Molecular Epidemiology of *Pseudomonas syringae* pv. *syringae* Causing Bacterial Leaf Spot of Watermelon and Squash in Florida

E. A. Newberry and B. Babu, North Florida Research and Education Center, University of Florida, Quincy; P. D. Roberts, Southwest Florida Research and Education Center, University of Florida, Immokalee; N. S. Dufault, E. M. Goss, and J. B. Jones,[†] Department of Plant Pathology, University of Florida, Gainesville; and M. L. Paret,[†] North Florida Research and Education Center, University of Florida, Quincy

Abstract

From 2013 to 2014, bacterial leaf spot epidemics incited by *Pseudomonas syringae* pv. *syringae* affected an estimated 3,000 ha of watermelon and squash in Florida, and caused foliar blighting and transplant losses in severely affected fields. To investigate the diversity of the causal agent, we isolated 28 *P. syringae* strains from diseased plants grown in 10 Florida and Georgia counties over the course of 2 years. Strains were confirmed as *P. syringae* through sequence analysis of the 16S ribosomal RNA, phenotypic, and biochemical profiling; however, 20 displayed an atypical phenotype by exhibiting nonfluorescent activity on King's medium B agar and being negative for ice-nucleating activity. Multilocus sequence analysis and BOX

polymerase chain reaction revealed the presence of two haplotypes among the collected strains that grouped into two distinct clades within *P. syringae* phylogroup 2. Pathogenicity testing showed that watermelon, cantaloupe, and squash seedlings were susceptible to a majority of these strains. Although both haplotypes were equally virulent on cantaloupe, they differed in virulence on watermelon and squash. The distribution of one haplotype in 9 of 10 Florida and Georgia counties sampled indicated that these epidemics were associated with the recent introduction of a novel clonal *P. syringae* lineage throughout major watermelon production areas in Florida.

Florida is consistently among the top producers of watermelon nationally, with 8,500 to 10,500 ha harvested in the state from year to year (FDACS 2012, 2014). Numerous fungal and viral diseases are common to Florida watermelon production; however, bacterial diseases are sporadic and generally considered to be of minor importance (Elwakil and Mossler 2013). In spring 2013, a new disease outbreak of bacterial leaf spot caused by *Pseudomonas syringae* was observed on watermelon and squash in major production areas throughout the state (Newberry et al. 2015). From 2013 to 2014, this disease affected an estimated 3,000 ha of the crop, with incidence reported from southern Georgia to southern Florida. In some fields, foliar blighting was observed at the early stages of seedling development and resulted in transplant losses and delayed fruiting of severely blighted seedlings.

Bacterial leaf spot of watermelon was first identified in Florida in 1963, where the causal agent was formally described as *P. syringae* pv. *lachrymans* (Hopkins and Schenck 1972; Mullin and Schenck 1963). Since that time, different members of the *P. syringae* species complex have been recovered periodically as leaf spot pathogens of watermelon, cantaloupe, and squash in the southeastern United States (Langston et al. 2003; Newberry et al. 2016). *P. syringae* is a gram-negative member of the class Gammaproteobacteria, and is responsible for numerous plant diseases worldwide (Bradbury 1986; Young 2010). Due to the inability to differentiate distinct pathogenic *P. syringae* populations with traditional biochemical and physiological tests, members of the species were classified at the infrasubspecific level into over 50 pathovars as defined by their distinctive pathogenicity and host range specialization (Young 2010). Worldwide, *P. syringae* pvs. *lachrymans*, *syringae*, and *aptata* have been identified as the causal agents of epidemics affecting various cucurbit species (Fatmi et al. 2008; Harighi 2007; Langston et al. 2003; Morris et al. 2000; Sedighian et al. 2014). In many cases, these epidemics were reported in a region where the

disease was previously unobserved, and were associated with greenhouse production or crops grown under plastic tunnels (Fatmi et al. 2008; Harighi 2007; Morris et al. 2000; Sedighian et al. 2014). Often, the disease did not reoccur the following season. However, there have been reports where the pathogen continued to be a problem, threatening the sustainability of cucurbit production in a region for subsequent seasons (Fatmi et al. 2008; Morris et al. 2000).

Seed infestation is recognized as a major factor contributing to long-distance dissemination of *P. syringae* in vegetable crops (Lamichhane et al. 2015). In 2011, the United States imported 146,000 kg of watermelon seed from 10 different countries, valued at \$269 million (USDA 2013). As global trade associated with agricultural production increases, so too does the potential for the unintentional introduction of seedborne pathogens into agricultural ecosystems (Schaad et al. 2014). *P. syringae*, an environmentally ubiquitous bacterial species, may have multiple sources of dissemination. Bacterial blight epidemics occurring in French cantaloupe fields were linked to precipitation and irrigation water rather than contaminated seed, thus suggesting that the epidemiological significance of environmental inoculum sources may be overlooked in bacterial disease outbreaks (Monteil et al. 2016; Riffaud and Morris 2002).

Over the past 15 years, phylogenetic analyses using various molecular markers have been conducted to investigate the relationships of *P. syringae* pathovars (Berge et al. 2014; Bull et al. 2011; Hwang et al. 2005; Sarkar and Guttman 2004; Yamamoto et al. 2000). These studies have shown *P. syringae* to be a phylogenetic complex of strains subdivided into 13 phylogroups on the basis of sequence divergence. Phylogroups 1 through 8 include most of the recognized pathovars and pathotype strains while the remaining phylogroups comprise strains primarily recovered from environmental sources (Berge et al. 2014). A survey of *P. syringae* strains isolated from symptomatic cucurbit tissue between 1999 and 2011 identified the population associated with disease in the United States to be genetically diverse, and primarily distributed among three clades within *P. syringae* phylogroup 2. This study also reported the presence of a novel *P. syringae* haplotype common to Florida, Georgia, and California that was isolated from several different cucurbit hosts (Newberry et al. 2016).

Recent epidemics of bacterial leaf spot affecting watermelon and squash in Florida were distinct from previously recorded disease outbreaks in the southeastern United States in terms of their scale and severity. The consequences associated with the introduction of exotic pathogens into agricultural ecosystems can be devastating for an

[†]Corresponding authors: M. L. Paret; E-mail: paret@ufl.edu; and J. B. Jones; E-mail: jbjones@ufl.edu

*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures are published online.

industry. Therefore, a knowledge of the pathogen diversity is essential to provide insight into potential modes of dissemination. To investigate the population structure of the pathogen associated with recent bacterial leaf spot epidemics, we collected 28 *P. syringae* strains from diseased fields in 10 different Florida and Georgia counties over the course of 2 years. Here, we provide evidence indicating that these epidemics were caused by two genetically distinct and clonal *P. syringae* lineages that were recently introduced and rapidly spread throughout major watermelon production areas in Florida.

Materials and Methods

Bacterial strain collection. Symptomatic leaf tissue was collected from commercial production fields of watermelon and squash grown in Florida and Georgia during the spring 2013 and 2014 seasons (Fig. 1). Sections (approximately 1 cm²) of leaf tissue were aseptically cut at the margin of lesions, surface sterilized by washing in 0.6% sodium hypochlorite for 1 min, and rinsed two times with sterile tap water. Leaf tissue was then macerated in sterile tap water and streaked on nutrient agar and King's medium B agar (KB). After 48 h of incubation at 28°C, single colonies were subcultured on KB and pure cultures were obtained for further characterization. Preliminary identification of the recovered bacterial strains was conducted through sequence analysis of the 16S ribosomal RNA (rRNA) and the ability to induce a hypersensitive response (HR) on tobacco leaves (Gürtler and Stanisich 1996). All strains that were identified as members of the *Pseudomonas* genus and HR-positive were preserved in a 30% glycerol solution at -80°C for long-term storage and, subsequently,

subjected to further analysis. The bacterial strains collected in this study are listed in Table 1.

Phenotypic characterization. Bacterial strains were assessed for fluorescence on KB and the LOPAT tests, which consist of levan production, oxidase reaction, potato soft rot ability, arginine dihydrolase production, and tobacco hypersensitivity (Lelliott et al. 1966). Copper sensitivity was determined according to a previously described method (Menkissoglu and Lindow 1991). First, the bacterial strains were cultured on plates of casitone yeast extract-glycerol agar (CYEG) amended with CuSO₄ · 5H₂O at 1 µg ml⁻¹. After incubation at 28°C for 24 h, bacterial cells were removed from the plates and suspended in sterile deionized water. The cell suspensions were adjusted to approximately 1 × 10⁸ CFU ml⁻¹ spectrophotometrically (optical density at 600 nm = 0.3), then diluted to a concentration of 10⁷ CFU ml⁻¹. Three 10-µl droplets were placed on CYEG agar plates amended with concentrations of CuSO₄ · 7H₂O at 0, 5, 10, 15, 20, 30, 40, 50, 60, and 70 µg ml⁻¹. Strains were incubated at 28°C and the plates were observed for growth after 48 h. Two *P. syringae* strains (DC3000 and A1513), which were previously identified as copper-sensitive and -resistant, respectively, were included as controls (Andersen et al. 1991; Feil et al. 2005). Ice nucleation activity (INA) of the bacterial strains was tested by culturing strains in a broth containing 0.01% casamino acids, 1.0% peptone, and 1.0% glucose at 24°C to the stationary growth phase. Bacterial suspensions were then kept at 4°C for 2 to 4 h prior to testing. Five 10-µl droplets of the colonized broth were placed onto aluminum foil on an ice-ethanol bath cooled to -12°C. Strains were considered to be INA positive if

Fig. 1. Map of Florida, with gray-shaded regions indicating the counties where the symptomatic leaf samples were collected. Numbers of strains isolated are indicated in parentheses ($n = 28$); strain 13-1 was collected from Tift County, GA and is not pictured here.

all five droplets froze within 30 s. Droplets of the uncultured broth were used as a negative control and all phenotypic tests were repeated twice.

Multilocus sequence-typing and analysis. Multilocus sequence-typing (MLST) was performed on the 28 *P. syringae* strains collected from the 2013–14 epidemic using four housekeeping genes (*gyrB*, *rpoD*, *gltA*, and *gap1*) according to the MLST schema described by Hwang et al. (2005). Amplicon sequences were trimmed to the same size, concatenated, and aligned with Mega 6 software (Tamura et al. 2013). A maximum-likelihood phylogeny was constructed with the sequences of 59 *P. syringae* strains from phylogroups 2 and 3 obtained from the Plant-Associated Microbes Database (PAMDB) and 3 additional strains from the National Center for Biotechnology Information (NCBI) GenBank. This analysis included publicly available sequences of 16 *P. syringae* strains previously isolated from symptomatic cucurbit tissue in the United States and France prior to 2013 (Table 2). Due to their association as cucurbit pathogens or genetic similarity to the strains sequenced in this study, the housekeeping gene sequence fragments of *P. syringae* strains HS191, CC457, and CC94 were extracted from the genomes available from NCBI with the following locus tags for each strain, respectively: *gyrB*: NZ_CP006256, AVEB02000356, and AVEA02000299; *rpoD*: NZ_CP006256, AVEB02000313, and AVEA02000508; *gltA*: NZ_CP006256.1, AVEB02000011, and AVEA02000519; and *gap1*: NZ_CP006256, AVEB02000099, and AVEA02000560. Model selection for the phylogenetic analysis was determined using the Akaike Information Criterion (Burnham and Anderson 2004), and the evolutionary history was inferred using the General Time-Reversible model (Nei and Kumar 2000). The multilocus sequence data for the strains collected in this study were deposited in the

PAMDB and NCBI databases with the following accession numbers: *gyrB* = MF370398 to MF370425, *rpoD* = MF370426 to MF370453, *gltA* = MF370454 to MF370481, and *gap1* = MF370482 to MF370509.

Genotypic characterization using BOX polymerase chain reaction. BOX polymerase chain reaction (PCR) analysis was conducted as described by (Versalovic et al. 1994) for all strains collected in this study. The PCR assay was performed in an Eppendorf Mastercycler gradient ES and the cycling conditions consisted of 1 initial cycle at 95°C for 7 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 65°C for 8 min; followed by a single final extension cycle at 65°C for 15 min. Template DNA consisted of cell suspensions adjusted to 10⁸ CFU ml⁻¹ and treated at 95°C for 10 min in sterile deionized water. The cell suspension (2 µl) was added to each 23-µl aliquot of reaction mixture, which contained 1× Gitschier buffer, 0.4 µM primer, 0.8 mM deoxynucleotide triphosphate (Sigma-Aldrich, St. Louis), 2.75 mM bovine serum albumin (Promega Corp., Madison, WI), 10% dimethyl sulfoxide (Sigma-Aldrich), and 2.5 U of DNA polymerase (Promega Corp.). DNA fragments were separated on a 1.5% agarose gel, stained with ethidium bromide, and photographed with a UV-gel documentation system (Alpha Imager; ProteinSimple, Santa Clara, CA).

Pathogenicity assays. The pathogenicity of the bacterial strains was evaluated on two-week-old seedlings of watermelon (*Citrullus lanatus* ‘Wrigley’; Seminis Vegetable Seeds, St. Louis), cantaloupe (*Cucumis melo* L. ‘Aphrodite’; Syngenta-U.S., Greensboro, NC), and squash (*Cucurbita pepo* ‘Conqueror III’; Seminis Vegetable Seeds). Inoculum was prepared from bacterial strains cultured on KB for 24 to 48 h at 28°C. Bacterial cells of cultures were suspended in sterile tap water and the resulting cell suspension was adjusted to

Table 1. *Pseudomonas syringae* strains collected in this study, including their geographical origin, year and host of isolation, phenotypic characteristics, multilocus haplotype, phylogroup classification, and the mean disease severity rating on watermelon (WM), cantaloupe (CL), and squash (SQ)^a

Strain	Source of strain										Mean severity rating ^b		
	County ^c	Year	Host	Fluor	INA	Hap	Phyl	WM (5.70)	CL (5.88)	SQ (4.96)			
13-1	Tift	2013	WM	–	–	1	2b	5.33 ± 0.82	7.83 ± 0.41**	6.83 ± 1.47**			
13-127A	Jackson	2013	WM	–	–	1	2b	4.50 ± 1.87	5.67 ± 0.52	5.17 ± 1.33			
13-139A	Suwannee	2013	WM	+	+	2	2a	7.17 ± 1.17**	7.33 ± 0.82	4.00 ± 0.63			
13-139B	Suwannee	2013	WM	+	+	2	2a	6.67 ± 1.03	6.33 ± 1.51	4.67 ± 1.03			
13-140A	Suwannee	2013	WM	–	–	1	2b	5.33 ± 0.82	6.67 ± 1.21	5.00 ± 0.63			
13-141A	Suwannee	2013	WM	+	+	2	2a	5.83 ± 0.98	7.67 ± 0.52**	4.83 ± 1.47			
13-217A	Suwannee	2013	WM	+	+	2	2a	7.00 ± 0.89	6.83 ± 0.98	5.00 ± 0.00			
13-217B	Suwannee	2013	WM	+	+	2	2a	6.83 ± 1.60	5.00 ± 1.10	4.00 ± 0.00			
13-425	Levy	2013	WM	–	–	1	2b	6.33 ± 1.03	5.17 ± 0.41	4.83 ± 0.75			
13-426	Gilchrist	2013	WM	–	–	1	2b	5.50 ± 1.05	6.50 ± 1.38	4.83 ± 0.41			
13-427	Alachua	2013	WM	–	–	1	2b	3.50 ± 0.55*	7.00 ± 0.89	4.00 ± 0.89			
13-428	Alachua	2013	WM	+	+	2	2a	4.83 ± 1.72	4.67 ± 0.82*	3.50 ± 1.38			
13-429	St. Johns	2013	WM	+	+	2	2a	7.50 ± 0.84**	7.50 ± 0.84**	3.83 ± 0.75			
13-509A	Hamilton	2013	SQ	–	–	1	2b	0.00 ± 0.00*	3.00 ± 1.41*	2.67 ± 0.82*			
13-522C	Levy	2013	WM	–	–	1	2b	5.17 ± 0.75	6.00 ± 0.63	5.67 ± 0.52			
13-585	Suwannee	2013	WM	+	+	2	2a	7.50 ± 1.22**	6.17 ± 1.17	4.50 ± 0.84			
13-630A	Levy	2013	WM	–	–	1	2b	4.67 ± 0.52	7.00 ± 0.63	6.50 ± 1.05**			
13-C1	Hendry	2013	WM	–	–	1	2b	5.50 ± 0.84	5.83 ± 0.41	5.50 ± 0.55			
13-C2	Hendry	2013	WM	–	–	1	2b	6.00 ± 0.63	6.17 ± 0.41	5.17 ± 0.98			
14-31S	Collier	2014	WM	–	–	1	2b	6.50 ± 0.55	7.83 ± 0.41	6.67 ± 1.03*			
14-32	Collier	2014	WM	–	–	1	2b	6.00 ± 0.89	5.17 ± 1.17	4.83 ± 1.17			
14-410	Alachua	2014	WM	–	–	1	2b	6.67 ± 1.21	6.00 ± 1.41	6.00 ± 1.67			
14-Gil	Gilchrist	2014	WM	–	–	1	2b	5.00 ± 2.19	5.17 ± 1.94	5.00 ± 0.00			
14-Pol	Suwannee	2014	WM	–	–	1	2b	6.17 ± 1.72	5.50 ± 1.05	5.17 ± 0.41			
14-SQI	Collier	2014	SQ	–	–	1	2b	2.67 ± 1.51*	4.00 ± 1.26*	3.83 ± 1.47			
14-WMA	Collier	2014	WM	–	–	1	2b	6.00 ± 2.10	7.00 ± 1.10	5.67 ± 0.82			
14-WMC	Collier	2014	WM	–	–	1	2b	5.67 ± 0.82	6.83 ± 1.47	7.00 ± 0.63**			
14-WMI	Collier	2014	WM	–	–	1	2b	6.50 ± 0.84	6.33 ± 1.03	5.50 ± 0.55			

^a Fluorescence (Fluor) was assessed under UV light using a 48-h culture grown on King’s medium B agar. INA = ice nucleation activity, Hap = haplotype, and Phyl = phylogroup.

^b Mean severity rating and standard deviation from 0 to 8 taken 21 days after inoculation, *n* = 6 plants per strain–host combination. Asterisks indicate a severity rating was significantly lower (*) or significantly greater (**) than the overall mean severity based on a nonparametric analysis of means for each host (α = 0.05). The overall mean severity rating for each host is shown in parentheses.

^c All counties listed are located in Florida, with the exception of Tift County in Georgia.

approximately 1×10^8 CFU ml⁻¹. Each plant was spray inoculated with the bacterial suspension until run-off, placed in a transparent plastic bag for 72 h, and maintained in the greenhouse with average temperature at $24 \pm 4^\circ\text{C}$ and 70 to 90% relative humidity. The negative control consisted of plants sprayed with sterile tap water. Three weeks after inoculation, the total proportion of necrotic or symptomatic leaf tissue was rated from 0 to 8 using a modified Horsfall-Barratt scale (Horsfall and Barratt 1945) where 0 = no symptoms and 1 = 1 to 3, 2 = 3 to 6, 3 = 6 to 12, 4 = 12 to 25, 5 = 25 to 50, 6 = 50 to 75, 7 = 75 to 87, and 8 = 87 to 100% symptomatic. Three replications were included for each strain–host combination and the experiment was carried out twice. For confirmation of Koch's postulates, symptomatic leaf tissue was collected from a subset of plants at the end of the experiment and bacterial strains were reisolated from plant tissue according to the method described above. To test for differences in virulence among the two *P. syringae* haplotypes collected in this study, data were subjected to a two-way nonparametric analysis of variance with weighted means for unequal sample sizes. Pairwise comparisons were made with Tukey's honestly significant difference ($\alpha = 0.05$) and all statistical analysis was conducted using JMP Pro 13 (SAS Institute, Cary, NC).

Results

Identification and phenotypic characterization. Twenty-eight bacterial strains were collected from nine counties in Florida and one in Georgia between 2013 and 2014 (Table 1). All strains were identified as members of the *Pseudomonas* genus through sequence analysis of the 16S rRNA and were levan positive, oxidase negative, nonsoft rotting on potato, and arginine dihydrolase negative and produced an HR on tobacco after 24 h (data not shown). Variability was observed in the fluorescence and INA of the collected strains (Supplementary Fig. S1): 20 were nonfluorescent and INA negative, whereas the eight fluorescent strains were INA positive (Table 1). In the copper resistance assays, all strains behaved similarly to the sensitive control (*P. syringae* pv. *tomato* DC3000), with a minimum inhibitory concentration (MIC) of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ of $40 \mu\text{g ml}^{-1}$, with the exception of strain 13-428, for which the MIC of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was $30 \mu\text{g ml}^{-1}$. The resistant control (*P. syringae* A1513) grew at all concentrations tested (data not shown).

MLST and analysis. MLST identified two haplotypes among the collected strains; the 20 nonfluorescent strains were assigned to haplotype 1 and 8 fluorescent strains to haplotype 2 (Table 1). Additionally, a BLAST search of the NCBI database identified *P. syringae* strains CC457 and HS191 to be identical to multilocus haplotype 1 at the four sequenced loci. Multilocus sequence analysis (MLSA) of the concatenated housekeeping genes was consistent with previous studies, and all clades were well defined, with bootstrap support

of 92% or greater (Bull et al. 2011; Hwang et al. 2005). The maximum-likelihood phylogeny indicated that the collected strains were members of *P. syringae* phylogroup 2, whereby haplotype 1 clustered in clade 2b and haplotype 2 in clade 2a (Berge et al. 2014; Bull and Koike 2015). Members of the 2b clade included other genetically diverse cucurbit pathogens previously isolated in the United States over various years and strains recovered from unrelated hosts. Haplotype 1 branched from the pathotype strain *P. syringae* pv. *syringae* LMG1247; however, it also clustered with the *P. syringae* pvs. *aptata* CFBP1617, *lapsa* LMG2206, *japonica* M301072, *atrofaciens* LMG5095, *coryli* NCPPB4273, and *pisi* NCPPB2585, which are also members of phylogroup 2b. Members of the 2a clade included *P. syringae* strains isolated as pathogens of unrelated hosts and a multilocus haplotype previously recovered in Florida, Georgia, and California from watermelon, cantaloupe, and squash over various years (Fig. 2; Table 2).

BOX-PCR fingerprinting. BOX-PCR has previously been used as a tool to identify clonal *P. syringae* populations (Bull and Koike 2015; Louws et al. 1994), and correlated with the genetic relatedness inferred through DNA-DNA hybridization or MLSA (Goldberg et al. 2006; Marques et al. 2008). The BOXA1R profiles obtained here confirmed that the two multilocus haplotypes each corresponded to genetically distinct clonal lineages. The banding patterns among the 20 strains in haplotype 1 were indistinguishable (Fig. 3) and, although these strains shared two common fragments with *P. syringae* pv. *syringae* HS191, they were clearly not identical (Supplementary Fig. S2). Among the eight strains from haplotype 2, these also displayed identical banding patterns, with the exception of 13-428, which lacked one approximately 4-kb fragment and contained two additional bands not present in the others (Fig. 3).

Pathogenicity assays. The results from two independent greenhouse experiments were similar and the effect of experiment was not significant ($P = 0.4758$); therefore, the results were combined. All of the collected bacterial strains were found to be pathogenic on watermelon cultivar Wrigley, cantaloupe cultivar Aphrodite, and squash cultivar Conqueror III, with the exception of strain 13-509A, which failed to produce lesions on watermelon. In general, the bacterial strains were moderately to highly virulent and 24 of the 28 strains produced a mean severity rating of 5.0 or greater on at least two hosts. The two strains originally isolated from squash (13-509A and 14-SQI) were weakly virulent, with average severity ratings significantly lower than the overall mean on three and two hosts, respectively ($P < 0.05$) (Table 1). Symptoms typically appeared 3 days after inoculation and were similar to those previously described for *P. syringae* infecting watermelon, cantaloupe, and squash (Hopkins and Schenck 1972; Newberry et al. 2016). These included stunting, water soaking on the cotyledons, and circular

Table 2. *Pseudomonas syringae* strains isolated from symptomatic cucurbit tissue in the United States and France prior to 2013 included for phylogenetic analysis^a

Strain	Host	Origin	Year	Source	Accession number			
					<i>gyrB</i>	<i>rpoD</i>	<i>gltA</i>	<i>gap1</i>
03-19A	Cantaloupe	Florida	2003	Newberry et al. 2016	KT625904	KT625925	KT625985	KT625959
03-113A	Cantaloupe	Florida	2003	Newberry et al. 2016	KT625906	KT625926	KT625987	KT625961
04-1	Watermelon	Georgia	2004	Newberry et al. 2016	KT625896	KT625912	KT625977	KT625951
200-1	Squash	Georgia	2000	Newberry et al. 2016	KT625901	KT625924	KT625982	KT625956
BS2121	Squash	California	2006	Newberry et al. 2016	KT625885	KT625927	KT625966	KT62593
BS3062	Watermelon	California	2011	Newberry et al. 2016	KT625895	KT625936	KT625976	KT62595
03-A13	Cantaloupe	Florida	2003	Newberry et al. 2016	KT625909	KT625921	KT625990	KT625963
02-B4	Watermelon	Florida	2002	Newberry et al. 2016	KT625911	KT625923	KT625992	KT625965
CC94	Cantaloupe	France	1994	Morris et al. 2000	AVEA02000299	AVEA02000508	AVEA02000560	AVEA02000519
CC457	Cantaloupe	France	2003	Morris et al. 2010	AVEB02000356	AVEB02000313	NZ_CP006256	AVEB02000011
99-3	Watermelon	Georgia	1999	Newberry et al. 2016	KT625900	KT625915	KT625981	KT625955
11-0438	ND	Florida	2011	Newberry et al. 2016	KT625908	KT625920	KT625988	KT625962
08-1	Watermelon	Florida	2008	Newberry et al. 2016	KT625897	KT625913	KT625978	KT625952
1188	Zucchini	California	ND	Hwang et al. 2005	...	...	...	...
01-23-5	Watermelon	Florida	2001	Newberry et al. 2016	KT625899	KT625919	KT625980	KT625954
99-1	Watermelon	Georgia	1999	Newberry et al. 2016	KT625898	KT625914	KT625979	KT625953

^a ND = not determined.

necrotic lesions, as well as foliar blighting and marginal necrosis on the true leaves, which progressed over time. On watermelon, lesions were gray to black and often surrounded by a chlorotic halo whereas, on squash, they initially appeared as yellow spots accompanied by vein clearing. Necrotic brown lesions were observed on cantaloupe and, as expected, no symptoms developed on the seedlings sprayed

with sterile tap water. Bacterial strains were reisolated from a subset of symptomatic leaf tissue and the identity of the recovered strains was confirmed based on the ability to fluoresce on KB and sequence analysis of *gyrB* (Hwang et al. 2005). No bacterial strains identified as *P. syringae* were isolated from the negative controls. A two-way nonparametric analysis of variance showed significant differences

Fig. 2. Maximum-likelihood phylogeny based on an alignment of four concatenated housekeeping gene fragments—*gyrB*, *rpoD*, *gap1*, and *gltA* (2,010bp)—for 86 strains of *Pseudomonas syringae* phylogroup 2 and four strains of phylogroup 3 (*P. syringae* pv. *lachrymans*, *P. amygdali*, *P. savastanoi*, and *P. syringae* pv. *tabaci*). Strains isolated from symptomatic tissue of watermelon, cantaloupe, or squash are labeled based on their collection, and the phylogroups are labeled at the node of each clade (Bull and Koike 2015). The pathovar designation of the type (T), pathotype (PT), and other reference strains is listed where available. The scale bar indicates the number of substitutions per site and bootstrap values greater than 75% are shown.

in the virulence between the two haplotypes ($P < 0.0001$). Strain 13-509A was excluded from this analysis because it was weakly virulent or nonpathogenic on the three hosts. Multilocus haplotype 2 was highly virulent on watermelon and produced a significantly greater mean severity rating (6.96) than haplotype 1 (5.83). In contrast, haplotype 2 was moderately virulent on squash, with a mean severity rating of 4.51, whereas haplotype 1 was highly virulent on this host (5.70). Both haplotypes were highly virulent on cantaloupe, and no significant differences between the mean severity ratings were observed on this host (Fig. 4).

Discussion

P. syringae is a genetically diverse pathogen of various cucurbit species and known to be disseminated through multiple sources into agricultural ecosystems (Monteil et al. 2016; Newberry et al. 2016; Słomnicka et al. 2015). A polyphasic characterization of the *P. syringae* population isolated from fields of diseased watermelon and squash throughout Florida revealed the presence of two clonal *P. syringae* lineages associated with recent disease outbreaks. Haplotype 1 was the most widely distributed and recovered from nine different Florida and Georgia counties between 2013 and 2014, whereas haplotype 2 was recovered from Suwannee, Alachua, and St. Johns Counties in 2013 (Table 1). All 20 strains which composed haplotype 1 displayed a unique phenotype, in that they were nonfluorescent on KB and negative for ice-nucleating ability (Table 1). These strains also displayed identical BOXA1R fragment patterns, indicating that they represent a genotypically uniform *P. syringae* population (Fig. 3). Haplotype 2 included eight fluorescent and ice-nucleating strains that also displayed identical BOXA1R fragment patterns, except for strain 13-428, which was similar but exhibited variation (Fig. 3).

Pathogenicity analysis showed that watermelon, cantaloupe, and squash seedlings were susceptible to a majority of the collected strains (Table 1). In general, both haplotypes were capable of producing moderate to high levels of disease severity on the three hosts; however, it was interesting to note differences in virulence between them. Despite being less prevalent, haplotype 2 was more virulent on watermelon than haplotype 1 yet produced only moderately severe disease on squash and was less virulent on this host. No obvious differences in virulence between the two haplotypes were observed on cantaloupe seedlings, which were also highly susceptible (Fig. 4). It was interesting to note that strain 13-509A failed to induce bacterial leaf spot symptoms on watermelon and was weakly virulent in general (Table 1); however, it could not be distinguished from other highly virulent strains using MLST or BOX-PCR (Table 1; Fig. 3). It is possible that there may be a genetic determinant, independent

of the evolution of the core genome (i.e., plasmid or type 3 secreted effector), responsible for this qualitative difference in pathogenicity. Of more practical relevance, all of the strains examined here were found to be sensitive to copper in vitro, which is one of the few management options currently available for the disease (Newberry et al. 2017).

The two multilocus haplotypes collected here were found to be members of *P. syringae* phylogroup 2 and related to other *P. syringae* strains isolated from diverse plant species in clades 2a and 2b (Fig. 2). Therefore, it was curious to identify from the NCBI database two other *P. syringae* strains that were identical to multilocus haplotype 1 and associated with both similar and unrelated plant diseases (Fig. 2). These included *P. syringae* strain CC457, which was isolated from symptomatic cantaloupe in France nearly a decade before the epidemics in Florida (Morris et al. 2010), and *P. syringae* pv. *syringae* HS191, which was recovered in Australia from diseased proso millet in 1969 (Gross and DeVay 1977; Ravindran et al. 2015). Interestingly, both of these strains were also reported to be non-ice nucleating, and a 2-kb truncation in the gene which encodes the outer membrane protein that serves as a nucleus for ice crystal formation was found in the genome of *P. syringae* pv. *syringae* HS191 (Berge et al. 2014; Ravindran et al. 2015). This suggests that this trait was

Fig. 4. Mean bacterial leaf spot severity on a scale of zero to eight on watermelon cultivar Wrigley, cantaloupe cultivar Aphrodite, and squash cultivar Conqueror III among the two *Pseudomonas syringae* haplotypes collected from the 2013–14 epidemic. Multilocus haplotype 1 is indicated in black ($n = 114$) and haplotype 2 in gray ($n = 48$). Error bars indicate the standard error of the mean and letters over the bars show significant differences (Tukey's honestly significant difference, $\alpha = 0.05$).

Fig. 3. BOX-polymerase chain reaction profiles of the *Pseudomonas syringae* strains collected from the 2013–14 Florida epidemic. Lane L shows the 1-kb ladder (Sigma-Aldrich) and NTC indicates the nontemplate control. Strains are grouped according to their multilocus sequence type: samples 1 to 20: 13-1, 13-140A, 13-C1, 13-C2, 13-127A, 13-425, 13-426, 13-427, 13-509A, 13-522C, 13-630A, 14-31S, 14-32, 14-WMA, 14-WMC, 14-WMI, 14-Pol, 14-Gil, 14-410, and 14-SQI, respectively (group I); and samples 21 to 28: 13-428, 13-429, 13-139A, 13-139B, 13-141A, 13-217A, 13-217B, and 13-585, respectively (group II). Arrows show fragments discerning strain 13-428 (sample 21) from other members of group II.

inherited vertically among strains of this lineage, and is atypical because members of *P. syringae* phylogroup 2 are often active for ice nucleation (Berge et al. 2014).

The genetic homogeneity among the *P. syringae* population recovered in this study contrasted with the diversity among other *P. syringae* strains isolated as cucurbit pathogens previously in the United States (Fig. 2). The identification of a single clonal *P. syringae* lineage (haplotype 1) distributed throughout Florida and isolated from production fields in the state located nearly 800 km apart indicated that this lineage was recently introduced into major Florida watermelon production areas (Fig. 1), likely occurring through either contaminated seed or watermelon transplants. The second clonal lineage (haplotype 2) identified here branched from another genetically uniform group of strains in phylogroup 2a that were predominantly associated with the disease in the United States prior to 2013 (Fig. 2; Table 2). Therefore, it was unclear whether this may represent an endemic *P. syringae* population, or whether haplotype 2 was also recently introduced into Florida.

Although *P. syringae* phylogroup 2 comprises 11 recognized pathovars associated with diseases of diverse plant families (Bradbury 1986; Bull and Koike 2015), members of this phylogenetic species are often identified as *P. syringae* pv. *syringae* based on the production of the lipodepsinonapeptide plant toxin syringomycin (Berge et al. 2014; Young 2010). *P. syringae* pv. *syringae* possesses a notoriously large and undefined host range (Bradbury 1986; Young 2010), yet differences in pathogenicity and virulence have been documented among individual strains within *P. syringae* phylogroup 2 (Maraite and Weyns 1997; Ravindran et al. 2015). In preliminary pathogenicity testing, we inoculated *P. syringae* pv. *syringae* HS191 on watermelon and squash and found that it was capable of producing necrotic lesions; however, these did not expand into the foliar blighting induced by some of the highly virulent strains isolated from watermelon (data not shown). The BOXA1R banding profiles among the strains collected in this study were similar but not identical to *P. syringae* pv. *syringae* HS191; therefore, whole-genome comparisons may provide relevant insights into the evolution of these strains as epidemic pathogens of watermelon and squash.

In Florida, there are approximately 10 major transplant production facilities which produce over 1 billion vegetable seedlings per year (McAvoy and Ozores-Hampton 2015). Therefore, these disease outbreaks could not have been caused by a single introduction of the pathogen. It is likely that an unresolved taxonomy, coupled with phenotypic and genotypic heterogeneity, have enabled virulent *P. syringae* strains to go undetected in cucurbit propagating material, especially because these strains cannot be differentiated from pathogens of unrelated hosts based on the evolution of the core genome at the four housekeeping gene fragments analyzed here (Fig. 2). Following the spring 2014 season, bacterial leaf spot did not reoccur on watermelon or squash in Florida; however, it was reported in Georgia watermelon fields and from watermelon transplants in North Carolina (Dutta et al. 2016; Quesada-Ocampo 2016). This highlights a need for the development of a specific diagnostic assay for members of *P. syringae* phylogroup 2 which pose a risk to cucurbit production, so as to limit their dissemination through contaminated seed or transplants.

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