INITIAL GENETIC ANALYSIS OF *XYLELLA FASTIDIOSA* IN TEXAS

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ABSTRACT

Xylella fastidiosa (*Xf*) is the causative agent of Pierce's disease of grape. *Xf* genetics has not been evaluated in Texas despite growing financial risk to the US grape industry, a Texas population of the insect vector now spreading in California, and evidence that the bacterium is ubiquitous to southern states. Using sequences of conserved *gyrB* and *mopB* genes, we have established at least two strains in Texas, grape strain and ragweed strain, corresponding genetically with subspecies *piercei* and *multiplex*, respectively. The grape strain in Texas is found in *Vitis vinifera* varieties, hybrid vines and in wild *Vitis* near vineyards; whereas, the ragweed strain in Texas is found in annuals, shrubs and trees near vineyards or other areas. RFLP and QRT PCR techniques were used to differentiate grape and ragweed strains with greater efficiency than sequencing and are practical for screening numerous *Xf* isolates for clade identity.

INTRODUCTION

The bacterium, *Xylella fastidiosa* (Wells) (*Xf*) resides in the xylem tissue of many plant species and is moved within plant communities through insect transmission. While many plants are not impacted by *Xf* infection, colonization of the xylem vessels of several economically important plants including citrus, coffee, grape, almond, oleander and oak result in disease (Purcell 1997). Symptoms of *Xf* infection include leaf chlorosis, leaf scorch, crop loss and plant death and are most severe in non-native crops (Purcell 1997). *Vitis vinifera* grapevine varieties (Cabernet Sauvignon, Chardonnay, etc.) are not native to the Americas and are susceptible to *Xf* infections or Pierce's disease (PD) which causes the rapid decline and mortality of vines. PD occurs throughout the US and has consistently occurred along the Texas Gulf coast (Kamas et al. 2004) and intermittently in the California grape growing regions for more than one hundred years (Purcell 1997). In the 1990s, a PD epidemic in southern California's Temecula region resulted from the introduction and subsequent range expansion of the glassy-winged sharpshooter (GWSS), (*Homalodisca vitripennis*) (de Leon et al. 2004) creating an enormous PD risk to the California grape industry.

Investigation of disease ecology and epidemiology in Texas has scientific advantages over simply investigating PD in California where it poses the greatest financial threat. Genetic evidence used to develop a phylogeny of GWSS has revealed that populations of this insect pest introduced into California originated from Texas (de Leon et al. 2004). The temperate Gulf climate of Texas and large numbers of *Xf* positive native plants (Buzombo et al. 2006) suggest that the bacterium has a long evolutionary history in the region. Therefore, genetic variability among *Xf* strains in Texas is critical to understanding the natural history of the disease in the US. Additionally, PD is the major limiting factor in the Texas Hill Country, a major wine grape production area in Texas. Despite the importance of understanding the genetics of *Xf* in this region, there are no published reports on *Xf* diversity within the state of Texas.

Xf strains demonstrate variable host ranges with some strains causing disease symptoms only in a specific plant species and others in multiple species (Almeida and Purcell 2003). *Xf* strains can be separated into clades, the most valuable first step in determining strain diversity. Current genetic analysis suggests five main clades: 1. isolates causing citrus variegated chlorosis (CVC) and coffee leaf scorch (CLS), 2. isolates causing PD, 3. isolates causing oleander leaf scorch (OLS), 4. isolates causing Dixon-like almond leaf scorch (ALS), and 5. isolates infecting hardwood (angiosperm) trees including oak, maple, and mulberry (Chen et al. 2002, Hendson et al. 2001, Meinhardt et al. 2003, Rodrigues et al. 2003, Schaad et al. 2004). Several techniques have been used in determining these clades genetically including REP-PCR and RAPD-PCR, (Chen et al. 2002, Hendson et al. 2001, Pooler et al. 1995, Rodrigues et al. 2003) and CHEF analysis (Hendson et al. 2001). Recently, three subspecies of *Xf* have been named and these subspecies are consistent with three of identified clades (excluding ALS and OLS) (Schaad et al. 2004). The PD strain has been labeled "subsp. *piercei*"; the hardwood group, peach and plum group "subsp. *multiplex*" and CVC strain group is "subsp. *pauca*. As these three clades have clear genetic differences and are differentially pathogenic we consider these to be distinct strains

Most genetic diversity studies have included sequencing a particular DNA fragment to determine phylogenetic relationships between isolates including the 16S-23S rDNA intergenic spacer region, 16S rDNA gene (Hendson et al. 2001) and the gyrase B (*gyrB*) gene (Rodrigues et al. 2003). Another gene useful for phylogenetic analysis is the *mopB* gene. The mopB protein is an outer membrane protein of bacteria with a fairly conserved sequence within the OmpA family of proteins (Fjellbirkeland et al. 2000).

OBJECTIVES

1. Analyze a preliminary collection of *Xf* isolates from Texas vineyards (grapevines and surrounding vegetation) as an initial screen of genetic diversity. Using *mopB* and *gryB* sequences, determine whether *Xf* isolates belonged to the grape clade (subsp. *piercei*), the hardwood mulberry clade (subsp. *multiplex*), OLS or ALS groups.
2. Determine if restriction fragment length polymorphism (RFLP) digestion and quantitative real-time polymerase chain reaction (QRT PCR) analyses of *gyrB* were consistent with gene sequence data.

RESULTS

Sequences of the *gyrB* and *mopB* genes were conserved within two strain groupings. One group of isolates showed perfect alignment with the PD strain 'Temecula' using BLAST and was designated the 'grape' strain. Another strain group aligned perfectly with the 'multiplex Dixon' strain and was designated the 'ragweed' strain. There was no genetic variability between 'grape' strains or 'Texas ragweed' strains for these two conserved genes and no evidence of OLS, CVC, or Coffee strains in this analysis of 14 isolates. Grape strain was identified from a variety of grapevines including: mustang grape (*Vitis mustangensis*), SO4 rootstock (*V. cinerea var. helleri* x *V. riparia*), Black Spanish/Lenoir (*V. aestivalis* hybrid with *V. cinerea* and *V. vinifera* parentage) and several European grape varietals (*V. vinifera*) (Table 1). The ragweed strain was identified from weed and woody species including: redspike Mexican hat flower (*Ratibida columnifera*), western ragweed (*Ambrosia psilostachya*), giant ragweed (*Ambrosia trifida var. texana*), annual sunflower (*Helianthus annuus*), sea myrtle (*Baccharis halimifolia*), cedar elm (*Ulmus crassifolia*) and heartleaf ampelopsis (*Ampelopsis cordata*) (Table 1).

Restriction digests of the *gry*B PCR amplicons indicated differential banding patterns consistent with predicted digestion patterns given by ChromasPro. Digestion of the *gyrB* PCR amplicons (408 bp) with the restriction enzyme BsrD1 showed a single cut (at bp 174) resulting in two bands for grape strain DNA. Ragweed DNA had no cuts for BsrD1 and one band for ragweed strains (data not shown). The enzyme Taq1 showed the opposite reaction. Taq1 cleaved grape strain *gyrB* amplicons, but did digest ragweed PCR products (Table 1).

QRT-PCR of the internal *gyr*B gene of these isolates showed Tm differences between grape and ragweed strains that were statistically distinct with grape strain DNA melting approximately 0.4-0.5^{o}C lower than DNA from ragweed strains ($P<0.0001$). Tukey's mean separation test showed strains could be placed into grape or ragweed categories based on Tm. Five strains showed variability in at least one to two runs such that they could not be placed statistically into one category (Table 1 on next page).

CONCLUSIONS

This initial investigation into the genetic variability of *Xf* strains in Texas indicates that there are two main strains in and near central Texas vineyards, subsp. *piercei* and subsp. *multiplex*. The strain isolated from grapevines in Texas is genetically identical to the Temecula PD strain in California. The grape strain was isolated from multiple European *V. vinifera* varieties, from hybrid *Vitis* species and rarely from wild *Vitis* species. Our preliminary data suggests that native *Vitis* species around the vineyard can harbor the grape strain and therefore are potential reservoirs for novel PD infections. With our current selection of plants in and around vineyards it is not possible to determine if the *Xf* isolated from wild and hybrid grapevines originated from infected vineyards or if the *Xf* isolated now in vineyards came from nearby wild or hybrid grapevines. We plan to analyze wild vines many kilometers from established vineyards for the presence and identity of *Xf* strains to help answer this question.

The ragweed strain (subsp. *multiplex*) is present in numerous weed species surrounding Texas vineyards. This strain is common in a large diversity of plants from annuals such as giant ragweed, annual sunflower, and redspike Mexican hat to perennial shrubs (sea myrtle) and woody trees (cedar elm). Interestingly, the ragweed strain was also found in peppervine (*Ampelopsis cordata*) a member of the Vitaceace, but not in the genus *Vitis*. Future work is planned to isolate and characterize Texas isolates from symptomatic oleander and stone fruit trees. Previous work using a multigenic approach indicate that *Xf* clades split 15,000 years ago and despite low variability within all subspecies, the subsp. *multiplex* shows the greatest variability, presumably because of a large host range (Schuenzel et al. 2005). Likewise, the California PD strain (subsp. *piercei*) has the lowest genetic variability and is speculated to have the narrowest host specificity (Schuenzel et al. 2005).

RFLP digestion of PCR amplicons was useful for distinguishing an isolate as either grape or ragweed. RFLP requires standard PCR and a 5-hour digestion, faster than the cloning methods used for sequencing. Even faster still was the comparison of the Tm between grape and ragweed strains after QRT PCR reactions (Bextine, data not shown). The QRT

PCR process allows for Tm to be measured for 96 samples in 1 hour allowing for a quick initial screen of multiple strains. QRT PCR occasionally resulted in samples not falling into either grape or ragweed categories with statistical certainty but was very useful for an initial screen of clade identity. QRT PCR requires lower DNA concentrations so strains could be identified directly from insect gut or sap. Although clearly advantageous for its speed we suggest QRT PCR be combined with RFLP for increased accuracy and that both techniques include controls of strains with known sequence identity.

Table 1. List of *Xf* isolates and strain designation as G = 'grape' (subsp. *piercei*) or R = 'ragweed' (subsp. *multiplex*) based on gyrB and mopB sequences, RFLP and QRT PCR analysis.

Isolate #	Host plant	Scientific name, host plant	Texas county	*gyrB* **	*mopB* **	RFLP	QRT PCR
VAL VAL 048	Mustang grape	*Vitis mustangensis*	Val Verde	G EU026151	G EU019700	G	G*
LLA FAL 634	SO4 rootstock	*Vitis cinerea var. helleri* x *Vitis riparia*	Llano	G EU026150	G EU019703	G	G*
BLA TEX 001	Cabernet Sauvignon	*Vitis vinifera*	Blanco	G EU026149	G EU19705	G	G
TRA FLA 300	Torringa Nacional	*Vitis vinifera*	Travis	G EU026148	G EU019702	G	G
VAL VAL 036	Black Spanish	*Vitis aestivalis* hybrid	Val Verde	G EU026147	G EU019701	G	G
GIL BEC 631	Wine grape	*Vitis vinifera*	Gillespie	G EU026146	G EU019704	G	G
HAR UHD 001	Sea myrtle	*Baccharis halimifolia*	Harris	R EU026138	R EU019694	R	R
TRA FLA 420	Redspike Mexican hat	*Ratibida columnifera*	Travis	R EU026139	R	R	R
LLA FAL 749	Western ragweed	*Ambrosia psilostachya*	Llano	R EU026142	R EU019696	R	R
LLA FAL 752	Giant ragweed	*Ambrosia trifida* var. *texana*	Llano	R EU026140	R EU019695	R	R
GIL BEC 626B	Giant ragweed	*Ambrosia trifida* var. *texana*	Gillespie	R EU026141	R EU019699	R	R
LLA FAL 650	Annual sunflower	*Helianthus annuus*	Llano	R EU026143	R EU019698	R	R*
LLA FAL 651	Heartleaf ampelopsis	*Ampelopsis cordata*	Llano	R EU026144	R EU019697	R	R*
UVA TAM 241	Cedar elm	*Ulmus crassifolia*	Uvalde	R EU026145	R EU019693	R	R*

* indicates that on at least one of two runs the strain indicated could not be categorized statistically as grape or ragweed (ANOVA, $p < 0.05$)

** indicates NCBI accession number after strain designation

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