Angular Leaf Spot of Cucurbits is Associated With Genetically Diverse *Pseudomonas syringae* Strains

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Abstract

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Angular leaf spot of cucurbits is generally considered to be caused by *Pseudomonas syringae* pv. *lachrymans*. It has a worldwide distribution and has been observed to emerge sporadically under humid and wet conditions. Reports of multiple *P. syringae* pathovars associated with the disease and lack of molecular analysis has left the true diversity of populations in the United States unclear. In this study, we collected 27 *P. syringae* strains causing foliar lesions and blighting on watermelon, cantaloupe, and squash in Florida, Georgia, and California over several years. Strains were fluorescent on King's medium B agar and displayed the typical phenotypic and biochemical characteristics of *P. syringae*.

P. syringae pv. *lachrymans* is a member of genomospecies 2. However, the genetic profiles obtained through both MLSA (*gyrB*, *rpoD*, *gapA*, and *gltA*) and BOX-PCR (BOXAIR) identified 26 of the *P. syringae* strains to be distributed among three clades within genomospecies 1, and phylogenetically distinct from genomospecies 2 member *P. syringae* pv. *lachrymans*. A novel MLSA haplotype of the pathogen common to all states and cucurbit hosts was identified. Considerable genetic diversity among *P. syringae* strains infecting cucurbits is associated with the same disease, and reflects the larger ecological diversity of *P. syringae* populations from genomospecies 1.

Angular leaf spot of cucurbits is generally considered to be caused by *Pseudomonas syringae* pv. *lachrymans* (Bradbury 1986; Zitter et al. 1996). It has a worldwide distribution, and has been observed to emerge sporadically under humid and wet conditions (Bhat et al. 2010; Riffaud et al. 2003; Zitter et al. 1996). Previous epidemics of angular leaf spot and similar cucurbit diseases have resulted in 80 to 100% disease incidence, and in severe cases, 100% yield loss due to bacterial blight (Harighi 2007; Langston et al. 2003; Morris et al. 2000). The symptoms may vary depending on the host and environmental conditions, and are initially observed as circular necrotic lesions. As the disease progresses, lesions may become irregular in shape and delineated by leaf venation, thus producing an angular appearance. Coalescence of the lesions results in a foliar blighting and destruction of the leaf canopy (Zitter et al. 1996). Bradbury (1986) reported that the host range of *P. syringae* pv. *lachrymans* included the larger Cucurbitaceae including *Citrullus lanatus* (watermelon), *Cucumis sativus* (cucumber), *C. melo* (cantaloupe or muskmelon), *Cucurbita maxima* (squash), and *C. pepo* (squash or pumpkin).

Until the early 2000s, reports of bacterial epidemics on cucurbits were almost always reported as *P. syringae* pv. *lachrymans* (Fatmi et al. 2008; Hopkins and Schenk 1972; Riffaud et al. 2003). The use of various tests to differentiate pathovars is based on the assumption that a pathovar represents a physiologically and genetically homogeneous taxonomic unit (Morris et al. 2000). Contradictory to this notion,

P. syringae pv. *lachrymans* has been reported to naturally infect unrelated perennial plant species, and is heterogeneous in terms of virulence and molecular features (Lamichhane et al. 2015; Marcelletti and Scortichini 2014; Slomnicka et al. 2014). Characterization of strains recovered from more recent epidemics occurring on cantaloupe and squash have identified *P. syringae* pvs. *aptata* and *syringae* as the causal agents, and a correlation between the level of virulence of a strain and its corresponding host range (Langston et al. 2003; Morris et al. 2000; Sedighian et al. 2014).

Genomic comparisons of *P. syringae sensu lato* have revealed nine distinct genomospecies within this pathogen complex, and serves as a framework to examine the taxonomic and phylogenetic relationships among strains associated with a specific family of hosts (Gardan et al. 1999). *P. syringae* pv. *lachrymans* has been placed into genomospecies 2 within this scheme; however, it is unclear if this is the only organism responsible for angular leaf spot of cucurbits (Marcelletti and Scortichini 2014). Taken together, these observations indicate that pathovar classification fails to precisely describe *P. syringae* pathogens of cucurbits.

Recent studies have demonstrated that the phylogroups identified through multilocus sequence analysis (MLSA) correspond to the demarcated genomospecies confirmed by DNA-DNA hybridization and whole genome sequencing (Berge et al. 2014; Bull et al. 2011; Marcelletti and Scortichini 2014). A large part of the genetic diversity within *P. syringae sensu lato* is represented by strains isolated from environmental and nonagricultural habitats (Berge et al. 2014). *P. syringae* strains from these environmental reservoirs may contribute to the emergence of new and virulent strains of the pathogen (Monteil et al. 2013). Although the pathovar concept is not defined by the phylogeny inferred through MLSA, this genetic structure can be used to identify novel *P. syringae* strains (Bull et al. 2011). Both *P. syringae* pvs. *lachrymans* and *syringae* have been identified as cucurbit pathogens in the United States, but molecular methods that discriminate genomospecies were not employed (Hopkins and Schenk 1972; Langston et al. 2003). The identification of *P. syringae* strains

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*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary figure is included in the online edition.

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responsible for angular leaf spot epidemics within this taxonomic framework will be useful for resistance breeding efforts and developing effective management practices.

Disease outbreaks on annual crops due to *P. syringae* pathogens are increasing in frequency (Lamichhane et al. 2015). Despite extensive characterization of the genetic diversity within *P. syringae sensu lato* (Berge et al. 2014; Bull et al. 2011; Gardan et al. 1999; Marcelletti and Scortichini 2014), limited molecular analysis data on *P. syringae* strains specifically causing angular leaf spot on watermelon, cantaloupe, and squash are currently available, and none from major cucurbit producing regions in the United States. Florida, Georgia, and California are consistently among the top producers of watermelon, cantaloupe, and squash with a combined farm gate value of \$530 million (USDA 2015). In this study, we demonstrate that different populations of *P. syringae*, distinct genetically from *P. syringae* pv. *lachrymans*, cause angular leaf spot on watermelon, cantaloupe, and squash in Florida, California, and Georgia. We also present data that indicates the presence of a novel MLSA haplotype in all three states. Preliminary results were previously presented (Rubio et al. 2012).

Materials and Methods

Bacterial strain collection. Twenty-seven *P. syringae* strains were collected between 1999 and 2011 from leaves of symptomatic watermelon, cantaloupe, and squash in Florida, Georgia, and California (Table 1). The pathotype strains were provided from Collection Française de Bactéries Phytopathogènes (CFBP); the International Collection of Micro-organisms from Plants, Landcare Research; the Laboratorium voor Microbiologie, University of Gent;

and the National Collection of Plant Pathogenic Bacteria (NCPBP) (Table 2). Bacterial strains were stored at -80°C in 50% glycerol and 50% nutrient broth or 30% glycerol in sterile deionized water. Strains were routinely cultured on King's Medium B (KMB) (King et al. 1954) at 28°C , and pure colonies were obtained for characterization.

Phenotypic characterization. Bacterial strains were assessed for fluorescence on KMB (King et al. 1954), levan production, oxidase reaction, potato soft rot ability, arginine dihydrolase activity, and tobacco hypersensitive response (HR) according to the LOPAT scheme for fluorescent pseudomonads (Lelliott et al. 1966). Ice nucleation activity (INA) of bacterial strains was tested according a previously published method (Cintas et al. 2002).

Confirmation of Koch's postulates. Pathogenicity of the bacterial strains was evaluated on watermelon (*Citrullus lanatus* cvs. Troubadour [Harris Seeds, Rochester, NY] and Wrigley [Seminis Vegetable Seeds, St. Louis, MO]), cantaloupe (*Cucumis melo* L. cv. Aphrodite [Syngenta-U.S., Greensboro, NC]), and squash (*Cucurbita pepo* cv. Conqueror III [Seminis Vegetable Seeds]). For confirmation of Koch's postulates, a subset of strains representative of each multi-locus haplotype was inoculated onto their original host of isolation, or if the original host of isolation was not specified then they were inoculated onto watermelon as follows. Two seedlings with one to two true leaves were tested per strain and four seedlings were used as negative controls. The negative controls consisted of plants inoculated with sterile tap water. Inoculum was prepared from bacterial cultures grown on KMB for 24 to 48 h at 28°C . Cells were suspended in sterile tap water and adjusted with a spectrophotometer to an optical density of 0.3 at a wavelength of 600 nm, corresponding

Table 1. List of bacterial strains collected in this study and characterization

Strain	Source of strain			Genomospecies ^c	MLSA haplotype ^d	LOPAT test results ^e					INA ^f	Disease severity rating ^g		
	State ^a	Year	Host ^b			L	O	P	A	T		WM	CL	SQ
99-1	GA	1999	WM	1(2b)	1	+	-	-	-	+	+	*(2)	*(2)	*(4)
99-3	GA	1999	WM	1(2b)	2	+	-	-	-	+	-	*(4)	*(3)	*(3)
200-1	GA	2000	SQ	1(2a)	3	-	-	-	-	+	+	*(4)	**(5)	**(5)
04-1	GA	2004	WM	1(2a)	3	-	-	-	-	+	+	*** (5)	*** (5)	*(4)
08-1	GA	2008	WM	1(2b)	4	+	-	-	-	+	+	*(2)	*(3)	*(3)
01-23-1	FL	2001	WM	1(2b)	5	+	-	-	-	+	-	*(4)	*** (6)	**(5)
01-23-1P	FL	2001	WM	1(2b)	5	+	-	-	-	+	-	*(3)	*** (4)	*(3)
01-23-2P	FL	2001	WM	1(2b)	5	+	-	-	-	+	-	*(3)	*** (6)	*(4)
01-23-5	FL	2001	WM	1(2b)	5	+	-	-	-	+	-	*(3)	*** (6)	*** (5)
02-B3	FL	2002	WM	1(2d)	6	-	-	-	-	+	+	*** (6)	*** (5)	*(4)
02-B4	FL	2002	WM	1(2d)	6	-	-	-	-	+	+	*** (5)	*** (5)	*(4)
03-113A	FL	2003	CL	1(2a)	3	-	-	-	-	+	+	*(3)	*** (6)	*** (5)
03-19A	FL	2003	CL	1(2a)	3	-	-	-	-	+	+	*(4)	*** (6)	*** (5)
03-A6	FL	2003	CL	2(3)	7	+	-	-	-	+	+	-	*** (5)	*(3)
03-A13	FL	2003	CL	1(2d)	8	+	-	-	-	+	+	*** (6)	*** (8)	*** (5)
11-0438	FL	2011	NA	1(2b)	9	+	-	-	-	+	+	*** (5)	*(4)	*(3)
BS3058	CA	2011	WM	1(2d)	10	+	-	-	-	+	+	*** (5)	*(4)	*(4)
BS3059	CA	2011	WM	1(2d)	10	+	-	-	-	+	+	*** (5)	*(3)	*(4)
BS3060	CA	2011	WM	1(2d)	10	+	-	-	-	+	+	*** (6)	*(3)	*(4)
BS3061	CA	2011	WM	1(2d)	10	+	-	-	-	+	+	*** (5)	-	*(3)
BS3062	CA	2011	WM	1(2d)	10	+	-	-	-	+	+	*** (5)	*(2)	*** (4)
BS2121	CA	2006	SQ	1(2a)	3	-	-	-	-	+	+	*** (5)	*(3)	*** (5)
BS2122	CA	2006	SQ	1(2a)	3	-	-	-	-	+	+	*** (5)	*(2)	*** (5)
BS2123	CA	2006	SQ	1(2a)	3	-	-	-	-	+	+	*** (5)	*(3)	*** (5)
BS2124	CA	2006	SQ	1(2a)	3	-	-	-	-	+	+	*** (5)	*(4)	*** (5)
BS2125	CA	2006	SQ	1(2a)	3	-	-	-	-	+	+	*** (6)	*(3)	*** (5)
BS2126	CA	2006	SQ	1(2a)	3	-	-	-	-	+	+	*** (5)	*(3)	*** (5)

^a GA = Georgia, FL = Florida, and CA = California.

^b Host: WM = watermelon, SQ = squash, CL = cantaloupe, and NA = not available.

^c Genomospecies according to Gardan et al. (1999) and in parentheses are phylogroups according to Hwang et al. (2005) as shown by Bull and Koike (2015).

^d MLSA haplotype: haplotype ID for the strains collected in this study.

^e LOPAT test results: L = levan, O = oxidase, P = potato soft rot, A = arginine dihydrolase, T = tobacco HR.

^f INA = Ice nucleation activity.

^g Disease severity rating on watermelon cvs. Troubadour and Wrigley (WM), cantaloupe cv. Aphrodite (CL), and squash cv. Conqueror III (SQ). Plants were observed for 14 to 21 days, (-) = no symptoms, * = low (1 to 24%), ** = medium (25 to 49%), *** = high (50 to 100%). The average of the Horsfall-Barratt rating is in parentheses.

to approximately 1×10^8 CFU/ml. Each plant was spray inoculated with the bacterial suspension until run-off. Plants were placed in transparent plastic bags for 72 h, and then maintained in the greenhouse or growth chamber at 20 to 25°C and 70 to 90% relative humidity with a 12-h photoperiod. Treatments were evaluated every other day for symptoms for a period of 14 to 21 days. Phytopathogenic bacteria were reisolated from the symptomatic tissue as follows. Approximately 1 cm² cut section of symptomatic leaf tissue was surface sterilized by washing in 0.6% sodium hypochlorite for 1 min and rinsing three times with sterile tap water. It was then macerated in sterile tap water and a loop-full of the resulting suspension was streaked onto KBC medium (Mohan and Schaad 1987) and incubated at 28°C. After 48 h, colonies were purified by subculturing onto KMB. Fluorescence and induction of a hypersensitive reaction (HR) of the recovered strains were evaluated and compared with the strain originally used for inoculation. In addition, for some strains, the *gyrB* locus was amplified and sequenced to confirm the strain identity (Hwang et al. 2005).

Host range and virulence tests. To assess the host range of the collected strains, pathogenicity assays were performed on two-leaf stage seedlings of watermelon, cantaloupe, and squash. Three seedlings of each host were tested per strain, and pathogenicity tests were repeated twice as described above. After 14 to 21 days, disease severity was assessed visually, and the proportion of symptomatic tissue was rated from 0 to 11 using the Horsfall-Barratt (HB) scale (Horsfall and Barratt 1945). A strain was considered to be pathogenic to a particular host genotype if an HB rating of 2 or greater was produced on at least half of the plants tested. Severity ratings were converted to midpoint averages of disease severity, and the virulence of a strain was categorized based the final percentage of foliar symptoms as: - = no symptoms, * = low (1 to 24%), ** = moderate (25 to 49%), and *** = high (50 to 100%).

Phylogenetic analysis. Multilocus sequence analysis was performed on the 27 *P. syringae* strains by sequencing four housekeeping genes: *gyrB* (gyrase B), *rpoD* (RNA polymerase sigma⁷⁰ factor), *gapA* (glyceraldehyde-3-phosphate dehydrogenase A), and *gltA* (citrate synthase), using the MLST schema described by Hwang et al. (2005). Amplicons were sequenced bidirectionally, aligned, and edited using the Muscle tool available within MEGA 6 (Tamura et al. 2013). Alignments were trimmed to the same size, and sequences were deposited into the National Center for Biotechnology Information GenBank (NCBI) database (<http://www.ncbi.nlm.nih.gov>) with the following accession numbers: *gyrB*, KT625885 to KT625911; *rpoD*, KT625912 to KT625938; *gapA*, KT625939 to KT625965; and *gltA*, KT625966 to KT625992. The multiple sequence alignment of concatenated sequences was deposited to Tree Base with the accession number S18260 (<https://treebase.org/treebase-web/home.html>). Phylogenetic analysis included *P. syringae* pathotypes (Bull et al. 2011) and other selected sequences of *P. syringae* strains from various hosts and locations displaying at most a 0.002 maximum composite pairwise distance between any strain collected in this study. Sequences were obtained from Plant Associated Microbes Database (PAMDB) (<http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl>) and whole genome shotgun alignments from GenBank (Accession numbers WP_003340175, WP_003411788, WP_002552974, and WP_024643214). Fifty sequences were included in the analysis. A maximum likelihood phylogeny was constructed using the general time reversible method (Nei and Kumar 2000). Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method (Saitou and Nei 1987) to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach (Tamura et al. 2004).

Genotypic characterization using BOX-PCR. To confirm the relationships inferred by MLSA, the genetic relatedness among a subset of strains representative of the different MLSA haplotypes recovered from each host and geographic region was assessed using BOX-PCR. *P. syringae* pathotype strains from genomospecies 1 were used for reference (Bull et al. 2011). Generation of amplicons for the BOXA subunit of the BOX element was accomplished using the BOXA1R primer (5' CTACGGCAAGGCGACGCTGACG 3')

(Rademaker et al. 2004). Genomic DNA was isolated using NucleoSpin Tissue kits (Macherey-Nagel, Deer Park, NY) from each strain and subjected to BOX-PCR analysis. DNA fragments were separated on a 1.5% agarose gel, stained with GelRed (Biotium, Hayward, CA), and visualized on a UV transilluminator (UVP, Upland, CA). The image was acquired and saved using Doc-ItLS Image Acquisition Software v. 7.1 (UVP). Genetic distances of bacterial strains were calculated as Pearson's product-moment correlation coefficients between densitometric curves generated from the fingerprint patterns using Bionumerics software version 4.602 (Applied Maths Inc., Kortrijk, Belgium). The optimal curve-based analytical parameters were utilized as suggested by the Bionumerics manual, and banding patterns were normalized in relation to the 1kb molecular marker (Life Technologies, Carlsbad, CA). A dendrogram was produced using unweighted pair group method with arithmetic mean (UPGMA) based on a matrix of genetic distances (Sneath and Sokal 1973).

Results

Phenotypic characterization. All strains collected in this study displayed traits typical of *P. syringae* such as fluorescence on KMB, absence of a terminal cytochrome oxidase, non-soft rotting on potato, arginine dihydrolase negative, and produced an HR on tobacco after 24 h. Variability was observed in the levan reaction and ice nucleation activity. Twelve strains did not produce levan from sucrose and five strains were non-ice nucleating (Table 1).

Confirmation of Koch's postulates and pathogenicity assays. All strains were pathogenic to their original host of isolation. Each strain reisolated from symptomatic tissue was confirmed to be identical to the original strain used for inoculation based on fluorescence, induction of HR, and sequence analysis of the *gyrB* locus, thus confirming Koch's postulates. No bacteria were isolated from the negative controls. Foliar symptoms included circular necrotic spots, marginal necrosis, foliar blighting, and water soaking, which were observed on all three hosts. On cantaloupe, lesions were tan, irregular, and had an angular appearance, while on watermelon they appeared as dark circular lesions, which sometimes appeared to have a white to tan center with chlorotic halo. Lesions on watermelon similarly became irregular and somewhat angular. Squash symptoms differed from watermelon and cantaloupe, and included yellow, water soaked, circular spots often associated with vein clearing, and resulted in necrosis (Supplementary Fig. S1). No differences in the symptoms produced by the different strains were observed other than the level of virulence in these assays.

Regarding the host range, all strains were pathogenic on watermelon, cantaloupe, and squash with the exception of BS3061 and

Table 2. List of type and pathotype stains of *Pseudomonas syringae* and related pathogens used for genetic comparison

Type, pathotype	Strain number	Genomospecies ^a
<i>P. syringae</i>		
pv. <i>aptata</i>	CFBP 1617 ^{PT}	1(2b)
pv. <i>atrofaciens</i>	CFBP 2213 ^{PT}	1(2b)
pv. <i>syringae</i>	LMG 1247 ^{PT}	1(2b)
pv. <i>lapse</i>	LMG 2206 ^{PT}	1(2b)
pv. <i>pisi</i>	LMG 5079 ^{PT}	1(2b)
pv. <i>coryli</i>	NCPBP 4273 ^{PT}	1(2b)
pv. <i>aceris</i>	LMG 2106 ^{PT}	1(2d)
pv. <i>solidagae</i>	ICMP 16925 ^{PT}	1(2d)
pv. <i>dysoxylis</i>	LMG 5062 ^{PT}	1(2a)
pv. <i>papulans</i>	LMG 5076 ^{PT}	1(2a)
pv. <i>lachrymans</i>	CFBP 6463 ^{PT}	2(3)
pv. <i>savastanoi</i>	LMG 2209 ^{PT}	2(3)
pv. <i>tabaci</i>	NCPBP 1427 ^{PT}	2(3)
<i>P. congelans</i>	LMG21466 ^T	1(2c)
<i>P. amygdali</i>	CFBP3205 ^T	2(3)

^a Genomospecies according to Gardan et al. (1999) and phylogroups according to Hwang et al. (2005) as shown by Bull and Koike (2015) are in parentheses.

03-A6 (Table 1), which were pathogenic against only two of the three hosts. The negative controls did not display any symptoms. Variability was observed in the level of virulence of a particular strain on different hosts; 16 strains were moderately (25 to 49%) to highly (>50%) virulent on two or three hosts; eight were moderately to highly virulent on only one host; and three were weakly virulent on all three hosts causing less than 25% disease severity. In general, bacterial strains were moderately or highly virulent on their original host of isolation with the exception of strains 01-23-1, 01-23-1P, 01-23-2P, and 01-23-5. These strains were isolated from watermelon, but found to be weakly virulent on watermelon and moderately to highly virulent primarily on cantaloupe. Among the three hosts tested, watermelon displayed the greatest degree of susceptibility with 16 strains categorized as moderately to highly virulent, 12 strains were moderately to highly virulent on cantaloupe, and 13 strains were moderately virulent on squash with none categorized as highly virulent (Table 1).

A large variability in virulence was observed for those strains isolated from watermelon. Georgia strains 99-1, 99-3, and 08-1 were weakly virulent on all hosts tested and 04-1 was moderately virulent on watermelon and cantaloupe. California strains BS3058, BS3059, BS3060, BS3061, and BS3062 were moderately to highly virulent on watermelon. These strains were weakly virulent on cantaloupe and squash, and BS3061 produced no symptoms on cantaloupe. Among the Florida strains, 02-B3 and 02-B4 were moderately to highly virulent on watermelon and cantaloupe. All strains recovered from cantaloupe were moderately to highly virulent on at least one

host. Strain 03-A6 was moderately virulent on cantaloupe, and produced only minor marginal necrosis on one watermelon seedling. No symptoms were observed on other watermelon plants, and it was therefore categorized as nonpathogenic on this host. Strains 03-113A and 03-19A from cantaloupe were moderately to highly virulent on two hosts and 03-A13 on three. Squash strain 200-1 collected in Georgia was moderately virulent on squash and cantaloupe, while all strains recovered from squash in California were moderately to highly virulent on watermelon and squash (Table 1).

MLSA. Phylogenetic analysis of four housekeeping genes indicated that all strains with the exception of 03-A6 were members of genomospecies 1, *P. syringae sensu stricto*, and were distributed among three clades within phylogroup 2 (2a, 2b, and 2d) (Fig. 1). Each clade displayed high statistical support, and the topology of the trees when comparing individual loci was consistent with the concatenated data set for each strain (data not shown). Strain 03-A6 was a member of genomospecies 2 and identical to *P. syringae* pv. *tabaci* at the four loci sequenced. Phylogroup 2b consisted of eight strains representing five MLSA haplotypes (1, 2, 4, 5, and 9) recovered from watermelon in Florida and Georgia, as well as *P. syringae* pvs. *aptata*, *atrofaciens*, *coryli*, *lapsa*, *pisi*, and *syringae*. Among these, strains 01-23-1, 01-23-1P, 01-23-2P, 01-23-5, 99-1, and 08-1 formed a subgroup with *P. syringae* pvs. *aptata* and *atrofaciens* with a bootstrap value of 75. Four of these strains (01-23-1, 01-23-1P, 01-23-2P, and 01-23-5) shared MLSA haplotype 4, and included in this subgroup were *P. syringae* strains 1188 and FF5 previously recovered from zucchini and pear, respectively (Hwang et al. 2005; Peñaloza-Vázquez

Fig. 1. Maximum likelihood phylogenetic tree (1,000 bootstrap value) of concatenated *Pseudomonas syringae* strains with *P. syringae* pathotypes (Bull et al. 2011), and selected sequences obtained from the GenBank database according the Hwang et al. (2005) scheme for multilocus sequence analysis. All strains collected in this study are indicated by (*), pathotype strains - PT, and type strains - T. Phylogroups are denoted at the node of each clade according to Bull and Koike (2015). The place and source of isolation of each strain is listed. ND = not determined.

et al. 2004). Also within phylogroup 2b, strain 99-3 grouped with *P. syringae* pv. *lapsea* and *P. syringae* strains B64 and DSM5025 recovered from wheat (Hwang et al. 2005). Strain 11-0438 was distinct from any sequence included in this clade.

Phylogroup 2d contained eight strains representing three multilocus haplotypes (6, 8, and 10) recovered from watermelon and cantaloupe in Florida and California. Strains BS3058, BS3059, BS3060, BS3061, and BS3062 associated with outbreaks on watermelon grown in California shared MLSA haplotype 10 and grouped with 03-A13 isolated from cantaloupe grown in Florida, and *P. syringae* pvs. *aceris* and *solidagae*. Related to this group were strains 02-B3 and 02-B4 recovered from watermelon in Florida, and *P. syringae* CC94 recovered from cantaloupe in France (Morris et al. 2000). These strains formed a subgroup with a bootstrap value of 99. The remaining 10 strains shared an identical MLSA haplotype (3) and were members of phylogroup 2a. These strains were recovered in California, Florida, and Georgia in 2000, 2003, 2004, and 2006 from watermelon, cantaloupe, and squash (Fig. 2). They clustered with *P. syringae* pvs. *papulans* and *dysoxylis*, *P. syringae* Cit7 from asymptomatic citrus, and *P. syringae* B76, a tomato pathogen (Hwang et al. 2005).

Genetic relatedness based on BOX-PCR. The dendrogram produced from the BOX-PCR fingerprinting profiles was congruent to that of MLSA and confirmed the phylogroup and genomospecies designations. All strains grouped into the same respective clades represented by pathotype strains (Table 2) as inferred through MLSA; however, the topology of the individual strains within a clade was slightly different (Fig. 2). Representative of phylogroup 2b, strains 01-23-1 and 99-1 were most closely related to *P. syringae* pv. *aptata*, while strains 08-1 and 99-3 grouped with *P. syringae* pv. *atrofaciens*. This clade also contained *P. syringae* pv. *syringae*, and strain 11-0438, which were more distantly related. Phylogroup 2d was represented by *P. syringae* pvs. *aceris* and *solidagae*; strains BS3058, BS3059, and BS3060 displayed identical fingerprinting patterns and grouped with 03-A13 and *P. syringae* pv. *solidagae*. Strain 02-B3 grouped with *P. syringae* pv. *aceris* in this clade. Representatives of the 10 strains that displayed identical MLSA haplotypes in phylogroup 2a were most closely related to *P. syringae* pv. *dysoxylis*. Among these strains, 03-113A, 200-1, and BS2122 displayed identical banding patterns, and 04-1 was closely related. Strain 03-A6 grouped apart from other strains in genomospecies 1 (Fig. 2).

Discussion

The genetic profiles obtained by MLSA and BOX-PCR identified the *P. syringae* strains infecting cucurbits in Florida, Georgia, and California to be predominantly distributed among three clades within genomospecies 1 phylogroup 2 (2a, 2b, and 2d; Gardan et al. 1999; Hwang et al. 2005). Genomospecies 1 is synonymous with phylogroup 2, and will be referred to as such in the following discussion (Bull and Koike 2015). One strain (03-A6) was a member of genomospecies 2 and identical to *P. syringae* pv. *tabaci* at the four loci sequenced. This is in contrast to the causal agent commonly attributed to angular leaf spot of cucurbits, *P. syringae* pv. *lachrymans*, which was phylogenetically distinct from all strains collected in this study (Bull et al. 2011; Gardan et al. 1999) (Fig. 1). *P. syringae* strains from phylogroup 2 previously were described to comprise a ubiquitous population including nonpathogens and strains isolated from environmental samples (Berge et al. 2014; Clarke et al. 2010). All strains included in this study were obtained from surface sterilized symptomatic cucurbit tissue, and using Koch's postulates we confirmed them to be pathogens of their original host.

Considering the genetic diversity observed among cucurbit pathogens in phylogroup 2, it is interesting to note that most of these strains were genetically distinct from any pathotype strain previously associated with cucurbit diseases except for *P. syringae* pv. *aptata* in phylogroup 2b. Six strains (01-23-5, 01-23-1, 01-23-1P, 01-23-2P, 99-1, and 08-1) collected in this study formed a subgroup with *P. syringae* pv. *aptata*, along with *P. syringae* 1188 (recovered from zucchini) and FF5 (a pathogen of ornamental pears) (Fig. 1) (Hwang et al. 2005; Peñaloza-Vázquez et al. 2004). This indicates

that genetic variants of *P. syringae* pv. *aptata* could be pathogenic on cucurbits, particularly on cantaloupe. However, genetic variants of this pathovar such as *P. syringae* FF5 were reported to infect unrelated hosts (Peñaloza-Vázquez et al. 2004). Strain 99-3 is also a member of phylogroup 2b and part of a subgroup of strains recovered from wheat and corn such as *P. syringae* strains B64, DSM5025, and *P. syringae* pv. *lapsea* (Hwang et al. 2005) (Fig. 1). Although 99-3 was weakly virulent on the three hosts tested, this is another example of a *P. syringae* strain identified as naturally infecting cucurbit tissue that was phylogenetically similar to unrelated phytopathogens.

Phylogroup 2d included strains isolated from watermelon and cantaloupe in Florida and California. Two of these strains (02-B3 and 02-B4) clustered with *P. syringae* CC94, which was recovered from cantaloupe in France, and characterized as *P. syringae* pv. *aptata* based on biochemical and host range tests (Morris et al. 2000). This group of strains was most closely related to *P. syringae* pvs. *aceris* and *solidagae*, and was phylogenetically distinct from *P. syringae* pv. *aptata*. This observation supports the notion that *P. syringae* pathovars associated with cucurbit diseases do not represent homogenous taxonomic units. Additionally, *P. syringae* CC94 shared the same MLSA haplotype as other *P. syringae* strains that were recovered from fresh snow fall (C. E. Morris, personal communication). The identification of other related cucurbit pathogens in this group supports the observations of Morris et al. (2008) that *P. syringae* strains recovered from precipitation are genetically similar to pathogens from disease outbreaks.

Given the genetic diversity of strains found in clades 2b and 2d, it was unexpected to find a single MLSA haplotype (3) common to all hosts and geographic regions sampled in phylogroup 2a. All strains in this group were levan negative and most closely related to the *P. syringae* pvs. *papulans* and *dysoxylis* (Fig. 1 and 2). These strains were moderately to highly virulent on more than one host; however, the level of virulence on a specific host was not consistent for individual strains. Those recovered in California were weakly virulent on cantaloupe, while those from Florida and Georgia were moderately to highly virulent on this host (Table 1). Differences in the cultural characteristics of these strains on nutrient agar were observed as well; California strains displayed a yellow pigment, while those from Florida and Georgia produced creamy white colonies typical of *P. syringae* (data not shown). Previous studies reported geographic locations and environmental conditions impact the phenotype of *P. syringae* strains (Morris et al. 2010), and this may account for the differences observed in colony morphology and virulence.

P. syringae pathogens of cucurbits can be transmitted to seedlings by contaminated seed (Leben 1981; Shila et al. 2013). Seed infestation has been demonstrated to be the main cause of long distance pathogen dissemination (Lamichhane et al. 2015), and the lack of genetic diversity in geographically segregated populations may indicate this (Parkinson et al. 2009). The BOX-PCR banding patterns produced by three of the strains recovered from Florida, Georgia, and California support the clonal nature of this MLSA haplotype (Fig. 2). Additionally, phenotypic traits are variable among and within phylogroups (Berge et al. 2014), and the fact that all strains displayed an atypical levan negative phenotype further suggests they are of the same clonal lineage.

The observation that most of these strains were recovered from different hosts in different states raises other possibilities for the consistent recovery of this haplotype. Sarkar and Guttman (2004) recovered strains with identical MLSA haplotypes isolated from the same host, as well as strains recovered from unrelated hosts such as paper mulberry and tobacco in geographically distant regions at different times. Interestingly, *P. syringae* strain KOZ8101 from paper mulberry is also a member of phylogroup 2a (Sarkar and Guttman 2004), as well as other well studied epiphytes such as *P. syringae* Cit7 (Fig. 1) (Orser et al. 1985). This relationship suggests that members of this clade may represent an ecological niche, well adapted to the leaf surface. A portion of the *P. syringae* life cycle can be carried out in the phyllosphere as an epiphyte on asymptomatic tissue (Hirano and Upper 2000). An epiphytic bacterium in a given leaf ecosystem has an extremely small possibility of causing a lesion, and this is

conditioned by host susceptibility, environmental conditions, intraspecific competition, and numerous other variables (Hirano and Upper 2000). The consistent recovery of this haplotype from symptomatic cucurbit tissue may indicate the identification of a common epiphyte that is also a successful pathogen of watermelon, cantaloupe, and squash.

Phylogenetic data alone are not sufficient for pathovar designation as pathovars must differ in their pathogenicity profiles on different hosts (Dye et al. 1980). For example, even phylogenetically related pathovars such as *P. syringae* pvs. *aptata* and *atrofaciens* have been differentiated by host range (Maraite and Weyns 1997). Recently, a

Fig. 2. A, DNA fragment-banding patterns from DNA amplified using BOX-A1R primers. Bacterial strains isolated from watermelon, cantaloupe, and squash are compared with pathotype strains in genomospecies 1. **B**, Unweighted pair group method with arithmetic mean (UPGMA) tree of cucurbit and pathotype strains in genomospecies 1. The dendrogram was constructed from a matrix of distances calculated as Pearson's product-moment correlation between densitometric curves produced by the BOX-PCR fingerprinting patterns in part A. Phylogroups are denoted according to Bull and Koike (2015).

pathogen of zucchini squash (*P. syringae* pv. *peponis*) was described from France (Manceau et al. 2011). The sequence homology of the *gyrB* and *rpoD* loci published for one strain of this new pathovar indicated that it is most closely related to *P. syringae* pvs. *lapsa* and *dysoxyli* respectively, both of phylogroup 2. This pathovar *peponis* is invalid because it was not proposed according to the standards for naming pathovars (Dye et al. 1980; Young et al. 2001).

The pathogenicity assays utilized in this study demonstrated that genetically different populations displayed similar symptoms and host range. Several strains (99-1, 99-3, and 08-1) were only weakly virulent on the three hosts tested and are associated with the disease, but may not be the causal agent of symptoms observed in the field. However, highly virulent strains were also identified in each of three clades from phylogroup 2 known to contain plant pathogens (2a, 2b, and 2d) (Berge et al. 2014). More extensive host range studies and comparison with pathotype strains may reveal that the strains collected here to represent different pathovars. However, because many of these strains form statistically supported subgroups with unrelated phytopathogens, epiphytic strains, and strains recovered from precipitation, we suggest that watermelon, cantaloupe, and squash are susceptible to a diverse population of *P. syringae*, and pathovar classification may be of limited utility for this group of pathogens. It is interesting to note that while strains from genomospecies 1 are genetically diverse, the only strain recovered from genomospecies 2 displayed an identical MLSA haplotype to *P. syringae* pv. *tabaci*. The lack of strains from genomospecies 2 suggests this genetic group may play a minor role in cucurbit diseases. Similar conclusions were reached for populations recovered from cucumber (Słomnicka et al. 2015).

In summary, we demonstrated that genetically diverse *P. syringae* strains are associated with angular leaf spot on cucurbits from Florida, Georgia, and California. These strains were phylogenetically distinct from *P. syringae* pv. *lachrymans*, which has been recognized as the causal agent of angular leaf spot. Among these genetically diverse *P. syringae* strains, we identified a number of MLSA haplotypes that were phylogenetically distinct from all previously identified pathovars associated with foliar diseases of cucurbits (Bradbury 1986). Moreover, a novel haplotype of the pathogen was common to three major cucurbit-producing states and multiple cucurbit hosts. The genetic diversity among these strains reflects the larger ecological diversity of *P. syringae* populations in phylogroup 2, and for this reason pathovar classification may not be appropriate for accurate and precise identification of pathogenic strains within this genomospecies. Further investigation of the host range for strains from genomospecies 1 and whole genome sequencing is needed to establish a pathovar for this genetic group, and to identify factors that may indicate epidemic potential among highly virulent types. These findings validate the need for routine characterization of *P. syringae* strains to understand the etiology of disease outbreaks, so that the risks associated with seed/transplant level introduction of the pathogen into major cucurbit production areas can be minimized.

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