ABSTRACT

MYERS, ASHLEY LAUREL. Pierce's disease of grapevines: Identifying the Primary Vectors in the Southeastern United States. (Under the direction of Dr. Turner Bond Sutton).

In the past 10 years the winegrape industry in the Southeastern United States has experienced rapid growth. However, further expansion may be inhibited by Pierce's disease (PD), caused by the bacterium *Xylella fastidiosa* that is transmitted from reservoir hosts to grapevines by sharpshooters and spittlebugs. Epidemiological studies were conducted to identify the primary vectors of *X. fastidiosa* to grapes in the Southeast by surveying sharpshooter populations in the eastern Piedmont and Coastal Plain of North Carolina where PD is most threatening, identifying potential sharpshooter vectors by PCR assays, conducting greenhouse experiments with potential vectors to determine transmission ability, and performing phylogenetic analyses of *X. fastidiosa* PCR products to provide information on what populations of *X. fastidiosa* sharpshooters in NC are carrying. In 2004 and 2005, leafhoppers were trapped in three vineyards in the eastern Piedmont and one vineyard in the northeastern Coastal Plain. Four insects have been identified as most abundant, *Oncometopia orbona*, *Graphocephala versuta, Paraphlepsius irroratus, and Agalliota constricta*. Specimens of *O. orbona*, *G. versuta*, and *P. irroratus* were tested for the presence of *X. fastidiosa* using a vacuum extraction method and nested PCR. Over the two seasons 27% of the *O. orbona*, 24% of the *G. versuta*, and 33% of the *P. irroratus* trapped were positive for *X. fastidiosa*. Transmission experiments were conducted with field-caught *O. orbona* and *G. versuta.* One hundred sixty-six vines used in transmission experiments were assayed for the presence of *X. fastidiosa* by ELISA. Bacterial DNA from an additional

sample (n = 6) of symptomatic plants was subjected to two-step PCR to confirm ELISA results. Data indicate both *G.versuta* and *O.orbona* transmit *X. fastidiosa* to grape. Phylogenetic analysis of *X. fastidiosa* DNA from insects and sequences obtained *in silico* using Neighbor-Joining of 1000 bootstraps resulted in one most parsimonious tree with three populations grouping by host. SNAP workbench analyses collapsed sequences into to 12 haplotypes and Hudson's ranked Z statistic showed no population subdivision between insect hosts.

PIERCE'S DISEASE OF GRAPEVINES: IDENTIFYING THE PRIMARY VECTORS IN THE SOUTHEASTERN UNITED STATES.

By

ASHLEY LAUREL MYERS

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

PLANT PATHOLOGY

Raleigh

2005

APPROVED BY

Dr. Turner B. Sutton
Chair of Advisory Committee

Dr. George G. Kennedy
Member of Advisory Committee

Dr. David F. Ritchie
Member of Advisory Committee

DEDICATION

To Laurel Gray Vineyards, my inspiration and my home.

BIOGRAPHY

Ashley Laurel Myers was born on August 26, 1981, in Winston-Salem, North Carolina. While attending Starmount High School in North Carolina, she became interested in biology after serving as North Carolina Health Occupations Students of America State President. Ashley pursued her interest during her undergraduate study at North Carolina State University. During the spring of 2001, Ashley's parents planted Vinifera vines in the Yadkin Valley of North Carolina establishing Laurel Gray Vineyards. As a direct result, her interest became focused on plant science and she spent the summer of 2002 doing apple and grape research for Dr. Turner B. Sutton. Ashley graduated Summa Cum Laude with a B.S. degree in Biological Sciences at North Carolina State University in 2003. She began to work on a Master of Science degree in Plant Pathology at North Carolina State University under the direction of Dr. Turner B. Sutton in 2003.

ACKNOWLEDGMENTS

To Mom and Dad, as I travel through life you are always there, and that is a comfort to me, because part of me will always be your little girl.

To my brother, Taylor, for always being on my side.

To Jay Bond, for all that you have done for us and for all the times you have made me smile.

To Dr. Turner Sutton, for quietly pushing me in the right direction.

To Dr. Jorge Abad, for going the extra mile to help me understand and succeed. Thank-you for your patience and kindness.

To Dr. Sam Anas, for always being ready to lend a helping hand.

To Drs. Turner Sutton, Dave Ritchie and George Kennedy, for serving on my graduate committee.

To Leah Floyd and Guoling Luo, for all of your help.

To the Moyer lab, for letting me invade for two years.

To Tam and Pam Cloer; Wally Butler of Silk Hope Vineyards, Debbie and Gene Stikeleather of Iron Gate Vineyards; and David Martin of Martin Vineyards for allowing me the use of your vineyards for my research.

To Tommy Grandy, for your interest in this project and assistance in replacing and collecting traps.

To Mukund Patel and Julie Miranda, for the friendship, sympathetic ears, and expert advise.

To Dr. Eugenia Gonzalez, for being a role model and friend.

To Luann Brown, for showing me what I am capable of.

TABLE OF CONTENTS

Page

LISTS OF FIGURES

LIST OF TABLES

INTRODUCTION

Pierce's disease of grapevines (PD) is caused by strains of the bacterium *Xylella fastidiosa* (Wells et al., 1987), an endophytic bacterial pathogen that resides in the xylem of plants (Esau, 1948), and is transmitted plant to plant by xylem-feeding insects such as sharpshooters (subfamily Cicadellinae in leafhopper family Cicadellidae) and spittlebugs (family Cercopidae) (Frazier & Freitag, 1946). Diseases caused by *X. fastidiosa* occur in tropical or subtropical environments of North America, Central America, and South America, and *X. fastidiosa* diseases appear to be rare or absent in cooler climates (Purcell, 1980). Within the United States, the incidence of PD ranges from Florida to Texas and into California, and decreases with increasing distance from the Gulf of Mexico (Hopkins & Purcell, 2002). Outside of the Americas, *X. fastidiosa* diseases have been reported only in Taiwan (Leu & Su, 1993) and the Kosovo region of the Balkans (Berisha et al., 1998).

Xylella fastidiosa has detrimental effects on many agriculturally important plants and many forest trees including oak, elm, oleander, maple, and sycamore (Hearon et al., 1980). Some of the most important *X. fastidiosa* diseases are Pierce's disease of grapevines (Davis et al., 1978), almond leaf scorch (Davis et al., 1980), alfalfa dwarf (Thomson et al., 1978), phony peach (Wells et al., 1983), plum leaf scald (Wells et al, 1981), oleander leaf scorch (Purcell et al., 1999), and citrus variegated chlorosis (Chang et al., 1993). Pierce's disease has caused an estimated $13 million in losses in California's Temecula Valley alone (Wine

Institute, revised 2002; Pierce's Disease Update, www.wineinstitute.org/communications/pierces_disease/pierces_disease_update.htm) and in one vineyard in the eastern Piedmont of North Carolina, the incidence of seriously affected vines or vine death due to PD increased from 24% in 2001 to 54% in 2002 (T.B. Sutton, personal communication).

Over 30 families of monocotyledons and dicotyledons are thought to be hosts to *X. fastidiosa* (Huang, 2004). The College of Natural Resources, University of California, Berkeley website (College of Natural Resources, revised 2005; Xylella Web Site, www.cnr.berkeley.edu/xylella) lists 145 natural or experimental hosts for PD strains of *X. fastidiosa* alone. However, it is probable that different plant species vary in their importance as a source plant for vector spread of *X. fastidiosa* (Purcell & Hopkins, 1996). Plants that support systemic bacterial movement can maintain and increase inoculum during periods of vector scarcity (Purcell & Hopkins, 1996), although nonsystemic hosts can serve as sources of inoculum (Hopkins & Purcell, 2002).

Xylella fastidiosa invades the host by inoculation via sharpshooter vectors (Frazier & Freitag, 1946) and spittlebugs (Severin, 1950). Sharpshooters, formally Cicadellinae leafhoppers, have an inflated clypeus enclosing strong musculature connected to the cibarium or pumping diaphragm, which enables the insects to feed on xylem (Redak et al., 2004). As of 2004, 39 species and 19 genera of Cicadellinae have been shown to vector *X. fastidiosa* (Redak et al., 2004). Most all sucking insects that feed in the xylem sap are potential vectors but vector species differ in their transmission efficiency or competence (Purcell & Hopkins, 1996). There is a very short latent period, if at all, and vectors retain the ability to transmit

the bacterium for indefinite periods following acquisition, however molting causes loss of infectivity (Purcell & Hopkins, 1996). Vector species trapped during the same acquisition or inoculation periods, acquire and inoculate *X. fastidiosa* with similar efficiencies (Purcell & Hopkins, 1996).

The red-headed sharpshooter, *Xyphon* (*Carneocephala*) *fulgida* (Nottingham); green sharpshooter, *Draeculacephala minerva* (Ball); blue-green sharpshooter (BGSS), *Graphocephala atropunctata* (Signoret); glassy-winged sharpshooter (GWSS), *Homoladisca coagulata* (Say); and *Oncometopia* spp. are abundant vectors often found in affected crops or adjacent fields (Redak et al., 2004), and are the most important vectors in the spread of PD in California and the Southeast (Adlerz & Hopkins, 1979; Wrinkler, 1949). Prior to the introduction of the glassy-winged sharpshooter (GWSS), PD in California only occurred in "hot spots" adjacent to overwintering or breeding habitats of *X. fulgida*, *D. minerva*, and the BGSS (Hopkins & Purcell, 2002). This lack of vine-to-vine spread of PD in California may be explained by vector feeding preference near tips of the growing shoots, where the bacteria must travel farther to reach vine tissue not removed during winter pruning (Hopkins & Purcell, 2002). There is also evidence that *X. fastidiosa*'s ability to survive winters decreases in smaller shoots (Feil & Purcell, 2001; Purcell, 1981).

In California, the GWSS was first reported in vineyards in the Temecula Valley, where winegrapes and citrus are the main crops. By 1999, the incidence of PD had reached alarming levels (Hopkins & Purcell, 2002). Unlike traditionally important vectors, GWSS feed at the base of new shoots and on dormant vines. The inoculation of woody portions of shoots may increase the likelihood of chronic infections because bacteria do not have as far

to spread to reach permanent tissue (Hopkins & Purcell, 2002). The introduction of GWSS into California has caused millions in losses and has prompted a resurgence of PD research (Wine Institute, revised 2002; Pierce's Disease Update, www.wineinstitute.org/communications/pierces_disease/pierces_disease_update.htm).

Once inside the host plant, bacteria multiply within the vascular system, plugging the xylem vessels (Esau, 1948). Symptoms of Pierce's disease, first described by Newton Pierce in 1892 (Pierce, 1892), are similar to the effects of water stress and include: decline of vigor, marginal necrosis or scorching of leaves along margins, decreased production, small fruit, (Hopkins, 1977), irregular maturing of the bark (Hopkins, 1981), and leaf blade abscission with petioles remaining attached to the cane (Gubler et al., 2005). Symptoms first appear mid to late summer and continue to develop through fall. Vine death may occur as early as 2 years after initial infection (Gubler et al., 2005).

Recently winegrape production in North Carolina and other states of the Southeast has rapidly expanded to include cultivation of *Vitis vinifera* and French-American hybrid grapes. There were 128 commercial vineyards in North Carolina in 1998 and there are currently 350 (NC Wine & Grape Council, revised 2005; Discover NC Wines, www.ncwine.org). Much of the expansion has been in the central and western Piedmont, and has lead to pests and disease problems in vineyards, which are endemic on native plants and wild grapevines. Consequently, growers must be prepared to face the challenge of producing winegrapes in a novel environment. The most significant of these challenges in the Southeast is Pierce's disease of grapevines. PD is the single most formidable obstacle to growing Vinifera grapes (The College of Natural Resources, revised

2005; Xylella Web Site, www.cnr.berkeley.edu/xylella) and limits the areas of North Carolina where production of *V. vinifera* and French-American hybrids are viable (Wolf and Poling, 1996; Southeastern Grape IPM, http://www.cals.ncsu.edu/plantpath/ExtensionPro/grapes/2004).

Much of the literature on Pierce's disease of grapevines, its causal organism *X. fastidiosa*, and its vectors is from California and Brazil, where *X. fastidiosa* causes citrus variegated chlorosis disease (CVC), which is devastating the citrus industry. Within the southeastern United States most work has been done on *V. rotundifolia* and little is known about the vectors, reservoir hosts of *X. fastidiosa*, and methods of controlling PD on *V. vinifera*.

A better understanding of the biology and epidemiology of Pierce's disease on *V. vinifera* in the Southeast would greatly enhance growers' abilities to manage Pierce's disease in their vineyards. Unfortunately, many factors affecting the development of Pierce's disease in North Carolina are unknown. The most notable lack of information is the identity of the vectors. Consequently, the objectives of this study were to better understand the epidemiology of Pierce's disease in the Southeast by (i) surveying sharpshooter populations in the eastern Piedmont and Coastal Plain of North Carolina where PD is most threatening, (ii) identifying potential sharpshooter vectors by PCR assays, (iii) conducting greenhouse experiments with potential vectors to determine transmission ability, and (iv) performing phylogenetic analysis of *X. fastidiosa* PCR products to provide information on the populations of *X. fastidiosa* that sharpshooters in NC are carrying.

MATERIALS AND METHODS

2.1 Insect surveys in four North Carolina vineyards. In order to determine the leafhopper species present in vineyards in North Carolina, from 13 May (day 134) to 10 September (day 254), 2004 and 6 April (day 96) to 22 August (day 234), 2005 yellow sticky traps (15.3 x 30.6 cm) (Great Lakes IPM, Vestaburg, MI) were placed in three vineyards in the eastern Piedmont (Vineyard 1, Wake Co.; Vineyard 2, Chatham Co.; and Vineyard 3, Alamance Co.) and one vineyard in the northeastern Coastal Plain (Vineyard 4, Currituck Co.), where PD has been well-documented (Harrison, et al., 2002). Vineyard 1 is a 5-yr-old Vinifera vineyard near Raleigh, NC ~ 1.7 ha in size with 1,586 vines. Vineyard 2 is a 7-yr-old vineyard near Pittsboro, NC of ~ 1 ha comprising 614 Vinifera and French-American hybrid grapevines. Vineyard 3, in Mebane, NC, is ~ 1.7 ha and contains 3,459 4-yr-old Vinifera and French-American hybrids. Vineyard 4 is a 14-yr-old Vinifera, French American hybrid, and muscadine vineyard located near the Outer Banks of NC in the northeastern Coastal Plain.

Trapping was initiated earlier in 2005 because data collected in 2004 indicated that leafhoppers were present prior to May and early season infection is reported to be most significant (Feil, 2003). Traps were prepared by placing a 4-cm strip of clear, fibrous tape (Clear Duck Tape®, Henkel CA, Inc., Avon, OH) on the tops of both sides of the trap to prevent tearing in strong winds. Eight traps were placed along the perimeter of each vineyard (Appendix 6.3,6.4,6.5), positioned on the cordon wires (~1 m above ground) and fastened with two binder clips on the upper left and right corners of the trap.

Traps were replaced every 14 days and stored at 4°C. Each trap was examined for presence of leafhoppers and the most abundant leafhoppers were counted and recorded. A subsample (the size of the subsample varied depending on insect availability but ranged from two to eight insects per trap per trapping period) of each species was selected arbitrarily and removed from traps, using Histoclear (RA Lamb LLC, Apex, NC) to dissolve the adhesive, then stored at -20°C for PCR analysis. Another sub-sample (n ~ 144) from 2004 was preserved in 70% ethanol for identification. The leafhoppers were initially identified to the genus level and the four most abundant leafhoppers were identified to the species level under the direction of personnel at the North Carolina State University Plant Disease and Insect Clinic using Cicadellinae references (Delong, 1948; Young, 1968; Young 1977). A more recent catalogue was checked to get consulted generic assignments (Poole, et al., 1997), and the vineyard specimens were compared to specimens in the NCSU Insect Collection.

2.2 Identification of potential vectors with nested PCR. The sharpshooters *Oncometopia orbona* (F.), *Graphocephala versuta* (Say) and *Paraphlepsius irroratus* (Say) were tested for presence of *X. fastidiosa*. Insect heads were severed from their bodies and pinned through their mouthparts with #3 stainless steel insect pins (Morpho®, Czech Republic) according to the protocol developed by Bextine et al. (2004). Pinned heads were placed into 1.5mL microcentrifuge tubes with 250µL phosphate-buffered saline (PBS; pH 7.0) and incubated at -20°C overnight. Bacterial DNA was extracted using vacuum infiltration as a pre-extraction method (Bextine et al., 2004). Briefly, lids to microcentrifuge tubes containing pinned insects were opened and placed into the vacuum chamber. A vacuum

was applied at 20 bars for 15 s then released slowly to separate the bacteria from the insect mouthparts. This procedure was repeated twice. After vacuum pre-extraction, DNA extraction was completed by using the DNA insect tissue extraction procedure from the Qiagen DNeasy Tissue Kit (Qiagen Inc., Hercules, CA, USA).

Nested-PCR (Pooler et al., 1997) was used to maximize and visualize the DNA amplification. Using as a template 5µL of DNA extracted from the insect mouthparts, DNA specific to *X. fastidiosa* was amplified using two pairs of oligonucleotide primers (Invitrogen Corporation, Frederick, MD) developed by Pooler and Hartung (1995). The external primers; 272-1 and 272-2, generate a 700-nucleotide amplicon, while internal primers, 272-1-int and 272-2-int, amplify a 500-nucleotide PCR product. Amplifications were performed in a 25µL volume containing: sterile distilled water, 2.5 mg 10x polymerase buffer, 4 mM dNTP's each, 0.15 µg each primer, 2.5% $MgCl_2$, and 1 U Taq polymerase (Promega, Madison, WI). Magnesium chloride (2.4%) was used in the nested amplification (J. Abad, personal communication). Positive controls consisted of 4µL water and 1µL *X. fastidiosa* PCR positive isolated from an isolate of *X. fastidiosa* from grape growing on PD2 agar medium (Davis et al., 1981). Negative controls were 5µL sterile water with PCR master mix. Preparation of the master mix and aliquoting of samples was done in The Clone Zone with HEPA Filter (USA Scientific, Inc., Ocala, FL) for maximum sterilization. For both amplifications the same PTC-100 Thermal Cycler (MJ Research Inc., Watertown, PA) profile was used (Pooler et al, 1997). Five µL of nested PCR product was analyzed by 1% agarose horizontal gel electrophoresis in TBE buffer. Gels were stained with ethidium bromide and bands were visualized under UV light. Amplicons were characterized as positive or negative.

DNA began to degrade during testing of *P. irroratus* and the amount of extracted DNA utilized as a template was reduced to 2.5µL.

2.3 Greenhouse experiments. Seedlings of the *X. fastidiosa* susceptible cultivar Chardonnay were used in the greenhouse transmission experiments. Some seedlings were 1-yr-old vines planted during summer of 2004 and pruned back to two or three buds during March 2005 to generate new growth. The grapevines were grown in 15 cm clay pots in a greenhouse with temperatures maintained at ~ 25ºC. Grapevines were treated every 14 days with an insecticide until 3 months before transmission experiments began. *O. orbona* and *G. verusta* were selected for the greenhouse experiments because (i) both genera have been shown to transmit the PD strain of *X. fastidiosa* (Alderz & Hopkins, 1979), (ii) both species have been shown to transmit *X. fastidiosa* to peach (Turner & Pollard, 1959a; Turner & Pollard, 1959b), and (iii) both *O. orbona* (personal observation) and *G.versuta* reproduce on grape (Alderz & Hopkins, 1979).

Field captured sharpshooters were used in transmission experiments to test for natural infectivity. Adult sharpshooters used for infectivity tests were collected from vineyard 1. Thirty-six additional adult *G. versuta* were captured at vineyard 3. Sharpshooters were collected multiple days during the period of peak trap catches in 2005.

O. orbona were typically captured on the base of new shoots by tapping them into sweep nets. *G. versuta* were caught with a 225 cm diameter sweep net by sweeping the upper canopy of the vine. Once caught, the insects were placed into plastic bags and stored in the shade until transferred within 2 hours to the experimental plants. To maximize feeding, insects were fasted during the time of transport from field to lab.

Fifteen-centimeter diameter plastic cages with mesh or nylon tops caged insects, so that insects had access to the entire plant. The soil of potted plants used in the *G. versuta* transmission experiments was covered with one layer of cheesecloth to facilitate removal of insects. Five sharpshooters were caged on the majority of plants; however one to seven insects were placed on some plants depending on size and the available supply of the insect. *O. orbona* were taken from the bags and placed manually onto the plant. *G. versuta* were aspirated into a 250mL Erlenmeyer flask and the flask was placed in the cage along with the vine to allow the insects to escape. Insects were allowed to feed undisturbed for 6 d in order to maximize acquisition and inoculation efficiencies. On day 6, sharpshooters were removed from test plants and stored at -20ºC for further testing. Caged plants with five and seven insects were placed into plastic bags and exposed to CO_2 for easier removal of insects. After exposure to the insects, egg masses found on the plants were manually removed and vines were treated with imidacloprid (Admire® 2F, Bayer CropScience, Durham, NC) to prevent reinfestation with nymphs. Inoculated plants were kept in propagation cages covered with 500 µm Nitex Bolting Cloth (Wilco®, Buffalo, NY) until all testing was complete in order to prevent possible inoculation of healthy plants in the greenhouse. Within 1 week of insect removal vines were treated with myclobutanil (Nova 40W, DowAgrosciences, Indianapolis, IN) and azoxystrobin (Abound, Syngenta Inc., Greensboro, NC) to control powdery mildew. All experiments had at least two negative controls, which were not exposed to insects.

Plants were held for ~ 4 months, watered daily, and monitored weekly for symptom development. Insecticidal sprays were applied every 14 days once all sharpshooters were removed from plants and mycobutanil was applied as needed for powdery mildew

control. Plants were scored for PD symptoms using a rating scale developed for PD severity based on typical symptoms where 0 = no symptoms, 1 = sporadic marginal necrosis on < 25% of leaves, 2 = necrosis of leaves on entire shoots (equivalent to 25% - 50% leaves with symptoms), 3 = the appearance of bladeless petioles with the majority of leaves necrotic (50% - 75% with symptoms), 4 = vines defoliated and fruit shriveling (75% - 100% leaves necrotic), and d = died within the season (Appendix 6.2).

To confirm visual ratings of greenhouse symptoms, leaves from each plant were collected 3 months post-inoculation. Symptomatic leaves were chosen based on feeding preferences of insects. Nonsymptomatic leaves for testing from plants used in *O. orbona* experiments were chosen from the base of vines because of basal shoot feeding preferences of the insect. Nonsymptomatic leaves for testing from *G. versuta* experiments were collected arbitrarily from the entire plant because *G. versuta* prefers to feed on leaf tissue. Samples were stored at 4°C until tested for *X. fastidiosa*.

A commercially available double-antibody sandwich ELISA test kit (AgDia Inc., Elkhard, IN) was used to test the 166 grapevines from the greenhouse experiments. Tissue consisting of 0.3 to 0.5 g was obtained from petioles collected from each vine. If symptoms were present, petioles from symptomatic leaves were used. Using a sterile razor blade and cutting board, samples were sliced lengthwise, down the center of the petiole and one half of each petiole was stored at 4°C for further testing with PCR. The remaining pieces were cut widthwise into several very small pieces ~ 1 mm in length. Samples were placed into centrifuge tubes with screw caps (Sarstedt Ag & Co, Germany) with 5mL AgDia grape extraction buffer (AgDia Inc., Elkhard, IN). Tissue was macerated with Brinkmann PTMR

3000 Homogenizer (Biomatic Technologies, Stoughton, MA) and ELISA was performed according to test kit instructions. One hundred microliters of the prepared sample was dispensed into test wells. Positive and negative controls were included. Results were quantified by an EMAX Precision Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA) set at a wavelength of 490nm. Test results were only valid if negative and positive controls were clear. To determine the positive cutoff value, three times the standard deviation of all known negative controls was added to the mean of all known negatives (J. Abad, personal communication). A sample with an OD above this cutoff value was considered positive and below the cutoff value, negative.

Immunocapture of *X. fastidiosa* followed by nested PCR was performed on a sample (n = 6) of ELISA positive plants to confirm the validity of the ELISA tests. Fresh petioles were collected from symptomatic tissue, sliced lengthwise and into 1mm discs and covered with 50mmol 1^{-1} Tris-Cl, pH 7.5 buffer in a 1.5mL microcentrifuge tube. Samples were incubated overnight at 4°C. Vacuum extraction was performed as described above (Bextine et al., 2004). After the vacuum extraction, the buffer was pipetted into clean 1.5mL microcentrifuge tubes and plant debris was discarded. Immunocapture of *X. fastidiosa* was conducted according to methods developed by Pooler et al. (1997). Antibodies to *X. fastidiosa* strain CVC5 were obtained from Cocalico Biologicals, Inc (Reamstown, PA). Whole antibody serum was diluted 1:200 (v/v) in PBS, pH 7.4. One hundred microliters of diluted antibody was added to 300µL of plant sample then incubated at room temperature for 30 min with gentle shaking on an orbital shaker. The sample was centrifuged for 2 min and the supernatant was discarded. The sample was washed twice with 300µL PBS/0.1% BSA

(w/v) to remove all unbound antibody. Pellets were resuspensed in 300µL PBS/0.1% BSA (w/v). Five µL Dynabeads M-280 (6-7 x 10^8 beads ml^{-1}) bound with sheep anti-rabbit IgG (Dynal, Lake Success, NY) were added to the suspension. The mixture was then incubated at room temperature for 30 min with gentle shaking on an orbital shaker. The Dynabead/bacteria complex was separated from the mixture with a large magnet, which drew the beads to the side of the tube. The supernatant was removed by pipette and discarded. The bead/bacteria complex was washed once with 300µL PBS, suspended in 5µL sterile distilled water, and the DNA was exposed by heat shocking the bacteria for 2 min at 98°C, then 2 min on ice, repeated three times.

One microliter of the DNA elute was then added to the PCR master mix as described above for the insect assays. Positive controls consisted of 4µL plant tissue extract and 1µL *X. fastidiosa* obtained from bacteria growing on PD2 agar medium (Davis et al., 1981). Negative controls were 5µL sterile water with no bacteria or plant tissue and 5µL plant tissue extract from experimental controls. PCR and visualization of PCR results were conducted as described above.

2.4 Phylogenetic analysis of sequences from NC insects. Amplified PCR products from insect assays were sequenced in both orientations. Sequencing was conducted following the specifications of the N.C. State University Genomic Research Laboratory (GRL). Nested PCR products corresponding to a fragment of the hypothetical protein gene of *X. fastidiosa*, were cleaned with the Qiagen PCR Purification Kit (Qiagen Inc., Hercules, CA, USA). Three microliters of purified DNA was used as a template in a 10 µL reaction containing: sterile water, BigDye mix/dilution buffer (1:1), and 0.15 µg internal primer, either 272-1-int

or 272-2-int (Invitrogen Corporation, Frederick, MD). Sequencing reactions were done with the PTC- 100 Thermal Cycler (MJ Research Inc., Watertown, PA) using the *X. fastidiosa* profile described above. After amplification, 10 µL DI water was used to bring the volume to 20 µL. Cleanup of sequencing reactions was done following the Qiagen DyeEx (Qiagen Inc., Hercules, CA, USA) kit instructions. The clean sequencing reactions were taken to the GRL to be run on capillary sequencers.

Sequences were assembled with the program Vector NTI (Invitrogen Corp., Carlsbad, CA). Sequences of each sample of *X. fastidiosa* were compared with sequences obtained *in silico* from GenBank and NCBI BLAST (Table 7). Multiple sequence alignments of nucleotides were performed using CLUSTAL X (Thompson et al., 1997) and Bioedit (Hall, 1999) with default parameters. Phylogenetic trees were obtained from the data by the Neighbor-joining method of pairwise comparison using 1000 bootstrap iterations and visualized with the program MEGA version 2.01 (Kumar et al., 1993). The nucleotide sequences are accessible in GenBank.

Further analyses were conducted in SNAP Workbench (Price & Carbone, 2005). Sequences were imported into SNAP Workbench in Fasta format, aligned with CLUSTAL W version 1.7 (Thompson et al., 1994) and converted to Phylp format (Felsenstein, 1993). SNAP Map (Aylor et al., 2004) collapsed sequences into haplotypes while removing indels and infinite site violations. A phylogenetic analysis with unweighted parsimony performed with PAUP 4.0 (Swofford, 1998) yielded one most parsimonious tree visualized in Treeview (Page, 1996). In examining the possibility of recombination, SNAP Clade (Markwordt et al.,

2004) was used to generate a site compatibility matrix. The compatibility matrix was visualized in SNAP matrix (Markwordt et al., 2004) and one variable site creating homoplasy was removed with no affect on the distribution of haplotypes.

To test for pairwise population subdivision between hosts, SNAP Map (Aylor et al., 2004) was used to generate the sequence file and Seqtomatrix (Hudson et al., 1992) converted the sequence file into a distance matrix. Permtest, based on nonparametric permutations of Monte Carlo simulations (Hudson et al., 1992), Nearest Neighbor Statistic (Hudson, 2000), and ranked Z (Hudson et al., 1992) calculated Hudson's test statistics K_{ST}, K_S, K_T, χ^2, Z, H_{ST}, H_S, H_T, and S_{nn}; where $K_{ST} = 1 - (K_S/K_T)$, K_S = average number of differences between sequences within subpopulations, K_T = average number of differences between sequences regardless of locality, χ^2 = test of allele frequencies in samples from different localities, Z = weighted sum of Z_1 and Z_2, where Z_i is the average of the ranks of all the $d_{ij,lk}$ values for pairs of sequences from within locality i, $H_{ST} = 1 - (H_S/H_T)$, H_S = weighted average of estimated haplotypes diversities in subpopulations, H_T = estimation of haplotypes diversity in the total population, and S_{nn} = how often the "nearest neighbor" (in sequence space) of sequences are from the same locality in geographic space. Sequenced-based statistics K_{ST}, K_S, K_T, and Z were chosen for the analysis because hosts sample sizes varied from 1 to 24 and sequenced-based statistics are more powerful when sample sizes are low (Hudson et al., 1992). In addition, guidelines in Hudson et al. (1992) suggest placing the most confidence in the Z statistic because the calculated $H_T > 0$ ($H_T > 1$-[1/min(sample sizes)]) and sample sizes are unequal. Host sample sizes of one do not provide statistical output, therefore only pairwise differences between insect species were examined.

RESULTS

3.1 Insect surveys in four North Carolina vineyards. In 2004, sticky traps caught up to nine species of leafhoppers and one species of spittlebug at each vineyard surveyed. Three leafhopper species, identified as *Graphocephala versuta*, *Agalliota constricta*, and *Paraphlepsius irroratus*, were the most abundant species trapped and each exceeded > 2% of the leafhoppers trapped in all 8 experimental years (two years x four vineyards) (Table 1). *Oncometopia orbona* populations were also ≥ 2% of the total population of leafhoppers in 6 of the 8 experimental years, and therefore, it was also included (Table 1). Populations of all other leafhopper and spittlebug species comprised 2% of the population and were grouped into the category, other.

Populations of *O. orbona* in 2004 were highest in all vineyards during the first two trapping periods, spanning 13 May to 9 June (Fig. 1A). In 2005 populations were highest during trapping periods extending from 17 May to 28 June (Fig. 1B). In 2005, traps were placed in the vineyards just prior to budburst on 6 April, and a few *O. orbona* were trapped in all vineyards except vineyard 4. The population of *O. orbona* was generally higher in vineyard 1 and lowest in vineyard 4 during the 2 years.

In 2004 populations of *G. versuta* began increasing in late May and peaked in mid to late June in each vineyard (Fig. 2A). In 2005 the population also began to increase in late May and in all vineyards but vineyard 3 the population peaked about 2 weeks later than 2004 (Fig. 2B). Very large numbers were trapped in vineyard 3 both seasons, with traps averaging over 2,200 individuals when the population level was highest. Similar to *O. orbona*, the fewest individuals of *G. versuta* were trapped in vineyard 4 in both seasons.

Populations of *P. irroratus* peaked in May. In 2004 the highest trap catches were recorded during the trapping period extending from 13 May to 27 May, the first period that traps were in the vineyards (Fig. 3A) and in 2005 the population increased rapidly in mid-May and was highest from 17 May to 14 June (Fig. 3B). Populations were lowest in vineyards 2 and 4 each year.

In 2004 populations of *A. constricta* began to increase in late May and peaked in mid to late June in each vineyard (Fig. 4A). Populations had a second, smaller peak during trapping periods extending from 30 July to 26 August. In 2005 the population once again began to increase in late May, however in all vineyards but vineyard 3 populations peaked 1 week later than in 2004 (Fig. 4B). Smaller population peaks were observed on trapping dates 6 April to 20 April and 9 August to 22 August in 2005. Very large numbers were trapped in vineyard 3 in both seasons, with 2004 traps averaging over 1,150 individuals and 2005 traps averaging over 2,250 individuals during the peak trapping periods. Similar to the other leafhoppers, vineyard 4 had the lowest populations in both years.

Species of leafhoppers caught on yellow-sticky traps during 2004 (Fig. 5) and 2005 (Fig. 6) in the central Piedmont (A) and Coastal Plain (B) differ in percent composition. In 2004 (Fig. 5), 54% of the leafhoppers caught in central Piedmont vineyards were *G. versuta*, compared to only 16% in the Coastal Vineyard. On the other hand, 64% of the leafhoppers trapped in the Coastal Plain were *A. constricta* compared to 38% in the Piedmont. The relative proportion of *P. irroratus* was greater in the Coastal Plain vineyard. *O. orbona* composed ~2% of the population in both locations. In 2005, the relative proportion of each species trapped in the Piedmont vineyards was similar. In the Coastal Plain vineyard in 2005

proportionately fewer *A. constricta* were captured and more *O. orbona*, *P. irroratus*, and *G. versuta* were captured than 2004.

3.2 Identification of potential vectors with nested PCR. Thirty-two percent and 21% of the *O. orbona* (Table 2) tested positive for *X. fastidiosa* in 2004 and 2005, respectively, yielding a 500 base pair amplicon in the nested PCR. In 2004, most positives (7 of 11) were from the trapping date 13 May to 27 May while in 2005 all insects tested (n = 7) from 20 April to 3 May were positive. The number of *O. orbona* and number testing positive decreased in late May. In 2004, 36% (n = 14) of the *O. orbona* tested from vineyard 1, 20% (n = 10) from vineyard 2, and 40% (n = 10) from vineyard 3 were positive for *X. fastidiosa.* No *O. orbona* from vineyard 4 were tested in 2004. In 2005, 10% (n = 20) of *O. orbona* tested from vineyard 1, 22% (n = 23) from vineyard 2, 41% (n = 22) from vineyard 3, and 0% (n = 12) from vineyard 4 were positive for *X. fastidiosa*. Assay results from trapping date 3 May through 17 May were discarded due to an error in testing.

Thirty-eight percent and 19% of the *G. versuta* from 2004 and 2005 respectively tested positive for *X. fastidiosa* (Table 3). In 2004 most positives (7 of 15) were from the trapping date 13 May to 27 May while in 2005 the most positives (4 of 6) was from 6 April to 20 April. None of the insects tested from July 2004 were positive. Of the *G. versuta* tested in 2004, 25% (n = 20) were positive from vineyard 1, 33% (n = 21) positive from vineyard 2, and 56% (n = 20) positive from vineyard 3. No *G. versuta* from vineyard 4 were tested in 2004. In 2005, 23% (n = 26) of *G. versuta* tested from vineyard 1, 15% (n = 26) from vineyard 2, 23% (n = 26) from vineyard 3, and 15% (n = 20) from vineyard 4 were positive for *X. fastidiosa*. Within vineyard 4, dates for the capture of individuals tested were unknown due to a sampling error.

Forty-eight percent and 18% of *P. irroratus* tested positive for *X. fastidiosa* in 2004 and 2005, respectively (Table 4). In 2004, the most positives (8 of 12) were from the 13 May to 27 May trapping period. The number of positives decreased after May however 27% of *P. irrroratus* tested after 27 May was found positive. Thirty-three percent (n = 12) were positive from vineyard 1, 69% (n = 16) positive from vineyard 2, and 33% (n = 12) positive from vineyard 3. No *P. irroratus* from vineyard 4 were tested in 2004. In 2005, 13% of the *P. irroratus* from vineyard 1 caught on trapping dates 17 – 31 May and 14 – 28 June tested positive (2 positives, n = 16), and 25% were positive from vineyard 4 (3 positives, n = 12). None of the individuals (n = 31) from vineyards 2 and 3 tested positive. Dates of capture from vineyard 4 are unknown due to a sampling error.

3.3 Greenhouse experiments. Samples from plants inoculated by *O. orbona* and *G. versuta* were analyzed separately on two ELISA plates. A sample with an optical density reading above the calculated cutoff value was considered positive and below the cutoff value considered negative. Positives cutoff values for *O. orbona* and *G. versuta* were 0.118 and 0.209, respectively (Appendix 6.6,6.7).

Fifty-eight of the 93 vines inoculated by *O. orbona* tested positive for *X. fastidiosa* (Table 5). The highest percentage of transmissions (83%) occurred in tests conducted from 17 May (10 of 12 plants ELISA positive). The transmission efficiency of *O. orbona* was 69% as determined by ELISA. Replicates from June were not included in the calculation of transmission efficiency because they consisted of five *O. orbona* per plant.

Three of the 55 vines inoculated by *G. versuta* tested positive for *X. fastidiosa* (Table 6). The only positives were from the 24 June replicate.

Thirty-seven *O. orbona* inoculated vines (Table 5) had visual symptoms of 1 or 2 on the rating scale (Appendix 6.2). An additional 13 vines were classified as having questionable symptoms (1?). Fifteen *G. versuta* (Table 6) inoculated vines were showing visual symptoms of 1 or 2 on the rating scale and 10 additional vines were classified as questionable symptoms (1?). Visual symptoms did not necessarily represent presence of the bacteria as determined by ELISA.

A sample of three symptomatic plants from transmission experiments with *G. versuta* and three symptomatic plants from transmission experiments with *O. orbona* were tested by immunocapture (Pooler et al., 1997) followed by nested PCR (J. Abad, personal communication) to confirm ELISA results (Appendix 6.8). Two plants inoculated by *O. orbona* with ELISA optimal density readings of 0.31 and 0.123 tested positive for *X. fastidiosa* and one with an optimal density of 0.143 tested negative. Two plants inoculated by *G. versuta* with ELISA optimal density readings of 0.244 and 0.277 tested positive for *X. fastidiosa*, a third with an optimal density of 0.224 tested negative.

3.4 Phylogenetic analysis of sequences from NC insects. Nested PCR products isolated from insects collected in North Carolina, corresponding to a 431 base pair region, and containing an open reading frame fragment of the hypothetical protein gene of *X. fastidiosa* and a 3' flanking region, were amplified during the sequencing reaction using primers 272-1-int and 272-2-int as markers. All 48 sequences matched known *X. fastidiosa* strains from NCBI BLAST and additional sequences were obtained *in silico* from isolates from grapevine (PD), almond, oleander, citrus, coffee, and Japanese beech bonsai (Table 7). Phylogenetic trees were obtained from the data by the Neighbor-joining method of pairwise comparison using 1000 bootstrap iterations and visualized with the program MEGA version

2.01 (Kumar et al., 1993). The results are shown in Fig. 7 using the South American CVC strain (*X. fastidiosa* 9a5c) as the outgroup. The dendogram shows three well-defined clades statistically supported by bootstrap procedures. The clades appeared to correspond to host: citrus/coffee group, almond/oleander group, and grape/NC insect group. All insect isolates, with the exception of B1 2005, grouped with the known PD strain. Isolate B1 2005 grouped in the almond/oleander clade. In the analysis, *X. fastidiosa* Ann-1 ctg268 is more closely related to the grape/NC insect clade than to the almond/oleander clade. Within the grape/NC insect clade, insects were not differentiated by species, location, or trapping date. In addition, the subpopulation in the grape/NC insect clade includes isolates from *O. orbona* and *G. versuta*, all three locations, and multiple trapping dates. Neither insects from vineyard 4 nor isolates obtained from *P. irroratus* were used in the sequence analyses.

SNAP Workbench (Price & Carbone, 2005) analyses confirmed the distribution of clades by grouping isolates into 12 haplotypes and 3 clades (Fig. 8). One clade was made up of haplotypes 1, 6, and 2. Haplotype 1 was comprised of isolate B1 2005, a NC insect isolate that grouped more closely to haplotypes 6 and 2, isolated from oleander/almond and Japanese beech bonsai hosts than to other NC insect isolates. The coffee and citrus isolates grouped closely within a second clade as haplotypes 3 and 5. A single oleander isolate (AAAM03000001.1) made up haplotype 7. The third clade was composed of NC insect isolates from *O. orbona* and *G. versuta* and the known PD strain isolated from grape (NC 004556.1).

The p-value ($p > 0.05$) for testing for pairwise genetic differentiation between insects with Hudson's tests ranked Z (Appendix 6.14) and K_{ST} (Appendix 6.13) was not significant,

indicating that isolates from *O. orbona* and *G.versuta* are genetically similar (Hudson et al., 1992).

DISCUSSION

The four most abundant species of leafhoppers trapped in vineyards in the central Piedmont and northeastern Coastal Plain of North Carolina were *G. versuta*, *A. constricta*, *P. irroratus*, and *O. orbona* In total, nine leafhopper and one spittlebug species were detected. Each species was captured in each of the eight sampling years, although in different amounts. Because our trapping results were consistent between years, we feel it is a good estimation of leafhopper species richness in vineyard canopies and therefore, includes the potential leafhopper vectors of *X. fastidiosa*.

Over the two seasons 27% of the *O. orbona*, 24% *G. versuta*, and 33% *P. irroratus* trapped tested positive for *X. fastidiosa*. Additionally *O. orbona* and *G. versuta* transmitted *X. fastidiosa* to grape under greenhouse conditions. These results are not surprising, as work done by others has shown that *O. orbona* and other members of the genera *Oncometopia* and *Graphocephala* are vectors of *X. fastidiosa* to grape (Adlerz & Hopkins, 1979; Frazier & Freitag, 1946; Kaloostain, 1962). *O. orbona* and *G. versuta* have previously been reported as vectors of *X. fastidiosa* to peach (Turner & Pollard, 1959b), and both *O. orbona* (personal observation) and *G. versuta* (Adlerz & Hopkins, 1979) reproduce on grape. Transmission studies were not performed with *P. irroratus.* *P. irroratus* has been shown to transmit phytoplasmas (Chiykowski, 1965; Gilmer et al., 1966) but not *X. fastidiosa*.

Our data suggest that *O. orbona* transmits *X. fastidiosa* to grape more efficiently than *G. versuta*. However, the *O. orbona* transmission experiments were initiated earlier resulting

in 1 additional month for symptom development, which may have resulted in the higher number of *O. orbona* inoculated plants testing positive for *X. fastidiosa*. In order to provide definitive evidence that *O. orbona* is a more efficient transmitter, experiments need to be repeated allowing an equivalent time for symptom development, controlling *X. fastidiosa* source tissue, insect acquisition periods, and reducing variability associated with insects by using source plants artificially inoculated with *X. fastidiosa* and maintained in the greenhouse. Studies done with *Homolodisca coagulata* (Almeida and Purcell, 2003) and *G. atropunctata* (Hill and Purcell, 1995) where source plant variability was reduced, resulted in up to 19.6 and 92% inoculation efficiencies for *H. coagulata* and *G. atropunctata*, respectively. Additionally, the transmission efficiency of *O. orbona* and *G. versuta* may be higher than found in our tests because plants used in transmission experiments were accidentally exposed to glyphosate and excessive water stress during a 2-day period, causing partial defoliation and stunting of some plants Consequently, symptom development on grapevines in the greenhouse was not always representative of typical symptoms of PD and did not correlate with presence of *X. fastidiosa* as determined by ELISA testing.

The population size of *G. versuta, A. constricta*, *P. irroratus*, and *O. orbona* varied between sampling years, however their relative abundance in central Piedmont vineyards was similar in 2004 and 2005. *G. versuta* and *A. constricta* were the most abundant species comprising 54 and 38% and 48 and 43% of the populations in 2004 and 2005, respectively. *P. irroratus* and *O. orbona* composed ~5 and 2% of the populations respectively each year. *A. constricta* was the most abundant species in the vineyard in the northeast Coastal Plain, comprising 64 and 51% of the population in 2004 and 2005, respectively. Populations of *G. versuta*, *P. irroratus*, and *O. orbona* averaged ~ 18, 7, and 2% respectively of the Coastal

Plain vineyard population each year. Coincidentally, although the vineyard in the Coastal Plain is located in a high-risk area for Pierce's disease (Harrison et al., 2002), the incidence of PD is low (Sutton, personal communication).

In insectary life history studies, *O. orbona* has been shown to complete two generations and a partial third (Turner & Pollard, 1959a) and *G. versuta* has been shown to complete three generations annually with evidence for a partial fourth (Turner & Pollard, 1959a). At least one generation of *O. orbona*, *P. irroratus*, and *G. versuta* was identified by our trap catches. Two generations of *A. constricta* were identified in 2004; however in 2005 a second generation was not clear, possibly because sampling was terminated too early. The seasonal patterns of *O. orbona* and *G. versuta* we observed on grape in North Carolina are similar to those found on peach (Turner & Pollard, 1959a) and grape (Krewer et al., 2002; Yonce, 1983) in Georgia. Turner and Pollard (1959a) found that *O. orbona* and *G. versuta* move onto peach trees in March and early April and move back to woods to overwinter in October. However, numbers of *O. orbona* and *G. versuta* trapped in vineyards in Georgia were much lower than we trapped in North Carolina vineyards (Krewer et al., 2002; Yonce, 1983). Little is known about the biology of *A. constricta* and *P. irroratus.*

Insecticides were applied in the vineyards, with the exception of vineyard 2, after the peak number of catches for *O. orbona* and *P. irroratus* but during the peak number of catches for *G. versuta* and *A. constricta* in 2004 and 2005. At vineyard 2, carbaryl (Sevin®, Bayer CropScience, Durham, NC) was applied weekly during April, May, June, July, and October of 2004 and 2005 to control for general insect pests. Insecticide use at vineyards 1, 3, and 4 consisted of one to three applications of carbaryl (Sevin®, Bayer CropScience, Durham, NC) for Japanese beetle control. Additionally, during 2005 one application of phosmet (Imidan

70-W, Gowan Company L.L.C., Yuma, AZ) and one application of fenpropathrin (Danatol 2.4 EC, Sumitomo Chemical Company, Ltd.) were applied at vineyard 3. Applications of insecticides may have affected the total leafhopper populations of *G. versuta* and *A. constricta* but should not have affected the time of populations' peaks.

The species composition within vineyards may reflect the surrounding vegetation. All vineyards were located near stands of hardwood forest with herbaceous understory and nearby grassy fields. Additionally, vineyard 1 had a small group (~10) of peach trees and ample landscape ornamentals along one side of the perimeter and vineyard 4 was located on an island near the Outer Banks of North Carolina and was in close proximity to peach and apples orchards, and a pumpkin patch. The leafhoppers may use the herbaceous and/or woody plants near to vineyards as secondary or oviposition hosts. Turner and Pollard (1959a) found that *O. orbona* and *G. versuta*, vectors of *X. fastidiosa* to peach, overwinter in woods, and are general feeders with many trees and shrubs included among their hosts. The leafhoppers trapped in low numbers (< 2%) may have been caught in vineyards during their migration between hosts. More research is needed to identify the host range of these insects.

Purcell (1975) found that populations of the blue-green sharpshooter (*Graphocephala atropunctata* Signoret) were highest near the perimeter of the vineyard early in the growing season. Later, newly matured adults were more evenly distributed within the vineyard. Because the yellow-sticky traps used in this study were only located along the perimeter of each vineyard, traps in future studies should be located throughout the vineyard in order to fully understand the seasonal dynamics of leafhoppers in North Carolina.

Patterns of detection of *X. fastidiosa* from insect mouthparts collected in 2004 and 2005 indicate that the overwintering generations of *O. orbona* and *G. versuta* are most

infective. The percentage of *O. orbona* with *X. fastidiosa* detected in their mouthparts was greatest prior to 27 May in 2004 and from 20 April to 3 May in 2005. After May in both years, detection of *X. fastidiosa* from insect mouthparts decreased to almost zero. Detection of *X. fastidiosa* from *G. versuta* was greatest from 6 Apr to 27 May. Decline in the number of individuals positive for *X. fastidiosa* later in the season, most likely reflects the mortality of overwintering adults. Other studies (Freitag & Frazier, 1954; Purcell, 1975) have found that a high percentage of sharpshooters are capable of transmitting *X. fastidiosa* in early spring, followed by a decline in individuals testing positive during periods of nymphal development. As newly molted adults acquire *X. fastidiosa* from infected plants, percentages of infective individuals increase into the fall. Based on this information the most important time to control leafhopper vectors of *X. fastidiosa* in North Carolina is during the months of May and June.

Leafhoppers enter the vineyard as overwintering adults (Freitag & Frazier, 1954), and depending on time of arrival and abundance play an important role in establishment of Pierce's disease (Alderz & Hopkins, 1979). Early season infection is more likely to lead to chronic infection of vines (Feil et al., 2003; Purcell, 1981). In North Carolina, *O. orbona* and *P. irroratus* appear to enter vineyards in late April and May, and reach their population peaks by mid-May through early June. Populations of *O. orbona* and *P. irroratus* were not as large as those of *G. versuta* and *A. constricta*. However, we noticed while trapping insects for the transmission studies that a higher population of *O. orbona* was present in the vineyard than was reflected on sticky traps. The high numbers of *G. versuta* and *A. constricta* were due to a rapid population increase typically during the last weeks of June and mid-June through mid-July, respectively. Large numbers of *A. constricta* (subfamily Agallinae), which are not

considered sharpshooters, were observed in grasses within vineyards; however, none were seen on grapevines or caught in sweep net samples of grapevine foliage.

Phylogenetic analyses using 272-1 and 272-2 primers as genetic markers amplified a portion of the hypothetical protein gene and a 3' noncoding region. Isolates, examined with the Neighbor-joining method of 1000 bootstrap iterations, grouped into 3 clades and 1 subpopulation within the largest clade. Clades appeared to group by host with a citrus group, almond/oleander group, and NC insect/grape group, suggesting that this marker can differentiate genetically distinct populations of *X. fastidiosa* according to the host. An unrooted haplotype tree generated by SNAP workbench analyses confirmed the distribution of clades. The branching resolved by these analyses is similar to and supported by major phylogenetic groups identified in other studies based on unrelated markers (Chen & Civerolo, 2004; Lin & Walker, 2004; Nunney, 2004). All but one North Carolina isolate grouped with the known Pierce's disease strain from California, providing evidence that leafhoppers in North Carolina carry the grape strain of *X. fastidiosa*. One North Carolina isolate grouped into the almond/oleander clade suggesting that some strains of *X. fastidiosa* in native or ornamental plant hosts nearby the vineyards are similar to almond or oleander strains from California. These strains may have coevolved or may have been introduced by interstate plant transport. Isolates of *X. fastidiosa* within North America (North American isolates do not include the citrus and coffee isolates from Brazil) do not appear to differentiate based on geographic location. Nunney (2004) found no evidence of geographical structure within the grape and oleander clades suggesting strong, possibly host driven selection. Hudson's ranked Z and K_{ST} statistical tests, indicate that isolates from *O.*

orbona and *G. versuta* are genetically similar. From this information, we can speculate that *O. orbona* and *G. versuta* feed on the same plant species. Deeper resolution needs to be obtained by analyzing additional loci and multiple isolates per plant host and geographic location. Phylogenetic analyses with multiple loci and/or satellite data may change these conclusions, as data from one locus may be due to random events.

Knowledge of the identity of the vectors of *X. fastidiosa* in the Southeast and their population dynamics will aid winegrape growers in managing Pierce's disease by enabling them to make better management decisions. Control of Pierce's disease in California is currently based on preventing the establishment of the disease in the vineyard through vegetation management and insecticide applications (Agriculture and Natural Resources, revised 2005; UC Statewide IPM Program, www.ipm.ucdavis.edu; College of Natural Resources, revised 2005; Xylella Web Site, www.cnr.berkeley.edu/xylella). Growers in the Southeast must be especially vigilant in early spring when Pierce's disease infection is thought to be most important (Purcell, 1975) and when populations of known vectors, *O. orbona* and *G. versuta*, enter the vineyard from their overwintering hosts. Systemic insecticides (imidacloprid) are currently the most effective treatment for glassy-winged sharpshooters (Agriculture and Natural Resources, revised 2005; UC Statewide IPM Program, www.ipm.ucdavis.edu). However, effectiveness of systemic insecticides on *O. orbona* and *G. versuta* has not been fully explored. Preliminary trials showed imidacloprid applications only extended the life of the vineyard by 1 year (Krewer et al., 2002). Because insecticidal sprays and rouging symptomatic vines are not highly efficient (Agriculture and Natural Resources, revised 2005; UC Statewide IPM Program, www.ipm.ucdavis.edu;

Purcell, 1975) other strategies for managing Pierce's disease need to be designed and implemented.

The majority of research on Pierce's disease has been in California. Studies need to address concerns specific to the development of Pierce's disease in the Southeast. In addition to continuing to identify and monitor vectors, a list of the most important plant hosts of *X. fastidiosa* and the insect vectors in the Southeast needs to be documented. By determining what plants serve as sources of *X. fastidiosa* and as hosts of the insect vectors, growers can more efficiently control Pierce's disease by removing source plants. The epidemiological importance of summer inoculations in the Southeast needs to be determined. In California, summer inoculations are not thought to contribute to chronic Pierce's disease development (Feil et al., 2003). Cooler nights and lower summer temperatures decrease rates of *X. fastidiosa* multiplication in California therefore slowing the colonization of summer infections (Feil and Purcell, 2001). In the Southeast, warm nighttime temperatures and high temperatures into late autumn need to be considered as factors increasing *X. fastidiosa* colonization and escalating the importance of summer inoculations. Should summer inoculations prove to epidemiologically important in the Southeast, the critical time of vector control would be extended.

When the expansion of the grape industry in North Carolina brought Pierce's disease to the attention of growers and researchers, very little was known about the epidemiology of Pierce's disease in the Southeast. From this study, we now know that three of the four most abundant leafhoppers present in North Carolina vineyards, *O. orbona*, *G. versuta*, and *P. irroratus* carry *X. fastidiosa* in their mouthparts, and *O. orbona* and *G. versuta* transmit *X.*

fastidiosa to grape. *O. orbona* is most likely the vector of greatest concern because it enters vineyards early in the spring and feeds on shoots, allowing *X. fastidiosa* more time to colonize the grapevine. Additional tests need to be done to determine if *P. irroratus* can also transmit *X. fastidiosa*.

LITERATURE CITED

Alderz, W. C., and Hopkins, D. L. 1979. Natural infectivity of two sharpshooter vectors of Pierce's disease of grape in Florida. J. Econ. Entomol. 72:916-919.

Almeida, R. P. P. and Purcell, A. H. 2003. *Homalodisca coagulata* (Hemiptera, Cicadellidae) Transmission of *Xylella fastidiosa* to Almond. Plant Dis. 87: 1255-1259.

Aylor, D., Price, E. W. and Carbone, I. 2004. SNAP Combine and Map. Distributed over the Internet, http://snap.cifr.ncsu.edu, Department of Plant Pathology, North Carolina State University.

Berisha, B., Chen, Y. D., Zhang, G. Y., Xu, B., and Chen, T. A. 1998. Isolation of Pierce's disease bacterium from grapevines in Europe. Eur. J. Plant Pathol. 104: 427-433.

Bextine, B., Blua, M.J., and Redak, R. 2004. Developing a method to detect *Xylella fastidiosa* in the glassy-winged sharpshooter. Pages 249-252 in: Proceedings of the 2004 Pierce's Disease Research Symposium.

Carbone, I., Liu, Y., Hillman, B. I., and Milgroom, M. G. 2004. Recombination and migration of *Cryphonectria hypovirus 1* as inferred from gene genealogies and the coalescent. Genetics 166: 1611-1629.

Chang, C. J., Garnier, M., Zreik, L., Rossetti, V., and Bove, J. M. 1993. Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. Curr. Microbiol. 27: 137-142.

Chen, J. and Civerolo E. L. 2004. Developing a microarray-PCR-based identification and detection system for *Xylella fastidiosa* strains important to California. Pages 171-174 in: Proceedings of the 2004 Pierce's Disease Research Symposium.

Chiykowski, L. N. 1965. Transmission of clover phyllody virus by the leafhopper *Paraphlepsius irroratus* (Say). Can. Ent. 97: 1171-1173.

Davis, M. J., French, W. J., and Schaad, N. W. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr. Microbiol. 6: 309-314.

Davis, M. J., Purcell, A. H., and Thomson, S. V. 1978. Pierce's disease of grapevines: Isolation of the causal bacterium. Science 199: 78-77.

Davis, M. J., Thomson, S. V., and Purcell, A. H. 1980. Etiological role of a xylem-limited bacterium causing Pierce's disease in almond leaf scorch. Phytopathology 70: 472-475.

DeLong, D.M. 1948. The leafhoppers, or Cicadellidae, of Illinois (Eurymelinae-Balcluthinae). Ill. Nat. Hist. Surv. Bull. 24(2): 91-376.

Esau, K. 1948. Anatomic effects of the virus of Pierce's disease and phony peach. Hilgardia. 18: 423-82.

Feil, H., and Purcell, A. H. 2001. Temperature-dependent growth and survival of *Xylella fastidiosa* in vitro and in potted grapevines. Plant Dis. 85: 1230-1234.

Feil, H., Feil, W. S., and Purcell, A. H. 2003. Effects of date of inoculation on the within-plant movement of *Xylella fastidiosa* and persistence of Pierce's disease within field grapevines. Phytopathology 93: 244-251.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Frazier, N. W. and Freitag, J. H. 1946. Ten additional leafhopper vectors of grape as determined by insect transmission. Phytopathology 36: 634-637.

Freitag, J. H. and Frazier, N. W. 1954. Natural infectivity of leafhopper vectors of Pierce's disease virus of grape in California. Phytopathology 44: 7-11.

Gilmer, R. M., Palmiter, D. H., Schaefers, G. A., and McEwen, F. L. 1966. Leafhopper transmission of X-disease virus of stone fruits in New York. N.Y. State Agric. Exp. (Geneva) Bull. 813: 22.

Griffiths, R. and P. Marjoram, 1996 Ancestral inference from samples of DNA sequences with recombination. J. Comp. Biol. 3: 479-502.

Gubler, W. D., Stapleton, J. J., Leavitt, G. M., Purcell, A. H., Varela, L. G., and Smith, R. J. 2005. UC IPM Pest Management Guidelines: Grape. UC ANR Publication 3448.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

Harrison, U. J., Anas, O., and Sutton, T.B. 2002. Geographical distribution of Pierce's disease in North Carolina's winegrowing region. Phytopathology 92: S35.

Hearon, S. S., Sherald, J. L., and Kostka, S. J. 1980. Association of xylem-limited bacteria with elm, sycamore, and oak leaf scorch. Can. J. Bot. 58: 1986-1993.

Hill, B. L. and Purcell, A. H. 1995. Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. Phytopathology 85: 209-212.

Hopkins, D. L. 1977. Diseases caused by leafhopper-borne rickettsia-like bacteria. Annu. Rev. Phytopathol. 17: 277-294.

Hopkins, D. L. 1981. Seasonal concentration of the Pierce's disease bacterium in grapevine stems, petioles, and leaf veins. Phytopathology 71: 415-418.

Hopkins, D. L. and Purcell, A. H. 2002. *Xylella fastidiosa*: Cause of Pierce's disease of grapevines and other emergent diseases. Plant Dis. 86: 1056-1066.

Huang, Q. 2004. First report of *Xylella fastidiosa* associated with leaf scorch in black oak in Washington, D.C. Plant Disease 88: 224.

Hudson, R. R., Boos D. D., and Kaplan N. L., 1992. A statistical test for detecting geographic subdivision. Mol. Biol. Evol. 9: 138-151.

Hudson, R. R. 2000. A new statistic for detecting genetic differenatiation. Genetics155: 2011-2014.

Kaloostian, G. H., Pollard, H. N., and Turner, W. F. 1962. Leafhopper vectors of Pierce's disease virus in Geogria. Plant Disease Rptr. 46(4): 292.

Krewer, G., Dutcher, J. D., and Chang, C. J. 2002. Imidacloprid insecticide slows development of Pierce's disease in bunch grapes. J. Entomol. Sci. 37: 101-112.

Kumar, S., Tamura, K., and Nei, M. 1993. MEGA: Molecular Evolutionary Genetics Analysis. Pennsylvania State University, University Park, P.A.

Lu, L. S., and Su, C. C. 1993. Isolation, cultivation, and pathogenicity of *Xylella fastidiosa*, the causal bacterium of pear leaf scorch disease in Taiwan. Plant Dis. 77: 642-646.

Lin, H. and Walker, A. 2004. Development of SSR markers for genotyping and assessing the genetic diversity of *Xylella fastidiosa* in California. Pages 206-209 in: Proceedings of the 2004 Pierce's Disease Research Symposium.

Loomis, N. H. 1958. Performance of *Vitis* species in the south as an indication of their relative resistance to Pierce's disease. Plant Dis. Rep. 42: 833-836.

Markwordt, J. D., Doshi, R. and Carbone, I. 2004. SNAP Clade and Matrix. Distributed over the Internet, http://snap.cifr.ncsu.edu, Department of Plant Pathology, North Carolina State University.

Nunney, L. 2004. Genome-wide identification of rapidly evolving genes in *Xylella fastidiosa*: Key elements in the systematic identification of host strains, and in the search for plant-host pathogenicity candidate genes. Pages 220-224 in: Proceedings of the 2004 Pierce's Disease Research Symposium.

Page, R. D. M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12: 357-358.

Pierce, N. B. 1882. The California vine disease. U.S. Dep. Agric., Div. Veg. Pathol. Bull. No. 2.

Poole, R. W., Garrison, R. W., McCafferty, W. P., Otte, D., and Stark, B. P. 1997. Nomain Insecta Nearctica, A Check List of the Insects of North America. Vol. 4: Non-Holometabolous Orders. Entomological Information Services, Rockville, M.D. 731.

Pooler, M. R. and Hartnung, J. S. 1995. Specific PCR detection and identification of *Xylella fastidiosa* strains causing citrus variegated chlorosis. Current Microbiology 31: 377-381.

Pooler, M. R., Myung, I. S., Bentz, J., Sherald, J., and Hartung, J. S. 1997. Detection of *Xylella fastidiosa* in potential insect vectors by immunomagnetic separation and nested polymerase chain reaction. Letters in Applied Microbiology 25: 123-126.

Price, E.W. and Carbone, I. 2005. SNAP: workbench management tool for evolutionary population genetic analysis. Bioinformatics 21: 402-404.

Purcell, A. H. 1975. Role of the blue-green sharpshooter, *Hordnia circellata*, in the epidemiology of Pierce's disease of grapevines. Environ. Entomol. 4: 745-752.

Purcell, A. H. 1980. Environmental therapy for Pierce's disease of grapevines. Plant Dis. 64: 388-390.

Purcell, A. H. 1981. Vector preference and inoculation efficiency as components of resistance to Pierce's disease in European grape cultivars. Phytopathology 71: 429-435.

Purcell, A. H. and Hopkins, D. L. 1996. Fastidious xylem-limited bacterial plant pathogens. Annu. Rev. Phytopathol. 34:131-151.

Purcell, A. H. and Saunders, S. R. 1999. Fate of Pierce's disease strains of *Xylella fastidiosa* in common riparian plants in California. Plant Dis. 83: 825-830.

Purcell, A. H., Saunders, S. R., Hendson, M. E., Grebus, M. E., and Henry, M. J. 1999. Causal role of *Xylella fastidiosa* in oleander leaf scorch disease. Phytopathology 89: 53-58.

Redak, R. A., Purcel, A. H., Lopes, J. R. S., Blua, M. J., Mizell, R. F., and Anderson, P. C. 2004. The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. Annu. Rev. Entomol. 49: 243-270.

Severin, H. H. P. 1950. Spittle-insect vectors of Pierce's disease virus. II. Life histories and virus transmission. Hilgardia 19: 357-382.

Swofford, D. L. 1998. PAUP*. Phylogenetic Analysis Using Parsimony (* and other Methods), Version 4.0. Sinauer Associates, Sunderland, MA.

Thomson, S. V., Kloepper, M. J., and Purcell, A. H. 1978. Alfalfa dwarf: Relationship to the bacterium causing Pierce's disease of grapevines. (Abstr.) Proc. Intl. Congr. Plant Pathol., 3rd. 3:65.

Thompson, J. D., Higgins, D.G., and Gibson T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nuc. Acids Res. 22: 4673-4680.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tolls. Nucleic Acids Res. 24: 4876-4882.

Turner, W. F. and Pollard, H. N. 1959a. Life histories and behavior of five insect vectors of phony peach disease. U.S. Dep. Agric. Tech. Bull. No. 1188.

Turner, W.F. and Pollard, H. N. 1959b. Insect transmission of phony peach disease. U.S. Dep. Agric. Tech. Bull. 1193.

Wells, J. M., Raju, B. C., Hung, H. Y., Weisburg, W. G., Mandelco-Paul, L., and Brenner, D. J. 1987. *Xylella fastidiosa* gen. Nov. sp. Nov.: Gram-negative, xylem-limited fastidious plant bacteria related to *Xanthomonas* spp. Int. J. Syst. Bacteriol. 37: 136-143.

Wells, J. M., Raju, B. C., and Nyland, G. 1983. Isolation, culture, and pathogenicity of the bacterium causing phony disease of peach. Phytopathology 73: 859-862.

Wells, J. M., Raju, B. C., Thompson, J. M., and Lowe, S. K. 1981. Etiology of phony peach and plum leaf scald diseases. Phytopathology 71: 1156-1161.

Wrinkler, A. J. 1949. Pierce's disease investigations. Hilgardia 19: 207-264.

Yonce, C. E. 1983. Geographical and seasonal occurrence, abundance, and distribution of phony peach disease vectors and vector response to age and condition of peach orchards and a disease host survey of Johnsongrass for rickettsia-like bacteria in the Southeastern United States. J. Geogria Entomol. Soc. 18: 410-418.

Young, D.A. 1968. Taxonomic study of the Cicadellinae (Homoptera: Cicadellidae). Part 1: Proconiini. Smithson. Inst. Bull. 261- 287.

Young, D.A. 1977. Taxonomic study of the Cicadellinae (Homoptera: Cicadellidae). Part 2: New World Cicadellini and the genus Cicadella. N.C. Agr. Exp. Sta. Bull., Raleigh, N.C. 235, 1135.

Table 1. Number of adult leafhoppers trapped in four North Carolina vineyards in 2004 and 2005, and the percentage composition of the most abundant species

	2004			2005		
Leafhopper species	Vineyard[y]	Number trapped	Percent	Vineyard[y]	Number trapped	Percent
Graphocephala versuta	1	2206	0.51	1	1848	0.50
	2	2240	0.64	2	2198	0.63
	3	5076	0.51	3	4560	0.40
	4	138	0.16	4	113	0.18
Oncometopia orbona	1	264	0.06	1	142	0.04
	2	56	0.02	2	102	0.03
	3	50	0.01	3	161	0.01
	4	20	0.02	4	58	0.09
Paraphlepsius irroratus	1	291	0.07	1	452	0.12
	2	165	0.05	2	102	0.03
	3	252	0.03	3	380	0.03
	4	74	0.09	4	88	0.14
Agalliota constricta	1	1142	0.26	1	1068	0.29
	2	965	0.27	2	1027	0.29
	3	4433	0.45	3	6213	0.54
	4	535	0.64	4	290	0.47
Other species [z]	1	128	0.03	1	167	0.05
	2	98	0.03	2	72	0.02
	3	127	0.01	3	113	0.01
	4	74	0.09	4	72	0.12

[y] Vineyards 1, 2, and 3 were located in central North Carolina. Vineyard 4 was located in the northeastern Coastal Plain of North Carolina.

[z] Five leafhopper species and one spittlebug species making up < 2% relative abundance were grouped as other species.

Table 2. Number of *Oncometopia orbona* positive for *X. fastidiosa* from insects trapped in vineyards in 2004 and 2005 when tested by nested PCR

2004				2005			
Dates	**Vineyard**	**Tested**	**Positive**	**Dates**	**Vineyard**	**Testeed**	**Positive**
5.13-5.27	1	7	3	4.06-4.20	1	2	0
	2	4	2		2	2	0
	3	4	2		3	2	0
5.27-6.9	1	4	2	4.20-5.03	1	1	1
	2	2	0		2	2	2
	3	4	2		3	4	4
6.9-6.21	1	2	0	5.03-5.17[z]	1	—	—
	2	2	0		2	—	—
	3	2	0		3	—	—
6.21-7.2	1	0	—	5.17-5.31	1	4	1
	2	1	0		2	4	0
	3	0	—		3	4	2
7.2-7.15	1	1	0	5.31-6.14	1	8	0
	2	1	0		2	8	3
	3	0	—		3	8	3
				6.14-6.28	1	4	0
					2	4	0
					3	4	0
				6.28-7.12	1	1	0
					2	3	0
					3	0	—
				4.6-7.1	4	12	0

[z] Tests from *O. orbona* collected on trapping period 5.03-5.17 2005 were not included in this table due to an error in testing.

Table 3. Number of *Graphocephala versuta* positive for *X. fastidiosa* from insects trapped in vineyards in 2004 and 2005 when tested by nested PCR

2004				2005			
Dates	**Vineyard**	**Tested**	**Positive**	**Dates**	**Vineyard**	**Tested**	**Positive**
5.13-5.27	1	5	2	4.06-4.20	1	2	2
	2	5	2		2	2	1
	3	5	3		3	2	1
5.27-6.9	1	4	2	4.20-5.03	1	4	2
	2	4	1		2	4	2
	3	4	2		3	4	2
6.9-6.21	1	5	0	5.03-5.17	1	4	0
	2	6	3		2	4	0
	3	5	3		3	4	0
6.21-7.2	1	4	1	5.17-5.31	1	4	1
	2	4	1		2	4	0
	3	4	3		3	4	2
7.2-7.15	1	1	0	5.31-6.14	1	4	1
	2	1	0		2	4	0
	3	1	0		3	4	1
7.15-7.30	1	1	0	6.14-6.28	1	4	0
	2	1	0		2	4	1
	3	1	0		3	4	0
				6.28-7.12	1	4	0
					2	4	0
					3	4	0
				4.6 - 7.30	4	20	3

Table 4. Number of *Paraphlepsius irroratus* positive for *X. fastidiosa* from insects trapped in vineyards in 2004 and 2005 when tested by nested PCR

2004				2005			
Dates	**Vineyard**	**Tested**	**Positive**	**Dates**	**Vineyard**	**Tested**	**Positive**
5.13-5.27	1	4	2	5.03-5.17	1	4	0
	2	4	3		2	4	0
	3	4	3		3	4	0
5.27-6.9	1	4	1	5.17-5.31	1	4	1
	2	4	4		2	4	0
	3	4	1		3	3	0
6.9-6.21	1	3	0	5.31-6.14	1	4	1
	2	4	2		2	4	0
	3	3	0		3	4	0
6.21-7.2	1	1	1	6.14-6.28	1	4	0
	2	4	2		2	0	0
	3	1	0		3	4	0
				6.28-7.12	1	0	0
					2	4	0
					3	0	0
				4.6 - 7.30	4	12	3

Table 5. Results of the greenhouse transmission experiments with *Oncometopia orbona*. Insects were caged on Chardonnay grapes for 6 days. Date corresponds to days insects were caged on test plants. Visual ratings were scored according to a 0 to 5 rating scale [y]. ELISA tests with an optimal density (OD) value ≥ 0.118 were considered positive.

Date	Vine	Number [x]	Visual [y]	ELISA	Date	Vine	Number [x]	Visual [y]	ELISA
5.17-5.23	A01	1	0	+	5.24-5.30	A43	1	0	-
5.17-5.23	A02	1	0	+	5.24-5.30	A44	1	1?	+
5.17-5.23	A03	1	1?	+	5.24-5.30	A45	1	1?	+
5.17-5.23	A04	1	1?	+	5.24-5.30	A46	1	0	+
5.17-5.23	A05	1	0	+	5.24-5.30	A47	1	0	+
5.17-5.23	A06	1	1	+	5.25-5.31	A48	1	0	-
5.17-5.23	A07	1	1?	+	5.25-5.31	A49	1	2	+
5.17-5.23	A08	1	1	+	5.25-5.31	A50	1	1	
5.17-5.23	A09	1	1	-	5.25-5.31	A51	1	1	+
5.17-5.23	A74	1	1?	+	5.25-5.31	A52	1	1?	+
5.17-5.23	A75	1	0	+	5.25-5.31	A53	1	1?	+
5.17-5.23	A78	1	1	+	5.25-5.31	A54	1	0	+
5.19-5.26	A73	1	0	+	5.25-5.31	A55	1	1	-
5.19-5.26	control	0	0	-	5.25-5.31	A56	1	0	-
5.19-5.26	A76	1	0	+	5.25-5.31	A57	1	1	+
5.19-5.26	A77	1	0	+	5.25-5.31	A58	1	1	+
5.19-5.26	A79	1	2	-	5.25-5.31	A59	1	1	+
5.24-5.30	control	0	0	-	5.25-5.31	A60	1	1	-
5.24-5.30	A11	1	0	-	5.25-5.31	A61	1	1?	+
5.24-5.30	A12	1	2	+	5.25-5.31	A62	1	1	+
5.24-5.30	A13	1	2	+	5.25-5.31	A63	1	1	+
5.24-5.30	A14	1	1	-	5.25-5.31	A64	1	0	+
5.24-5.30	A15	1	0	-	5.25-5.31	A65	1	0	-
5.24-5.30	A16	1	1	-	5.25-5.31	A66	1	0	+
5.24-5.30	A17	1	1?	-	5.25-5.31	A67	1	2	
5.24-5.30	A18	1	0	+	5.25-5.31	A68	1	0	+
5.24-5.30	A19	1	1	+	5.25-5.31	A69	1	0	+
5.24-5.30	A20	1	1?	+	5.25-5.31	A70	1	0	+
5.24-5.30	A21	1	2	-	5.25-5.31	A71	1	0	-
5.24-5.30	A22	1	0	-	5.25-5.31	A72	1	1	+
5.24-5.30	A23	1	0	+	6.7 - 6.13	A01	5	0	-
5.24-5.30	A24	1	1	+	6.7 - 6.13	A02	5	0	-
5.24-5.30	A25	1	1	-	6.7 - 6.13	A03	5	1	-
5.24-5.30	A26	1	0	+	6.7 - 6.13	A04	5	1	-
5.24-5.30	A27	1	1	+	6.8 -6.14	A05	5	0	-
5.24-5.30	A28	1	0	+	6.8 -6.14	A06	5	0	-
5.24-5.30	A29	1	1	+	6.8 -6.14	A07	5	0	-
5.24-5.30	A30	1	1	+	6.8 -6.14	A08	5	0	-
5.24-5.30	A31	1	1	+	6.9-6.15	A09	5	1?	-
5.24-5.30	A32	1	1?	+	6.9-6.15	A10	5	1	+
5.24-5.30	A33	1	0	-	6.10-6.16	A11	5	0	+
5.24-5.30	A34	1	0	+	6.10-6.16	A12	5	0	+
5.24-5.30	A35	1	0	-	6.10-6.16	A13	5	0	-
5.24-5.30	A36	1	0	+	greenhouse 1	control [z]	0	0	-
5.24-5.30	A37	1	1	-	greenhouse 2	control	0	0	-
5.24-5.30	A38	1	2	-	greenhouse 3	control	0	0	-
5.24-5.30	A39	1	2	+	greenhouse 4	control	0	0	-
5.24-5.30	A40	1	1	+	greenhouse 5	control	0	0	-
5.24-5.30	A41	1	2	-	greenhouse 6	control	0	0	-

[x] represents the number of insect per vine.

[y] 0 = no symptoms, 1? = questionable symptoms, 1 = sporadic marginal necrosis on < 25% of leaves, 2 = necrosis of leaves on entire shoots (equalivant to 25 - 50% leaves with symptoms), 3 = the appearance of bladeless petioles and the majority of leaves necrotic (50 - 75% with symptoms), 4 = defoliation occurring and fruit shrivel (75 - 100% leaves necrotic), d = died within the season

[z] greenhouse controls represent grapevines exposed to greenhouse conditions.

Table 6. Results of the greenhouse transmission experiments with *Graphocephala versuta*. Insects were caged on Chardonnay grapes for 6 days. Date corresponds to days insects were caged on test plants. Visual ratings were scored according to a 0 to 5 rating scale [y]. ELISA tests with an optimal density (OD) value ≥ 0.209 were considered positive.

Date	Vine	Number [x]	Visual [y]	ELISA	Date	Vine	Number [x]	Visual [y]	ELISA
6.21-6.27	A01	5	0	-	6.24-6.30	A33	7	1	-
6.21-6.27	A02	5	0	-	6.24-6.30	A34	7	1?	-
6.21-6.27	A03	5	0	-	6.24-6.30	A35	7	2	-
6.21-6.27	A04	5	0	-	6.24-6.30	A36	7	1?	-
6.21-6.27	A05	5	0	-	6.24-6.30	control	0	0	-
6.21-6.27	A06	5	1?	-	6.24-6.30	A37	7	0	-
6.21-6.27	A07	5	0	-	6.30-7.6	A38	7	1	-
6.21-6.27	control	0	0	-	6.30-7.6	A39	7	0	-
6.23-6.29	C08	5	0	-	6.30-7.6	A40	7	0	-
6.23-6.29	C09	5	0	-	6.30-7.6	A41	7	2	-
6.23-6.29	C10	5	1	-	6.30-7.6	A42	7	0	-
6.23-6.29	C12	5	0	-	6.30-7.6	A43	7	0	-
6.23-6.29	control	0	0	-	6.30-7.6	A44	7	0	-
6.23-6.29	C13	5	0	-	6.30-7.6	A45	7	2	-
6.23-6.29	C14	5	0	-	6.30-7.6	A46	7	0	-
6.23-6.29	A15	7	0	-	6.30-7.6	A47	7	0	-
6.24-6.30	A16	7	0	-	6.30-7.6	A48	7	1	-
6.24-6.30	A17	7	0	-	6.30-7.6	A49	7	0	-
6.24-6.30	A18	7	0	+	6.30-7.6	A50	7	0	-
6.24-6.30	A19	7	0	+	6.30-7.6	A51	7	1	-
6.24-6.30	A20	7	1	-	6.30-7.6	A52	7	1?	-
6.24-6.30	A21	7	1?	-	6.30-7.6	A53	7	1	-
6.24-6.30	A22	7	2	-	6.30-7.6	A54	5	0	-
6.24-6.30	A23	7	1	-	7.5-7.11	A55	5	0	-
6.24-6.30	A24	7	1?	-	7.5-7.11	control	0	0	-
6.24-6.30	A25	7	1	-	7.5-7.11	A56	5	2	-
6.24-6.30	A26	7	0	-	7.5-7.11	A58	7	0	-
6.24-6.30	A27	7	1?	-	7.6-7.12	A59	5	0	-
6.24-6.30	A28	7	1?	-	7.6-7.12	control	0	0	-
6.24-6.30	A29	7	0	+	greenhouse 1	control [z]	0	0	-
6.24-6.30	A30	7	1?	-	greenhouse 2	control	0	0	-
6.24-6.30	A31	7	1	-	greenhouse 3	control	0	0	-
6.24-6.30	A32	7	1?	-	greenhouse 4	control	0	0	-
					greenhouse 5	control	0	0	-

[x] number of insects per vine.

[y] 0 = no symptoms, 1? = questionable symptoms, 1 = sporadic marginal necrosis on < 25% of leaves, 2 = necrosis of leaves on entire shoots (equalivant to 25 - 50% leaves with symptoms), 3 = the appearance of bladeless petioles and the majority of leaves necrotic (50 - 75% with symptoms), 4 = defoliation occurring and fruit shrivel (75 - 100% leaves necrotic), d = died within the season

[z] Greenhouse controls represent grapevines exposed to greenhouse conditions.

Table 7. Host, haplotypes, isolate name, and source of 46 isolates from NC sharpshooters and eight sequences obtained from GenBank

Host	Haplotype (frequency)	*X.fastidiosa* isolate names	Source
Oncometopia orbona	1(1) 4(2) 8(1) 10(18) 11(1) 12(1)	A1 2004, B1 2004, C1 2004, A1 2005, B1 2005, C1 2005	NC vineyards
Graphocephala versuta	4(4) 9(1) 10(17)	A4 2004, B4 2004, C4 2004, A4 2005, B4 2005, C4 2005	NC vineyards
Japanese beech bosnai	2(1)	*X. fastidiosa* strain JB-USNA	gb AY196792.1
Coffee	5(1)	*X. fastidiosa* strain Found-4	gb AF344190.1
Citrus	5(1) 3(1)	*X. fastidiosa* strain Found-5 *X. fastidiosa* 9a5c	gb AF344191.1 ref NC 002488.3
Oleander	6(1) 7(1)	*X. fastidiosa* Ann-1 ctg125 *X. fastidiosa* Ann-1 ctg268	gb AAAM03000127.1 gb AAAM03000001.1
Grape	10(1)	*X. fastidiosa* Temecula1	ref NC 004556.1
Almond	6(1)	*X. fastidiosa* Dixon ctg86	gb AAAL02000008.1

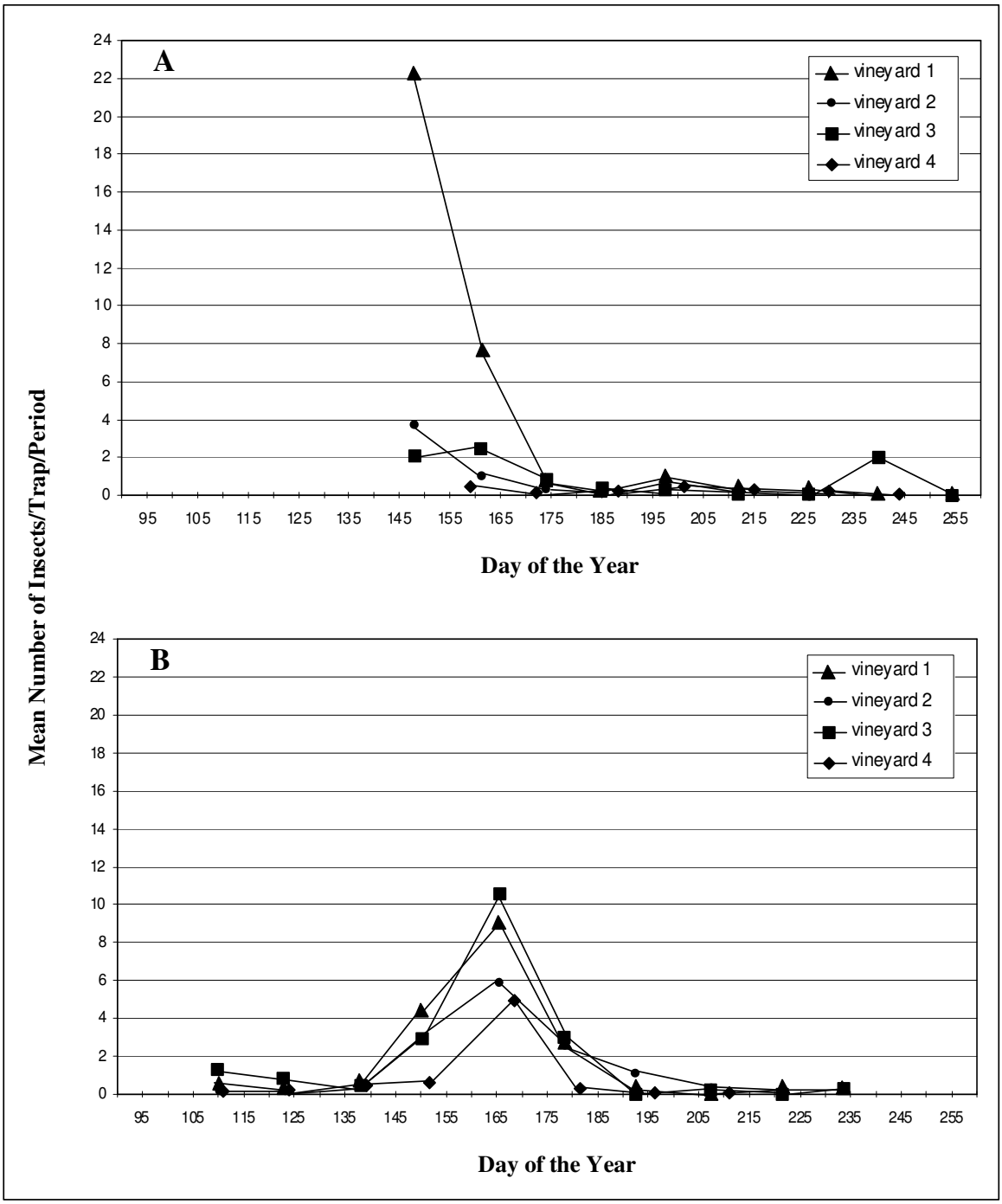


Figure 1. Populations of adult *Oncometopia orbona* in vineyards 1, 2, 3, and 4 during 2004 (A) and 2005 (B). Each point represents mean number of insects caught per trap during each trapping period. Trapping periods in vineyards 1, 2, and 3 were days 134-148, 148-161, 161-173, 184-197, 197-212, 212-226, 226-239, 239-254 in 2004 and 96-110, 110-123, 123-137, 137-150, 150-165, 165-179, 179-193, 193-207, 207-221, 221-234 in 2005. Trapping periods in vineyard 4 were days 146-159, 159-173, 173-188, 202-215, 215-230, 230-244, 244-259 in 2004 and 96-111, 111-124, 124-138, 138-152, 152-168, 168-182, 182-196, 196-211 in 2005.

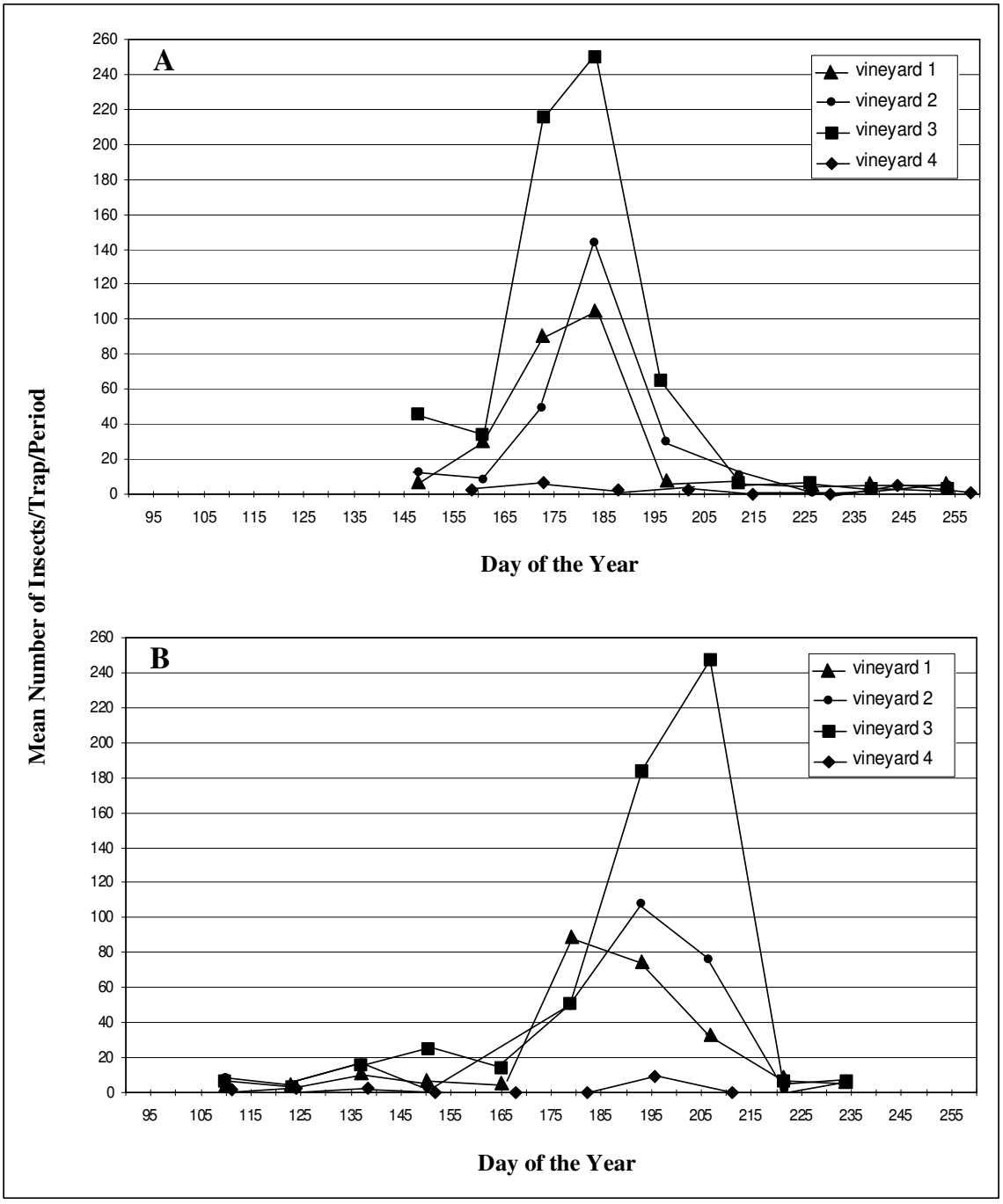


Figure 2. Populations of adult *Graphocephala versuta* in vineyards 1, 2, 3, and 4 during 2004 (A) and 2005 (B). Each point represents mean number of insects caught per trap during each trapping period. Trapping periods in vineyards 1, 2, and 3 were days 134-148, 148-161, 161-173, 184-197, 197-212, 212-226, 226-239, 239-254 in 2004 and 96-110, 110-123, 123-137, 137-150, 150-165, 165-179, 179-193, 193-207, 207-221, 221-234 in 2005. Trapping periods in vineyard 4 were days 146-159, 159-173, 173-188, 202-215, 215-230, 230-244, 244-259 in 2004 and 96-111, 111-124, 124-138, 138-152, 152-168, 168-182, 182-196, 196-211 in 2005.

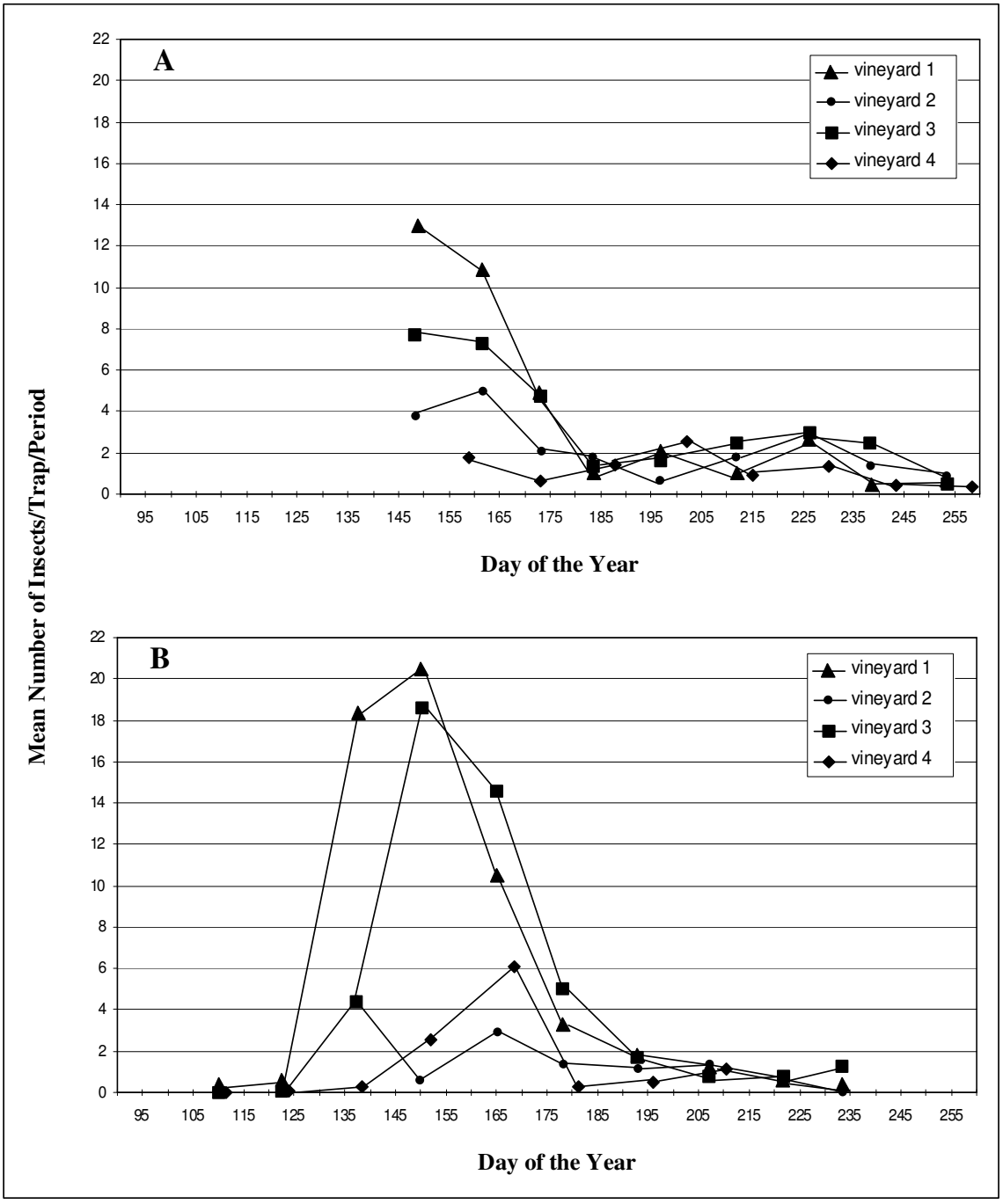


Figure 3. Populations of adult *Paraphlepsius irroratus* in vineyards 1, 2, 3, and 4 during 2004 (A) and 2005 (B). Each point represents mean number of insects caught per trap during each trapping period. Trapping periods in vineyards 1, 2, and 3 were days 134-148, 148-161, 161-173, 184-197, 197-212, 212-226, 226-239, 239-254 in 2004 and 96-110, 110-123, 123-137, 137-150, 150-165, 165-179, 179-193, 193-207, 207-221, 221-234 in 2005. Trapping periods in vineyard 4 were days 146-159, 159-173, 173-188, 202-215, 215-230, 230-244, 244-259 in 2004 and 96-111, 111-124, 124-138, 138-152, 152-168, 168-182, 182-196, 196-211 in 2005.

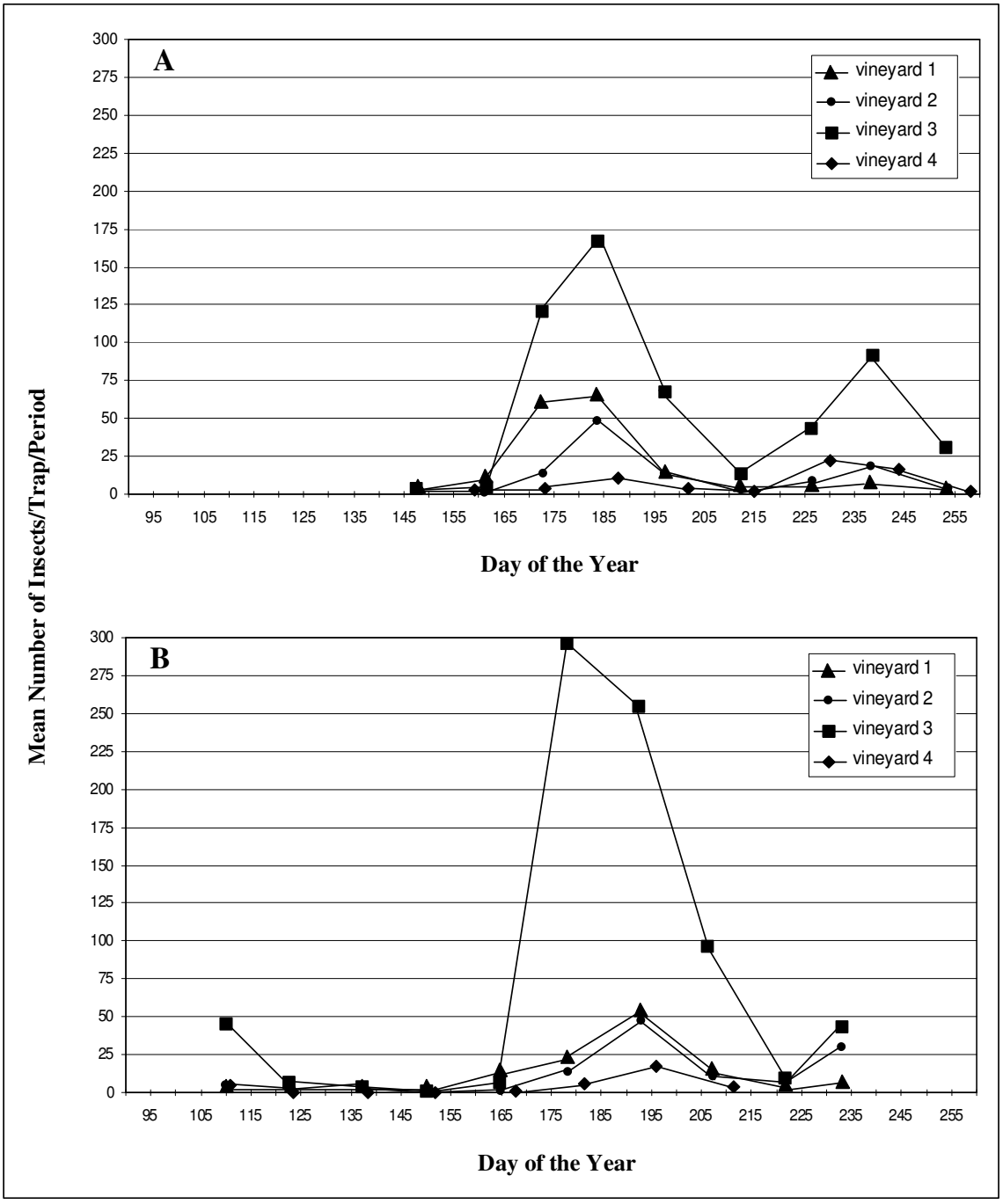


Figure 4. Populations of adult *Agalliota constricta* in vineyards 1, 2, 3, and 4 during 2004 (A) and 2005 (B). Each point represents mean number of insects caught per trap during each trapping period. Trapping periods in vineyards 1, 2, and 3 were days 134-148, 148-161, 161-173, 184-197, 197-212, 212-226, 226-239, 239-254 in 2004 and 96-110, 110-123, 123-137, 137-150, 150-165, 165-179, 179-193, 193-207, 207-221, 221-234 in 2005. Trapping periods in vineyard 4 were days 146-159, 159-173, 173-188, 202-215, 215-230, 230-244, 244-259 in 2004 and 96-111, 111-124, 124-138, 138-152, 152-168, 168-182, 182-196, 196-211 in 2005.

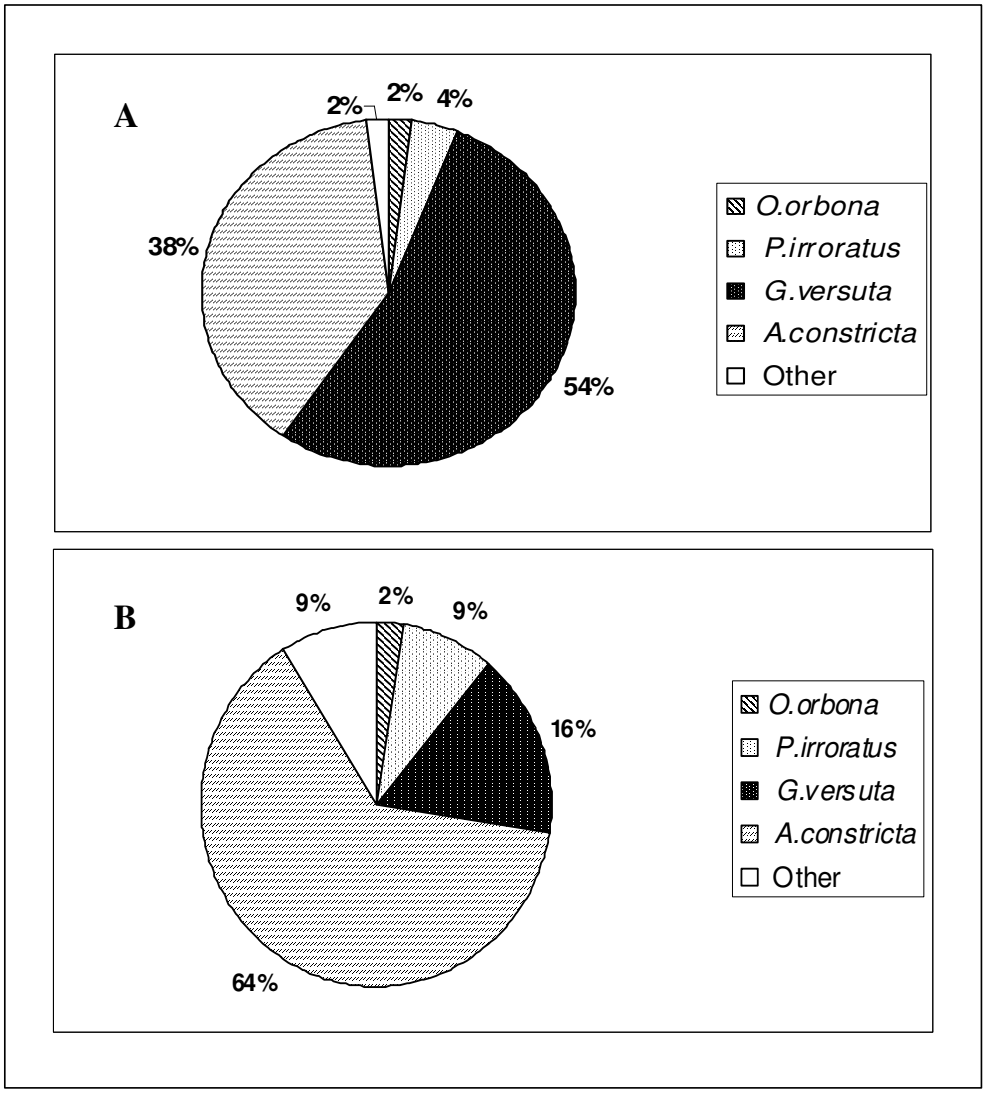


Figure 5. The relative proportion of leafhoppers trapped in 2004 from central Piedmont (A) and the Coastal Plain vineyard (B). The percentages in A represent the mean of each insect species from the three central Piedmont vineyards.

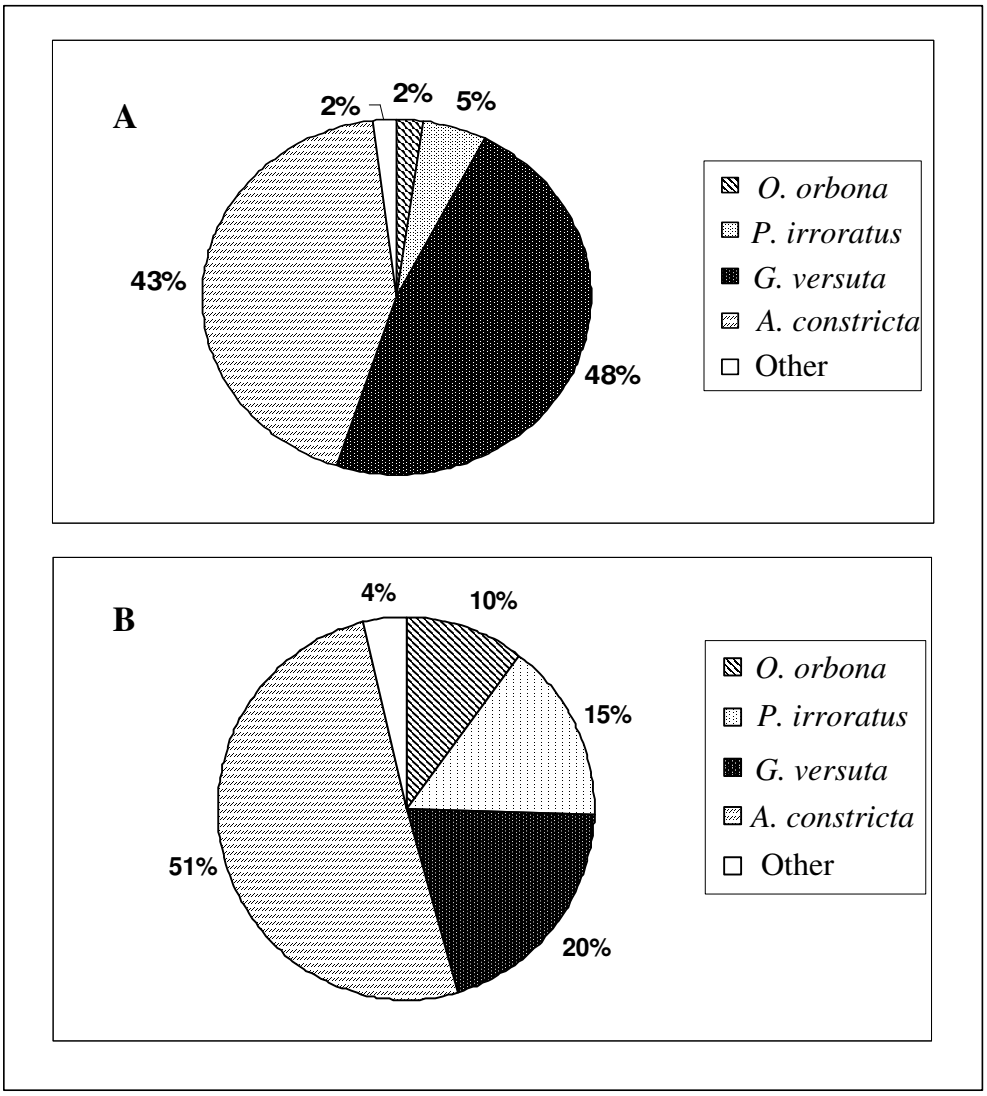


Figure 6. The relative proportion of leafhoppers trapped in 2005 from central Piedmont (A) and the Coastal Plain vineyard (B). The percentages in A represent the mean of each insect species from the three central Piedmont vineyards.

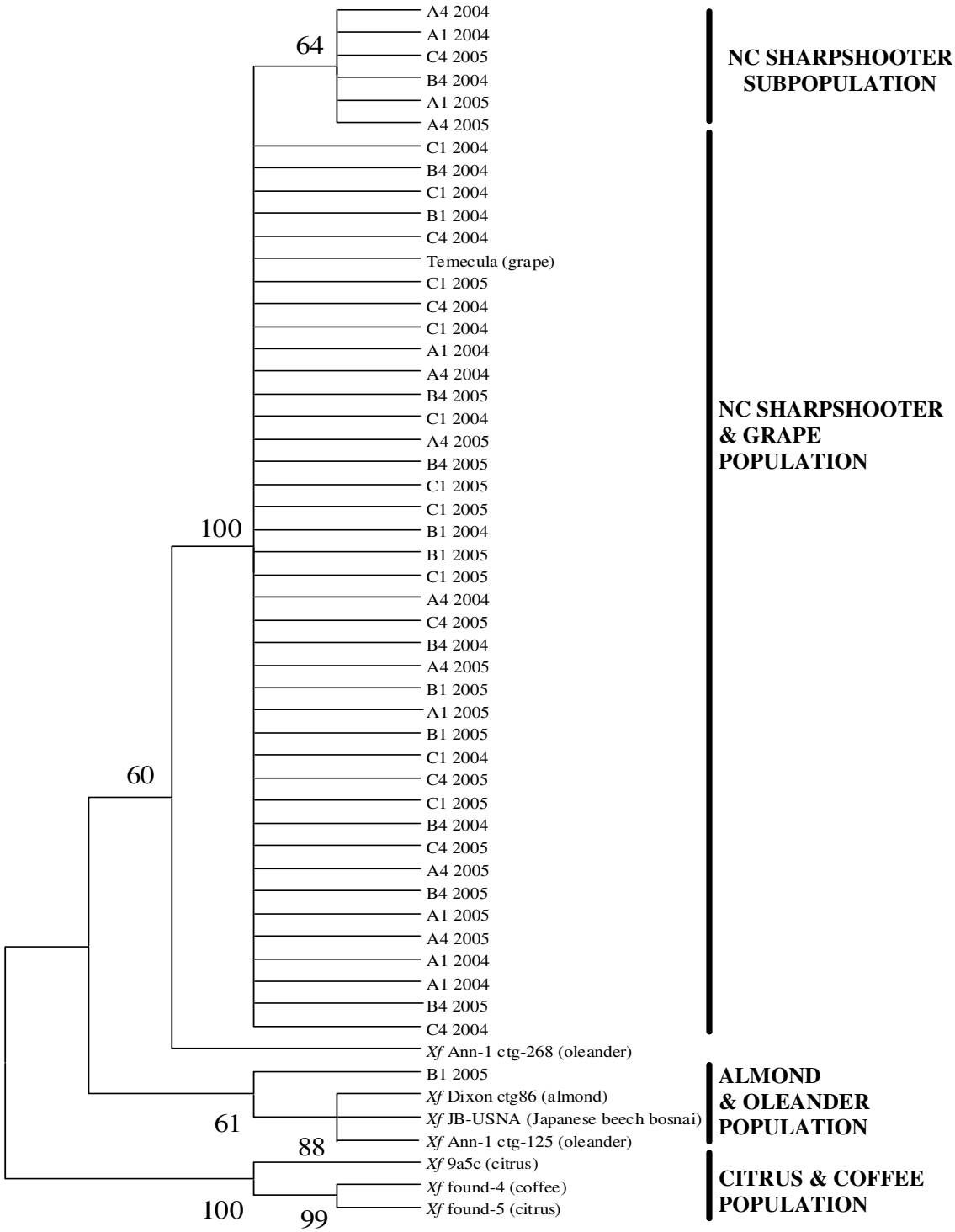


Figure 7. Dendogram of *X. fastidiosa* isolates by Neighbor-Joining method. The dendogram shows relationships among 46 isolates of *X. fastidiosa* from NC sharpshooters and 8 *X. fastidiosa* isolates from host plants obtained from Genebank. A, B, and C represent vineyards 1, 2, and 3, respectively; 1 and 4 represent the sharpshooter species *Oncometopia orbona* and *Graphocephala versuta*, respectively; and 2004, 2005 the year isolates were collected. Isolates were amplified with 272-1-int and 272-2-int primers.

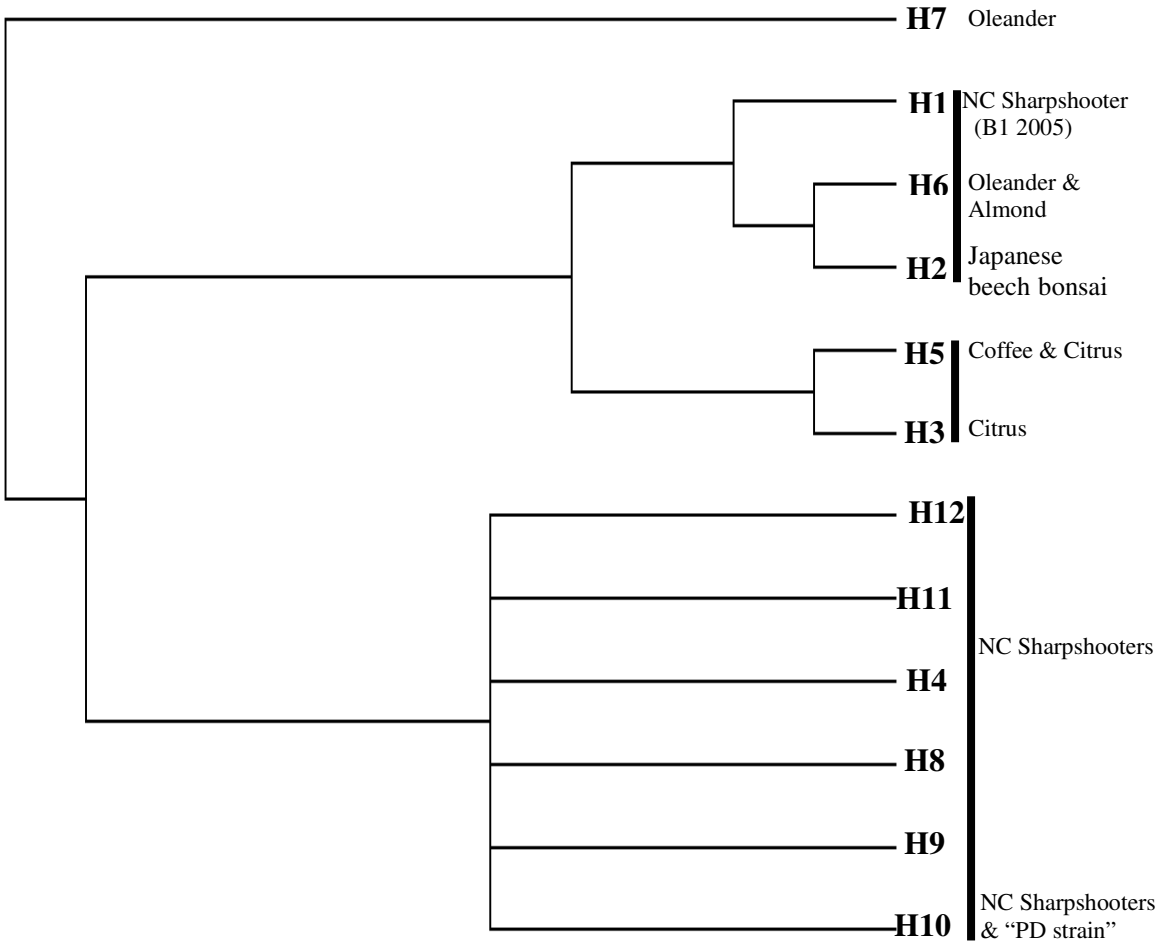


Figure 8. Unrooted haplotype cladogram of *X. fastidiosa* isolates. Indels and variable positions violating infinite sites were removed. One site of homoplasy was detected and removed with no affect on haplotypes distribution. Haplotypes group into three clades and are represented by host.

APPENDIX

Appendix 6.1 Pierce's disease severity in three vineyards in the central Piedmont of North Carolina

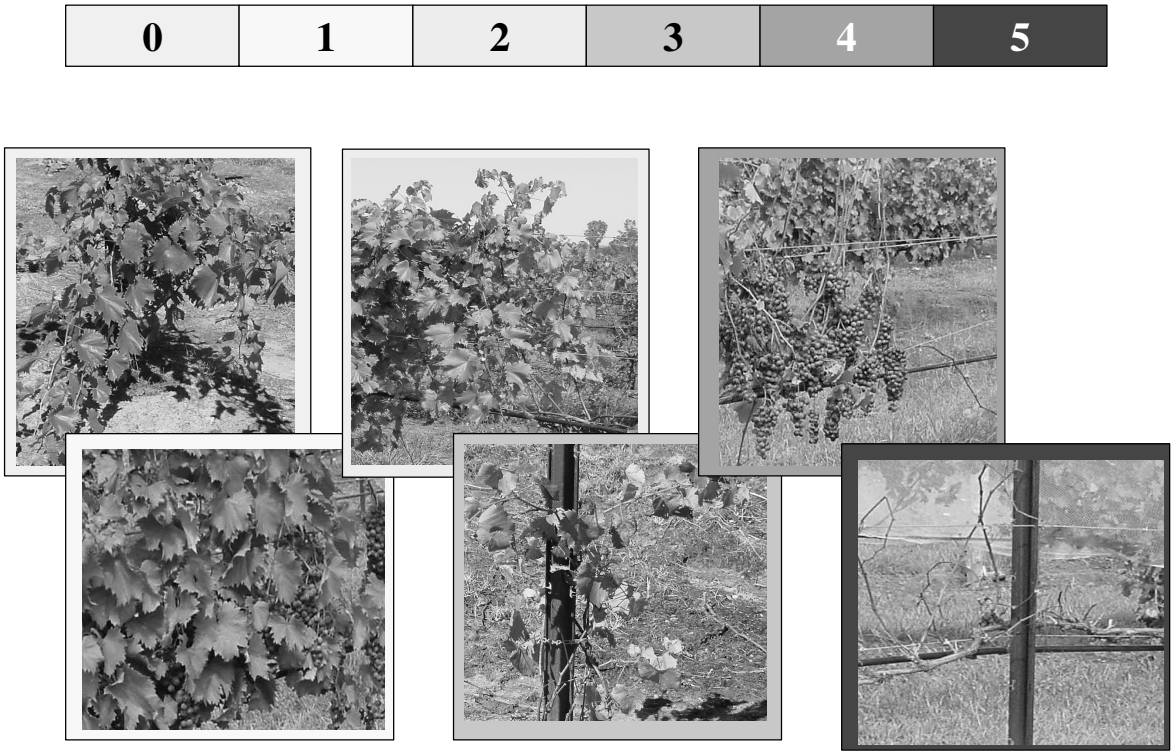
INTRODUCTION

Incidence of PD has been documented as function of vector abundance (Purcell, 1981). To determine if there is a relationship between disease incidence, vineyard sticky trap counts, and the composition of surrounding vegetation, the severity of PD was mapped in each of the three vineyards in the eastern Piedmont during September 2004.

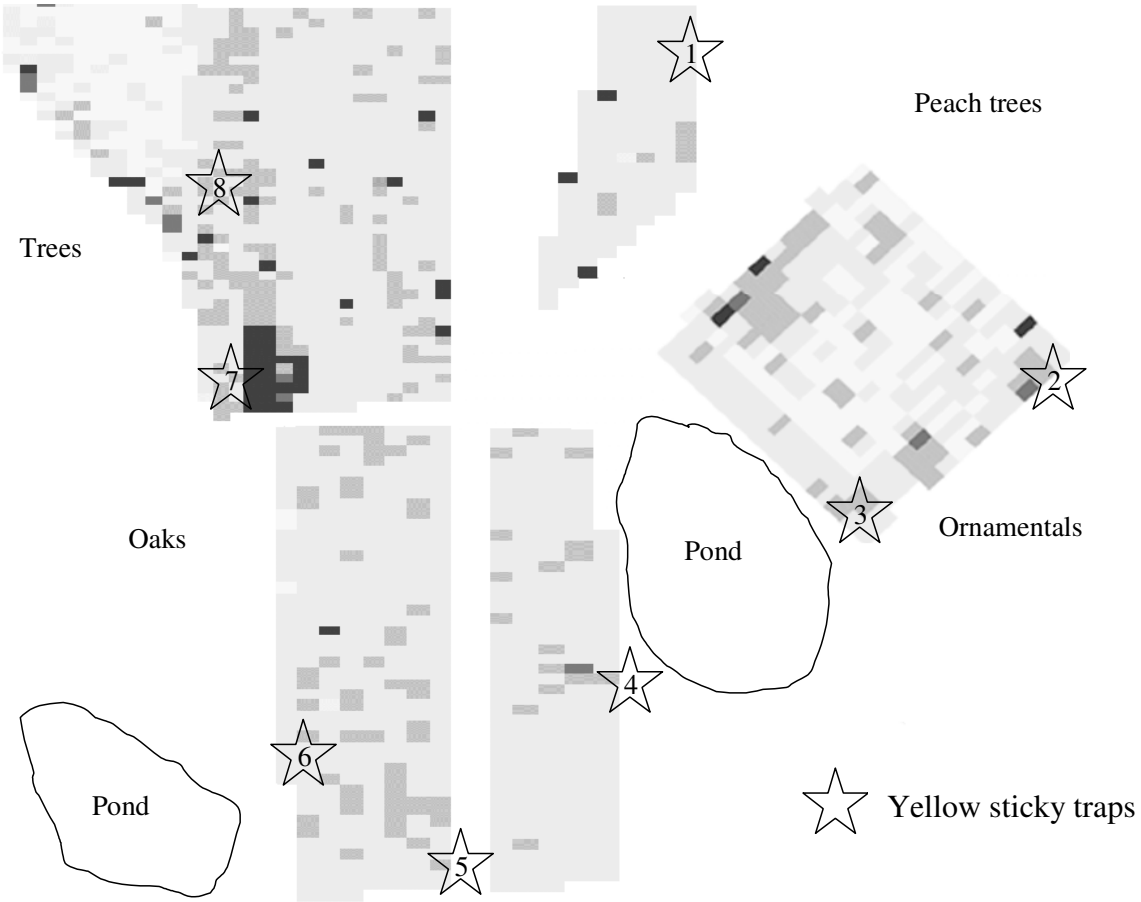
MATERIALS AND METHODS

Vines were rated in September when plants were showing optimal symptoms. A rating scale was developed for PD severity based on typical symptoms (Hopkins, 1981) where 0 = no symptoms, 1 = sporadic marginal necrosis on < 25% of leaves, 2 = necrosis of leaves on entire shoots (equivalent to 25% - 50% leaves with symptoms), 3 = the appearance of bladeless petioles with the majority of leaves necrotic (50% - 75% with symptoms), 4 = vines defoliating and fruit shrivel (75% - 100% leaves necrotic), and d = died within the season (Appendix 6.2). Most trellising systems in the vineyards consisted of bilateral cordons with vertical shoot positioning. Each cordon on a plant was assessed separately and the two ratings were averaged for a whole vine rating. Maps were made of each vineyard showing disease severity for each vine, along with yellow sticky trap placement and the location of perimeter vegetation (Appendix 6.3, 6.4, 6.5).

Appendix 6.2 Rating scale for Pierce's disease severity. 0 = no symptoms, 1? = questionable symptoms, 1 = sporadic marginal necrosis on < 25% of leaves, 2 = necrosis of leaves on entire shoots (equivalent to 25 - 50% leaves with symptoms), 3 = the appearance of bladeless petioles and the majority of leaves necrotic (50 - 75% with symptoms), 4 = defoliation occurring and fruit shrivel (75 - 100% leaves necrotic), 5 = died within the season.

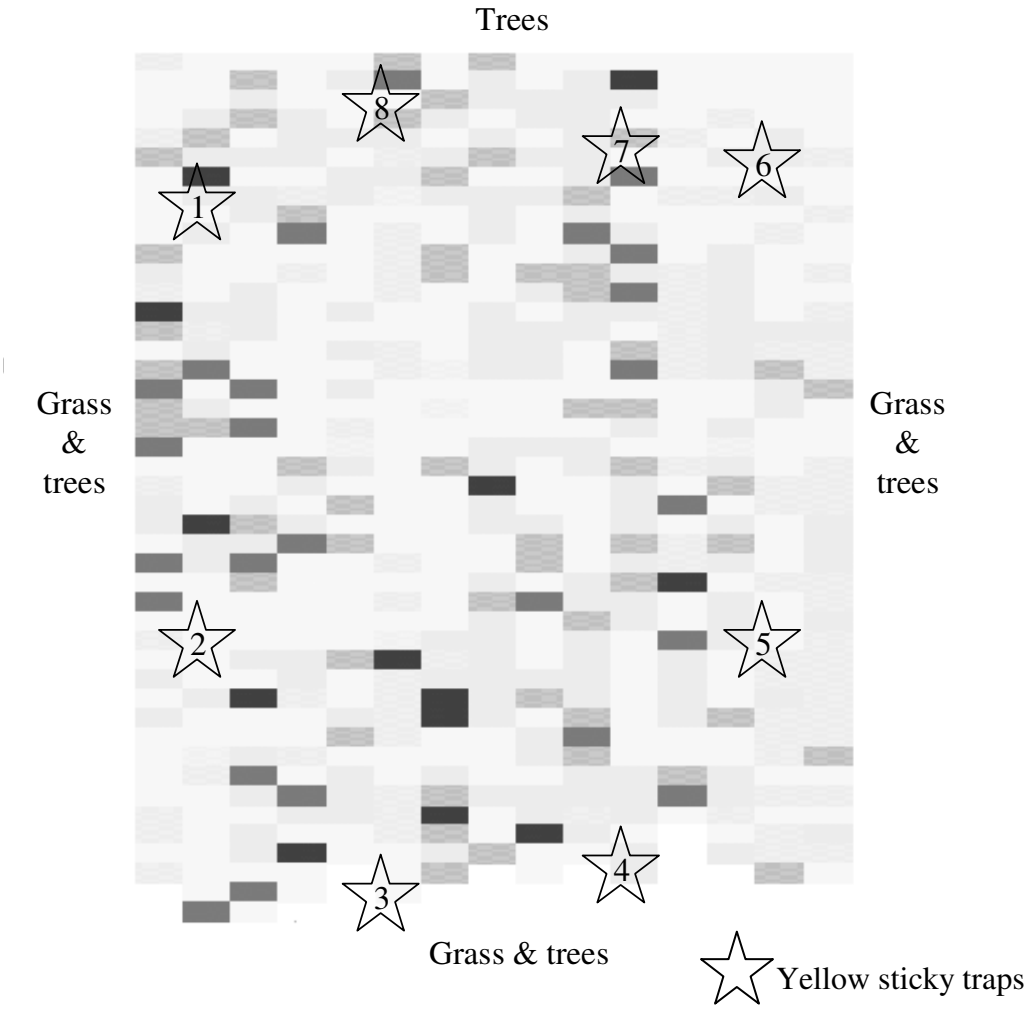


Appendix 6.3 Presence of Pierce's disease in vineyard 1 in 2004 based on visual disease symptoms. Each rectangle represents an individual vine, color-coded to correspond to its disease severity rating. The eight stars represent the placement of eight yellow-sticky traps. Landmarks and perimeter vegetation are labeled. The rating scale depicts the severity ratings of the visual disease symptoms and the number of vines in 2004 with each rating.



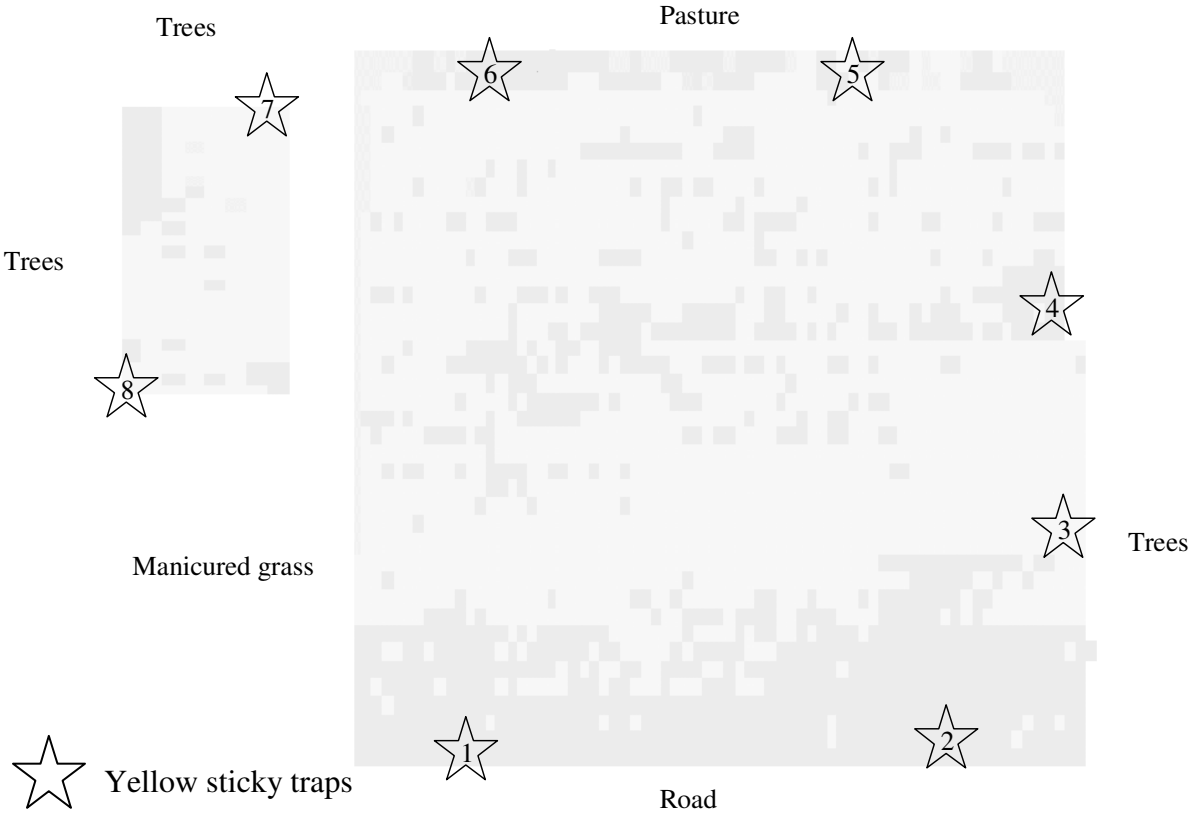
Rating	0	1	2	3	4	5
Vines (#)	7	231	1116	204	10	15

Appendix 6.4 Presence of Pierce's disease in vineyard 2 in 2004 based on visual disease symptoms. Each rectangle represents an individual vine, color-coded to correspond to its disease severity rating. The eight stars represent the placement of eight yellow-sticky traps. Landmarks and perimeter vegetation are labeled. The rating scale depicts the severity ratings of the visual disease symptoms and the number of vines in 2004 with each rating.



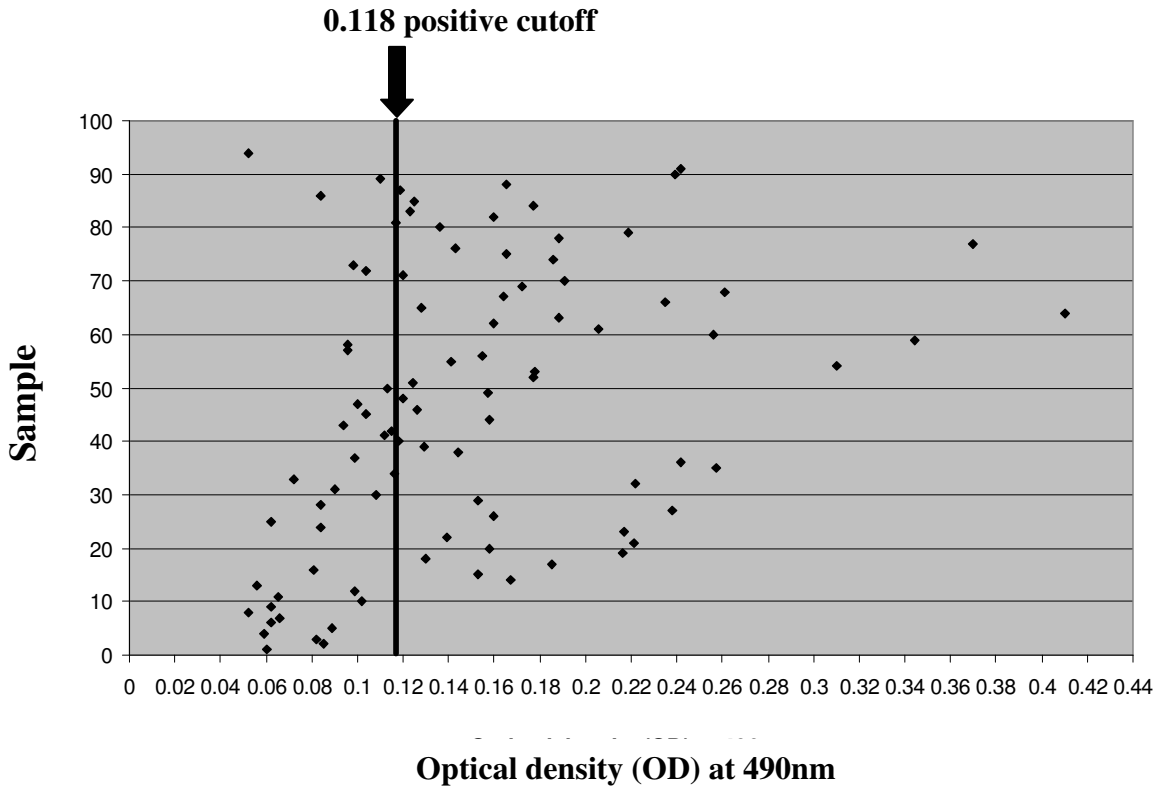
Rating	0	1	2	3	4	5
Vines (#)	61	298	161	48	26	13

Appendix 6.5 Presence of Pierce's disease in vineyard 3 in 2004 based on visual disease symptoms. Each rectangle represents an individual vine, color-coded to correspond to its disease severity rating. The eight stars represent the placement of eight yellow-sticky traps. Landmarks and perimeter vegetation are labeled. The rating scale depicts the severity ratings of the visual disease symptoms and the number of vines in 2004 with each rating.

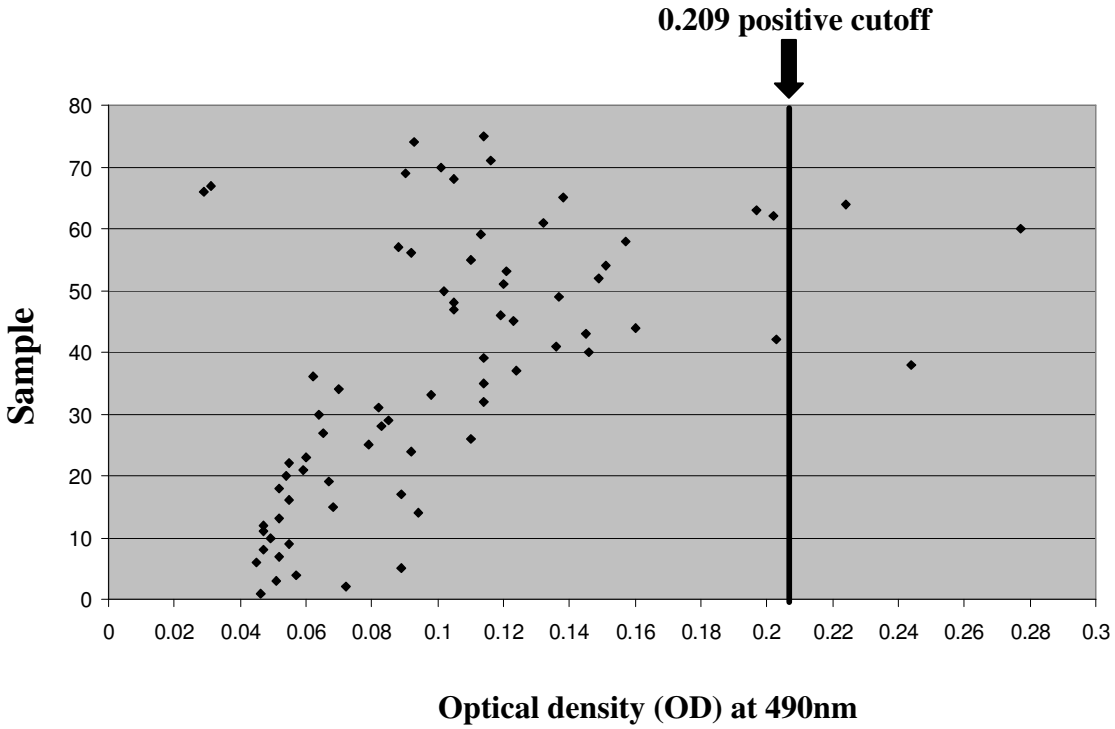


Rating	0	1	2	3	4	5
Vines (#)	7	231	1116	204	10	15

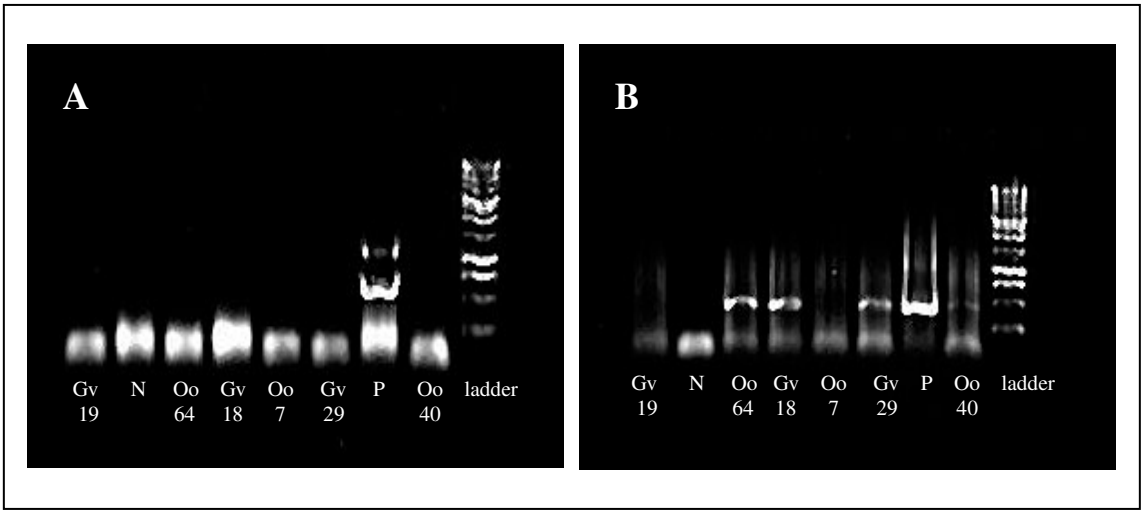
Appendix 6.6 Scatterplot of ELISA results from tests of *Oncometopia orbona* inoculated plants from transmission studies. Optical density (OD) readings were taken at 490nm. Each point represents OD values from individual plants. A positive cutoff, calculated from known negatives' OD values, of 0.118 yields 57 positive readings and 36 negative readings.



Appendix 6.7 Scatterplot of ELISA results from tests of *Graphocephala versuta* used in transmissions studies in greenhouse experiments. Optical density (OD) readings were taken at 490nm. Each point represents OD values from individual plants. A positive cutoff, calculated from known negatives' OD values, of 0.209 yields 3 positive readings and 69 negative readings.



Appendix 6.8 Horizontal gel electrophoresis of *X. fastidiosa* PCR products from *Onocemtopia orbona* and *Graphocephala versuta* transmission studies. First round PCR (A) with 272-1 and 272-2 primers amplified only the positive control. Second round or nested PCR with 272-1-int and 272-2-int primers showed two positives from *O. orbona* inoculated plants (Oo 64; Oo 40) and two positives from *G. versuta* inoculated plants (Gv 18; Gv 29), as well the positive control amplicon (P). N = negative control.



Appendix 6.9 Ouput from SNAP workbench SNAP Map.

```
Position
1111111111122222222222222222222222222222222222222222222222222233333333
33

1112344567888903335578899012233333333444444455555555566666777777788888
8888999134445678

5701573560790690225948207282578123456781234579012345789035790234678923
456789123332470298

Site Number
1111111111222222222233333333334444444444555555555566666666667777777777
888888888

1234567890123456789012345678901234567890123456789012345678901234567890
123456789012345678

Consensus
CCTCTACCCAGCACTGTACAGGCCGTGCCTTCTCACACAGAACCAACACATC
ATGACGGCCC
          TAGAAATCTTGCGGACTCCAGGTGAC
Site Type
ttvtttvvvttttttvvvttvvvvvvtttvvtvtvvvvvvtvtvvvttttvtvvvvtvtvtvvvvvvvvtttvvvvtvvvvttvtttv
t
Character Type  --------iiii-iii-i--iii-i-i-i----------------------------------------------i--i-i---
H1      (  1) ..……..CCA.T.A..……..C..
H2      (  1) T.……..C..CCA.T.A.……..A….C…
H3      (  1)
.T.TCGAAAGATGTCT.C..CC..T….ACACACACATATGAACGTGTCCGTATGAAC
TGGGCCTTTCTC
            ACACACGA....AC...
H4      (  6) ..G.……..A.……..C.….
H5      (  2) ……AGAT.TCTGC..CC.AT.……..CACT
H6      (  2) ……..C..CCA.T.A.……..A….C…
H7      (  1) ……..C..CC...C.T.……..C...
H8      (  1) ……..T.……..A.……..C.….
H9      (  1) ……..G.……..A.……..C.….
H10     ( 36) ……..A.……..C.….
H11     (  1) ……..A.……..T.C.….
H12     (  1) ……..A.……..GC.….
```

H1 B1 2005
H2 *Xf* JB-USNA (Japanese beech bonsai)
H3 *Xf* 9a5c (citrus)
H4 A4 2004, C4 2004, B4 2004, A1 2004, A4 2005, A1 2004
H5 *Xf* found-4 (coffee), *Xf* found-5 (citrus)
H6 *Xf* Ann-1 ctg- 125 (oleander), *Xf* Dixon ctg86 (almond)
H7 *Xf* Ann-1 ctg-268 (oleander)
H8 C1 2005
H9 A4 2005
H10 B4 2005, C1 2005, C4 2004, B1 2004, A1 2005, B4 2004, C4 2004, B1 2005, A1 2004, A4 2005, C1 2005, B4 2005, C4 2005, C1 2004, C1 2005, B1 2005, B4 2004, A1 2004, A4 2004, A4 2004, B4 2005, C1 2004, B1 2005, C1 2004, A4 2005, B4 2005, B1 2004, A1 2005, C4 2004, C1 2004, C4 2005, C1 2004, B4 2004, A4 2005, C4 2005, Temecula (grape)
H11 A1 2004
H12 C1 2005

Appendix 6.10 Output from SNAP workbench for Hudson's chi-squared permutation based statistic testing for population subdivision between hosts.

```
Sample configuration: 23  23  1  1  2  2  1  1
Test of Roff and Bentzen MBE 6: 539-45
Number of permutations: 1000
Observed values of statistics:
Number of alleles: 12. Ht: 0.547869  Chi: 196.043478 ( p-value: 0.002000)

 1 2: Number of alleles: 7. Ht: 0.410628  Chi: 5.695238  ( p-value: 0.697000)
 1 3: Number of alleles: 7. Ht: 0.503623  Chi: 24.000000 ( p-value: 0.213000)
 1 4: Number of alleles: 7. Ht: 0.503623  Chi: 24.000000 ( p-value: 0.208000)
 1 5: Number of alleles: 8. Ht: 0.543333  Chi: 25.000000 ( p-value: 0.058000)
 1 6: Number of alleles: 8. Ht: 0.543333  Chi: 25.000000 ( p-value: 0.045000)
 1 7: Number of alleles: 6. Ht: 0.442029  Chi: 0.347826  ( p-value: 1.000000)
 1 8: Number of alleles: 7. Ht: 0.503623  Chi: 24.000000 ( p-value: 0.178000)
 2 3: Number of alleles: 4. Ht: 0.423913  Chi: 24.000000 ( p-value: 0.094000)
 2 4: Number of alleles: 4. Ht: 0.423913  Chi: 24.000000 ( p-value: 0.079000)
 2 5: Number of alleles: 5. Ht: 0.470000  Chi: 25.000000 ( p-value: 0.015000)
 2 6: Number of alleles: 5. Ht: 0.470000  Chi: 25.000000 ( p-value: 0.012000)
 2 7: Number of alleles: 3. Ht: 0.358696  Chi: 0.274600  ( p-value: 1.000000)
 2 8: Number of alleles: 4. Ht: 0.423913  Chi: 24.000000 ( p-value: 0.076000)
 3 4: Number of alleles: 2. Ht: 1.000000  Chi: 2.000000  ( p-value: 1.000000)
 3 5: Number of alleles: 3. Ht: 1.000000  Chi: 3.000000  ( p-value: 1.000000)
 3 6: Number of alleles: 3. Ht: 1.000000  Chi: 3.000000  ( p-value: 1.000000)
 3 7: Number of alleles: 2. Ht: 1.000000  Chi: 2.000000  ( p-value: 1.000000)
 3 8: Number of alleles: 2. Ht: 1.000000  Chi: 2.000000  ( p-value: 1.000000)
 4 5: Number of alleles: 2. Ht: 0.666667  Chi: 0.750000  ( p-value: 1.000000)
 4 6: Number of alleles: 3. Ht: 1.000000  Chi: 3.000000  ( p-value: 1.000000)
 4 7: Number of alleles: 2. Ht: 1.000000  Chi: 2.000000  ( p-value: 1.000000)
 4 8: Number of alleles: 2. Ht: 1.000000  Chi: 2.000000  ( p-value: 1.000000)
 5 6: Number of alleles: 4. Ht: 1.000000  Chi: 4.000000  ( p-value: 1.000000)
 5 7: Number of alleles: 3. Ht: 1.000000  Chi: 3.000000  ( p-value: 1.000000)
 5 8: Number of alleles: 3. Ht: 1.000000  Chi: 3.000000  ( p-value: 1.000000)
 6 7: Number of alleles: 3. Ht: 1.000000  Chi: 3.000000  ( p-value: 1.000000)
 6 8: Number of alleles: 2. Ht: 0.666667  Chi: 0.750000  ( p-value: 1.000000)
 7 8: Number of alleles: 2. Ht: 1.000000  Chi: 2.000000  ( p-value: 1.000000)
```

Appendix 6.11 Output from SNAP workbench for Hudson's nearest neighbor statistic testing for population subdivision between hosts.

```
Sample configuration: 23  23  1  1  2  2  1  1
Number of permutations: 1000
 Global test:
      Snn:   0.408598 ( p-value: 0.055000)

 Pairwise tests of samples:
     1 2:   Snn:   0.483851 ( p-value: 0.643000)
     1 3:   Snn:   0.916667 ( p-value: 0.246000)
     1 4:   Snn:   0.958333 ( p-value: 0.173000)
     1 5:   Snn:   0.960000 ( p-value: 0.007000)
     1 6:   Snn:   0.900000 ( p-value: 0.033000)
     1 7:   Snn:   0.907407 ( p-value: 1.000000)
     1 8:   Snn:   0.916667 ( p-value: 0.293000)
     2 3:   Snn:   0.958333 ( p-value: 0.100000)
     2 4:   Snn:   0.958333 ( p-value: 0.060000)
     2 5:   Snn:   0.960000 ( p-value: 0.005000)
     2 6:   Snn:   1.000000 ( p-value: 0.008000)
     2 7:   Snn:   0.914474 ( p-value: 1.000000)
     2 8:   Snn:   0.958333 ( p-value: 0.068000)
     3 4:   Snn:   0.000000 ( p-value: 1.000000)
     3 5:   Snn:   0.333333 ( p-value: 0.658000)
     3 6:   Snn:   0.333333 ( p-value: 0.661000)
     3 7:   Snn:   0.000000 ( p-value: 1.000000)
     3 8:   Snn:   0.000000 ( p-value: 1.000000)
     4 5:   Snn:   0.166667 ( p-value: 1.000000)
     4 6:   Snn:   0.666667 ( p-value: 0.346000)
     4 7:   Snn:   0.000000 ( p-value: 1.000000)
     4 8:   Snn:   0.000000 ( p-value: 1.000000)
     5 6:   Snn:   0.750000 ( p-value: 0.329000)
     5 7:   Snn:   0.333333 ( p-value: 0.671000)
     5 8:   Snn:   0.333333 ( p-value: 0.663000)
     6 7:   Snn:   0.666667 ( p-value: 0.342000)
     6 8:   Snn:   0.166667 ( p-value: 1.000000)
     7 8:   Snn:   0.000000 ( p-value: 1.000000)
```

Appendix 6.12 Output from SNAP workbench for Hudson's H_{ST}, H_T, H_S statistics testing for population subdivision between hosts.

```
Sample configuration: 23  23  1  1  2  2  1  1
Number of permutations: 1000  weighting constant: 2
Observed values of statistics:
 Hst: nan , Hs: nan  Ht: 0.547869 ( p-value: 0.000000)

 1 2:  Hst: -0.010695, Hs: 0.415020  Ht: 0.410628 ( p-value: 0.779000)
 1 3:  Hst: nan,       Hs: nan       Ht: 0.503623 ( p-value: 0.000000)
 1 4:  Hst: nan,       Hs: nan       Ht: 0.503623 ( p-value: 0.000000)
 1 5:  Hst: 0.156139,  Hs: 0.458498  Ht: 0.543333 ( p-value: 0.053000)
 1 6:  Hst: 0.156139,  Hs: 0.458498  Ht: 0.543333 ( p-value: 0.042000)
 1 7:  Hst: nan,       Hs: nan       Ht: 0.442029 ( p-value: 0.000000)
 1 8:  Hst: nan,       Hs: nan       Ht: 0.503623 ( p-value: 0.000000)
 2 3:  Hst: nan,       Hs: nan       Ht: 0.423913 ( p-value: 0.000000)
 2 4:  Hst: nan,       Hs: nan       Ht: 0.423913 ( p-value: 0.000000)
 2 5:  Hst: 0.209486,  Hs: 0.371542  Ht: 0.470000 ( p-value: 0.015000)
 2 6:  Hst: 0.209486,  Hs: 0.371542  Ht: 0.470000 ( p-value: 0.012000)
 2 7:  Hst: nan,       Hs: nan       Ht: 0.358696 ( p-value: 0.000000)
 2 8:  Hst: nan,       Hs: nan       Ht: 0.423913 ( p-value: 0.000000)
 3 4:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 3 5:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 3 6:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 3 7:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 3 8:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 4 5:  Hst: nan,       Hs: nan       Ht: 0.666667 ( p-value: 0.000000)
 4 6:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 4 7:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 4 8:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 5 6:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 5 7:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 5 8:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 6 7:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
 6 8:  Hst: nan,       Hs: nan       Ht: 0.666667 ( p-value: 0.000000)
 7 8:  Hst: nan,       Hs: nan       Ht: 1.000000 ( p-value: 0.000000)
```

Appendix 6.13 Output from SNAP workbench for Hudson's K_{ST}, K_T, K_S statistics testing for population subdivision between hosts.

```
Sample configuration: 23  23  1  1  2  2  1  1
Number of permutations: 1000  weighting constant: 2
Observed values of statistics:
 Kst: nan , Ks: nan  Kt: 5.852551 ( p-value: 0.000000)

 1 2:  Kst: -0.001748, Ks: 0.754941  Kt: 0.753623  ( p-value: 0.677000)
 1 3:  Kst: nan,       Ks: nan       Kt: 1.934783  ( p-value: 0.000000)
 1 4:  Kst: nan,       Ks: nan       Kt: 2.615942  ( p-value: 0.000000)
 1 5:  Kst: 0.862097,  Ks: 1.122530  Kt: 8.140000  ( p-value: 0.002000)
 1 6:  Kst: 0.521649,  Ks: 1.122530  Kt: 2.346667  ( p-value: 0.010000)
 1 7:  Kst: nan,       Ks: nan       Kt: 1.076087  ( p-value: 0.000000)
 1 8:  Kst: nan,       Ks: nan       Kt: 1.851449  ( p-value: 0.000000)
 2 3:  Kst: nan,       Ks: nan       Kt: 1.289855  ( p-value: 0.000000)
 2 4:  Kst: nan,       Ks: nan       Kt: 1.956522  ( p-value: 0.000000)
 2 5:  Kst: 0.948672,  Ks: 0.387352  Kt: 7.546667  ( p-value: 0.006000)
 2 6:  Kst: 0.779914,  Ks: 0.387352  Kt: 1.760000  ( p-value: 0.005000)
 2 7:  Kst: nan,       Ks: nan       Kt: 0.373188  ( p-value: 0.000000)
 2 8:  Kst: nan,       Ks: nan       Kt: 1.206522  ( p-value: 0.000000)
 3 4:  Kst: nan,       Ks: nan       Kt: 16.000000 ( p-value: 0.000000)
 3 5:  Kst: nan,       Ks: nan       Kt: 49.333333 ( p-value: 0.000000)
 3 6:  Kst: nan,       Ks: nan       Kt: 4.666667  ( p-value: 0.000000)
 3 7:  Kst: nan,       Ks: nan       Kt: 11.000000 ( p-value: 0.000000)
 3 8:  Kst: nan,       Ks: nan       Kt: 1.000000  ( p-value: 0.000000)
 4 5:  Kst: nan,       Ks: nan       Kt: 42.000000 ( p-value: 0.000000)
 4 6:  Kst: nan,       Ks: nan       Kt: 12.000000 ( p-value: 0.000000)
 4 7:  Kst: nan,       Ks: nan       Kt: 19.000000 ( p-value: 0.000000)
 4 8:  Kst: nan,       Ks: nan       Kt: 15.000000 ( p-value: 0.000000)
 5 6:  Kst: nan,       Ks: nan       Kt: 39.166667 ( p-value: 0.000000)
 5 7:  Kst: nan,       Ks: nan       Kt: 51.333333 ( p-value: 0.000000)
 5 8:  Kst: nan,       Ks: nan       Kt: 48.666667 ( p-value: 0.000000)
 6 7:  Kst: nan,       Ks: nan       Kt: 8.000000  ( p-value: 0.000000)
 6 8:  Kst: nan,       Ks: nan       Kt: 4.000000  ( p-value: 0.000000)
 7 8:  Kst: nan,       Ks: nan       Kt: 10.000000 ( p-value: 0.000000)
```

Appendix 6.14 Output from SNAP workbench for Hudson's ranked Z statistic testing for population subdivision between hosts.

```
Sample configuration: 23  23  1  1  2  2  1  1
Number of permutations: 1000  weighting constant: 2
Observed values of statistics:
 Zst: nan , Zs: nan  Zt: 715.000000 ( p-value: 0.000000)

 1 2:  Zst: -0.002881, Zs: 548.936759  Zt: 547.359903  ( p-value: 0.639000)
 1 3:  Zst: nan,       Zs: nan         Zt: 638.634058  ( p-value: 0.000000)
 1 4:  Zst: nan,       Zs: nan         Zt: 646.429348  ( p-value: 0.000000)
 1 5:  Zst: 0.173701,  Zs: 584.316206  Zt: 707.148333  ( p-value: 0.002000)
 1 6:  Zst: 0.128331,  Zs: 584.316206  Zt: 670.341667  ( p-value: 0.010000)
 1 7:  Zst: nan,       Zs: nan         Zt: 574.427536  ( p-value: 0.000000)
 1 8:  Zst: nan,       Zs: nan         Zt: 634.034420  ( p-value: 0.000000)
 2 3:  Zst: nan,       Zs: nan         Zt: 574.483696  ( p-value: 0.000000)
 2 4:  Zst: nan,       Zs: nan         Zt: 581.719203  ( p-value: 0.000000)
 2 5:  Zst: 0.207088,  Zs: 513.557312  Zt: 647.685000  ( p-value: 0.006000)
 2 6:  Zst: 0.159884,  Zs: 513.557312  Zt: 611.293333  ( p-value: 0.005000)
 2 7:  Zst: nan,       Zs: nan         Zt: 506.817029  ( p-value: 0.000000)
 2 8:  Zst: nan,       Zs: nan         Zt: 569.711957  ( p-value: 0.000000)
 3 4:  Zst: nan,       Zs: nan         Zt: 1284.500000 ( p-value: 0.000000)
 3 5:  Zst: nan,       Zs: nan         Zt: 1349.000000 ( p-value: 0.000000)
 3 6:  Zst: nan,       Zs: nan         Zt: 971.833333  ( p-value: 0.000000)
 3 7:  Zst: nan,       Zs: nan         Zt: 1237.500000 ( p-value: 0.000000)
 3 8:  Zst: nan,       Zs: nan         Zt: 827.500000  ( p-value: 0.000000)
 4 5:  Zst: nan,       Zs: nan         Zt: 1026.666667 ( p-value: 0.000000)
 4 6:  Zst: nan,       Zs: nan         Zt: 1200.666667 ( p-value: 0.000000)
 4 7:  Zst: nan,       Zs: nan         Zt: 1321.500000 ( p-value: 0.000000)
 4 8:  Zst: nan,       Zs: nan         Zt: 1279.500000 ( p-value: 0.000000)
 5 6:  Zst: nan,       Zs: nan         Zt: 1290.583333 ( p-value: 0.000000)
 5 7:  Zst: nan,       Zs: nan         Zt: 1367.500000 ( p-value: 0.000000)
 5 8:  Zst: nan,       Zs: nan         Zt: 1346.500000 ( p-value: 0.000000)
 6 7:  Zst: nan,       Zs: nan         Zt: 1099.333333 ( p-value: 0.000000)
 6 8:  Zst: nan,       Zs: nan         Zt: 803.000000  ( p-value: 0.000000)
 7 8:  Zst: nan,       Zs: nan         Zt: 1173.500000 ( p-value: 0.000000)
```