DIFFERENTIATION AND ANALYSIS OF *Xylella fastidiosa* SUPBSPECIES *fastidiosa* CULTURES ISOLATED FROM A SINGLE TEXAS VINEYARD USING SIMPLE SEQUENCE REPEAT MARKERS

A Thesis

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ABSTRACT

Xylella fastidiosa subspecies *fastidiosa* is the causative agent of Pierce's disease of grape and has caused significant crop stress and loss in vineyards throughout Texas. While multiple techniques are available to identify subspecies of *X. fastidiosa*, only simple sequence repeat markers can be used for the differentiation of isolates within individual subspecies. In this research, SSR markers were utilized to demonstrate the diversity of subsp. *fastidiosa* isolates from within a single vineyard. The distributions of strains defined within subsp. *fastidiosa* were also compared to epidemiological data to clarify any relationships.

Initial results from isolation attempts indicate disease severity to have the largest impact on the success of isolation attempts with 7% of samples rated as 'Healthy' and 83% of samples rated as 'Advanced' producing successful isolations. A conventional PCR protocol employing 5 SSR markers was used to generate banding profiles for 97 isolates collected from 7 grape varieties planted in 5 blocks throughout a single Texas vineyard. SPSS statistical program was used to execute a hierarchical cluster analysis to produce a dendrogram which grouped isolates into 3 strain groups with 7% or 15% dissimilarity. Of the 3 epidemiological factors analyzed, the distribution of strains showed significant dependence on grape variety while having no dependence on disease severity or location within the vineyard.

To The Generations Before

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CHAPTER I

INTRODUCTION

The bacterium *Xylella fastidiosa* is a bacterial species which has been identified as the causative agent of diseases for numerous agricultural crops and landscape plants. One of the most notorious of these is Pierce's disease of grape. As a species, *X. fastidiosa* is endemic to several continents as well as many states in the continental U.S. and has caused major economic losses of several food crops. For these reasons, this bacterium has been extensively studied.

History of Pierce's Disease

First reported by N.B. Pierce in a 1892 bulletin by the U.S. Bureau of Agriculture (35), Pierce's disease of grape has long been considered a major problem to the agriculture production of grapes and is particularly notorious in California. The pathogen went from virus status, as demonstrated by experiments in 1949 showing it was transmitted via leaf hoppers by Severin (45), to being identified as a rickettsia-like bacterium in 1973 by Hopkins and Mollenhauer (22). The ambiguity of the organism's classification was mainly due to the inability to isolate a pure culture. It was not until 1978 when Davis et al. (15) developed a complex media which facilitated the isolation of the bacteria associated with Pierce's disease of grape and almond leaf scorch, that more in-depth research into the pathogen was able to progress with greater ease. The next landmark for this organism's classification came in 1987 when Wells et al. (54)

proposed a new genus and species, *Xylella fastidiosa*, as the taxonomic name for this pathogen. Since then, a great deal of emphasis has been placed on understanding the various subspecies of *X. fastidiosa* that are an important feature of the population structure of the pathogen. These efforts were greatly facilitated by the completion of genomic sequencing and annotation of several strains of the bacteria including; 9a5c which causes citrus variegated chlorosis (47) as well as strains which cause disease on almond and oleander plants (4, 5).

The Disease

A pathogen for many plants, *X. fastidiosa* affects several agricultural crops of economic value such as coffee, almond, citrus, alfalfa, and grape. It also causes disease in shade trees such as oak and elm, and shrubs including oleander (23). In most cases, symptoms will include leaf scorch, stunting of new growth, and eventually death. In grapes, Pierce's disease of grape symptoms also include irregular maturation of the stem or 'green islands', petiole retention, and desiccation of grape bunches or 'raisining'. However, the most characteristic symptom of Pierce's disease of grape is the pattern of leaf scorch. In the early stages of disease, scorch will develop at the very tips of the leaf and move to encompass the entire margin of the leaf blade with no regard to leaf veins. At the innermost edge of necrosis, there will be a zone of yellow or red coloration depending on the variety of grape. White grape varieties develop a yellow line and red varieties have a red line. Variety will also affect the progression of disease symptom expression as some grapes have demonstrated resistance or tolerance to *X. fastidiosa*.

Vitis species varieties, such as Viogner, tend to be more susceptible and succumb to the disease more quickly, those crossed with native Amercian grapes, such as the French American hybrid Blanc du Bois have been known to express fewer symptoms and continue to produce high yields (J. Kamas, personal communication).

Another factor affecting disease progression through a vineyard is the insect vector. Movement of the bacteria is mostly facilitated through xylem sap-feeding insects including sharpshooters, leafhoppers, and spittle bugs (9). There are a continually growing number species with the potential to serve as vectors with varying degrees of transmission efficiency. The most notorious, however, is the glassy-winged sharpshooter (*Homalodisca vitripennis*). After its introduction in the early 1990's (48), the glassy-winged sharpshooter began to dramatically change the epidemiology of Pierce's disease of grape in Southern California (6) by increasing the seasonal occurrence of vine-to-vine spread (39). This increase is attributed to the habit of glassy-winged sharpshooters to feed on the stems of vines rather than new growth as well as feeding more readily on dormant vines (51). Due to the rise of disease incidence caused by the glassy-winged sharpshooter, and its increasing range, the California Department of Food and Agriculture established the Pierce's Disease Control Program which coordinates the state-wide management of this insect vector (http://www.cdfa.ca.gov/pdcp/).

Control methods for Pierce's disease of grape are currently dependent on managing the insect vector to prevent the spread of disease and managing infected and infested plant material to minimizing inoculum sources. Several pesticides, including

systemic and non-systemic formulations, have been developed to reduce the number of insect vectors within and around the vineyard. Managing native vegetation and minimizing other insect habitats is doubly important because *X. fastidiosa* also has a number of supplemental hosts which do not express symptoms. In order to reduce inoculum sources, weeds and infected grapevines should be promptly removed. Heavy pruning has also been observed to reduce, but not eliminate inoculum in areas which experience colder winter climates (37). This phenomenon known as 'cold curing' is most effective when the bacterium is localized to new growth. The best method for preventing the introduction of Pierce's disease of grape is planting resistant varieties and choosing a vineyard site with low disease pressure. Unfortunately, resistant varieties are often not those most desired for wine production.

Impact

The wine and grape industry is a large and growing business with an economic footprint of over a $162 billion nationally (30). In Texas alone, the industry has grown from $1.35 billion in 2007 to $1.7 billion in 2009 (31). With over 3,000 bearing acres, Texas is America's fifth largest grape and wine producing state and hosts 190 wineries as last recorded by the Texas Wine and Grape Growers' Association (www.txwines.org/facts.asp). Texas also plays host to the bacterium *X. fastidiosa* and many of the xylem-feeding insects which have been implicated in the spread of Pierce's disease of grape.

While not the only challenge to the wine and grape industry, Pierce's disease of grape has become a major limiting factor to long-term production in Texas causing over 80% mortality in some cases (28). In a study commissioned by the California Department of Food and Agriculture, Pierce's disease of grape caused a 36% loss of gross to the state's agriculture income (46). A study by Appel et al. (unpublished) followed the progression of disease for 5 years in a single block of Viogner grapes. During the course of this study, disease began appearing in patches the first year after planting and steadily increased each subsequent year. At the end of a 5 year period, a high percent of grapevine mortality caused a significant loss of yield and the block was abandoned. This pattern in yield loss has been observed in several vineyards which grow susceptible varieties (2).

The Pathogen

An aerobic, rod-shaped, Gram negative, xylem-inhabiting bacterium, *X. fastidiosa* is relatively small, ranging in size from 0.2-0.4 by 1.0-4.0μm and is not visible using standard light microscopy (21). With an optimal growth temperature range of 26-28°C, it is considered a nutritionally fastidious organism and is notoriously difficult to grow in axenic culture. Isolation requires a rigorous aseptic technique and surface sterilization procedure as well as a specialized complex media (13). In addition to its nutritional requirements, it is also a slow growing organism, taking 7-14 days for a cultured colony to reach the size of a pin head, and is often overtaken by fungi and other contaminates further complicating isolation attempts. While it does not possess a

flagellum, it does produce several pili with various functions, including Type IV pili which facilitate the movement of the bacterium both up- and downstream through the xylem vesicles (25).

Similar to the xanthan gum produced by *Xanthomonas campestris* pv. *campestris* *X. fastidiosa* is also a producer an extracellular polysaccharide. This substance contributes to cell aggregation and biofilm development which can cause blockages impeding the movement of fluid and nutrients throughout the plant and contributing to symptom development (24). Biofilm development, as regulated by a diffusible signaling factor, has been shown to affect the transmission efficiency of vectors by facilitating the colonization of the bacterium in the foregut (33).

There are several major differences among the species *X. fastidiosa* cultures which can be used to categorize isolates including host range, pathogenicity, nutritional requirements, and genetic composition (38). In addition to the infection of multiple types of plants where disease is expressed, *X. fastidiosa* can also survive as an endophyte in many hosts which remain asymptomatic. For just those isolates which cause Pierce's disease, at least 145 plants have been identified as hosts (23). There is also a degree of host-specificity observed in the various strains isolated from different hosts leading to the classification of pathovar types. However, these relationships are complex (23). For example, almond trees can be infected by strains isolated from grapes or oleanders but the grape and oleander strains are not reciprocal and do not infect each other's host. While the mechanisms for host-specificity have yet to be identified, each host presents a different micro-habitat for *X. fastidiosa* growth. As a result, there exist small differences

in the nutritional requirements needed for optimal growth by various strains of *X. fastidiosa*. Several complex media recipes have been used to culture *X. fastidiosa* strains, each originally designed for isolation from a specific host. These media can be used to categorize isolates into two groups based on nutritional fastidiousness. Most isolates will fall into the 'Phony Peach' (PP) group and will grow on the following specialized medias: periwinkle wilt (PW)(13), buffered charcoal-yeast extract (BCYE)(55), and CS-20 (8). Isolates in the PP group, however, will not grow on Pierce's Disease 2 media (PD2)(14). Those that grow on PD2 are clustered into the 'Pierce's Disease' (PD) group and will grow on all four media. These isolates will also cause disease symptoms to be expressed on grapes (21). All isolates of *X. fastidiosa* will grow on PW media. The last method of categorization is based on genetic variation and analysis. All strains of *X. fastidiosa* share a high degree of DNA-DNA homology ranging from 75-100% (20). However, through the use various techniques and assays, it is possible to differentiate isolates into definitive subspecies groups. It is also through the analysis of genetic variation that we are able to better understand the relationship of strains to hosts and disease.

Classification of *Xylella fastidiosa*

As previously mentioned, *X. fastidiosa* is classified as a single species which includes a variety of pathogenic and non-pathogenic strains. It is also the only taxonomic species listed within the genus which was first suggested in 1984 by Wells et al. (53). In this study fatty-acid analysis, guanine to cytosine content ratios, and 16S

ribosomal ribonucleic acid (rRNA) sequencing data were used as evidence to classify *X. fastidiosa* as a novel genus and species. The same study also determined *Xylella* as being most closely related to *Xanthomonas* and other bacteria in the gammaproteobacterium group. While the composite results from this research were able to describe a novel genus, individual strains within the species were unable to be identified and has been the focus of many studies since.

The first attempt to categorize this large and encompassing group of xylem-limited bacteria into smaller groups was conducted by Chang and Schaad in 1982 (7) before *X. fastidiosa* was the accepted classification. In their research, they used sodium dodecyle sulfate-poly-acrylamide-gel electrophoresis (SDS-PAGE) to perform an analysis of the total cell envelope protein profiles. Their results indicated enough variability to distinguish various pathogenic strains into groups based on one of five hosts used for isolation while maintaining a high similarity among groups. Wichman and Hopkins (56) conducted a similar study in 2002 with the whole-cell protein profiles and were able to identify four distinct pathogenic groups and two miscellaneous groups based on isolation hosts, including the following: 1) grape, 2) elderberry, 3) oak, 4) oleander and 5/6) almond, blackberry, lupine, mulberry, periwinkle, elm, and plum.

Many other efforts have since been made to categorize and differentiate *X. fastidiosa* into smaller groups, such as pathovars or subspecies, with most giving similar results and following general pathogenic distinctions. In 1992, Restriction Fragment-Length Polymorphisms (RFLP) were used on twenty-four strains isolated from eight hosts by Chen et al. (10) to better understand the amount of diversity within the genome

of *X. fastidiosa*. However, their study only showed the existence of an RFLP group for Pierce's disease isolates and was not able to distinguish other pathogenicity groups with any distinction. Pooler and Hartung (36) used Random Amplified Polymorphic DNA (RAPD) -Polymerase Chain Reaction (PCR) in 1995 to divide the species into five host groups including: citrus, plum-elm, grape-ragweed, almond, and mulberry. Their results reaffirmed those previously achieved using RFLPs, but were faster and less expensive. In 2001, Hendson et al. (17) completed the first comprehensive study to identify distinct subspecies within *X. fastidiosa* by using a battery of techniques to analyze the genome. These techniques included RAPD-PCR, enterobacterial repetitive intergenic consensus sequence, repetitive extragenic palindromic (REP) elements, contour-clamped homogeneous electric field (CHEF) gel electrophoresis, plasmid content, and 16S-23S rRNA intergenic spacer region sequencing. Analysis of each technique individually produced strain delineations similar to each other with variations on the number of host-associated groupings defined.

It was not until 2004, when Schaad et al. (43) completed a second comprehensive analysis of *X. fastidiosa* populations, that novel subspecies classifications were proposed. In this work, twenty-six strains from ten hosts were analyzed using DNA-DNA homology assays and sequenced 16S-23S intergenic spacer (ITS) regions. The results led to the recommendation for three novel subspecies to be identified as follows: A) subspecies *piercei* which includes isolates pathogenic to grape, alfalfa, maple and almond group II; B) subspecies *multiplex* which includes isolates pathogenic to peach, elm, plum, pigeon grape, sycamore, and almond group I; and C) subspecies *pauca* which

includes strains pathogenic to citrus. These subspecies classifications have been accepted with the exception of subspecies *piercei* being altered to subspecies *fastidiosa* (42). There have, however, been two additional subspecies added including subspecies *sandyi* which includes isolates pathogenic to oleander (44) and the proposed subspecies *tashke* which includes isolates pathogenic to chitalpa plants (40). Lastly, in 2007, it was determined by Wickert et al. (57) not to separate isolates causing disease on coffee from those which cause disease on citrus into a separate subspecies due to the high degree of genetic similarity and the inability to cluster isolates separately based on genetic analysis. This leaves both citrus and coffee isolates, neither of which has been isolated in North America, grouped into subspecies *pauca*.

Detection and Differentiation

While an enzyme-linked immunosorbent assay (ELISA) has been available for the detection of *X. fastidiosa* since 1980 (34), a PCR method for detection was not available until 1994 (29). Since then, there have been several protocols explored to develop an easy method for both the detection and the differentiation of *X. fastidiosa* subspecies. In 2005, Travensolo et al. (50) used sequence characterized amplified region (SCAR) markers to specifically identify Pierce's disease strains of *X. fastidiosa* from the strains of nine other hosts as well as a collection of various other Gram negative and positive bacteria. However, it was never developed for the identification of other *X. fastidiosa* isolates which do not cause Pierce's disease. In 2003, Rodrigues et al. (41) used the 16S rRNA and the *gyr*B gene to produce a multiplex PCR protocol for the

detection and differentiation of isolates of subspecies *fastidiosa* and *pauca* from all other strains. Similarly, in 2007, Bextine and Child (3) also used the *gyr*B gene, but this study used the complete sequences to demonstrate the use of DNA melting temperatures, as obtained through a SYBR Green-based quantitative real-time-PCR protocol, to differentiate Pierce's disease, almond leaf scorch, and oleander leaf scorch strains of *X. fastidiosa*. This method was not recommended as a definitive means of differentiation due to its sensitivity to genetic mutations.

Perhaps the most durable and applicable method for the detection and differentiation of subspecies was described by Hernandez-Martinez et al. (18) who developed a multiplex PCR assay for the detection and differentiation of the three subspecies most likely to be found in North America: subspecies *multiplex*, subspecies *fastidiosa*, and subspecies *sandyi*. Using three primer sets, the product from a single PCR procedure can be visualized through standard gel electrophoresis and the resulting DNA banding patterns will result in one of five patterns indicating the sample as one of the following possibilities: negative for *X. fastidiosa*, positive for *X. fastidiosa* subspecies *sandyi*, positive for *X. fastidiosa* subspecies *fastidiosa*, positive for an almond leaf scorch (ALS) group I isolate of *X. fastidiosa* subspecies *multiplex*, and positive for an ALS group II isolate of *X. fastidiosa* subspecies *multiplex*.

Short repetitive DNA sequences were also used to analyze *X. fastidiosa* population variation. Such sequences have been successfully used to study microbial evolution and epidemiology in various bacterial species as well as several eukaryotic organisms (52). The first use of such sequences to study a plant pathogen was

conducted on *X. fastidiosa* to study the variability in citrus and coffee isolates collected throughout Brazil (12) and later used to study isolates from several subspecies obtained throughout Brazil, Costa Rica, and the continental United States (32). Both of these studies were able to successfully group isolates by subspecies and showed a significant degree of variability within those subspecies based on geography and host origin. Lin et al. (26) identified thirty-four simple sequence repeat (SSR) loci and designed primers using *X fastidiosa* isolates from oleander, citrus, almond, and grape. With these primers, they were able to analyze the resulting gel electrophoresis banding profiles to successfully group isolates by subspecies, host origin, and region. The scope of this study included several counties across California and citrus isolates from Sao Paulo, Brazil. A later study used SSRs to analysis ninety-three grape isolates from five vineyards within Napa Valley, California (11). This study demonstrated a high degree of variability within isolates but did not find any relationship to geographic distribution. A high degree of SSR diversity was observed when compared to other pathogens and it was hypothesized this may be caused by a high rate of evolution. This phenomenon was discussed in previous publications (1, 58) and supports the use of SSR markers as a tool for studying variations within local populations over a short time scale.

Research Objectives

In an effort to further understand the diversity of *X. fastidiosa* and the impact that genetic variability may have on disease expression, this research will focus on

subspecies *fastidiosa* isolates collected from a single vineyard in the Texas Gulf Coast region with the following objectives:

1. To assemble a library of *X. fastidiosa* isolates from a single vineyard to represent a variety of epidemiological factors (grapevine variety, disease severity, and relative geography);

2. To demonstrate the diversity of *X. fastidiosa* subspecies *fastidiosa* isolates within a single vineyard sampling area; and

3. To analyze for a potential correlation between the diversity of *X. fastidiosa* subspecies *fastidiosa* and the several epidemiological factors in Objective 1.

CHAPTER II

MATERIALS AND METHODS

Sampling

During the summer of 2007, 337 grapevine samples were collected from 8 varieties growing in 8 blocks throughout a vineyard in Brenham, TX. The varieties included the following: Blanc du Bois, Cabernet Sauvignon, Merlot, Chambroucin, Primitivo, Shiraz, Muscat Blanc, and Ruby Cabernet. Each of these vineyard blocks had been previously surveyed for Pierce's disease symptoms by rating each vine on a scale of 1 (healthy, no symptoms) to 5 (dead). Samples consisted of five petioles harvested from vines demonstrating early symptoms of Pierce's disease or a vine which in 2006 had scored a '3' or '4' in a disease survey, indicating advanced symptoms and dieback occurred in the previous year. When available, petioles were taken from symptomatic leaves in the upper portion of the canopy and the most basal leaf of a symptomatic vine, resulting in two different samples from the same vine. After removing the petiole from the plant, leaf blades were discarded and petioles were stored in labeled zip-lock bags. Labels included vineyard name, variety, block number, row number, column number, and vine number as well as position within the vine where the sample was collected. Samples where then stored in a cooler with cold packs until returned to the laboratory and placed under refrigeration.

Vines were selected from throughout the vineyard to maximize the potential for culture isolation while ensuring diversity of varieties and areas represented. There were

three exceptions to this guideline. Block 7, comprised completely of Blanc du Bois, was sampled systematically to be consistent with a different, ongoing experiment in that block. Block 6 contained equal proportions of Merlot and Muscat Blanc. The section containing Merlot was also sampled systematically to be consistent with the previously mentioned ongoing experiment. Lastly, every fourth vine in the Muscat Blanc was sampled to compensate for previous difficulties in isolating *X. fastidiosa* from that variety and maximizing the potential for culture isolation.

Isolation and Culture Purification

Upon returning to the laboratory, samples were stored in a conventional refrigerator (4°C) until processed. The processing procedure was adapted from Schaad et al. (21) and included an intense surface sterilization process which consisted of the following steps:

1. Cut petioles into 1 inch pieces.
2. Rinse in 70% EtOH.
3. Soak in 1% NaHClO for 3 minutes.
4. Rinse with sterile and distilled H_2O.
5. Submerge in sterile, distilled H_2O in micro Petri dish until plating.

The plating process consisted of removing the terminal 5 mm of each piece of petiole and dissecting the tissue into 3-5mm segments. Each segment was squeezed in the center using forceps or pliers and the fluid which was discharged from each end of the segment was blotted onto PW (Periwinkle Wilt) media modified from Davis et al. (13).

Two plates of modified PW media were blotted for each sample processed, with a total of 10 – 12 attempts per sample. All processing was done aseptically, including use of a laminar flow hood (NuAire, Inc., Plymouth, MN 55447).

Plates were incubated at 28.0°C for 7-14 days. Suspected cultures retrieved from blotting were subjected to a series of isolation transfers for single cell isolation onto Periwinkle Wilt Media- Gelrite (PWM-G) and Pierce's Disease 3 (PD3) media modified from Davis et al. (15) which was dubbed PD3-G^{+}. Cultures were identified as *X. fastidiosa* if they exhibited the following morphological characteristics as identified in Wells et al. (1987): discreet, circular, entire, smooth, convex and opaque reaching approximately 0.6 mm diameter after 10 days. Once a pure culture was obtained, cultures were smeared onto three PD3-G^{+} media for overgrowth to be used for DNA extractions, ELISA testing, and frozen storage.

Frozen Storage of Isolates

A set of frozen cultures were made for each isolate collected. Using a 3mm inoculating loop, two clumps of bacteria were removed from the PD3-G^{+} media plates and thoroughly vortexed to suspend cultures in 0.75ml of PD3-G^{+} Broth. A 40% solution of glycerol in sterile, distilled H_2O was then added to each suspension to bring the volume to 1.5ml. Suspended cultures were frozen and stored at -80°C.

Species Confirmation

For each isolate two cell suspensions were prepared for species confirmation with double antibody sandwich- enzyme linked immunosorbant assay (DAS-ELISA). Using a 3mm inoculating loop, two clumps of bacteria were removed from the PD3-G^+ media plates and thoroughly vortexed in a 1.5 ml micro tube to suspend cultures in 1 ml of General Extraction Buffer as provided in the Agdia DAS-ELISA kit. Each cell suspension was tested using DAS-ELISA Kits (Agdia, Inc., Elkhart, IN 46514) using the manufactures protocol to identify isolates as *X. fastidiosa*. A test was considered positive if the specified test well emitted a blue color and generated an absorbance reading >0.3000 using 620nm as the measurement wavelength on the SPECTRAFlour (Tecan Inc., Durham, NC 27703).

DNA Extraction

Upon ELISA confirmation of the isolates as *X. fastidiosa*, three clumps of bacteria were removed from cultures grown in PD3-G^+ media plates using a 3mm inoculating loop and suspended in 300µl of MicroBead Solution (MO BIO Laboratories, Inc., Carlsbad, CA 92010) for DNA extraction. DNA extraction was completed using Ultraclean Microbial DNA extraction kits from the same source according to the instructions provided by the manufacturer. Three extractions were produced for each isolate and stored in a conventional freezer until needed. DNA from an additional three strains was provided by C.F. Gonzalez (Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843) to be used as a non-

subspecies *fastidiosa* control. These strains consisted of a Temecula strain (California grape strain), the Dixon strain (California almond strain), and another non-grape strain of unknown origin.

Table 1. List of primers used for subspecies identification.

Primer	Forward Sequence	Reverse Sequence
XF1968	GGAGGTTTACCGAAGACAGAT	ATCCACAGTAAAACCACATGC
XF2542	TTGATCGAGCTGATGATCG	CAGTACAGCCTGCTGGAGTTA
ALS	CTGCAGAAATTGGAAACTTCAG	GCCACACGTGATCTATGAA

Subspecies Identification

To confirm all isolates from the vineyard were *X. fastidiosa* subsp. *fastidiosa*, primer sets XF1968-L/R, XF2542-L/R, and ALM-1/2 (Table 1) were used in a multiplex PCR assay described by Hernandez-Martinez et al. (2006). DNA from Temecula (grape) and Dixon (almond) and another non-grape isolate were previously confirmed as subsp. *fastidiosa* and subsp. *multiplex*, respectively and were used as biological standards. Each 25µl PCR reaction mix contained 0.25µL of each primer, 18.5µl PCR Master Mix 2X (Promega Corporation, Madison, WI 53711), and 5µl of sample DNA. The PCR process was conducted using the following temperature series: 94°C for 5 min. (initial denaturation), 40 cycles of 94°C for 1min, 55°C for 1min, and 72°C for 1 min, 72°C for

10min (final extension), and 4°C hold. All PCR products were subjected to electrophoresis using a 1.5% agarose gel run at 80V to observe the number and size of bands produced by each isolate. Grape disease-causing strains, or *X. fastidiosa* subsp. *fastidiosa,* are indicated by a single band of ~412 base pairs.

Table 2. Simple Sequence Repeat Primer sequences used to differentiate *X. fastidiosa* isolates.

Primer	Forward Sequence	Reverse Sequence	Type of repeat motif
OSSR-9	TAGGAATCGTCTTCAAACTG	TTACTATCGGCAGCAGAC	$(TTTCCGT)^{13}$
GSSR-4	GCGTTACTGGCGACAAAC	GCTCGTTCCTGACCTGTG	$(ATCC)^{7}$
GSSR-7	ATCATGTCGTGTCGTTTC	CAATAAAGCACCGAATTAGC	$(GGCAAC)^{24}$
GSSR-14	TTGATGTGCTTTTGCGGTAAG	GACAGGTCCTCTCATTGCG	$(TCCCGTA)^{24}$
GSSR-19	GCCGATGCAGAACAAGAAC	TCAACTTCGCCACACCTG	$(GAAAAACAAG)^{19}$

Simple Sequence Repeat Markers

Using 5 simple sequence repeat (SSR) primers developed by Lin et al. (2005) (Table 2), a series of PCRs were completed for each isolate as well as the Temecula, Dixon, and other non-grape isolates. Each 25 µL PCR reaction consisted of 2 µL sample DNA, 50 pmole SSR primer, 12.5 µlPCR Master Mix 2X, and 9.5 µl nuclease free water. The PCR process was conducted using the following temperature series: 95°C for 6 min (initial denaturation), 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1

min, 72°C for 7 min (final extension), and 4°C hold. The full SSR-PCR products were mixed with 6 µl 10X loading Dye and 10 µl were loaded into a 2% agarose gel. Gel electrophoresis was conducted using 1 X TBE buffer for 3-4 hours at 80V and visualized with ethidium bromide.

Hierarchal Cluster Analysis

The size of each band (base pairs) was quantified using AlphaEase FC software (Alpha Innotech, San Leandro, CA 94577). A 1 kb ladder was used as the molecular weight standard and the Temecula strain was used to standardize all gels. Relationship distances among isolates were derived by calculating the summation of the absolute value for differences of each corresponding SSR markers for two isolates according to the following equation.

$$ABS(A_{SSR1}-B_{SSR1}) + ABS(A_{SSR2}-B_{SSR2})+\ldots ABS(A_{SSR5}-B_{SSR5});$$

Where A represents isolate i and B represents isolate j and

SSR # represents a particular marker.

Distances were used to create a distance matrix which was inserted in to SPSS v13.0 (SPSS Inc., Chicago, IL 60606) where a hierarchical cluster analysis was completed for each of the following cluster methods: between-group linkage, within-group linkage, nearest neighbor, furthest neighbor, and median cluster. All analysis utilized the squared Euclidean measure of distance. Cluster methods were evaluated for biological accuracy based on position of the non-grape and 'Dixon' strains in relation to 'Temecula' and *X. fastidiosa* subsp. *fastidiosa* isolates.

Epidemiological Data

Several epidemiological factors were analyzed to determine their influence, if any, on the occurrence of any potential sub-strains or isolates. These factors included location within the vineyard, grape variety, and disease severity. Vineyard location and grapevine variety were recorded when the sample was taken. Disease severity was determined through a visual inspection of each grapevine for characteristic symptoms and rating each vine on a scale of 1 - 7, where:

1- Healthy vine with no characteristic symptoms of Pierce's disease.

2- Incipient symptoms: 3 or more leaves showing foliar symptoms characteristic of Pierce's disease.

3- Advanced Symptoms: More than half the vine showing foliar symptoms characteristic of Pierce's disease or the plant is demonstrating petiole retention. May include 'raisining'.

4- Advanced w/ Dieback: Entire cordons or vines on plant are dead or dying. May also represent vines that have experienced the death of entire cordons or vines previously leading to growth closer to the main cane.

5- Dead

6- Stump: Plant has been pruned back to where only a stump exists.

7- Replant: Evidence that a previous grapevine has been removed and replaced with a new one within the past year.

The statistical program SPSS was used to compare the percentage of successful isolations to the following three parameters: disease severity, vineyard block, and grape variety. Each analysis produced Pearson's Chi-Squared tests with p-values <0.01 indicating a significant relationship between the percentage of successful isolation and all three factors (Appendix A: Analysis of Isolations).

CHAPTER III

RESULTS

Sampling and Isolation

A total of 329 grapevine samples, representing 204 plants, were processed for isolation of *X. fastidiosa*. Processing initially produced 120 isolates from 86 vines which exhibited characteristic culture morphology of *X. fastidiosa.* When transferred, several potential isolates were lost to poor growth resulting in a final total of 97 isolates. These surviving isolates were further analyzed for species and subspecies identification.

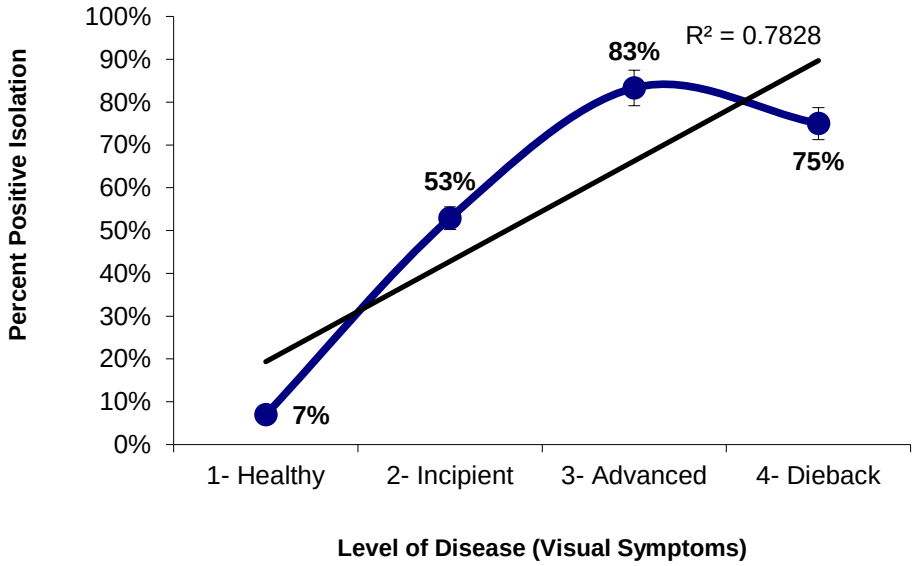

Fig. 1. Percent of successful isolations per disease index rating. Graph showing the relationship of successful isolation of *X. fastidiosa* versus the disease severity as demonstrated through visual symptoms of Pierce's Disease of grape in 2007.

A total of 278 vines where scored for disease severity at the time of sampling. Those vines with a rating of '4- Advanced with dieback' (n=48) had a 75% (n=36) rate of successful isolation, vines with a rating of '3- Advanced symptoms' (n=6) had an 83% (n=5) rate of successful isolation, vines with a rating of '2- Incipient symptoms' (n=138) had a 53% (n=73) rate of successful isolation, and vines with a rating of '1- Healthy vine' (n=86) had a 7% (n=6) rate of successful isolation. When comparing isolation attempts and disease severity, a positive correlation (R^2=0.7828) is observed between successful isolation and an increase in disease severity. This relationship is maintained until plants start expressing symptoms consistent with a severity rating or '4- Dieback' (Fig. 1).

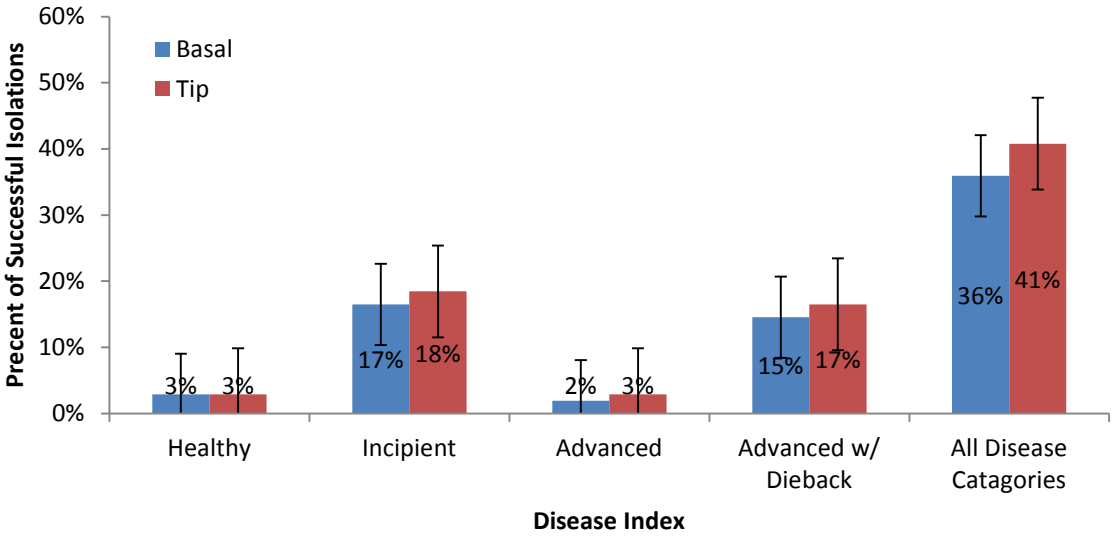

Fig. 2. Percent of successful isolations from tip or basal petioles per disease index rating. Graph showing the percentage of successful isolation attempts for each disease index category (Healthy, Incipient, Advanced, Advanced with Dieback, and All Categories) for petioles collected from the base or tip of a sample grapevine shoot.

A total of 103 vines had petiole samples collected from two locations on symptomatic shoots. The 'Basal' samples where those closest to the base of the symptomatic shoot and the 'Tip' samples where those furthest from the base but still fully formed. Basal petiole samples had a success rate of 36% (n=37) and Tip petiole samples had a success rate of 41% (n=42) (Fig. 2).

Of the 8 varieties sampled, Blanc du Bois was the only to produce no isolates (n=24 attempts). The highest frequency of successful isolation was from Chambroucin (73%, n = 33). The percentages of successful isolations for other varieties were as follows: Muscat Blanc 12% (n = 108), Merlot 33% (n = 38), Cabernet Sauvignon 43% (n = 14), Syrah 48% (n = 45), Primitivo 52% (n = 44), Ruby Cabernet 57% (n = 30), and an overall of 36% (n = 337) (Fig. 3).

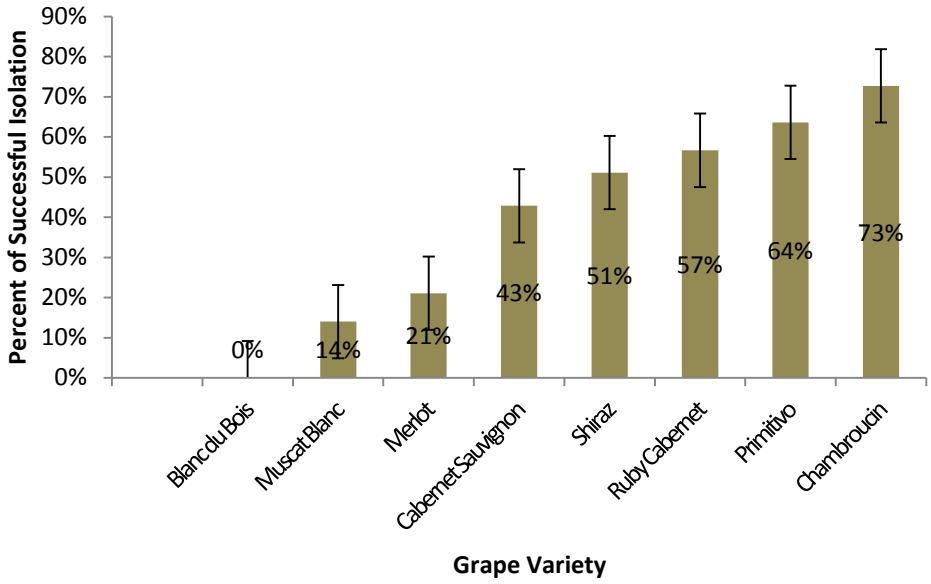

Fig. 3. Percent successful isolations per grape variety. Graph depicts the level of success for isolations attempts for each grape variety.

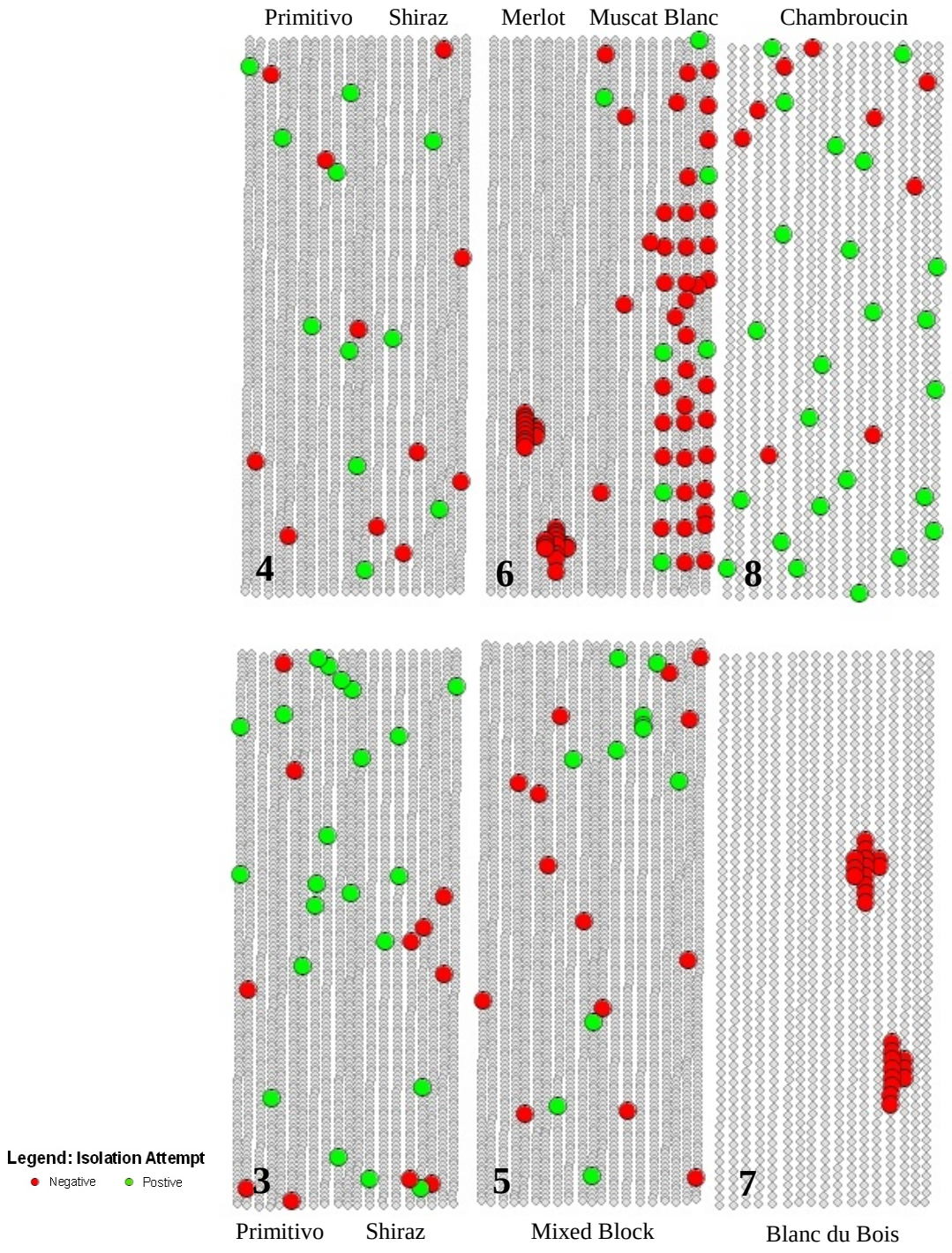

Fig. 4. Vineyard map of sample vines and positive isolates. Vines sampled and processed for the isolation of *X. fastidiosa* are depicted by red or green dots. Red dots indicate failed isolation attempts. Green dots indicate successful isolation of *X.fastidiosa.*

The location of positive isolations were from vines throughout all areas of the vineyard except block 7 (Blanc du Bois) and the Merlot (half of block 6) (Fig. 4). Block 3 produced the most isolates (n=40) at a 61% rate of success while the identical block 4 produced fewer isolates (n=11) at a lower rate of success, 48%. Both blocks are half Primitivo and half Shiraz. Block 6, the most heavily sampled, only produced 13 isolates with a 10% rate of successful isolation. Block 8 produced the most successful rate of isolation (73%, n=24) (Table 3).

Table 3. Isolation attempts for *X. fastidiosa* separated by vineyard block.

Location	Isolation		Total Attempts	Percent Successful Isolation
	Negative	Positive		
Block 3	26	40	66	61%
Block 4	12	11	23	48%
Block 5	34	32	66	48%
Block 6	103	13	116	11%
Block 7	25	0	25	0%
Block 8	9	24	33	73%
Total:	209	120	329	36%

Species and Subspecies Identification

All isolates analyzed tested positive using DAS-ELISA as *X. fastidiosa*. The typical, distinctive blue coloring of a positive reaction can be seen in each well of the reaction wells on a 96-well plate, with the exception of negative controls in the first row (Fig. 5).

In addition, all isolates analyzed using the multi-primer PCR assay generated one band at approximately 412bp indicating isolates belong to the subspecies *fastidiosa* (Fig. 6). Both non-grape and 'Dixon' DNA generated two bands at 638 and 521bp indicating they belong to the subspecies *multi-plex* as was expected. Images of the multi-primer gel electrophoresis products for all isolates can be found in Appendix B: Multiprimer Gel Photos.

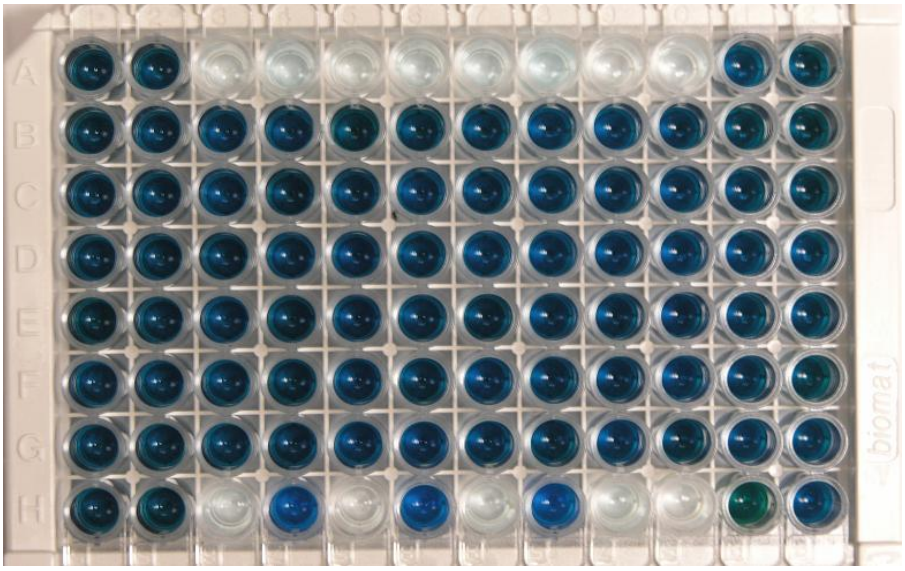

Fig. 5. DAS-ELISA of isolated cultures. Photograph depicts results of DAS-ELISA assay used to identify suspected cultures as *X. fastidiosa*. Blue color indicates a positive test where clear wells indicate a negative test. All clear wells in this photograph correspond to the negative controls.

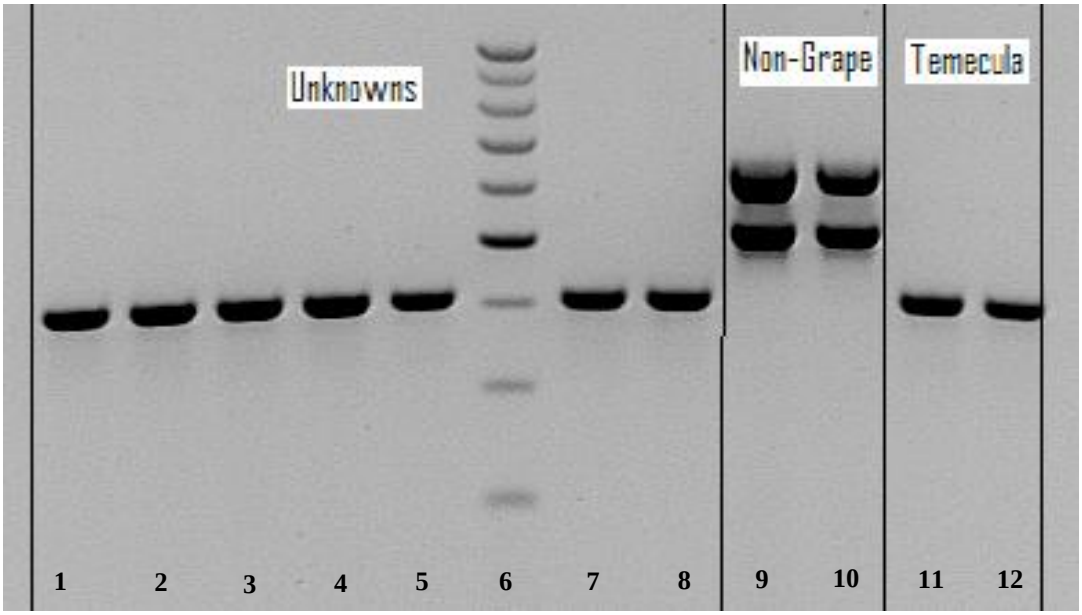

Fig. 6. Subspecies identification of *X. fastidiosa* isolates. A gel electrophoresis was run for the products from the multiplex PCR assays. Lanes 1-5, 7, & 8 are samples from seven unknown cultures isolated from field samples. These samples have a single band at 412bp. Lanes 9 & 10 are known non-grape isolates which have two bands at 512 and 638bp. Lanes 11 & 12 are known grape isolates (Temecula) with a single band at 412bp. Lane 6 is a 100bp DNA Ladder.

SSR and Hierarchical Cluster Analysis

All 97 isolates produced a single band for each SSR primer sets. The sizes of the PCR products ranged from 211bp to 429bp. Each SSR generated varying degrees of difference among DNA fragment sizes with primer set GSSR# 19 producing the most variability of 199bp between the most extreme values. Photos of the gel generated by each of the SSR markers for a subset of 18 isolates is pictured below (Fig. 7).

A cluster analysis using between-group linkages was performed and generated a dendrogram which mirrored the expected distribution of subspecies groups (Fig. 8). This dendrogram shows three major clusters with 14 - 44 isolates each. All clusters

include at least 3 varieties and 3 blocks with two clusters containing isolates from all 5 blocks and 6 or 7 cultivars. The cluster with the least diversity contains mostly isolates from Chambroucin, the variety with the highest rate of successful isolation. Lastly, 21 pairs of isolates extracted were from the same plant. Of these pairs 90% were sorted into the same cluster while 10% are separated into different clusters.

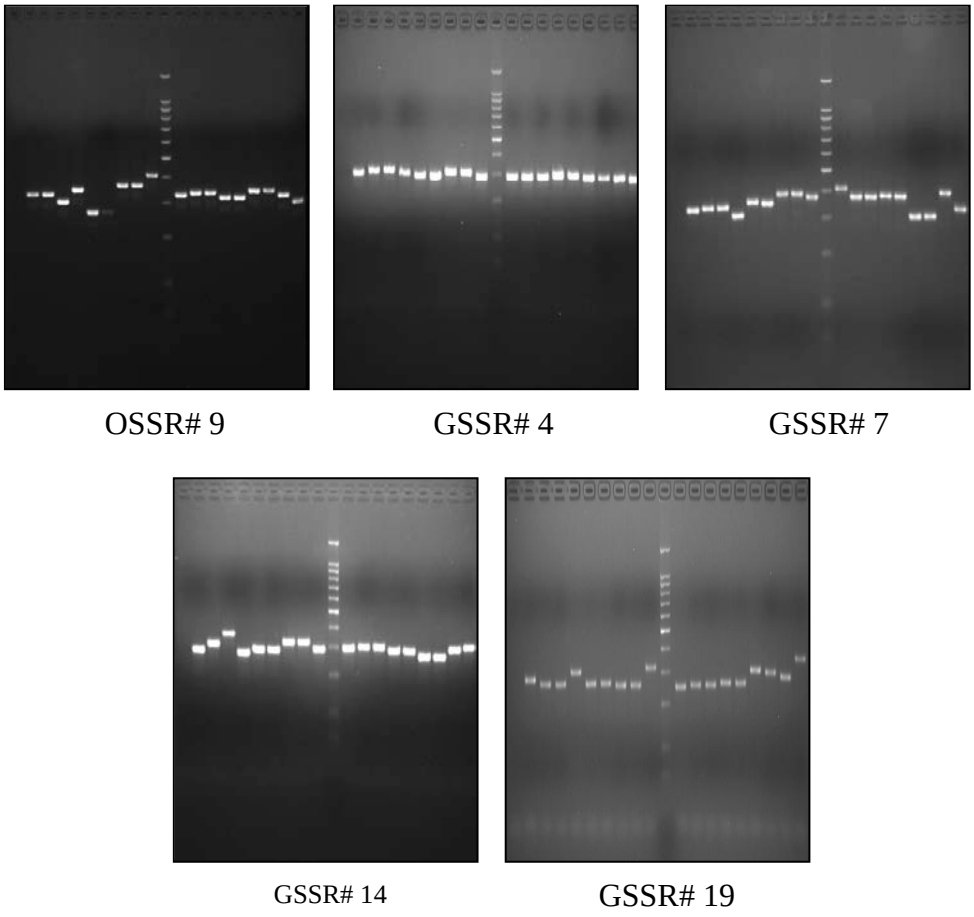

Fig. 7. Differentiation of *X. fastidiosa* subsp. *fastidiosa* isolates. Photographs depict gel electrophoresis results for the PCR products of 18 samples processed with 5 individual SSR markers. The center lane of each gel depicts a 100bp ladder.

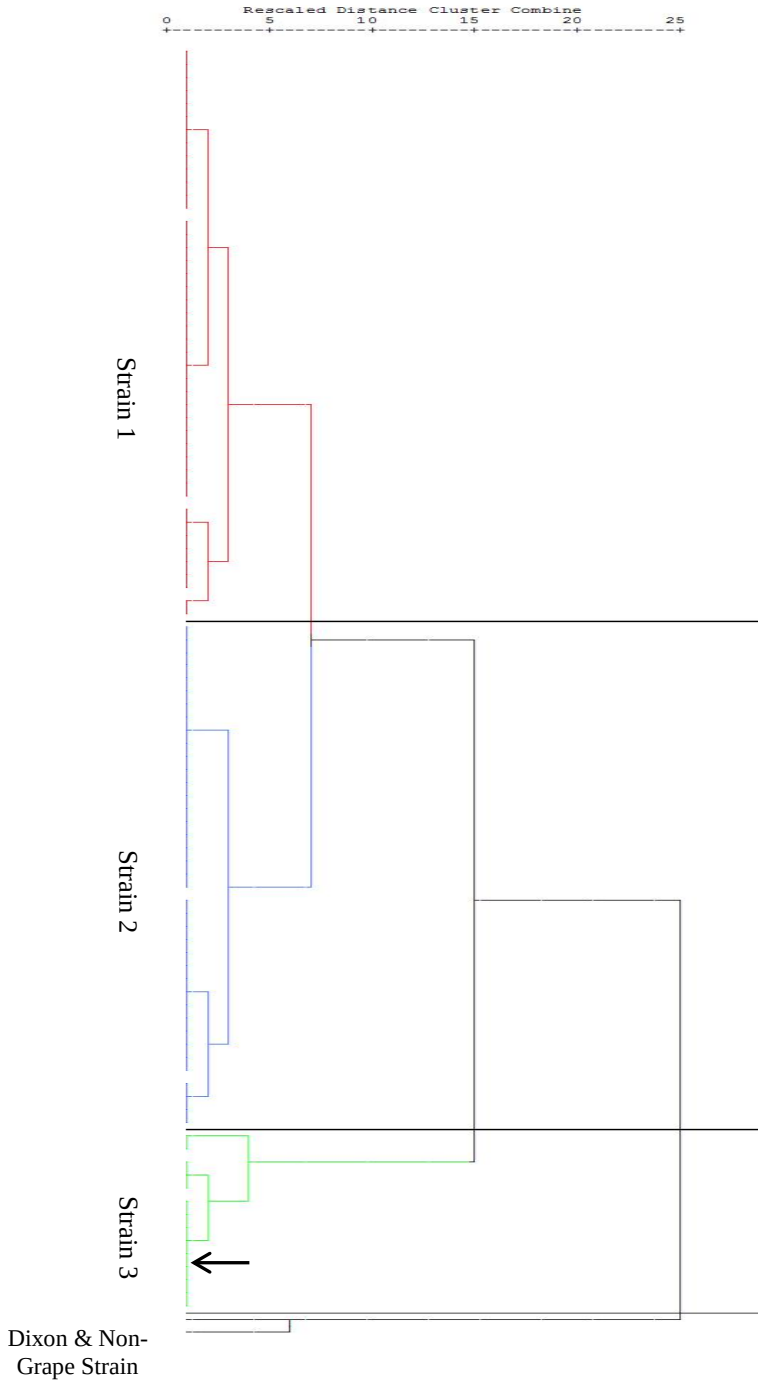

Fig. 8. Simplified dendrogram of strains. This dendrogram was generated by hierarchical cluster analysis of 97 isolates collected from a single vineyard. Each color represents a separate cluster or strain, which were arbitrarily named 1, 2, and 3. The Dixon and Non-Grape isolates were not grouped with any subspecies *fastidiosa* isolates while the Temecula isolate (see arrow) was grouped with Strain 3. A larger representation of this dendrogram can be found in Appendix B.

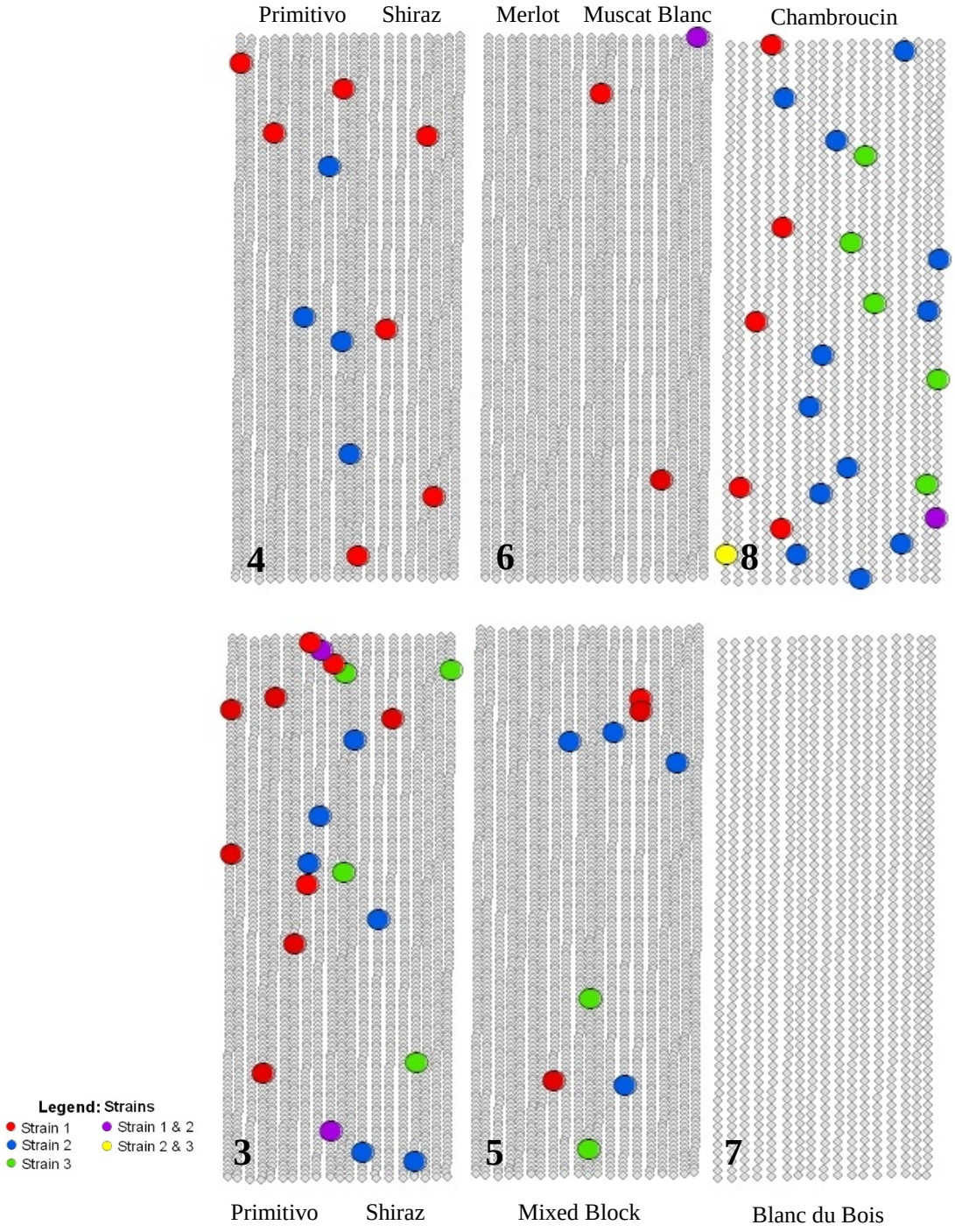

Fig. 9. Vineyard map depicting the distribution of strain groups. Strain groups were defined by cluster analysis of 97 *X. fastidiosa* subsp. *fastidiosa* isolates. Each dot represents a vine from which one or more successful isolates were obtained while the color defines the strain(s) isolated.

Analysis of Strain Groups

When viewing the dendrogram generated through the hierarchical cluster analysis of simple sequence repeat markers, three distinct strain groups are formed (Fig. 8). Arbitrarily, they were labeled strain 1 (n=39), strain 2 (n=44), and strain 3 (n=13) beginning from the top-most cluster on the dendrogram and are colored red, blue, or green respectively. Strain groups 1 and 2 are most closely related, merging at 7 units while strain group 3 does not merge until 15 units. All three strains merge with the non-grape isolates at 25 units. Units were calculated using squared Euclidean measure of distance and describe the relative amount of dissimilarity.

Observing the distribution of strains, it should be noted that all three strain groups are comprised of most blocks and varieties (Fig. 9). The only strain group that does not have representatives from each block is also comprised of the least varieties and is strain group 3. In addition, this group is the most distantly related and is where the Temecula type strain from California was clustered.

Epidemiological Data

In August of 2007, a field survey for disease severity was conducted for all but 10 rows of grapevines in block 5 (*personal communication*, D.N. Appel, Dept. of Plant Pathology and Microbiology, TAMU, College Station, TX 77843) (Fig. 10). When plotted with survey data from the previous 6 years, a more comprehensive view of disease progression can be observed (Fig. 11). Block 8, which consisted entirely of Chambroucin grapevines, had the greatest disease pressure as indicated by the

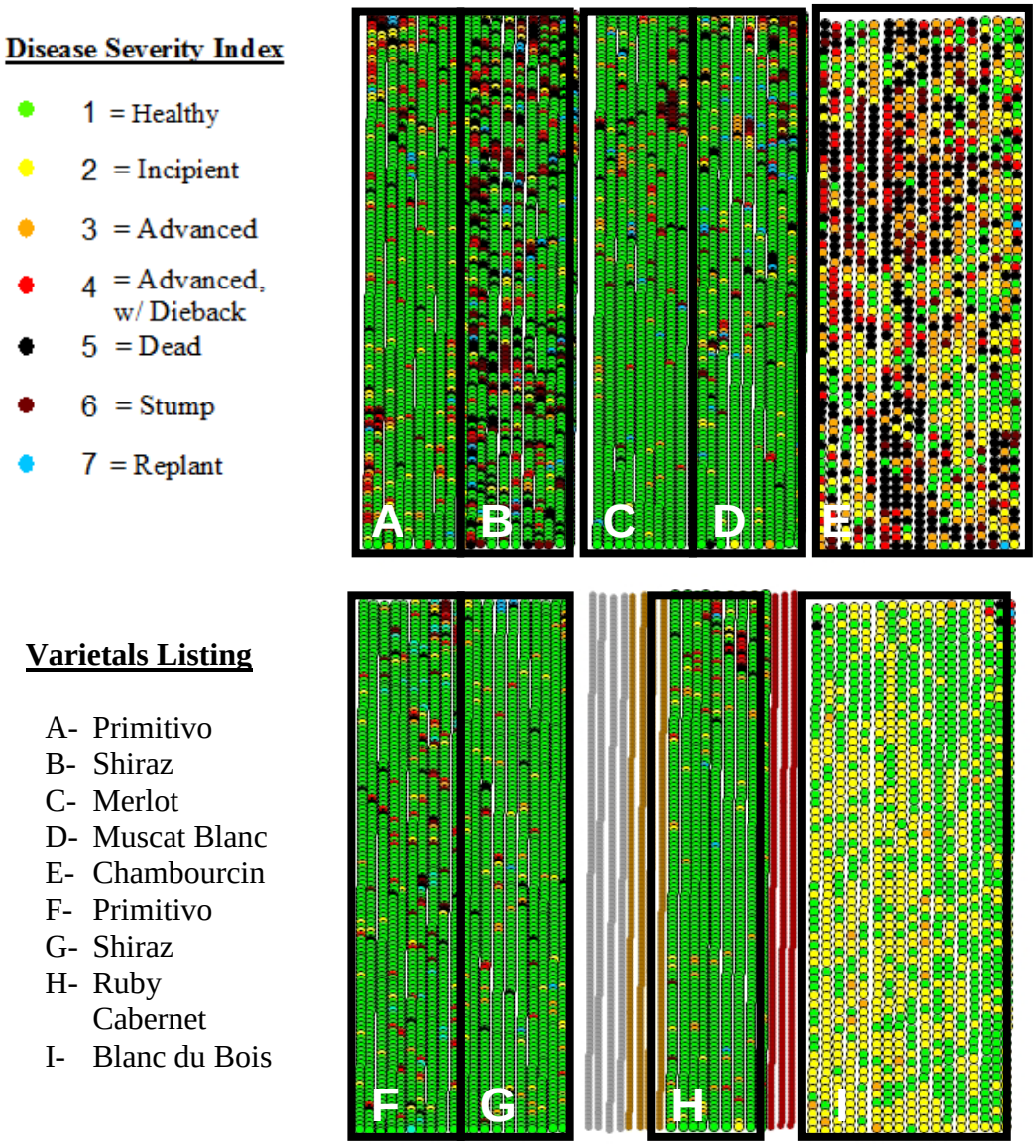

Fig. 10. Vineyard map of 2007 disease severity survey. Map depicts an ArcGIS visualization of disease severity. The top key indicates the disease severity index rating for each vine. The bottom key lists the varieties and indicates their location within the vineyard.

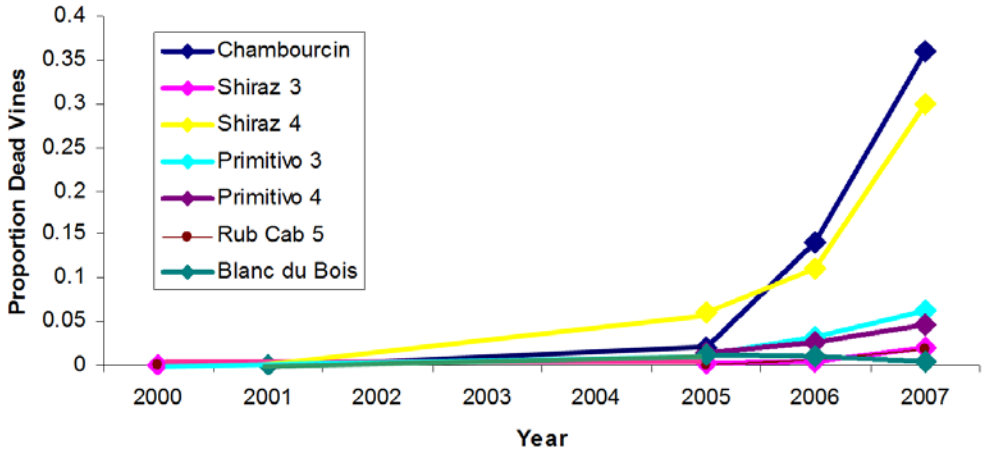

Fig. 11. Disease progress curve for vineyard from 2000 thru 2007. Graph shows the disease progress curve for several grape varieties by vineyard block. Data was collected through multiple disease severity surveys conducted from 2000 thru 2007 through a visual inspection for symptoms indicative of Pierce's Disease of grape.

proportion of dead vines. Chambroucin also showed the greatest increase in disease incidence over the past 3 years (2005-2007). The second highest proportion of dead vines occurred in the Shiraz half of block 4 followed by the Primitivo half of block 3.

The next step in this study was to compare strain groupings to several epidemiological factors using the Pearson's Chi-Square test. The first and least dependent pairing with a p-value = 0.303 was strain groups versus disease severity. Using the disease index, strain groupings were compared to healthy, mild symptoms, advanced and dieback symptoms, and dead, stump, or re-plants. The dead stump, and re-plant categories were combined because each would result from the death of the vine. While there is no statistical significance between these two variables, isolates collected from plants with incipient symptoms were overwhelmingly from strain 2 while those collected from plants that later died or were most likely removed where

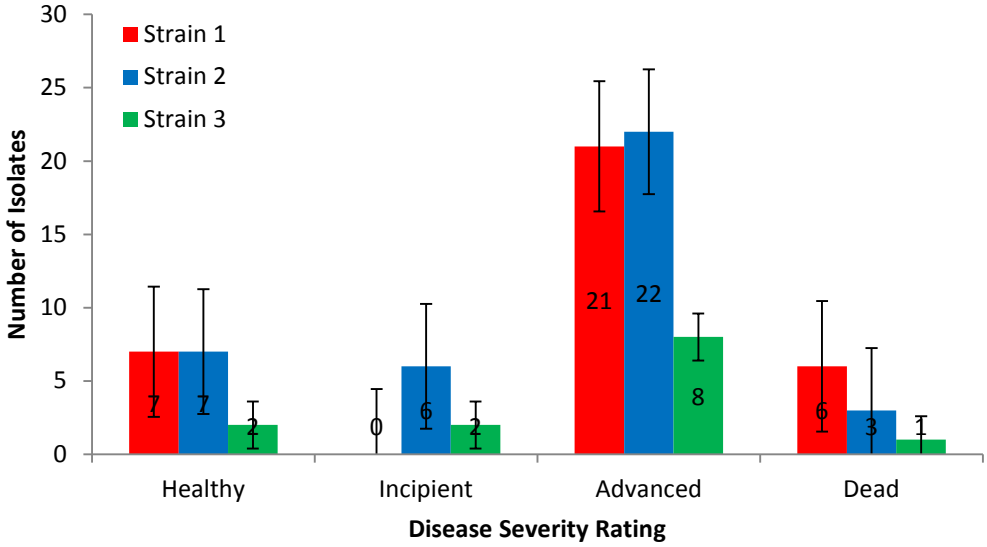

Fig. 12. Successful isolation per disease severity rating. Bar graph indicates the number of each isolate from strains 1, 2, and 3 from each disease index category (Healthy, Incipient, Advanced and Dieback, and Dead/stump/replant).

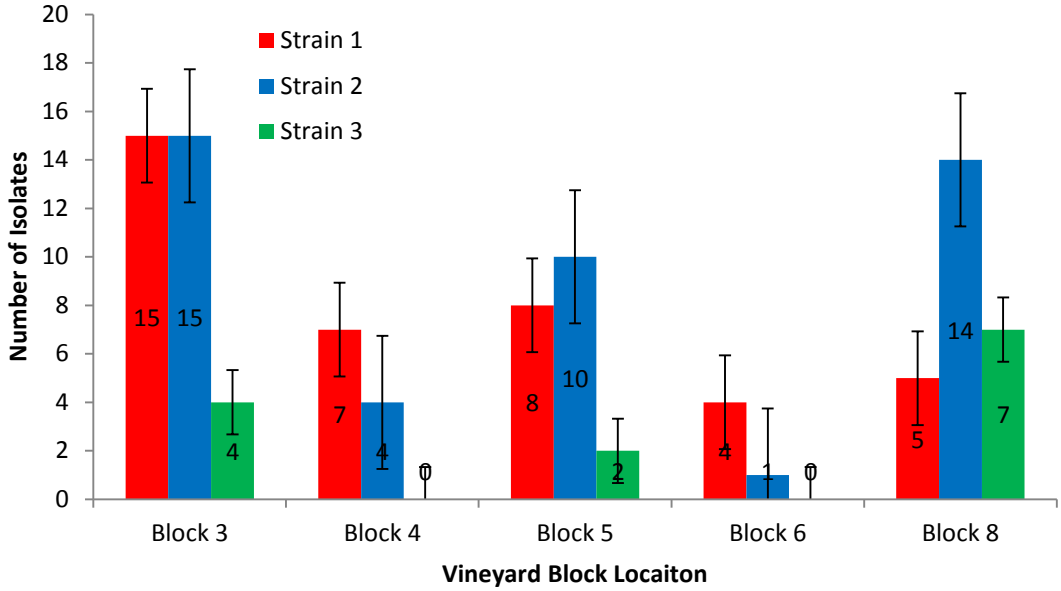

Fig. 13. Successful isolation per vineyard location. Bar graph indicates the number of isolates from strains 1, 2, and 3 from each vineyard block (3,4,5,6,or 8).

from strain 1 (Fig. 12). Healthy and advanced symptom vines produced isolates in relatively equal amounts of both strains 1 and 2 with much fewer of strain 3.

When comparing strain groups with vineyard block locations, a p-value of 0.091 was generated (Fig. 13). Strain 1 makes up over half of the isolates obtained from blocks 4 and 6 but falls within standard deviations. Strain 2 makes up over half of block 8 and falls beyond the standard deviations of both strain 1 and 3 for that black. Blocks 3 and 5 have no appreciable difference between strains 1 or 2, however, strain 3 falls well beyond the standard deviation of both. In all blocks, strain 3 is represented the least.

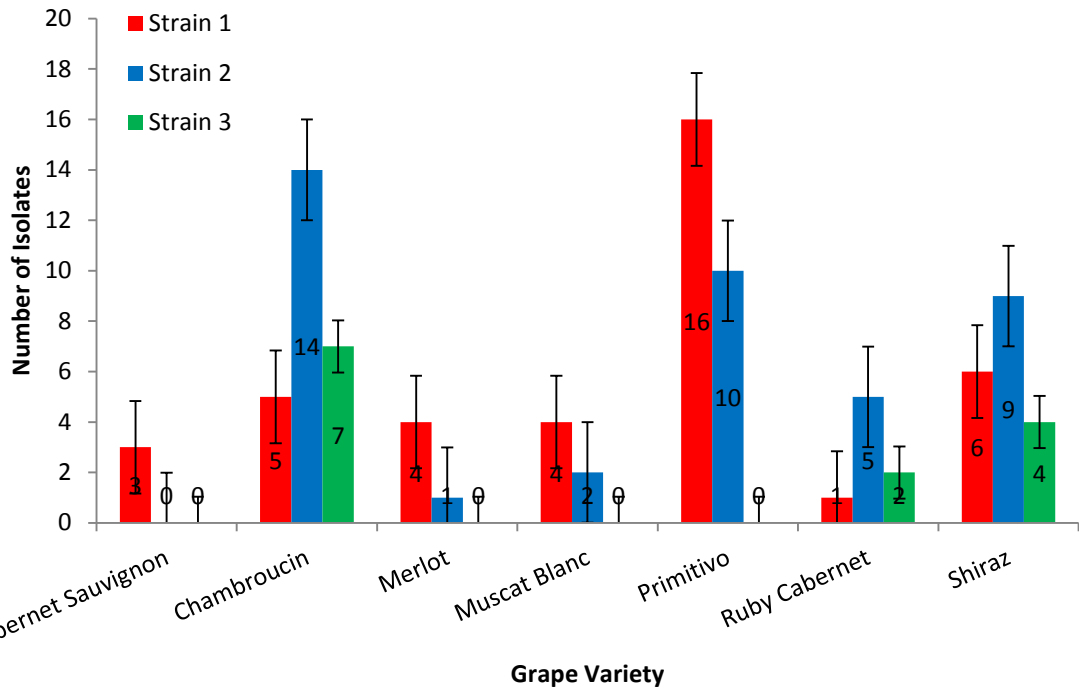

Fig. 14. Number of isolates per grape variety. Bar graph indicates the number of isolates from strains 1, 2, and 3 collected from each grape variety type.

The last comparison made was strain group to grapevine variety (Fig. 14) which showed a dependent relationship to strain identity with a p-value of 0.015. This dependence increased with a p-value = 0.009 if grapevine varieties were identified by vineyard block (Fig. 15). The major difference between the two categories was the effect of splitting the Shiraz and Muscat Blanc varieties into two blocks. Block 3 of Shiraz and block 5 of Muscat Blanc are dominated by strain 2 while block 4 Shiraz and block 6 of Muscat Blanc are dominated by strain 1. It is also important to note strain 3 is only present in the Shiraz half of block 3, Ruby Cabernet section of block 5, and the Chambroucin in block 8 of which none are adjacent nor do they show the same amount of disease pressure. It should be noted that each of these Pearson Chi-Square tests suffered from a small sample size due to the difficulty of collecting a larger number of successful isolations resulting in a less robust analysis.

Also of importance in this cluster analysis were 21 pairs of isolates gathered from single grapevines. These cultures were collected from different sections of the vine (tip or basal petioles) and isolated independently. 90% of these pairs were clustered within the same strain group. Of those distributed into separate strains, only one plant did not have both isolates in strain 1 and 2, the most closely related strain groups.

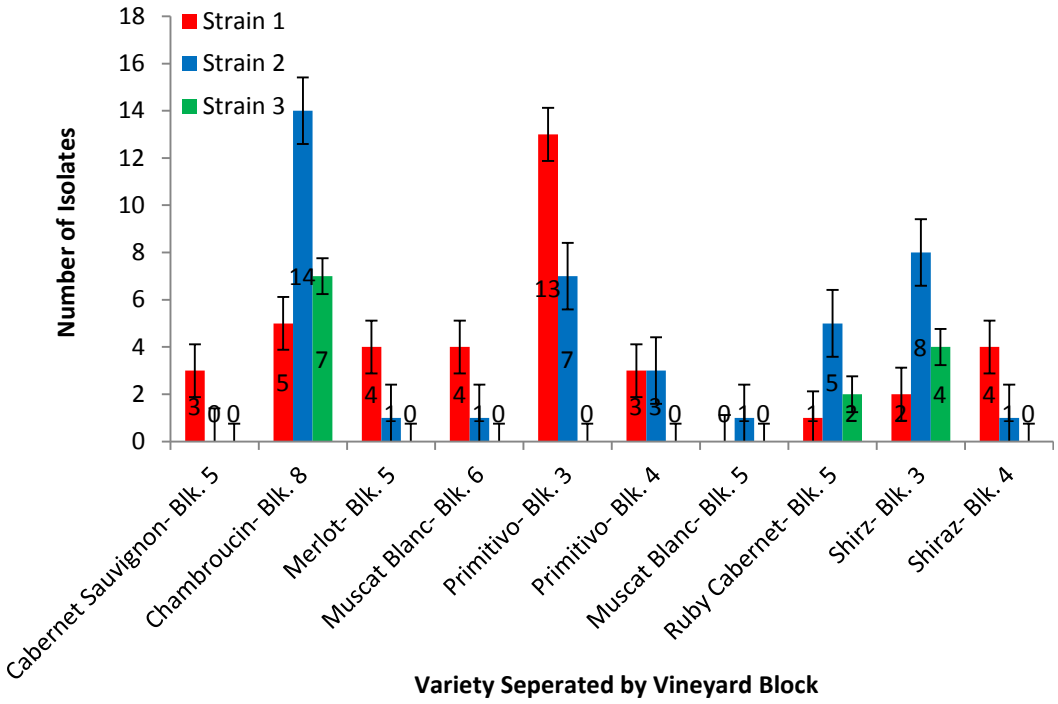

Fig. 15. Number of isolates per grape variety by vineyard block. Bar graph indicates the number of isolates from strains 1, 2, and 3 from each grape variety type when separated by vineyard block.

CHAPTER IV

DISCUSSION

Success and Implications of Culture Isolations

A library of 97 *Xylella fastidiosa* subspecies *fastidiosa* isolates was created through the sampling and isolation of a single vineyard. This is a result of less than half of isolation attempts proving successful. Of these successful isolation attempts, a pattern was observed for a positive correlation with disease severity (R^2=0.7828) (Fig. 1). This is to be expected due to the higher bacterial titer present in symptomatic grapevines (16, 19, 38). The reduction in successful isolations in plants exhibiting dieback could be a result of the reduced growth rate and viability of bacterium caused by the loss of available nutrients and optimal growing environment. An observation of note is the 7% of non-symptomatic grapevines which yielded *X. fastidiosa* isolates. Current management practices for Pierce's disease include reducing the bacterial population in the environment through the removal of infected plant tissue. This method relies on visual symptoms to identify those plants which pose a hazard to continued disease spread. The efficacy of this control method comes into question when the pathogen remains in the vineyard via non-symptomatic grapevines. Further research should be conducted to study how disease progression is affected by the presence of non-symptomatic, infected grapevines.

Success of isolation was also compared to the position on a grapevine from which a sample petiole was harvested. Common practice, based on other xylem-limited

bacteria, suggests the selection of petioles closest to the base of any shoot sampled (38). As *X. fastidiosa* produces biofilms which result in the aggregation of cells, it is believed these basal petioles will have a higher bacterial titer due to the larger flow of xylem fluid through the area, therefore increasing the probability of the successful isolation of the pathogen (49). This study did not find a significant advantage to sample position as the percent of successful isolations from basal petioles was within the standard of error for the percentage of successful isolations from tip petioles (Fig. 2). There was also no significance difference observed when petiole position was separated by disease index ratings. Given the demonstrated relationship between symptom severity and isolation success, a more robust experiment would compare the rates of successful isolation between petiole samples collected from leaves showing foliar symptoms and non-symptomatic leaves on the same shoot.

An analysis of isolation attempts for each variety showed a large degree of variability of success from 0% to 73% (Fig. 3). Most values fall within standard error, however, Blanc du Bois, Muscat Blanc, and Merlot resulted in fewer successful isolation attempts then all others. While Blanc du Bois and half of the Merlot plants available were sparsely sampled, Muscat Blanc was the most heavily sampled variety so this observation is not an artifact of sample size. A more probable explanation for this observation would be the varying degrees of disease incidence and severity. For example Chambroucin, the variety with the largest isolation success rate, was also the variety with the highest disease pressure.

Analysis of the location of the plant within the vineyard, as determined by the vineyard block where the plant was located, produced fairly uniform rates of isolation success with most falling between 48% and 73% , a difference of 25% (Table 4). The only exceptions to this distribution of were the comparatively low successful positives from blocks 6 and 7. This is likely due to the Merlot half of block 6 and the entirety of block 7 being largely ignored due to an ongoing experiment in these areas. Also, the Muscat Blanc half of block 6 and the Blanc du Bois in block 7 have shown low disease pressure in previous surveys (Fig. 10) and are considered resistant varieties. The most interesting results from the successful isolation data are those from blocks 3 (61%) and 4 (48%). Both of these blocks are half Primitivo and half Shiraz and are adjacent to each other, yet there is a 13% difference in success for isolation. There is also a large difference in the rate of disease progression for the Shiraz in block 3 and the Shiraz in block 4 from 2005 to 2007 (Fig. 11). While there are no factors recorded in this study that could contribute to this discrepancy, these blocks were planted a year apart (block 3 in 2000 and block 4 in 2001) and there is a slight incline from block 3 to 4. There has also been a larger occurrence of nutrient deficiencies occurring in block 4 (personal observation) and the two Primitivo sections utilize different root stocks (101-14 and SO4 for blocks 3 and 4 respectively). However, without further study of these sections, the cause for the difference in isolation success cannot be defined.

Given the studied parameters of disease severity, petiole position, grape variety, and grapevine location, the most influential factor to determine sample selection in order to increase the probability for the successful isolation of *X. fastidiosa* is disease severity.

Specifically, plants which are expressing advanced symptoms of Pierce's disease are most successful for pathogen isolation. Further research into which sample selection criteria would affect isolation success should include temporal relationships both throughout the day and year, the occurrence of other diseases and disorders, and the effects of rootstock.

Subspecies Identification and Differentiation

A theory which has never been addressed in current research is the possibility of multiple subspecies groups within *X. fastidiosa* colonizing grapevines in a natural environment and leading to variability in symptom expression and disease response (*Personal communication*, D.N. Appel, Dept. of Plant Pathology and Microbiology, TAMU, College Station, TX 77843). This phenomenon would result in a false positive diagnosis of Pierce's disease through ELISA and generalized PCR reactions, especially in plants that do not exhibit Pierce's disease symptoms. While some studies have proven the ability of isolates other than subspecies *fastidiosa* to colonize grapevines in a controlled environment (20), and others have studied the genetic diversity of *X. fastidiosa* cultures isolated from grapevines(11, 17, 26, 42, 44, 50, 58), this is the first study to specifically focus on the subspecies classification of isolates from naturally occurring *X. fastidiosa* populations in a vineyard setting. The results of this research indicate only *X. fastidiosa* subspecies *fastidiosa* cultures colonize grapevines in this natural environment. Alternatively, *X. fastidiosa* subspecies *fastidiosa* may be the most successful at colonizing grapevines in a natural environment and are therefore the most

likely to be isolated. If this were the case, however, there would be some variation in culture morphology when isolates were selected for purification, which was not observed. A more definitive study would attempt to identify subspecies from plant exudates without relying on purified isolations. However, there is no current protocol able to identify multiple subspecies of *X. fastidiosa* coexisting within a single sample.

Once all isolates were identified as *X. fastidiosa* subspecies *fastidiosa*, analysis was conducted to further differentiate isolates into strain groups (Fig. 7). This was accomplished using SSR markers previously developed by Lin et al. (27). While SSR markers have been shown to have a high rate of evolution, they can be used to view current trends in population distributions on a smaller genetic scale (11, 58). Using SSR data and hierarchical cluster analysis, multiple groupings were identified; however, three groups were distinguished as having more than 5% relative dissimilarity and arbitrarily labeled strains 1, 2, and 3. Of these three groups, strain 3 was the most distantly related with 15% relative dissimilarity while strains 1 and 2 had 7% relative dissimilarity. In contrast, two isolates which were not *X. fastidiosa* subspecies *fastidiosa* showed a 25% relative dissimilarity. Of particular interest is the inclusion of the Temecula type strain, an isolate from California, into strain group 3. This indicates a conservation of genetic structure among national isolates of *X. fastidiosa* subspecies *fastidiosa*. It also suggests the possibility of Texas specific strains of *X. fastidiosa* subspecies *fastidiosa* given the separation of strains 1 and 2 from strain 3. A more comprehensive study of isolates nationwide would be required to definitively identify any area specific strains. While previous research has shown diversity within *X. fastidiosa* subspecies *fastidiosa,* they

have been focused on differentiating between subspecies and determining the origins of *X. fastidiosa.* These studies have also used larger geographic scales ranging from intercontinental to statewide. This is the first indication of the genetic diversity that exists within the scale of a single vineyard. In addition, of the grapevines that resulted in multiple isolates, 10% of these plants contained isolates from different strains. This is the first look at the diversity of *X. fastidiosa* within a single grapevine and evidence for the co-colonization of *X. fastidiosa* strains within a single plant.

Relationship of Strain Groups to Epidemiological Factors

The distribution of strain groups was compared with several epidemiological factors including disease severity, grapevine location, grape variety, and grape variety separated by vineyard block. Of these factors, disease severity was the least dependent (p-value= 0.303) indicating no relationship between strain identification and disease expression. This suggests there is little or no difference in virulence among strains.

When strain distributions were compared to grapevine location, as indicated by the vineyard block from which the isolate originated, a weak dependence (p-value = 0.091) was observed. The main contributors to this association were the prevalence of strains 1 and 2 in blocks 3 and 5 and strain 2 in block 8 (Fig. 13). Strain 3 is also completely absent from blocks 4 and 6. While a minimal relationship between strain group distributions and grapevine location can be inferred from this data, there are many variables to consider when identifying the cause of this relationship to exist including the

following: elevation, relative distance to an inoculum source, relative distance to vector habitat, and distribution of disease incidence.

The last component analyzed was the various grape varieties planted throughout the vineyard. This factor showed a strong dependence (p-value = 0.015) when associated with strain distributions (Fig. 14). While not conclusive, these results suggest a possible preference for specific grape varieties. In addition, when grape varieties were separated by vineyard block (Fig. 15) this relationship became stronger (p-value = 0.009). Of significance are the two varieties most affected by this division, Muscat Blanc and Shiraz. Both of these varieties exist in two separate blocks, which were planted in consecutive years (2000 and 2001). For both varieties, those isolates from grapevines planted in 2000 are predominantly from strain 2 and those from grapevines planted in 2001 are predominantly from strain 1. This would suggest each strain was introduced in different years; however, when other varieties planted in those same years are reviewed, approximately half of the varieties are predominated by strain 1 isolates and half by strain 2 isolates. To further understand this event, a comparison between strain distributions and disease incidence and severity for each recorded year may enable an exact year of introduction to be detected for each strain. In addition, continued tracking of strain group spatial distribution over time might assist in clarify what causes the strong relationship between isolate strain and variety.

CHAPTER V

CONCLUSIONS

One of the limiting factors to studying *X. fastidiosa* has been the ability to successfully isolate and cultivate pure cultures. By assembling such a large library of isolates from a diverse sampling, several factors were able to be analyzed for their effect on sample selection and successful isolation. This research determined the most influential factor in sample selection for obtaining a pure culture to be disease severity and expression. This study did not, however, find any advantage to considering petiole location, grape variety type, or relative location within the vineyard when selecting samples for culture isolation. Moreover, *X. fastidiosa* was successfully isolated from non-symptomatic grapevines, stressing the need for a more thorough method for identifying plants which serve as potential sources of inoculum.

In addition to creating a library of isolates and defining criteria for sample selection, the diversity of *X. fastidiosa* was evaluated. It appears, while there are several subspecies of *X. fastidiosa* which can be found in Texas, the only one colonizing grapevines is subspecies *fastidiosa*. Moreover, a finer degree of differentiation can be delineated within the subspecies using Simple Sequence Repeat markers, clustering isolates in to strain groups. This observation is reinforced by previous research conducted on *X. fastidiosa* subspecies *fastidiosa*; however, this is the first study to restrict the geographic range to a single vineyard. Three distinct strains were differentiated with one strain being related to the Temecula type strain while the other

two were not. This demonstrates both the conservation of genetic material between Texas and California isolates of *X. fastidiosa* subspecies *fastidiosa* as well as the diversity and potential for area specific strains of *X. fastidiosa* subspecies *fastidiosa*. Another discovery gained from studying population diversity is the ability of two different strains of *X. fastidiosa* subspecies *fastidiosa* to coexist within the same plant, furthering the discussion on the effects of multiple genetically distinct isolates on plant disease expression.

With the diversity of *X. fastidiosa* subspecies *fastidiosa* defined, strain distributions were compared to several epidemiological factors to begin to understand the population dynamics of the pathogen and what possible effects there may be on disease expression. While there are many variables to be considered, this research focused on disease severity, grapevine location, and grape variety. This research found strain type to be independent of disease incidence, weakly dependent on grapevine location, and strongly dependent on grape variety. These results suggest several characteristics for strain type dynamics; that there is no difference is virulence among strain types; that strain distribution amongst the vineyard is not dependent on strain type; and that there may be a selection factor either within the pathogen or plant which determines strain/grape variety combination. Alternatively, there may be other factors which were not observed that may be affecting strain type/ grape variety associations.

In conclusion, the three objectives outlined for this project, to create a library of *X. fastidiosa* isolates, to demonstrate diversity within subspecies *fastidiosa*, and to analyze the population diversity against several epidemiological factors, have been

completed and expanded upon. Through this research, a more complete understanding of the population dynamics of *Xylella fastidiosa* subspecies *fastidiosa* has been outlined for this vineyard. In addition, the relationship between pathogen and host has been further analyzed providing insights into the expression of disease in a naturally infected vineyard. While several theories have been expanded upon or proven, the results from this thesis can also serve as a basis for exploring new topics of research, beginning new discussions and furthering the body of knowledge on Pierce's disease of grapes.

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APPENDIX A

ANALYSIS OF ISOLATES

1. Analysis of strain groups 1 (Red), 2 (Blue), and 3 (Green) against disease severity ratings.

Crosstab

			07 Rating				
			Healthy	Incipient	Advanced and advanced w/dieback	Dead, stump, or replant	Total
Strain	Red Group	Count	7	0	21	6	3
		Expected Count	6.4	3.2	20.4	4.0	34.
	Blue Group	Count	7	6	22	3	3
		Expected Count	7.2	3.6	22.8	4.5	38.
	Green Group	Count	2	2	8	1	1
		Expected Count	2.4	1.2	7.8	1.5	13.
Total		Count	16	8	51	10	8
		Expected Count	16.0	8.0	51.0	10.0	85.

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.194(a)	6	.303
Likelihood Ratio	9.993	6	.125
Linear-by-Linear Association	.548	1	.459
N of Valid Cases	85		

a 7 cells (58.3%) have expected count less than 5. The minimum expected count is 1.22.

2. Analysis of strain groups 1 (Red), 2 (Blue), and 3 (Green) against vineyard block.

Crosstab

			Block					
			3	4	5	6	8	Total
Strain	Red Group	Count	15	7	8	4	5	39
		Expected Count	13.8	4.5	8.1	2.0	10.6	39.0
	Blue Group	Count	15	4	10	1	14	44
		Expected Count	15.6	5.0	9.2	2.3	11.9	44.0
	Green Group	Count	4	0	2	0	7	13
		Expected Count	4.6	1.5	2.7	.7	3.5	13.0
Total		Count	34	11	20	5	26	96
		Expected Count	34.0	11.0	20.0	5.0	26.0	96.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	13.650(a)	8	.091
Likelihood Ratio	15.332	8	.053
Linear-by-Linear Association	5.637	1	.018
N of Valid Cases	96		

a 8 cells (53.3%) have expected count less than 5. The minimum expected count is .68.

3. Analysis of strain groups 1 (Red), 2 (Blue), and 3 (Green) against grape variety.

Crosstab

			Cultivar							
			Cabernet Sauv	Chambourcin	Merlot	Muscat Blanc	Primitivo	Ruby Cab	Shiraz	Total
Strain	Red Group	Count	3	5	4	4	16	1	6	39
		Expected Count	1.2	10.6	3.3	2.4	10.6	3.3	7.7	39.0
	Blue Group	Count	0	14	4	2	10	5	9	44
		Expected Count	1.4	11.9	3.7	2.8	11.9	3.7	8.7	44.0
	Green Group	Count	0	7	0	0	0	2	4	13
		Expected Count	.4	3.5	1.1	.8	3.5	1.1	2.6	13.0
Total		Count	3	26	8	6	26	8	19	96
		Expected Count	3.0	26.0	8.0	6.0	26.0	8.0	19.0	96.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	25.052(a)	12	.015
Likelihood Ratio	31.183	12	.002
N of Valid Cases	96		

a 15 cells (71.4%) have expected count less than 5. The minimum expected count is .41.

4. Analysis of strain groups 1 (Red), 2 (Blue), and 3 (Green) against grape variety and vineyard block.

			CultivarByBlk						
			Cabernet Sauv. Blk 5	Chambroucin Blk 8	Merlot Blk 5	Muscat Blanc Blk 6	Primitivo Blk 3	Primitivo Blk 4	Muscat Blanc Blk 5
Strain	Red Group	Count	3	5	4	4	13	3	0
		Expected Count	1.2	10.6	3.3	2	8.1	2.4	0.4
	Blue Group	Count	0	14	4	1	7	3	1
		Expected Count	1.4	11.9	3.7	2.3	9.2	2.8	0.5
	Green Group	Count	0	7	0	0	0	0	0
		Expected Count	0.4	3.5	1.1	0.7	2.7	0.8	0.1
Total		Count	3	26	8	5	20	6	1
		Expected Count	3	26	8	5	20	6	1

			CultivarByBlk			Total
			Ruby Cabernet Blk 5	Shiraz Blk 3	Shiraz Blk 4	
Strain	Red Group	Count	1	2	4	39
		Expected Count	3.3	5.7	2	39
	Blue Group	Count	5	8	1	44
		Expected Count	3.7	6.4	2.3	44
	Green Group	Count	2	4	0	13
		Expected Count	1.1	1.9	0.7	13
Total		Count	8	14	5	96
		Expected Count	8	14	5	96

Chi-Square Tests

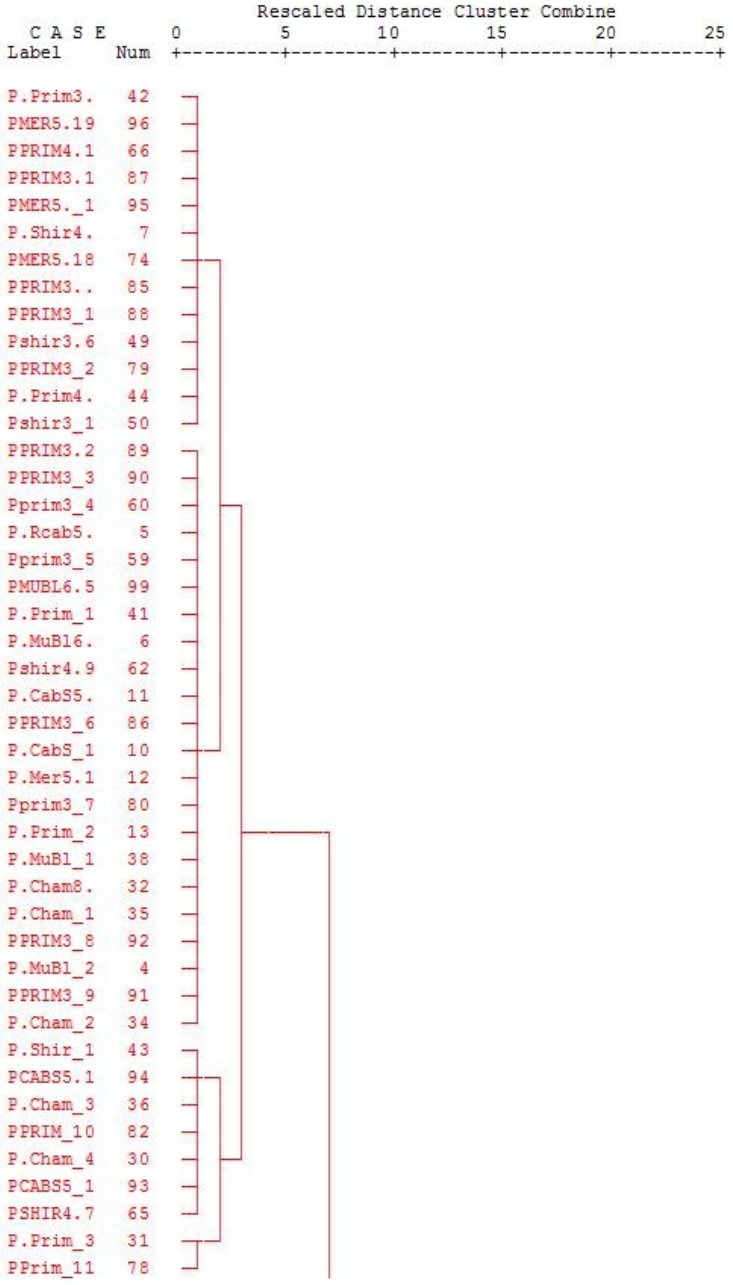
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	35.257(a)	18	.009
Likelihood Ratio	42.232	18	.001
Linear-by-Linear Association	.121	1	.728
N of Valid Cases	96		

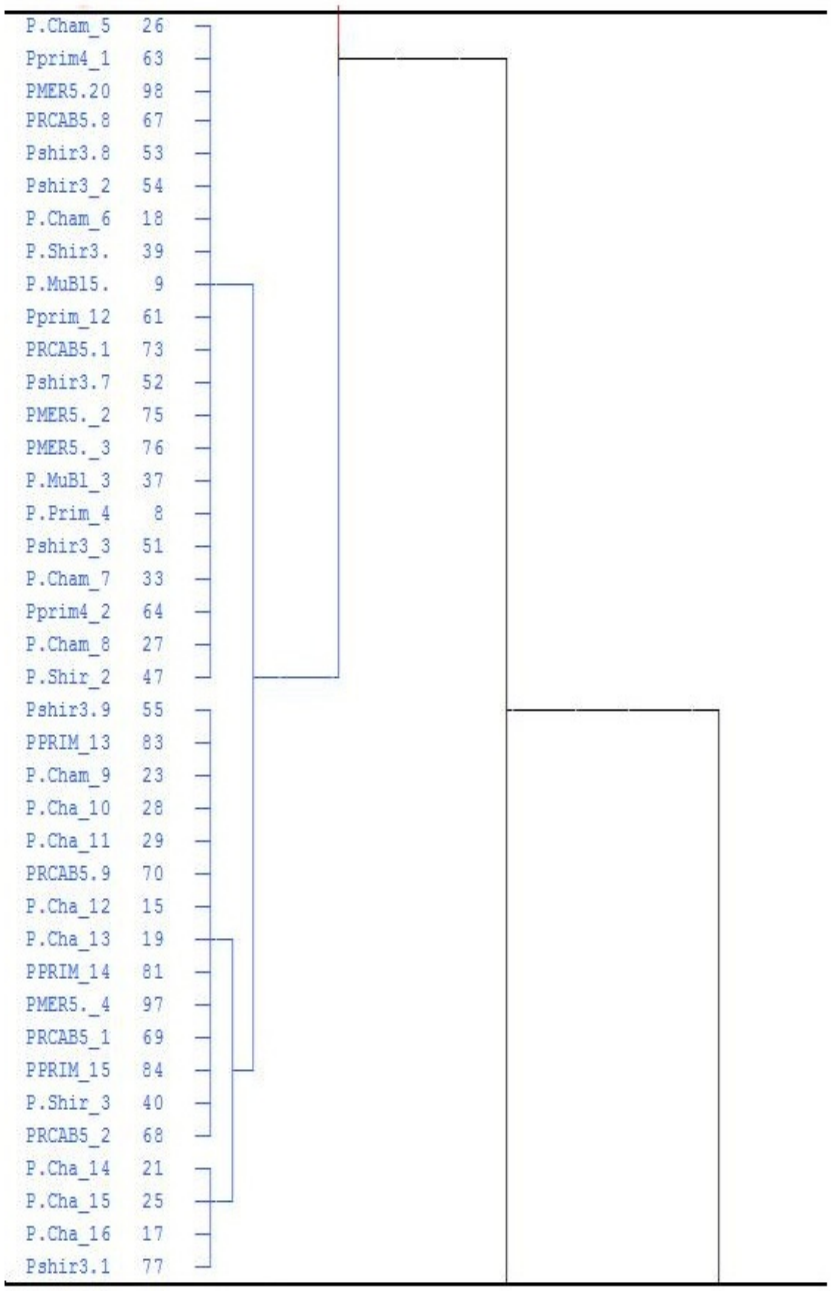
a 24 cells (80.0%) have expected count less than 5. The minimum expected count is .14.

APPENDIX B

FULL DENDROGRAM AND KEY

Dendrogram is broken into three sections. Strain 1 (Red), Strain 2 (Blue), and Strain 3 (Green).

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PRCAB5.8 67
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Pshir3_2 54
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P.Shir3. 39
P.MuBl5. 9
Pprim_12 61
PRCAB5.1 73
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PMER5._2 75
PMER5._3 76
P.MuBl_3 37
P.Prim_4 8
Pshir3_3 51
P.Cham_7 33
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P.Cham_8 27
P.Shir_2 47
Pshir3.9 55
PPRIM_13 83
P.Cham_9 23
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P.Cha_11 29
PRCAB5.9 70
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P.Cha_13 19
PPRIM_14 81
PMER5._4 97
PRCAB5_1 69
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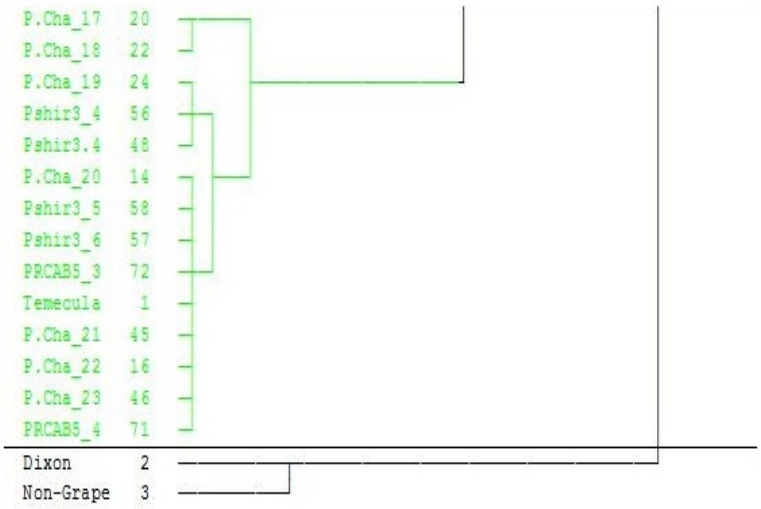
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Pshir3_6 57
PRCAB5_3 72
Temecula 1
P.Cha_21 45
P.Cha_22 16
P.Cha_23 46
PRCAB5_4 71
Dixon 2
Non-Grape 3

Abbreviated Name	Extended Name
P.CabS_1	P.CabS5.13.10.7.T
P.CabS5.	P.CabS5.13.10.7.B
P.Cha_10	P.Cham8.11.3.3
P.Cha_11	P.Cham8.12.1.4
P.Cha_12	P.Cham8.1.5.2
P.Cha_13	P.Cham8.4.72
P.Cha_14	P.Cham8.7.1.1
P.Cha_15	P.Cham8.9.6.7
P.Cha_16	P.Cham8.1.1.8.C
P.Cha_17	P.Cham8.17.1.4.C
P.Cha_18	P.Cham8.2.4.5
P.Cha_19	P.Cham8.6.4.6
P.Cha_20	P.Cham8.7.6.5
P.Cha_21	P.Cham8.8.5.4
P.Cha_22	P.Cham8.1.3.6
P.Cha_23	P.Cham8.1.1.8
P.Cha_24	P.Cham8.2.2.3
P.Cha_25	P.Cham8.17.1.4
P.Cham_1	P.Cham8.15.4.4
P.Cham_2	P.Cham8.14.7.9
P.Cham_3	P.Cham8.16.2.3
P.Cham_4	P.Cham8.13.1.7
P.Cham_5	P.Cham8.10.2.2
P.Cham_6	P.Cham8.4.1.5
P.Cham_7	P.Cham8.13.7.3
P.Cham_8	P.Cham8.10.3.9
P.Cham_9	P.Cham8.8.2.5
P.Cham8.	P.Cham8.13.5.6
P.Mer5.1	P.Mer5.18.10.3.T
P.MuBl_1	P.MuBl6.2.32.3.B
P.MuBl_2	P.MuBl6.5.6.4
P.MuBl_3	P.MuBl6.2.32.3.T
P.MuBl5.	P.MuBl5.3.3.10.T
P.MuBl6.	P.MuBl6.10.29.2
P.Prim_1	P.Prim3.13.1.2.T
P.Prim_2	P.Prim3.17.10.4.T
P.Prim_3	P.Prim4.20.31.1
P.Prim_4	P.Prim4.11.14.4
P.Prim3.	P.Prim3.14.7.7.T
P.Prim4.	P.Prim4.11.29.3
P.Rcab5.	P.Rcab5.6.2.6.T
P.Shir_1	P.Shir4.4.26.4
P.Shir_2	P.Shir4.10.8.2
P.Shir_3	P.Shir3.4.12.3.T
P.Shir3.	P.Shir3.9.3.3.T
P.Shir4.	P.Shir4.3.5.4
PCABS5.1	PCABS5.14.3.2.B
PCABS5_1	PCABS5.14.3.2.T
PMER5._1	PMER5.19.8.2.T
PMER5._2	PMER5.19.1.7.T
PMER5._3	PMER5.19.1.7.B
PMER5._4	PMER5.20.11.1.T
PMER5.18	PMER5.18.10.7.B
PMER5.19	PMER5.19.8.2.B
PMER5.20	PMER5.20.11.1.B
PMUBL6.5	PMUBL6.5.2.4.B
PPRIM_10	PPRIM3.17.10.4.B
PPrim_11	PPrim3.11.11.7.T
Pprim_12	Pprim3.12.1.4.T
PPRIM_13	PPRIM3.13.5.10.T
PPRIM_14	PPRIM3.12.4.10.B
PPRIM_15	PPRIM3.13.5.10.B
PPRIM3..	PPRIM3..13.6.4.T
PPRIM3.1	PPRIM3.16.2.4.T
PPRIM3.2	PPRIM3.20.2.7.T
PPRIM3_1	PPRIM3.16.2.4.B
PPRIM3_2	PPRIM3.12.1.4.T.2
PPRIM3_3	PPRIM3.20.2.7.B
Pprim3_4	Pprim3.11.1.7.B
Pprim3_5	Pprim3.11.1.7.T
PPRIM3_6	PPRIM3.14.7.7.B
Pprim3_7	Pprim3.12.1.4.B
PPRIM3_8	PPRIM3.20.5.8.B
PPRIM3_9	PPRIM3.20.5.8.T
PPRIM4.1	PPRIM4.17.27.1
Pprim4_1	Pprim4.12.25.1
Pprim4_2	Pprim4.14.16.2
PRCAB5.1	PRCAB5.12.3.5.B
PRCAB5.8	PRCAB5.8.3.3.T
PRCAB5.9	PRCAB5.9.12.4.B
PRCAB5_1	PRCAB5.9.12.4.T
PRCAB5_2	PRCAB5.8.3.3.B
PRCAB5_3	PRCAB5.10.12.1.T
PRCAB5_4	PRCAB5.10.8.10..B
Pshir3.1	Pshir3.12.3.B
Pshir3.4	Pshir3.4.10.2.A
Pshir3.6	Pshir3.6.2.9.T
Pshir3.7	Pshir3.7.7.1.B
Pshir3.8	Pshir3.8.12.1.T
Pshir3.9	Pshir3.9.3.3.B
Pshir3_1	Pshir3.6.2.9.B
Pshir3_2	Pshir3.8.12.1.B
Pshir3_3	Pshir3.7.7.1.T
Pshir3_4	Pshir3.10.1.9.T
Pshir3_5	Pshir3.10.6.1.B
Pshir3_6	Pshir3.10.6.1.T
PSHIR4.7	PSHIR4.7.15.3
Pshir4.9	Pshir4.9.2.2