Genotyping *Xylella fastidiosa* in Rabbiteye blueberry in Louisiana, USA

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Abstract Rabbiteye blueberry (*Vaccinium ashei* = *V. virgatum*) comprises much of the blueberry acreage in the southeastern USA states of Louisiana and Mississippi. Three genotypes of *X. fastidiosa* were identified from rabbiteye blueberry in Louisiana by multilocus sequence typing. A genotype that was found at two orchards, sequence type (ST) 42, was identical to one previously found in southern highbush blueberry in Georgia and two non-blueberry native species in Texas. Two newly identified genotypes shared most alleles with *X. fastidiosa* strains considered, like ST 42, to be part of a group that is believed to have resulted from recombination between *X. fastidiosa* subsp. *multiplex* and subsp. *fastidiosa*. These two genotypes each also had one newly identified allele. This work suggests that a narrow range of *X. fastidiosa* genotypes infect

rabbiteye blueberry in Louisiana but that rabbiteye blueberry may serve as an alternative host for *X. fastidiosa* strains that infect more susceptible southern highbush cultivars.

Keywords *Xylella fastidiosa* · Bacterial leaf scorch · Blueberry · *Vaccinium* · Multilocus sequence typing

Xylella fastidiosa Wells et al. has been verified as the cause of bacterial leaf scorch of southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) and has been detected in rabbiteye blueberry (*V. ashei* Reade = *V. virgatum* Aiton) in the southeastern USA in Alabama, Georgia, and Louisiana (Chang et al. 2009; Coneva et al. 2012; Ferguson et al. 2017; Parker et al. 2012). Rabbiteye blueberry is considered less susceptible to bacterial leaf scorch than southern highbush blueberry and is associated with less dramatic symptoms than those observed in some southern highbush cultivars (Brannen et al. 2016; Chang et al. 2009; Ferguson et al. 2017). However, in Louisiana, researchers found an association between *X. fastidiosa* infection and yield reduction (Ferguson et al. 2017).

X. fastidiosa has been reported from numerous wild and cultivated plants (EFSA 2018), but there is host specificity among strains of *X. fastidiosa* (Harris and Balci 2015; Nunney et al. 2013). Understanding the host range of strains is important (Hopkins and Purcell 2002), as host range is likely to impact the efficacy of disease management practices such as rogueing of alternative hosts and utilization of clean planting material.

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Within *X. fastidiosa*, there are two validly published subspecies (*fastidiosa* and *multiplex*) and at least four proposed subspecies (*morus*, *pauca*, *sandyi*, and *tashke*) (Bull et al. 2010; Nunney et al. 2014b; Randall et al. 2009; Schaad et al. 2004a, b; Schuenzel et al. 2005), although Marcelletti and Scortichini (2016) argue that strains previously classified as subspecies *sandyi* and *morus* actually belong in subsp. *fastidiosa*. Parker et al. (2012) and Van Horn et al. (2017) identified *X. fastidiosa* strains naturally occurring in blueberry as subsp. *multiplex*. When only one locus was compared, two isolates from blueberry were equally closely matched to strains identified as subsp. *multiplex* and subsp. *sandyi* (Nissen 2010; Nunney et al. 2013). Nunney et al. (2013) placed all seven *X. fastidiosa* isolates of blueberry origin that were included in their study into a group that, while considered to be part of subsp. *multiplex*, may have arisen from recombination with subsp. *fastidiosa* (Nunney et al. 2014a).

Scally et al. (2005) developed a multilocus sequence typing (MLST) scheme, modified by Yuan et al. (2010), which allows *X. fastidiosa* strains to be genotyped below the subspecies level. The MLST approach involves typing strains based on the exact nucleotide sequences at seven housekeeping loci (Scally et al. 2005). An additional locus, *pilU*, is sometimes utilized in conjunction with the MLST loci (Nunney et al. 2014a; Yuan et al. 2010). Parker et al. (2012) took a different multilocus approach to characterizing *X. fastidiosa* isolates, which they termed multilocus sequence analysis of environmentally mediated genes (MLSA-E). Their approach involved nine genes that had, on average, higher ratios of non-synonymous to synonymous substitution rates than those used in the Yuan et al. (2010) MLST scheme.

Seven blueberry isolates that have been typed by the MLST scheme have been identified as sequence type (ST) 42 or 43, which were among strains that were deemed to be recombinant subsp. *multiplex* (Nunney et al. 2013). By the MLSA-E analysis of Parker et al. (2012), seven strains from blueberry, including one from rabbiteye blueberry, grouped together in what they called clade A of *X. fastidiosa* subsp. *multiplex*. Nunney et al. (2013) included 13 of the 21 *X. fastidiosa* subsp. *multiplex* isolates (from blueberry and other hosts) used by Parker et al. (2012), and they found that the six that Parker et al. (2012) called clade A were all part of the group that Nunney et al. (2013) considered recombinant.

While these findings suggest the possibility that a narrow range of *X. fastidiosa* subsp. *multiplex* genotypes occur in naturally infected blueberry plants, southern highbush blueberry plants expressed symptoms of leaf scorch following inoculation with *X. fastidiosa* subsp. *fastidiosa* isolates from elderberry and lupine, as well as *X. fastidiosa* subsp. *multiplex* isolates from almond and blackberry (Hopkins et al. 2012). Oliver et al. (2015) found that four subsp. *fastidiosa* isolates from two species (grape and elderberry) were able to colonize southern highbush blueberry cultivar Rebel plants, although the symptoms they caused were less severe than those caused by two of three isolates of blueberry origin. Recently, Di Genova et al. (2019) reported natural and artificial infection of southern highbush blueberry plants in Georgia by subsp. *fastidiosa*.

The objective of this study was to determine what *X. fastidiosa* strains are found in rabbiteye blueberry plants. This relates to the practical questions of what plants might serve as alternative hosts for *X. fastidiosa* in rabbiteye blueberry and whether rabbiteye blueberry might serve as an alternative host of the bacterium for more susceptible southern highbush blueberry cultivars.

To determine what *X. fastidiosa* genotypes are found in rabbiteye blueberry, DNA extracted from the following sources was utilized for multilocus sequence typing: (i) *X. fastidiosa* isolate LA-Y3C cultured from a ‘Tifblue’ rabbiteye blueberry stem in an East Feliciana Parish, Louisiana, orchard, (ii) petiole/midrib tissue from ‘Tifblue’ rabbiteye blueberry plants from the same East Feliciana Parish orchard, and (iii) stem tissue of a rabbiteye blueberry plant from a Saint Landry Parish, Louisiana, orchard. Samples represented both of the two out of 17 Louisiana blueberry orchards sampled in which *X. fastidiosa* was detected (Ferguson 2016). All source plants had been confirmed as *X. fastidiosa*-positive by real-time PCR. DNA was subjected to PCR using primers for loci *leuA*, *petC*, *malF*, *cysG*, *holC*, *nuoL*, *gltT*, and *pilU*, based on the protocol outlined in Yuan et al. (2010). Between 0.7 and 5 µl of template DNA was used in each 25 µl reaction.

PCR was performed on an Applied Biosystems 2720 Thermal Cycler (Life Technologies, Carlsbad, California, USA) using the following program: 3 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 65 °C, and 1 min at 72 °C; and 10 min at 72 °C (Yuan et al. 2010). A 1% agarose gel and GelRed stain (Biotium, Hayward, California, USA) were used to check for the presence of products of the expected sizes. Beckman Coulter

Genomics (Danvers, Massachusetts, USA) or Macrogen USA (Rockville, Maryland, USA) sequenced PCR products using the Sanger method. Products were either purified prior to sending by using the QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA) or purified using an enzymatic method by the company performing sequencing.

Forward and reverse sequences were aligned using MAFFT software (<http://mafft.cbrc.jp/alignment/server/>). In MAFFT or Mesquite (<http://mesquiteproject.org>), aligned forward and reverse sequences were viewed together and then assembled. Chromas (<http://technelysium.com.au/wp/chromas/>) was used to resolve conflicts between base calls. For the seven MLST loci, the sequences were entered as queries in the *X. fastidiosa* MLST database (<http://pubmlst.org/xfastidiosa/>), using the BLAST function. When assembled sequences differed from established alleles, MAFFT was used to determine where they differed. For *pilU*, sequences were entered into the NCBI BLAST query tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the matching *pilU* allele was noted.

When sequences from MLST loci and *pilU* of *X. fastidiosa* from culture and infected plant samples were BLASTed into the MLST database (for MLST loci) or NCBI database (for *pilU*), exact matches were revealed for most sequences, and close matches were found for others (Table 1). Sequences of one isolate and two plant tissue samples from the East Feliciana Parish orchard, as well as one from a Saint Landry Parish

orchard, corresponded to the previously defined ST 42 and had *pilU* sequences identical to allele 9.

ST 42 was previously identified from blueberry in Georgia and from giant ragweed and western soapberry in Texas (Nunney et al. 2013). The latter two species, both native to the southern United States, are in the Asteraceae and Sapindaceae families, respectively. Other genotypes identified as ST 42 for which the non-MLST *pilU* locus identity was reported have also had *pilU* allele 9, as found in this study (Nunney et al. 2013).

Samples EF-H3 and EF-N3 shared some alleles with ST 42 but differed from it at two and four loci, respectively (Table 1). Two alleles, the *gltT* allele of EF-H3 and the *cysG* allele of EF-N3, did not match any that were previously defined in the MLST database. The EF-N3 *cysG* allele differed from alleles 12 and 14 by one base. The *gltT* sequence of EF-H3 was most similar to *gltT* allele 7 but differed at two bases. EF-H3 *gltT* and EF-N3 *cysG* alleles have been deposited in GenBank (accessions MG581179 and MG581180) and have been assigned allele numbers 16 and 33, respectively, in the MLST database (Table 1). The newly identified combinations of alleles were designated as STs 82 and 83 (Table 1).

Other than those newly established by this study, the alleles in STs 82 and 83 are found either in STs that, like STs 42 and 43, are believed to have resulted from recombination between *X. fastidiosa* subspecies *multiplex* and *fastidiosa*, or in both these and non-recombinant *X. fastidiosa* subsp. *multiplex* strains (Nunney et al. 2013, 2014a, b; Yuan et al. 2010).

Table 1 *X. fastidiosa* alleles at multilocus sequence typing (MLST) and *pilU* loci

Isolate or Plant ^a	Location (Parish)	Alleles ^b								ST ^b
		<i>leuA</i>	<i>petC</i>	<i>malF</i>	<i>cysG</i>	<i>holC</i>	<i>nuoL</i>	<i>gltT</i>	<i>pilU</i>	
EF-C3	East Feliciana	6	3	5	12	4	3	3	9	42
EF-H3	East Feliciana	3	3	5	12	4	3	16 ^c	9	82 ^c
EF-K2	East Feliciana	6	3	5	12	4	3	3	9	42
EF-N3	East Feliciana	6	3	5	33 ^c	7	4	7	9	83 ^c
LA-Y3C	East Feliciana	6	3	5	12	4	3	3	9	42
SL-6	St. Landry	6	3	5	12	4	3	3	9	42

^a DNA sources were rabbiteye blueberry petiole/midrib tissue except for LA-Y3C (an isolate) and SL-6 (extracted from stem tissue)

^b Alleles (except those of *pilU*, which is not an MLST locus) and sequence types (ST) correspond to those in the *X. fastidiosa* MLST database (<http://pubmlst.org/xfastidiosa/>). Alleles also correspond to the following GenBank accessions: *cysG* 12 = HM243602, *cysG* 14 = FJ610190, *cysG* 33 = MG581180, *gltT* 3 = FJ610215, *gltT* 7 = FJ610220, *gltT* 16 = MG581179, *holC* 4 = FJ610196, *holC* 7 = JX899403, *leuA* 3 = FJ610158, *leuA* 6 = JX899394, *malF* 5 = FJ610175, *nuoL* 3 = FJ610206, *nuoL* 4 = FJ610208, *petC* 3 = FJ610167, *pilU* 9 = JX899410

^c Alleles *cysG* 33 and *gltT* 16, as well as STs 82 and 83, were newly established based on this study

Because our sequences were generated from plant samples, there is a possibility that more than one strain of *X. fastidiosa* was present, and isolation of the bacteria is needed to firmly establish the existence of new genotypes.

The alleles found in the EF-H3 sample (ST 82) were the same as those associated with ST 22, except that ST 22 has *gltT* allele 3 (Nunney et al. 2013). ST 22 has been identified from giant ragweed and western ragweed in Texas (Nunney et al. 2013). The alleles identified from EF-N3 (ST 83), except at the *cysG* locus, are the same as those in ST 28, which has been identified from giant ragweed, annual sunflower, and seacoast sumpweed (Nunney et al. 2013). All of these ST 22 and ST 28 isolates came from Texas, where plants were collected around vineyards for *X. fastidiosa* isolation (Nunney et al. 2013). Additional efforts to type *X. fastidiosa* from wild plants might reveal that these STs are present in other species.

A limited number of *X. fastidiosa* STs have been identified from rabbiteye blueberry. However, considering that ST 42 has been found in giant ragweed and that recombinant genotypes similar to others identified from rabbiteye blueberry have been found in this and other Asteraceae family plants, the number of alternative hosts for strains of *X. fastidiosa* that infect blueberry may be considerable. Weed management compatible with good production practices could help minimize alternative hosts in the immediate vicinity of blueberry plants. Additional work to determine the sequence types of *X. fastidiosa* in wild plants would provide more insight on the likelihood of being able to aid management of *X. fastidiosa* by targeting certain plant species for removal. If it were determined that a limited number of common wild plants host genotypes of *X. fastidiosa* that infect blueberry, management of these could be prioritized. Costa et al. (2004) made a similar recommendation after identifying a limited number of wild plants as hosts of *X. fastidiosa* around vineyards in California.

The finding of ST 42 in rabbiteye blueberry does suggest that rabbiteye blueberry may serve as an alternative host of strains that are able to infect susceptible southern highbush blueberry cultivars. There may be strains of *X. fastidiosa*, however, that are identical at MLST loci but have differences at other loci that influence their abilities to infect certain hosts. Cross-inoculation studies might add some clarity regarding the cross-infectivity of isolates from rabbiteye and southern highbush blueberry plants. Considering that older rabbiteye plants do not always show distinctive

symptoms of *X. fastidiosa* infection (Ferguson et al. 2017), growers are advised to take care to use clean planting stock when introducing rabbiteye plants into orchards in which susceptible southern highbush cultivars are grown.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement The research reported in this article did not have human or animal subjects.

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