ROBUST ONE-HOUR GENOTYPING OF *XYLELLA FASTIDIOSA* STRAINS USING FRET PROBES

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ABSTRACT
Epidemiological studies of Pierce's disease (PD) can be confounded by a lack of genetic information on the bacterial causative agent, *Xylella fastidiosa* (*Xf*). PD in grape is caused by genetically distinct strains of *Xf* subsp. *fastidiosa* (*Xff*), but is not caused by numerous other strains or subspecies of *Xf* that typically colonize plants other than grape. Detection assays such as ELISA and qPCR are effective at detecting and quantifying *Xf* presence or absence, but offer no information on *Xf* subspecies or strain identity. Surveying insects or host plants for *Xf* by current ELISA or qPCR methods provides only presence/absence and quantity information for any and all *Xf* subspecies, potentially leading to false assessments of disease threat. This study provides a series of adjacent-binding fluorescence resonance energy transfer (FRET) DNA melt analysis probes (Cardullo et al. 1988) that are capable of efficiently discriminating *Xf* subspecies and strain relationships in one hour real-time PCR reactions.

LAYPERSON SUMMARY
Pierce's disease (PD) of grape is the single greatest factor limiting grape production in Texas. PD outbreaks have caused major economic loss to the grape industry in California as well. The disease is caused by a particular grape strain of a bacterium that is spread between plants by insects that feed on grapevines. Diagnostic tests to detect the bacterium will detect the grape strain as well as numerous other closely related strains of the bacterium that do not cause PD. Lack of an efficient means to distinguish the grape strain of the bacterium from other closely related strains that cause no harm to grape is hindering an understanding of the disease cycle. This project provides efficient DNA-based tests to distinguish the closely related bacterial strains from one another.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a xylem-limited bacterium that causes leaf scorch diseases in a wide array of plant species, and it is vectored by a number of xylophagous insects. Several subspecies of the bacterium have been named, including *Xf* subsp. *fastidiosa* (*Xff*) that causes PD in grape; *Xf* subspecies *sandyi* (*Xfs*) (Schuenzel et al. 2005) that causes oleander leaf scorch; *Xf* subsp. *pauca* (*Xfp*) that causes citrus variegated chlorosis, *Xf* subsp. *taschke* (*Xft*) that causes leaf scorch in chitalpa (Randall et al. 2007); and a genetically diverse subspecies, *Xf* subsp. *multiplex* (*Xfm*) that causes leaf scorch diseases in a large number of tree species (Schaad et al. 2004; Schaad et al. 2004). Many of the subspecies may occupy multiple hosts, but cause disease symptoms in only a select subset of potential plant hosts (Hopkins et al. 2002). While methods for efficient detection of the bacterium exist, such as ELISA and qPCR, epidemiological studies can be hindered because the detection assays commonly used detect all subspecies, but do not provide subspecies or strain identification. Isolating and culturing strains is a laborious and time-consuming process due to the fastidious nutritional requirements and slow growth habit of the bacterium. A multilocus sequence typing (MLST) system for *Xf* has been developed that is capable of generating sufficient genetic information to easily discriminate subspecies and strains (Scally et al. 2005), and although the method has been streamlined (Yuan et al. 2010), it remains a time-consuming process. In order to complement more informative and more time-consuming assays such as MLST, we have developed several real-time PCR probe sets capable of rapid and robust subspecies and strain identification by DNA melt analysis. The probe sets target many of the same genes utilized in the established MSLT assay, so that a rapid preview of important strain differences can be generated. These probe sets are shown to be capable of identifying *Xf* DNA polymorphisms even when the *Xf* DNA is a small proportion of a mixed DNA isolation containing plant, insect, and microbial DNA from other species.

OBJECTIVES
1. Develop rapid genotyping methods capable of distinguishing *Xf* subspecies and strains using plant and insect DNA extractions where the proportion of *Xf* DNA may be very small in relation to contaminating DNA.

RESULTS AND DISCUSSION
Ten genes that have been previously identified as highly conserved among *Xf* strains in previous work to develop a MLST assay (Yuan, Morano et al. 2010) were selected and aligned using VectonNTI (Invitrogen, Carlsbad CA) to visually select informative single nucleotide polymorphisms (SNPs). Alignments included the 26 stains used in the MLST project. AlleleID software (Premier Biosoft International, Palo Alto, CA) was used to align sequences and indicate SNPs that could potentially discriminate between the fully sequenced type strains for each *Xf* subspecies. AlleleID was then used to design adjacent-binding FRET probes for DNA melt assays to discriminate the SNP differences. Real-time PCR was carried out in a

384-well Roche LightCycler 480 real-time PCR instrument. Immediately following amplification, a DNA melt assay was conducted by annealing probe and amplicon, then ramping up sample temperature while continuously collecting fluorescence data. Melt curves and negative first derivative plots of DNA melt data were generated with the melt curve analysis module in the LightCycler software suite. An example of two SNPs used for *Xf* probe design are shown in **Figure 1**.

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                (1) 1         10        20        30        40        50        60        70        80        90        102
nuoN F primer   (1) ---GTGTACCTGGGGCTTGAGCTGTTG----------------------------------------------------------------------
nuoN R primer rc (1) ----------------------------------------------------------------------------------------------------
nuoN Quencher Probe rc (1) ----------------------------------------------TTGGGGTCAATCGCGATA----------------------------
nuoN Fluorescent Probe rc (1) ------------------------CGCTTTGTTCCTATGCGTTG----------------------------------------------
nuoN Dixon      (1) TGGTGTACCTGGGGCTTGAGCTGTTGGCGCTTTGTTCCTATGCGTTGGTTGGGGTCAATCGCGATAGCAAAATATCGACTGAGGCAGCGATGAAGTACATCG
nuoN Temecula   (1) TGGTGTACCTGGGGCTTGAGCTGTTGGCGCTTTGTTCCTATGCGTTGGTTGCGGTCAATCGCGATAGCAAAATATCGACTGAGGCAGCGATGAAGTACATCG
nuoN Ann-1      (1) TGGTGTACCTGGGGCTTGAGCTGTTGGCGCTTTGTTCCTATGCGTTGGTTGCGGTCAATCGTGATAGCAAAATATCGACTGAGGCAGCGATGAAGTACATCG
Consensus       (1) TGGTGTACCTGGGGCTTGAGCTGTTGGCGCTTTGTTCCTATGCGTTGGTTG GGTCAATCG GATAGCAAAATATCGACTGAGGCAGCGATGAAGTACATCG
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Figure 1. Partial DNA sequence alignment of the *Xf nuoN* gene. Forward primer, fluorescent probe, and quencher probe binding sites are shown above the type strains for *Xfm* (Dixon), *Xff* (Temecula), and *Xfs* (Ann-1). Identical sequence has a blue background while SNP positions have a white background.

For the *nuoN* gene, two variable sites are present within the sequence of the quencher probe. The probe is designed to have 100% identity to the *Xfm* strains described in the MLST work. The quencher probe has 1 G/C mismatch with the *Xff nouN* amplicon at the 15th nucleotide. The quencher probe has two mismatches with the *Xfs nuoN* amplicon, a C/T mismatch at the 5th nucleotide and the same G/C mismatch as with Temecula at the 15th nucleotide from 5' to 3' (quencher probe reverse complement is shown in **Figure 1**).

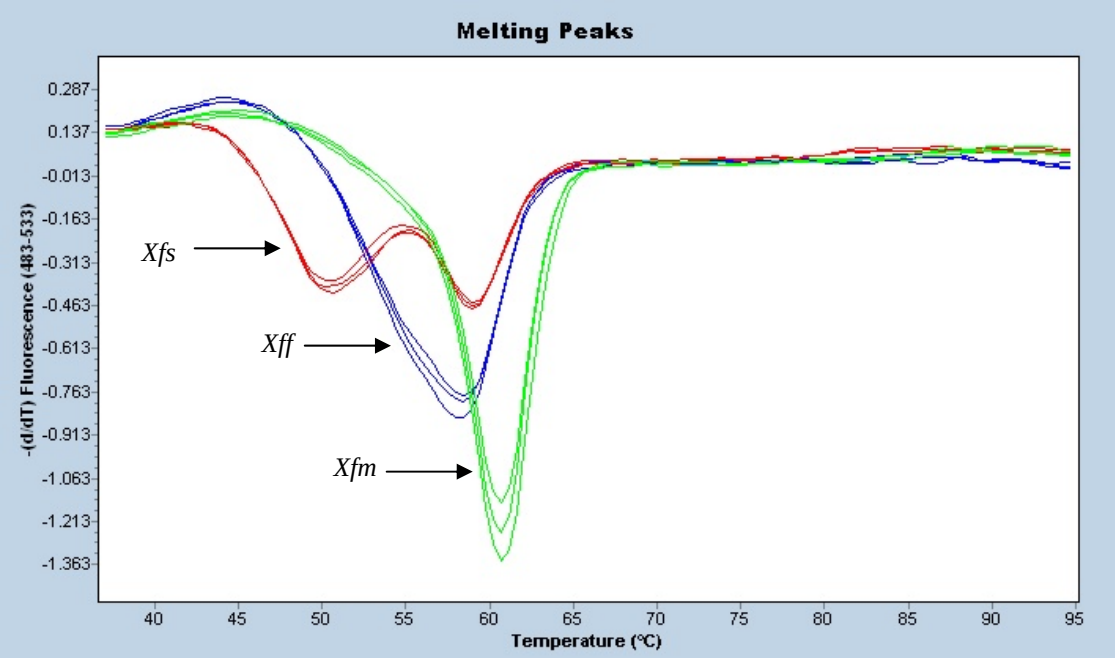

Figure 2. Melting analysis of *Xf* strains using *nuoN* FRET probes. The quencher probe has 100% sequence identity to the Dixon strain of *Xfm*, and the melt is indicated in green. The Temecula strain of *Xff* has a single base mismatch with the probe and its melt is indicated in blue. The Ann-1 strain of *Xfs* has two mismatches with the probe, and its melt is indicated in red.

The melting curves for the *nuoN* gene allow easy discrimination between the *Xfm*, *Xff*, and *Xfs* type strains by using only 2 adjacent-binding DNA FRET probes (**Figure 2**). As expected, the *Xfm* melt has the highest melting temperature (Tm) at 60.7° C, since the probe has 100% sequence identity to the Dixon *Xfm* strain. The Temecula *Xff* Tm is slightly lower at 59.07° C due to the 1 bp mismatch between the probe and *Xff* amplicon. The Ann-1 *Xfs* melt displays two Tm's, one at 59.07° C, equivalent to the *Xff* melt, and due to the same mismatch that occurs in *Xff*. The second *Xfs* Tm is at 50.53° C, and gives the *Xfs* melt profile two distinctive and characteristic troughs, allowing extreme ease of interpretation in scoring melt differences. Additionally, the *nuoN* probes function equally well when used on pure DNA from axenically-grown cultures, or when used on plant or insect DNA extractions where the proportion of *Xf* DNA is a very small part of a complex DNA sample.

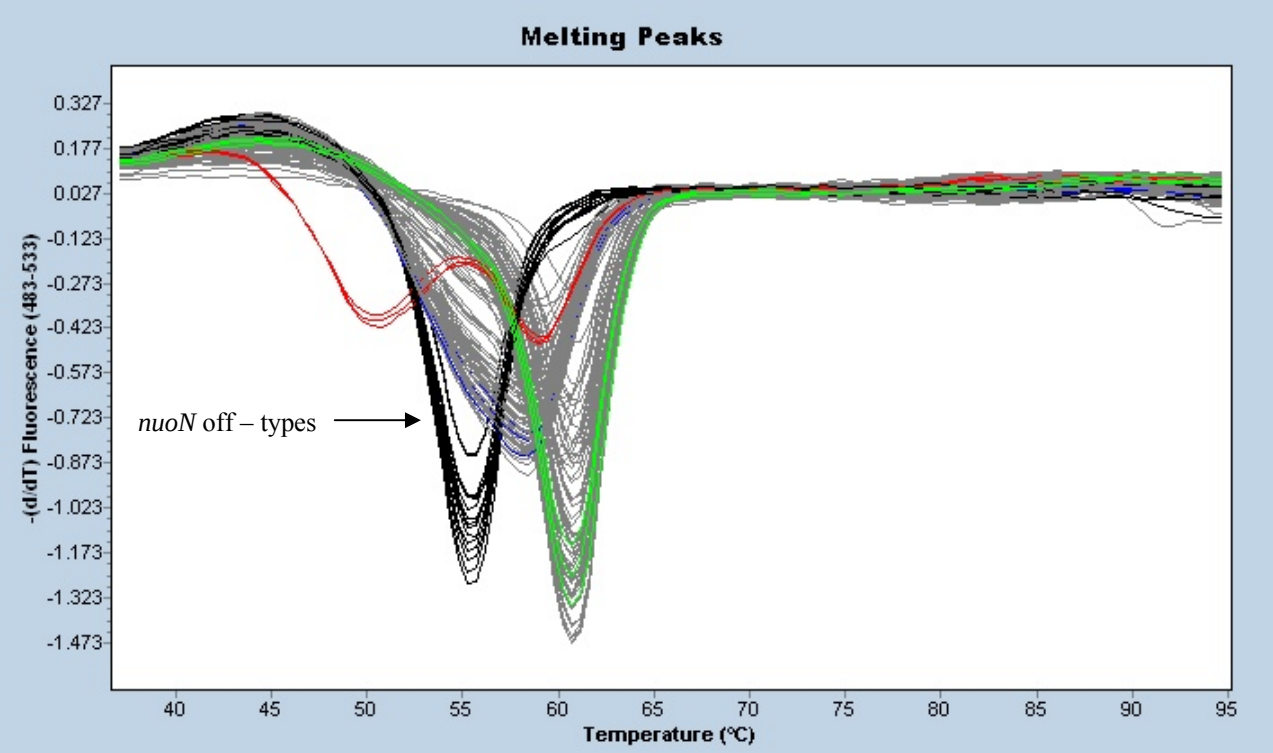

Figure 3. Melt analysis of 93 insect DNA extractions using the *nuoN* probe set. Melt standard curves included in the analysis are Dixon *Xfm* in green, Temecula *Xff* in blue, and Ann-1 *Xfs* in red. Insect sample melts are gray, except for several with unusual melt characteristics that are black and indicated by the arrow.

As a step in developing these assays, non-specific DNA binding dyes such as sybr green I and LCgreen plus were explored as an inexpensive option in developing a multilocus melt typing assay for *Xf*. These assays were unsatisfactory due to close and variable melting temperatures when using pure DNA from cultured type strains. The assays were simply unusable when amplifying *Xf* DNA from plant and insect DNA extractions, presumably due to melt variation caused by novel alleles not encountered in sequence databases when designing assays, as well as by inherent variation in melting PCR amplicons with non-specific DNA binding dyes. This probe-based genotyping method is robust in the face of unexpected genetic variation, and highly consistent due to short probe length. For example, **Figure 3** shows *nuoN* melting curves from a number of insect DNA extractions. A group of samples, all from Gillespie County Texas do not show the characteristic melt of *Xfm*, *Xff*, or *Xfs*. Instead, these samples all melt at 55.44° C. The *nuoN* assay alone would not be sufficient to group these samples with any of the identified subspecies. Additional probe melt assays with genes such as *lacF*, *petC*, *pilU*, and *gyrB* have identified these *nuoN* off-types as *Xfm* (**Figure 4)**. Thus, a novel *nuoN* allele has been identified for *Xfm* in insect samples from Gillespie County Texas. Probe-based melt analysis may be used in this way to quickly and efficiently screen for strains that should be given a more thorough genetic evaluation by additional methods.

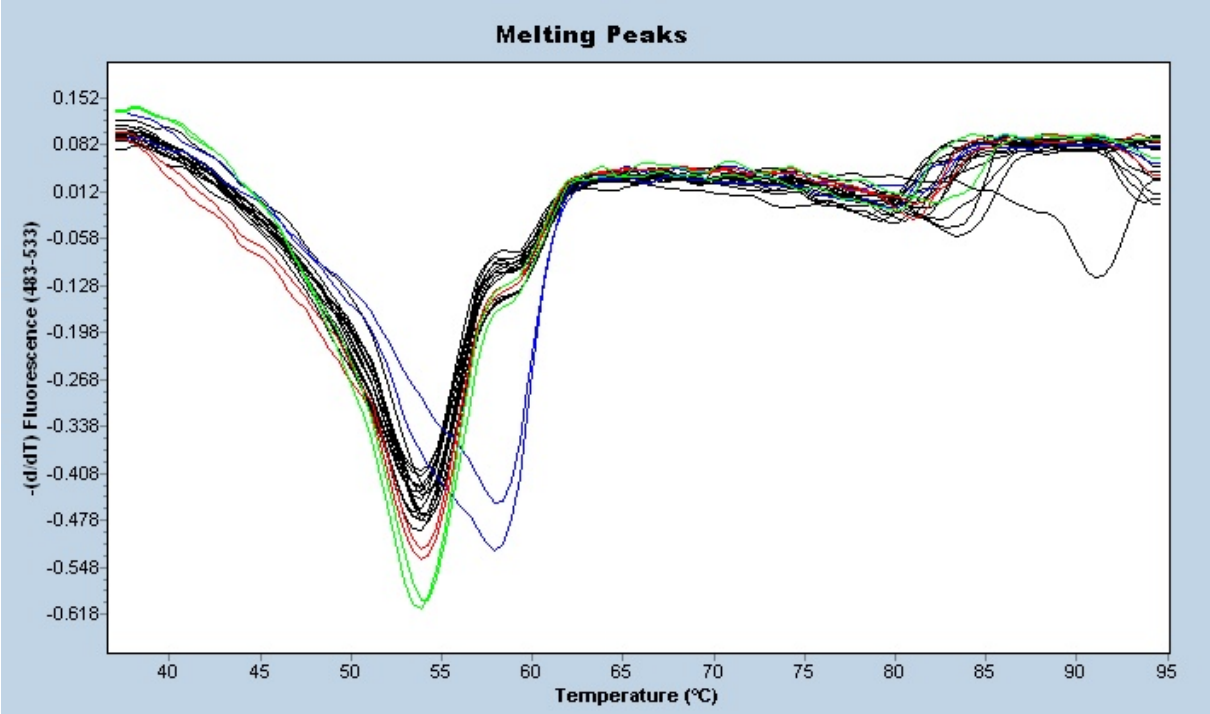

Figure 4. Melt analysis of *nuoN* off-types using the *gyrB* probe set. Melt standard curves included in the analysis are Dixon *Xfm* in green, Temecula *Xff* in blue, and Ann-1 *Xfs* in red. Off-types from the *nuoN* probe melt analysis are black. *Xfm* and *Xfs* have identical melts with the *gyrB* probe set, and all *nuoN* off-types show a non-*Xff* melt profile.

CONCLUSIONS

Probe-based DNA melt assays for several *Xf* genes have been developed that allow rapid discrimination of subspecies and strain relationships. The assays are efficient, requiring little DNA template, so that *Xf* DNA can be genotyped even if it is a small proportion of the DNA in a sample originating from a plant on insect DNA extraction. Many of the genes in the assay are already part of a MLST assay for *Xf*, so that the probe-melt assay may complement the MLST assay as an indicator of samples that require genotyping of greater depth.

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