negative controls. Results were recorded for each isolate using visual assessment and confirmed with a microplate reader (Beckman Coulter Inc., Brea, CA). Each ELISA test was performed in duplicate and repeated at least twice for each isolate. The ELISA kit was later discontinued so no isolates after 2008 were tested.

RESULTS

A total of 154 *X. campestris* isolates were collected over 10 years from grower fields or transplant production facilities. Isolates were evaluated with MLSA and a pathogenicity assay, and a subset were also evaluated with Rep-PCR using the BOX-A1R primer, and polyclonal antibodies (ELISA). A complete listing of the results for each isolate can be found in Supplementary Table S1.

MLSA analyses for *X. campestris*. In accordance with Fargier et al. (2011), all eight loci utilized in the MLSA scheme were highly polymorphic with *rpoD* having the most polymorphic sites at 72, and *efp* having the least at 10 (Table 1, Supplementary Table S2). Housekeeping genes *rpoD* and *tpiA* showed the greatest amount of variation with 7.13% and 5.47% of sites being polymorphic in each gene, respectively (Table 1). Sequence variation was approximately 3 to 4% for the remaining genes. No gaps or insertions were present in any of the sequenced genes. Concatenation of the eight loci could resolve the 154 *X. campestris* isolates into 64 haplotypes with a discriminatory power of 0.967 (Table 1). Of the 64 haplotypes,

41 haplotypes were associated with a single isolate. The most numerous haplotype (H) was H22 ($n = 18$), which was continually isolated over a period of years from 2006 to 2007 and from 2009 to 2013 (Table 2).

Phylogenetic analyses using the maximum likelihood method demonstrated that the New York *X. campestris* isolates were comprised of two distinct crucifer-infecting pathovars including the following: *X. campestris* pv. *campestris* ($n = 131$) and *X. campestris* pv. *raphani* ($n = 23$) (Table 2, Fig. 1). Furthermore, the phylogenetic analysis demonstrated that two subgroups were present within the New York *X. campestris* pv. *campestris* population (XCC group A and B) with *X. campestris* pv. *campestris* group B diverging from the single *X. campestris* pv. *raphani* cluster (Fig. 1). The majority of New York *X. campestris* pv. *campestris* isolates belonged to group A ($n = 103$) with the remaining isolates in group B ($n = 27$) or a singleton (H34, isolate 0682). No absolute associations with geographical origin, year, host, or symptoms could be observed between the two *X. campestris* pv. *campestris* groups. The 131 *X. campestris* pv. *campestris* isolates alone could be broken into 51 haplotypes with a discriminatory power of 0.956 when the eight loci were concatenated (Table 1).

Twenty-three *X. campestris* isolates were determined to be *X. campestris* pv. *raphani* based upon sequence data and pathogenicity assays (Tables 1 and 2). As opposed to the *X. campestris* pv. *campestris* isolates, housekeeping genes *tpiA* and *dnaK* showed the greatest amount of variation with 3.70 and 3.38% of sites being polymorphic in each gene within *X. campestris* pv. *raphani*, respectively (Table 1). When the eight loci were concatenated, the 23 *X. campestris* pv. *raphani* isolates could be separated into 13 haplotypes with a discriminatory power of 0.921 with the most numerous haplotype being H12 ($n = 5$) and nine haplotypes representing single isolates.

To infer associations and diversity among *X. campestris* between this study and previously published results, 18 *X. campestris* isolates (comprising of 15 *X. campestris* pv. *campestris* and three *X. campestris* pv. *raphani* isolates) that were collected from Europe, Australia, Africa, and North and South America (Fargier et al. 2011)

TABLE 1. Genetic variation in *Xanthomonas campestris* isolates determined from multilocus sequence analysis

Gene	Sequenced fragment (bp)	No. of isolates sequenced	No. of haplotypes	No. (%) of polymorphic sites	Discriminatory power
<i>X. campestris</i>					
<i>atpD</i>	767	154	13	30 (3.91%)	0.860
<i>dnaK</i>	711	154	8	25 (3.52%)	0.544
<i>efp</i>	419	154	8	10 (2.39%)	0.742
<i>fyuA</i>	761	154	12	26 (3.42%)	0.704
<i>glnA</i>	789	154	13	26 (3.30%)	0.719
<i>gyrB</i>	831	154	10	27 (3.25%)	0.710
<i>rpoD</i>	1,010	154	15	72 (7.13%)	0.788
<i>tpiA</i>	676	154	15	37 (5.47%)	0.855
Concat.	5,964	154	64	253 (4.24%)	0.967
<i>X. campestris</i> pv. <i>campestris</i>					
<i>atpD</i>	767	131	8	24 (3.13%)	0.812
<i>dnaK</i>	711	131	4	6 (0.844%)	0.470
<i>efp</i>	419	131	5	7 (1.67%)	0.654
<i>fyuA</i>	761	131	7	19 (2.50%)	0.601
<i>glnA</i>	789	131	8	13 (1.65%)	0.617
<i>gyrB</i>	831	131	7	17 (2.05%)	0.625
<i>rpoD</i>	1,010	131	11	61 (6.04%)	0.712
<i>tpiA</i>	676	131	11	22 (3.25%)	0.806
Concat.	5,964	131	51	169 (2.83%)	0.956
<i>X. campestris</i> pv. <i>raphani</i>					
<i>atpD</i>	767	23	5	22 (2.87%)	0.771
<i>dnaK</i>	711	23	5	24 (3.38%)	0.771
<i>efp</i>	419	23	4	7 (1.67%)	0.593
<i>fyuA</i>	761	23	5	12 (1.58%)	0.609
<i>glnA</i>	789	23	5	19 (2.41%)	0.771
<i>gyrB</i>	831	23	5	18 (2.17%)	0.557
<i>rpoD</i>	1,010	23	5	26 (2.57%)	0.806
<i>tpiA</i>	676	23	5	25 (3.70%)	0.771
Concat.	5,964	23	13	153 (2.56%)	0.921

were compared with the 64 New York haplotypes (Fig. 2). Phylogenetics demonstrated that the haplotypes from the separate populations were intermixed with no distinct clustering (Fig. 2). The Fargier et al. (2011) isolates similarly clustered among the two

distinct *X. campestris* pv. *campestris* subgroups (XCC group A and B) and the *X. campestris* pv. *raphani* cluster. In addition, *X. campestris* pv. *campestris* isolate 1711 (collected from France) had high sequence identity to the single New York *X. campestris* pv.

TABLE 2. Diversity observed among *Xanthomonas campestris* isolates based on phylogenetic subgroup, MLSA haplotype, symptoms observed during pathogenicity assays, and years collected

Phylogenetic subgroup ^a	Conc. haplotype	No. of isolates	Symptoms ^b	Years isolated
<i>X. campestris</i> pv. <i>campestris</i>				
A	H1	8	V	2004, 2011, 2013
A	H3	13	V	2004, 2005, 2007, 2010, 2011, 2012
A	H5	1	V	2004
A	H9	2	V	2005, 2011
A	H21	1	V	2006
A	H22	18	V	2006, 2007, 2009, 2010, 2011, 2012, 2013
A	H23	1	V	2006
A	H24	4	V	2006, 2008
A	H27	4	V	2006, 2008
A	H30	2	V	2006
A	H31	6	V	2004, 2006, 2007, 2013
A	H32	1	V	2006
A	H35	1	V	2006
A	H37	7	V	2008, 2009
A	H38	1	V	2008
A	H39	1	V	2008
A	H40	1	V	2008
A	H41	1	V	2008
A	H42	1	V	2008
A	H43	1	V	2008
A	H44	1	V	2008
A	H45	7	V	2009, 2011
A	H46	3	V	2009, 2011
A	H48	2	V	2009
A	H49	1	V	2009
A	H50	1	V	2009
A	H51	1	V	2010
A	H52	1	V	2010
A	H53	1	V	2010
A	H54	1	V	2010
A	H55	1	V	2010
A	H56	1	V	2010
A	H58	1	V	2011
A	H59	1	V	2011
A	H60	1	V	2012
A	H61	1	V	2012
A	H63	2	V	2013
A	H64	1	V	2013
B	H2	8	V	2004, 2006, 2008, 2011
B	H4	4	V	2004, 2006, 2007
B	H6	3	V	2004, 2008, 2013
B	H15	1	V	2005
B	H16	1	V	2005
B	H19	1	V	2006
B	H25	2	V	2006
B	H26	2	V	2006
B	H28	1	V	2006
B	H29	2	V	2006, 2008
B	H57	1	V	2011
B	H62	1	V	2013
Singleton	H34	1	V	2006
<i>X. campestris</i> pv. <i>raphani</i>				
C	H7	1	NS	2005
C	H8	2	NS	2005, 2009
C	H10	1	NS	2005
C	H11	1	NS	2005
C	H12	5	NS	2005, 2006, 2007
C	H13	3	NS	2005, 2007, 2009
C	H14	1	NS	2005
C	H17	1	NS	2005
C	H18	1	NS	2006
C	H20	1	NS	2006
C	H33	1	NS	2009
C	H36	4	NS	2007, 2009, 2012, 2013
C	H47	1	NS	2009

^a The *X. campestris* pv. *campestris* New York population could be divided into two subgroups (XCC A and XCC B) and a singleton, but the *X. campestris* pv. *raphani* New York population remained in a single group (XCR C).

^b Characteristic symptoms observed from greenhouse pathogenicity assays. The two symptoms observed included: V-shaped lesions on the margins of leaves (V) or necrotic spots covering the leaves (NS).

campestris isolate 0682 (H34), which was distinct from both *X. campestris* pv. *campestris* subgroups A and B (Figs. 1 and 2). Surprisingly, several New York *X. campestris* haplotypes had high sequence identity to the Fargier et al. (2011) isolates with zero

single nucleotide polymorphisms (SNPs) detected, even though isolates were collected decades apart from various countries (Table 3).

The DAPC analysis was consistent with the phylogenetic analyses for both New York (Fig. 1B) and combined haplotypes,

Fig. 1. A, Maximum likelihood (ML) phylogenetic tree for the concatenated genes (5,964 nucleotides)—*atpD*, *dnaK*, *efp*, *glnA*, *gyrB*, *rpoD*, *tpiA*, and *fyuA*—for New York *Xanthomonas campestris* haplotypes ($n = 64$) and reference strains *X. campestris* pv. *campestris* ATCC33913 and *X. campestris* pv. *raphani* 756C. The ML tree is rooted against reference strain *X. campestris* pv. *vesicatoria* 85-10 (root is not shown). Reference strains are designated with black triangles. The number of isolates per haplotype group (H) is displayed in parentheses. Bootstrap values are shown at the nodes if greater than 50%. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (Tamura et al. 2013). **B,** The same data set as A, plotted on the first two principal components of the DAPC with groups represented as inertia ellipses. Different shapes represent the three phylogenetic subgroups (A = circle; B = square; and C = triangle).

showing clear differentiation between the three groups. The two haplotypes, H34 and 1711, which were distinct from subgroups A and B in the phylogenetic analysis, clustered with group B in the DAPC. This is not surprising in that these haplotypes were most closely related to group B haplotypes in the phylogeny.

All MLSA haplotype gene sequences were deposited in GenBank (accession no.: *atpD* [KR922987 to KR922999], *dnaK* [KR922979 to KR922986], *efp* [KR922971 to KR922978], *fyuA* [KR922959 to KR922970], *glnA* [KR922946 to KR922958], *gyrB* [KR922936 to KR922945], *rpoD* [KR922921 to KR922935], and *tpiA* [KR922906 to KR922920]).

Pathogenicity assay. All of the 154 collected isolates caused either V-shaped lesions or leaf spotting when inoculated into young cabbage plants in the greenhouse (Table 2). The majority of isolates tested, 85% ($n = 131$), caused typical V-shaped lesions in the inoculated greenhouse cabbage plants and the remaining 15% ($n = 23$) caused leaf spotting (Table 2). Twenty-one of the 35 isolates collected from transplants in early spring caused leaf spotting reactions (*X. campestris* pv. *raphani*), while only two field isolates were found to cause leaf spotting indicative of *X. campestris* pv. *raphani*. These results were consistent between replications of the inoculations.

BOX-A1R PCR fingerprinting. A total of 15 BOX-A1R patterns—1 ($n = 4$), 2 ($n = 15$), 3 ($n = 8$), 4 ($n = 3$), 5 ($n = 6$), 6 ($n = 8$), 7 ($n = 2$), 8 ($n = 2$), 9 ($n = 12$), 10 ($n = 21$), 11 ($n = 1$), 12 ($n = 7$), 13 ($n = 2$), 14 ($n = 3$), and 15 ($n = 1$)—were observed among the 95 isolates of *X. campestris* collected between 2004 and 2008 that were tested using this method. There was no correlation between BOX-A1R patterns and their spatial distribution, and very little fingerprint overlap from year-to-year except for one farm where the same fingerprint (BOX-A1R pattern 2) was isolated over a five year period (isolates 0450, 0659, 0660, 0792, and 08306). Interestingly, BOX-A1R pattern 1 was characterized from several isolates in New York and from seed of the same variety obtained from a seed testing facility that year (isolates 0407, 0455, 0473, and 0499E3).

ELISA antigen assay. The serological data collected from five years of isolates showed 86% ($n = 91$) of the 106 isolates tested reacted only with the *X. campestris* pv. *campestris* antigen, while 12% ($n = 13$) were positive only with the *X. campestris* pv. *raphani* antigen. Two isolates reacted with both antigens. The pathogenicity assay results did not always correlate with the ELISA results. Based on ELISA, 24 isolates did not show the corresponding pathogenicity symptom typical of their pathovar. Results were consistent both within and between replications.

DISCUSSION

This study has enabled a better understanding of the population diversity of *X. campestris* isolates associated with symptomatic cruciferous crops, their frequency, persistence, and distribution statewide. Over the previous 10 years, 154 *X. campestris* isolates were collected and characterized from symptomatic seedlings, field plants, and stored cabbage heads. Because *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* are both present in New York, it was not surprising to find both pathogens in our survey. Interestingly, *X. campestris* pv. *raphani* was relatively uncommon in the field, but prevalent with transplants from production greenhouses. This distinction could be explained by the selection process of diseased field tissue (extension agents/growers would send tissue that had characteristic V-shaped lesions) and/or by that fact that *X. campestris* pv. *raphani* proliferates better in warm/humid environments such as greenhouses (Zhao et al. 2000).

The two pathovars were differentiated in greenhouse pathogenicity tests because *X. campestris* pv. *campestris* was systemic, forming V-shaped lesions with vein blackening, while *X. campestris* pv. *raphani* caused localized necrotic spots. For the majority of New

York isolates, a *X. campestris* pv. *campestris* positive ELISA test, which were performed the first 6 years, correlated with V-shaped lesions when tested on cabbage in the greenhouse, but systemic isolates were not always characterized as pv. *campestris* nor were necrotic spot-producing isolates always characterized as pv. *raphani*. Similar results were previously reported demonstrating the antibodies apparent lack of specificity between pathovars (Alvarez and Lou 1985; Alvarez et al. 1994). This could be explained by the fact that vascular and nonvascular Xanthomonads appear to have few differences in gene content even though they display differing tissue specificity; and symptom expression could be modified by pH, temperature, and other environmental conditions (Bogdanove et al. 2011; Chen et al. 1994; He et al. 2007).

The MLSA approach to genotype *X. campestris* isolates that infected *Brassica* vegetables provided an excellent view of the *X. campestris* population within New York State, and how these strains are spreading both spatially and temporally. *X. campestris* pv. *campestris* can be disseminated through infected seed and/or transplants, thereby, being a major concern to both growers and seed producers. Many *Brassica* vegetable growers in New York (and other states) outsource the growing and maintenance of seedlings to greenhouse transplant production facilities that are both in- and out-of-state. When transplants are ready for direct planting they are then shipped to their respective growers. In our study, infected transplants were received from greenhouse transplant production facilities in three states.

Interestingly, one of the greenhouse transplant facilities had several MLSA haplotypes of *X. campestris* pv. *raphani* ($n = 10$) present throughout the years. In 2005, seven different haplotypes of *X. campestris* pv. *raphani* were present at the transplant facility; however, many of those haplotypes were lost in the following years except for haplotype H12 (persisted from 2005 to 2007) and haplotypes H13 and H8, which both persisted until 2009. Subsequently, H36 became the prominent haplotype from 2007 to 2013. This haplotype could have survived on reused flats, benches or elsewhere in the environment of the facility, or could have been reintroduced on contaminated seed during that seven year period. None of the haplotypes that persisted at the facility were later isolated from fields in New York or the surrounding states.

X. campestris pv. *campestris* haplotype H45 was isolated from a specific cabbage variety in two greenhouse transplant production facilities located in disparate states in 2009. The simultaneous isolation of an identical haplotype from different greenhouse transplant production facilities (in different states) in the same year suggests that the hybrid seed could have been contaminated, thereby, spreading this unique haplotype to growers across several states. Haplotype H45 was later isolated from New York fields in 2011. Evidence for isolates moving into fields from infected seed was further documented with isolates characterized with the haplotype H1 and BOX-A1R pattern 1 (isolates 0407, 0455, and 0473), which were collected from three different New York counties in 2004 from the same cabbage variety. The identical MLSA haplotype and BOX-A1R pattern was also observed in an isolate collected from seed of the same cabbage variety obtained from a seed testing facility that year (isolate 0499E3). The haplotype was again isolated in New York in 2011 and 2013. The initial introduction event in 2004 appears to be seed-associated, however, the re-isolation of haplotype H1 in 2011 and 2013 could have been due to overwintering or a reintroduction event.

Two distinct crucifer-infecting pathovars, *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani*, were detected and differentiated by MLSA throughout our study; however, within *X. campestris* pv. *campestris*, two phylogenetically distinct subgroups (based on MLSA data) persisted throughout the years with subgroup B being more genetically similar to *X. campestris* pv. *raphani* than *X. campestris* pv. *campestris* subgroup A. No differences in symptoms,

disease progression or host range were observed between these two subgroups of *X. campestris* pv. *campestris*. The majority of *X. campestris* pv. *campestris* isolates clustered in subgroup A, in which an isolate was collected every year. No *X. campestris* pv. *campestris*

isolates from subgroup B were detected in 2009, 2010, or 2012. Interestingly, a New York *X. campestris* pv. *campestris* isolate with the unique haplotype H34 clustered as a singleton between *X. campestris* pv. *campestris* subgroup B and *X. campestris* pv. *raphani*.

Fig. 2. Maximum likelihood (ML) phylogenetic tree for the shortened concatenated genes (4,486 nucleotides)—*atpD*, *dnaK*, *efp*, *glnA*, *gyrB*, *rpoD*, *tpiA*, and *fyuA*—for unique New York *Xanthomonas campestris* haplotypes ($n = 64$), previously studied *X. campestris* sequence types ($n = 18$), and reference strains *X. campestris* pv. *campestris* ATCC33913 and *X. campestris* pv. *raphani* 756C. The ML tree is rooted against reference strain *X. campestris* pv. *vesicatoria* 85-10 (root is not shown). Reference strains are designated with black triangles, while unique sequence types from Fargier et al. (2011) are designated with black dots. Haplotype number and/or strain number are indicated with country of isolation in parentheses. Bootstrap values are shown at the nodes if greater than 50%. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (Tamura et al. 2013).

Two phylogenetically distinct subgroups of *X. campestris* pv. *campestris* have been identified in smaller populations of globally isolated *X. campestris* isolates (Alvarez et al. 1994; Fargier et al. 2011; Tsygankova et al. 2004). When several *X. campestris* isolates from the Fargier et al. (2011) study were included in our analysis, the majority of the *X. campestris* pv. *campestris* isolates (collected in Europe, Africa, and North and South America) clustered into subgroup A, while few isolates clustered into subgroup B (Fargier et al. 2011). However, evolutionary inferences between the *X. campestris* pv. *campestris* subgroups and *X. campestris* pv. *raphani* should be approached with caution when using MLSA data alone (Baltrus et al. 2014). Future work with isolates from each of the *X. campestris* pv. *campestris* subgroups using whole-genome sequencing should be implemented to identify relationships between the subgroups and their similarity to *X. campestris* pv. *raphani*. Even though they are phylogenetically similar, *X. campestris* pv. *raphani* and *X. campestris* pv. *campestris* have very distinct infection mechanisms (nonvascular versus vascular). Bogdanove et al. (2011) compared the two complete genomes of nonvascular (*X. campestris* pv. *raphani*) and (*X. oryzae* pv. *oryzicola*). They found that adaptations at the level of gene content account for host specificity and subtler adaptations at the level of amino acid or noncoding regulatory nucleotide sequences determine tissue specificity (Bogdanove et al. 2011).

MLSA haplotypes of New York isolates do not appear to be unique as no distinct clustering was present when the New York *X. campestris* haplotypes were compared with the globally collected haplotypes (Fargier et al. 2011). The global population of *X. campestris* pv. *campestris* appears to be very homogenous with dominant haplotypes persisting for extended periods. For example, numerous *X. campestris* pv. *campestris* haplotypes collected from 1958 to 2001 across Europe, Africa, and Australia had one or zero SNPs (between the eight concatenated housekeeping genes) when compared with New York strains collected between 2004 to 2013 (Table 3). More genetic variation appeared to exist between New York haplotypes than between globally collected strains isolated approximately 50 years ago. Granted, we are unable to say that the strains present in the mid-twentieth century are identical to the strains being recovered from grower's fields in New York, but it appears that several dominant haplotypes are persisting and being globally-disseminated.

An absolute correlation did not exist between MLSA and BOX-A1R for diverse isolates of *X. campestris*; however, associations were common. Both MLSA and BOX-A1R provide complementary approaches to fingerprinting isolates based on either SNPs (present

within the housekeeping genes) or differences in the spacing of repetitive sequences (dispersed throughout the genome), respectively. The utilization of both methods in combination provided greater differentiating capabilities, but as sequencing costs became lower and the number of BOX-A1R patterns increased it became clear that MLSA would be a more effective approach to characterize *X. campestris* populations because of its efficiency, speed, easier interlab reproducibility, and ability to quickly compare global populations (Tancos et al. 2015; Wicker et al. 2012).

Over the course of this study, 15 BOX-A1R patterns were identified among the subset of *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* isolates collected from 2004 to 2008. There was a wide variation of isolates across New York and new isolates were identified each year. While the rate at which new BOX-A1R patterns develop is unknown, the fact that isolates with the same BOX-A1R pattern (pattern 2) were identified at the same farm for 5 years (isolates 0450, 0659, 0660, 0792, and 08306) provides important evidence that BOX-A1R patterns are stable over the course of a single season (or even several seasons) in New York. The MLSA data agreed with the aforementioned BOX-A1R pattern by demonstrating that the isolates had nearly identical haplotypes (H31 differed from H44 [isolate 08306] by a single SNP). The isolates persisted on the same farm due to improper seedbed rotations and sanitation. Our BOX-A1R results are similar to what has been observed by others in many parts of the world studying diseases caused by bacterial phytopathogens (Hartung and Civerolo 1987; Norman et al. 2009; Schneider 1996). Studies of *X. campestris* from many countries in Europe, Asia, and Africa found, as we did, that BOX-A1R patterns did not correlate with race type or geographic location, but the technique was valuable for confirming pathogen diversity (Chidamba and Bezuidenhout 2012; Jensen et al. 2010; Lema et al. 2012; Massomo et al. 2003; Mulema et al. 2011; Rouhrazzi and Khodakaramian 2014; Singh et al. 2011; Valverde et al. 2007; Zaccardelli et al. 2008).

The data presented here demonstrate that the New York populations of *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* are highly diverse with 64% of the isolates being unique haplotypes that were represented only once. Conversely, some dominant haplotypes were present for five or more years (haplotypes H3 and H22) and were collected across New York State. *X. campestris* pv. *campestris* is capable of being maintained on the same farm for several years as previously noted; however, the appearance of novel haplotypes each year implies that overwintering of the pathogen is not the primary source of inoculum in New York, but that new haplotypes are being transported into the state each year.

TABLE 3. Sequence similarity compared between *Xanthomonas campestris* haplotypes collected in New York (present study) and globally (Fargier et al. 2011)

New York population ^a				Global population ^a				
Haplotypes	Year(s) isolated	Location ^b	No. of isolates ^c	Haplotypes	Year(s) isolated	Location ^b	No. of isolates ^c	No. of SNPs ^d
<i>X. campestris</i> pv. <i>campestris</i>								
H4	2004, 2006–07	NY	4	ST3	1958	UK	1	0
H24	2006, 2008	NY	4	ST16	1975, 1979	FR	3	0
H34	2006	NY	1	ST20	1958, 1967, 1975	FR, DE	4	0
H3	2004–05, 2007, 2010–12	NY	13	ST17	1967, 1976, 1999–00	CI, FR, BE, ES	9	0
H9	2005, 2011	NY	2	ST7	2000–01	ES	2	0
H19	2006	NY	1	ST5	unk	unk	1	0
H32	2006	NY	1	ST21	1975	AU	1	1
H45	2009, 2011	NY, MI, GA	7	ST22	1967, 1999	FR, BE	2	1
H22	2006–07, 2009–13	NY, PA	18	ST1	unk	unk	1	1
<i>X. campestris</i> pv. <i>raphani</i>								
H36	2007, 2009, 2012–13	NY	4	ST13	unk	US	1	2

^a Subset of unique haplotypes collected from New York (present study) or the Fargier et al. (2011) study and how the haplotypes compare.

^b Location where isolates were collected. FR = France, DE = Germany, UK = United Kingdom, CI = Cote d'Ivoire (Ivory Coast), BE = Belgium, ES = Spain, AU = Australia, US = United States, NY = New York, MI = Michigan, GA = Georgia, unk = unknown.

^c The number of isolates that comprise the respective haplotype group.

^d The number of single nucleotide polymorphisms (SNPs) detected in the eight concatenated housekeeping genes (4,486 nucleotides) that differentiate the two haplotype groups (New York population versus global population).

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