MULTI-LOCUS SIMPLE SEQUENCE REPEAT (SSR) MARKERS FOR GENOTYPING AND ASSESSING GENETIC DIVERSITY OF *XYLELLA FASTIDIOSA* IN CALIFORNIA

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ABSTRACT
We have designed and developed *Xylella fastidiosa* (*Xf*) Simple Sequence Repeat (SSR) primers. Thirty-four of them have been validated and are available to public (Lin et al. 2005). These primers are *Xf*-specific and powerful for detecting polymorphism among and within crop-associated *Xf* strains and can be used for *Xf* genotyping, population structure and genetic diversity studies. Recently, we used fluorescent-labeled primers for PCR and an ABI 3100 genetic analyzer in combination with our rapid sample preparation protocol to create a high-throughput *Xf* pathogen diagnostic and genetic analysis platform. We used this marker system to study the geographic population structures of grape *Xf* strains in California. We also used this marker system as a tool to study interactions between *Vitis* and *Xf* in Pierce's disease (PD) resistant and susceptible grapes.

INTRODUCTION
Understanding pathogen genetic diversity is critical in developing an effective disease control strategy. Host plant resistance is one of the most important components in integrated crop management. However, the durability of disease resistance depends upon the variability and adaptability of pathogen populations and their interactions with host plant resistance genes. Variation in pathogen population structure can lead to resistance breakdown and disease outbreak under suitable environmental conditions. The molecular basis of plant host-*Xf* interactions needs to be investigated to better understand the epidemiology of *Xf*-induced diseases and how *Xf* strains interact to cause PD in different grape cultivars. Given the fact that diseases caused by *Xf* are complex patho-systems with a wide range of symptomatic as well as asymptomatic hosts, a number of insect vectors with wide host ranges, and variable environmental factors, the genetic diversity and biological relationships of *Xf* strains with different grape cultivars needs to be better understood. The goal of this project is to develop a reliable marker system that unambiguously identifies *Xf* strains from various geographic locations and host plants. Coletta-Filho et al. (2001 and 2003) developed simple sequence repeat markers from CVC *Xf* sequences and used them for CVC *Xf* population genetic studies. Here we designed SSR markers from four available *Xf* genomic sequences that work with all *Xf* strains. We further developed this marker system into a fluorescent-based multiplex genotyping format. Using a rapid DNA isolation method, we directly analyze *Xf* from infected plant tissues therefore avoiding the time-consuming bacterial isolation step and reducing the chance of sample loss due to contamination and culture difficulties. This system has proven to be powerful and reliable for distinguishing genetic relatedness. The sensitivity, specificity, and power in detecting polymorphism, as well as its adaptability to a high through-put diagnostic platform makes this system an ideal tool for large scale studies of *Xf* population genetics and epidemiological risk assessment analyses.

OBJECTIVES
1. Develop a high through-put multi-locus *Xf* genetic analysis system for genotyping and analyzing population structures of *Xf* in California.
2. Analyze genetic diversity and structure of *Xf* populations. Construct a large *Xf* allele frequency database for use as an *Xf* strain identification system.
3. Use the SSR Marker analysis system to study the interactions between hosts and *Xf* including adaptation, host selection and pathogenicity of *Xf* strains.

RESULTS
Objective 1
To develop an accurate and high through-put system for *Xf* genetic analysis, we combined fluorescent- labeled primers for PCR and analyzed them with a 16-capillary DNA sequence analyzer (ABI 3100). Each dye set consists of four primers labeled with FAM, NED, HEX and VIC respectively (Figure 1A). Therefore, data output is four times (96 x 4 = 384) more than that obtained with a single dye (96 samples) per run. To be accurate in determining allele size, an internal molecular sizing marker labeled with LIZ was co-separated with samples through each capillary tube. GeneMapper software identifies

alleles generated by fluorescent-labeled amplicons and reports sizes in base pairs. Software automatically generates a data sheet for further analysis. Because the primers are *Xf* specific, we can directly analyze *Xf* from infected plant tissues, therefore significantly increasing the speed of the work and reducing chances of sample loss during the pathogen isolation process. This system is able to identify samples in which multiple strains coexist (Figure 1B). We have optimized the system for large scale sample processing and data analysis.

Objective 2

Genetic diversity and population structures of PD *Xf* were analyzed by SSR primers. Eighty-three *Xf* samples collected from California representing four geographic populations of Napa, Sonoma, Kern and Riverside counties. Depending on availability, multiple samples were collected from each vineyard and 2-8 vineyards were sampled from each county for this study. Haplotypes and allele frequencies from each population and subpopulations were recorded. Genetic distances among populations were estimated (Figure 2). This hierarchical dataset allows partitioning of the genetic differentiation among counties, among vineyards within a county, and among individuals (Table 1). A larger proportion of total genetic diversity (68.89 %) is attributed to genetic diversity among different host plants suggesting genetic differentiation of *Xf* was partly driven by host selection.

Objective 3

To study the interactions of *Xf* with grape hosts, three grapevines (PD resistant 9621-67, PD susceptible 9621-94, and highly susceptible Chardonnay) were used for this study. Two strains of *Xf*, Stag's leap and Talcott were used for single and mixed infections. Samples were harvested at 6 and 12 weeks post inoculation. ELISA assay and Mixed *Xf* SSR genotyping will be performed to evaluate interactions between host and pathogens in single and mixed strain infections in PD resistant and susceptible grapes. The last sample harvest was in September, 2005. We are currently working on sample assay.

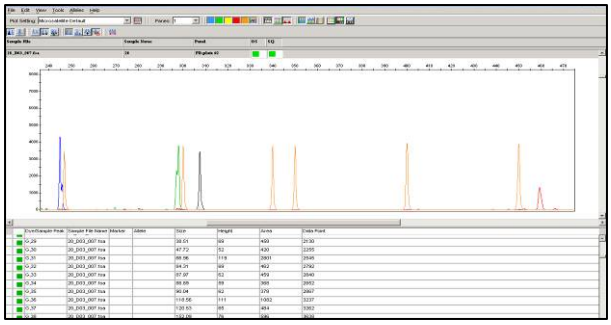

Figure 1A. Multi-locus genotyping analysis. Four alleles are presented in red, black, green, and blue peaks respectively. A molecular sizing standard is in yellow.

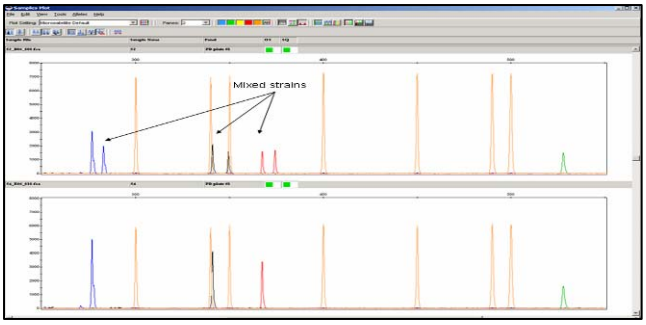

Figure 1B. Two mixed strains were detected by three primers as peaks shown in red, black and blue.

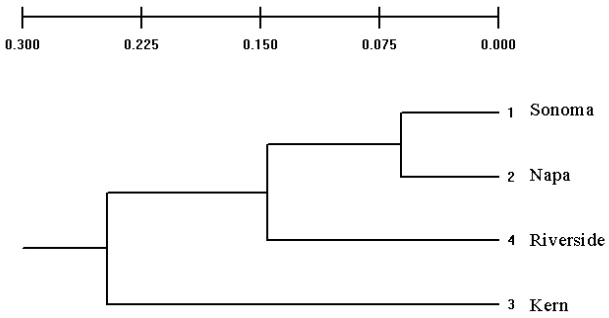

Figure 2. UPGMA cluster analyses using Nei's coefficient presented the genetic distances among four geographic populations.

Table 1. Analysis of molecular variance of the SSR haplotypes for *Xf* populations isolated from three grapevine growing counties in California. Total variance was partitioned into hierarchical components indicating larger proportion of total genetic diversity (68.89 %) is attributed to genetic diversity among different host plants

Source of variation	Sum of squares	Variance components	Percentage of variation*	Φ-statistics*
Among counties	203.997	2.06592	11.03	0.110
Among vineyards/counties	371.787	3.75837	0.07	0.225
Within vineyards	877.203	12.90004	68.89	0.311
Total	1452.988	18.72433	100	

* Probability of having larger variance component and Φ-statistic than the observed values by chance alone based on 1,000 random permutation (P<0.001).

CONCLUSIONS

In conclusion, the SSR marker system presented here is useful for strain identification, examining genetic diversity, and can aid in epidemiological and strain virulence studies. This multi-locus marker system is particularly suitable for studying *Xf* population genetics because it unambiguously reveals the variability of independent genetic loci. When this multiplex format is combined with a fluorescence-based automated sequencing analyzer, it provides an accurate and high-through-put platform for large scale pathogen detection. In addition, all the SSR primers we designed flank conserved sequence regions across *Xf* strains. Therefore, they are not only useful for *Xf* grape PD strains, but also for strains from other agronomic, horticultural and ornamental crops. Finally, each SSR primer detects a specific locus in the genome and the allelic information is recorded digitally. These features allow researchers at different sites, using different equipment, to share and compare results unambiguously when the same sets of multi-locus primers are used. Therefore, it is possible to compile global data sources for worldwide epidemiological and population genetic studies.

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