

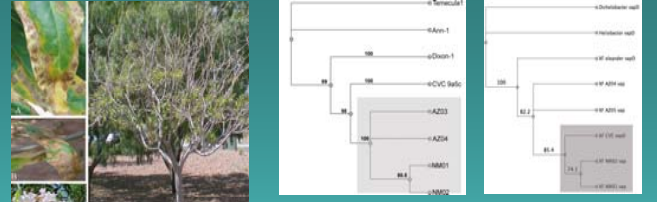
Genetic Analysis of a novel *Xylella fastidiosa* Subspecies found in the Southwestern US

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Abstract

Xylella fastidiosa, the causal agent of Pierce's disease, is associated with leaf scorch symptoms in *Chitalpa tashekensis*, a common ornamental landscape plant used throughout the Southwestern United States. Phylogenetic analysis of multiple loci was used to examine the *Xylella fastidiosa* inflicting chitalpa strains from New Mexico, Arizona and trees imported into New Mexico nurseries. These loci were compared with previously reported *X. fastidiosa* strains. Loci analyzed included the 16S ribosome, 16S-23S ribosomal intergenic spacer region, gyrB, single sequence repeat sequences, *X. fastidiosa*-specific sequences, and the virulence associated protein (VapD). This analysis indicates that the *X. fastidiosa* isolates associated with infected chitalpa trees in the Southwest are a highly related group that is distinct from the four previously defined taxa of *X. fastidiosa* subsp. *fastidiosa* (previously *X. fastidiosa* subsp. *fastidiosa* subsp. *sandyi*) and *X. fastidiosa* subsp. *paucica*. We propose classifying this chitalpa inflicting group as a new subspecies, *X. fastidiosa* subsp. *tashe*.

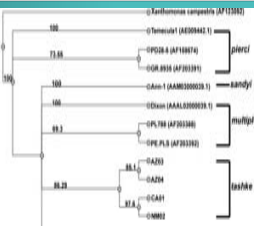


Introduction

Xylella fastidiosa is a gram negative system limited bacterium that causes serious disease problems in many diverse species. Diseases caused by *X. fastidiosa* include Pierce's disease in grapes (Dottori et al., 1978), citrus variegated chlorosis (CVCV) (Chang et al., 1993), coffee leaf scorch (Li et al., 2001), peach leaf scorch (Went and Heydrich-Allen, 2000), phony peach (Hartnett et al., 1983), gum leaf scorch (Raji et al., 1982), and almond leaf scorch (Abdelnabi and Pierce, 2004). *X. fastidiosa* is the causative agent of diseases found in landscape plants such as olivander leaf scorch (Parron et al., 1997), mulberry leaf scorch (Hernandez-Martinez et al., 2006), and oak leaf scorch (Hartnett et al., 1996). *X. fastidiosa* is transmitted by xylem feeding insect vectors such as sharpshooters, leafhoppers, and cicadas (Bridges et al., 2004).

Chitalpa (Chitalpa tashekensis Elias and Wisnisi) is an interactive hybrid between desert willow (*Chilopsis linearis* Cav.) and *Candollea bicolor* Willd. (Olson and Ramsey, 2006) that was developed for use as an ornamental landscape plant in the Southwest. *X. fastidiosa* was detected in chitalpa trees that displayed leaf scorch symptoms in southern New Mexico and Arizona. Chitalpa plants imported into New Mexico nurseries from California were the source of the infection. A multilocus phylogenetic analysis was performed to further characterize these strains of *X. fastidiosa*. This analysis indicates that the *X. fastidiosa* isolates inflicting chitalpa plants in New Mexico, Arizona and imported into nurseries from California are highly related to each other and are distinct from the previously described *X. fastidiosa* subspecies.

Chitalpa tashekensis exhibiting leaf scorch symptoms. (A) and (B) Leaf chlorosis and necrosis from symptomatic chitalpa trees. (C) Flowers of the chitalpa tree. (D) Symptomatic chitalpa tree exhibiting branch dieback.



Phylogram of concatenated sequences from eight different loci. These concatenations were used to make maximum likelihood and phylogenetic analyses using Genesoft Pro 4.0.4 and Paup 4.0. The maximum likelihood tree was bootstrapped 1000 times with consensus percentages shown at nodes. The sequences included ONSB, ANSB, CSNSB, ga casch, 27-2 (nucleo), RST, IRL, and TYPWS sequences that were separated by four new to split spatially analogous isolates included the NM and AZ chitalpa isolates and the four completely sequenced strains of *X. fastidiosa* CVCV6 (genbank AF098194), Tenevadil (AF089442), Chitalpa (AF098191), and almond (DQ06.AA.82000003.1). This analysis supports a new subspecies of *X. fastidiosa*.

Phylogram of VapD amino acid sequences. VapD translated sequences from the NM and AZ *X. fastidiosa* isolates were aligned with the vapD protein sequences from CV-93c5 (genbank NP_061707) and Olender (Amin-1, YP_001209). VapD from *Helicoverpa zea* (NP_207759) and *Dichelobacter* (YP_001209) were included with *Dichelobacter* as the out-group. The Jalco-Cantor neighbor-joining tree shown was bootstrapped 1000 times. VapD found in NM and AZ *X. fastidiosa* isolates.

Methods and Materials

New Mexico, California Chitalpa trees exhibiting leaf scorch symptoms from New Mexico, Arizona, sampled during the summer and fall of 2006. Samples were from the plant collection of nurseries, green houses, and trees. Samples were placed in plastic bags and stored at 4°C.

Bacterial culture from chitalpa leaves. Leaves were surface sterilized by washing sequentially in 70% ethanol and 1% sodium hypochlorite solution for 1 min. Leaves were then placed in sterile water and shaken for 1 min. The suspension was centrifuged at 10,000 rpm for 1 min. The supernatant was centrifuged at 10,000 rpm for 1 min. The pellet was resuspended in 100 µl of sterile nuclease-free phosphate buffer. The ground with a homogenizer. 10 µl of this extract was added to 90 µl of sterile nuclease-free phosphate buffer. Total RNA was extracted by using RNeasy spin columns.

Genotyping using PCR. PCR was performed on total DNA extracted from chitalpa trees, pruned branch systems (leafy shoot) that had been infected with *X. fastidiosa* in the past. 100 µl of total DNA was used for PCR. The primers for the PCR reaction included 16S PCR (forward: 5'-TGAAGGACAGGATGAC-3', reverse: 5'-GAGAGGAGGAGGAG-3', 16S-23S PCR (forward: 5'-TGAAGGACAGGATGAC-3', reverse: 5'-GAGAGGAGGAGGAG-3', gyrB PCR (forward: 5'-TGAAGGACAGGATGAC-3', reverse: 5'-GAGAGGAGGAGGAG-3', VapD PCR (forward: 5'-TGAAGGACAGGATGAC-3', reverse: 5'-GAGAGGAGGAGGAG-3'). PCR products were analyzed on 1% agarose gels and stained with ethidium bromide.

Phylogenetic analysis and sequence analysis. The PCR products were directly sequenced using Big Dye Terminator version 1.1.34 (Applied Biosystems). Sequencing reactions were purified using BigDye X100 purification columns (Applied Biosystems). Gels were run on a 3730XL DNA sequencer (Applied Biosystems). Sequence analysis was performed using Sequencing Analysis Software (Applied Biosystems). Phylogenetic analysis was performed using PhyML 2.4.4 (Guindon et al., 2005).

Target locus	Primer name	Primer sequence (5'→3')	Source
16S ribosome	16S forward	TGAAGGACAGGATGAC	Radionenko et al., 1998
	16S reverse	GAGAGGAGGAGGAG	Li and Dahlbeck, 1995
16S ITS - 23S	ITS forward	AGTCTGACAGGATGACGCT	Li and Dahlbeck, 1995
	ITS reverse	CTGCAAGGATGAC	
gyrB	gyrB forward	AGGAGGAGGAGGAGGAGGAG	Olson et al., 2006
	gyrB reverse	CTGCAAGGATGAC	
ANSB12	ANSB12 F	TGACATGATGATGATGATGATGAT	Olson et al., 2006
	ANSB12 R	CTGCAAGGATGAC	
SIB-OSB26	OSB26 F	TGACATGATGATGATGATGATGAT	Olson et al., 2006
	OSB26 R	CTGCAAGGATGAC	
SIB-OSB12	OSB12 F	TGACATGATGATGATGATGATGAT	Olson et al., 2006
	OSB12 R	CTGCAAGGATGAC	
PFR36	PFR36 F	TGACATGATGATGATGATGATGAT	http://www.campbellbiology.com/index.php
	PFR36 R	CTGCAAGGATGAC	
IL	IL F	AGGAGGAGGAGGAGGAGGAGGAG	Francis et al., 2004
	IL R	CTGCAAGGATGAC	
RST	RS1	GCTTATATTTTCAGGATGATGATGATGATGAT	Montgomery et al., 2004
	RS2	CAGTATGATGATGATGATGATGAT	
VapD	VapD F	CAGGAGGAGGAGGAGGAGGAGGAG	This study
	VapD R	CTGCAAGGATGAC	

Phylogram of the 16S ribosome and ITS region, and the phylogenetic tree are the isolates from chitalpa obtained from NM, AZ, and CA. The sequences for the subgroups *paucica*, *multiplis*, and *paucica* were taken from Shad 2004 and the sequences obtained from genbank are noted with the chitalpa accession numbers. The maximum likelihood method using Paup 4.0 was utilized to make the tree. The tree was bootstrapped 1000 times and bootstrapping consensus percentages are shown on the tree with *Leuonhosoma* as the outgroup. The *chitalpa* isolates comprise a new and separate subspecies as compared to those previously described.

- ## Conclusions
- Chitalpa trees in New Mexico, Arizona and imported into NM from nurseries in California are infected with *X. fastidiosa*.
 - Phylogenetic analysis of ten different loci of the *X. fastidiosa* isolates from the southwestern chitalpa samples revealed that these isolates are more genetically related to each other than previously described strains of *X. fastidiosa*.
 - Analysis of the 16S-23S ribosomal ITS based phylogeny indicates that the chitalpa isolates are different from the four established subspecies (*paucica*, *multiplis*, *sandyi*, and *multiplis*). The chitalpa isolates are a separate unique subspecies.
 - Concatamer analysis supports that the chitalpa NM and AZ strains comprise a new subspecies that is independent from the previously described *fastidiosa* subspecies.
 - Sequences potentially coding for virulence associated protein D are present in chitalpa *X. fastidiosa* strains.
 - Based on the differences noted above, we propose that the chitalpa strains are a unique subspecies that we propose calling *X. fastidiosa* subsp. *tashe* due to its discovery in and wide association with *Chitalpa tashekensis*.

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Acknowledgements

The authors would like to acknowledge Dr. Michael Hoverson for the use of the pressure chamber. We would also like to thank Bob Sautter and Jason Patten for their technical support. This work was supported by USDA grant 2006-2006-2006-2006-2006 grant #2006-02061-3.