FAST, EASY AND EFFICIENT DNA EXTRACTION AND ONE-STEP POLYMERASE CHAIN REACTION FOR THE DETECTION OF *XYLELLA FASTIDIOSA* IN POTENTIAL INSECT VECTORS

Q. Huang[1], J. Bentz[1] and J.L. Sherald[2]

[1] US Department of Agriculture, Agricultural Research Services, US National Arboretum, Floral and Nursery Plants Research Unit, Beltsville, MD 20705, USA
[2] US Department of Interior, National Park Service, Center for Urban Ecology, Washington, DC 20007, USA

SUMMARY

A quick, simple and efficient procedure for detecting *Xylella fastidiosa* in potential insect vectors is described. The procedure employs a commercially available DNeasy tissue kit for the extraction of high-quality DNA from the insect, followed by one-step polymerase chain reaction amplification using previously published oligonucleotide primers specific to *X. fastidiosa*. The procedure does not require the use of phenol, chloroform or alcohol for the precipitation of nucleic acids. Also it does not need additional purification or enrichment steps, and can be completed in less than a day. The procedure was used successfully in detecting *X. fastidiosa* in two potentially important leafhopper species, *Graphocephala versuta* and *G. coccinea,* and in a treehopper species *Entilia concisa*, collected from a nursery where bacterial leaf scorch disease caused by *X. fastidiosa* occurs.

Key words: Bacterial leaf scorch, DNA extraction, insect vectors, PCR detection, *Xylella fastidiosa.*

INTRODUCTION

Xylella fastidiosa Wells *et al.* is a fastidious bacterium that causes many economically important plant diseases including Pierce's disease of grapevine, citrus variegated chlorosis, and leaf scorch of elm, oak, oleander, sycamore and mulberry (Sherald, 2001; Hopkins and Purcell, 2002). *X. fastidiosa* is transmitted by xylem-feeding insects, such as sharpshooter leafhoppers (family Cicadellidae) and spittlebugs (family Cercopidae), see DeLong and Severin (1949, 1950). The vectors can acquire and transmit the bacterium in less than two hours (Purcell and Finley, 1979; Hill and Purcell, 1995). Adult carrier insects can transmit the bacterium for life but do not pass it to progeny (Purcell et al., 1979; Purcell and Finley, 1979).

Corresponding author: Q. Huang
Fax: +1.301.5045096
E-mail: huangq@ba.ars.usda.gov

Mention of a proprietary product does not constitute an endorsement or a recommendation by the USDA for its use.

Currently, no effective therapy for infected plants or a strategy for prevention of infection is available. Control of diseases caused by *X. fastidiosa* is limited to pruning of infected shoots, removal of infected plants, use of healthy plant materials for planting, and identification and control of insect vectors. Identification of insect vectors, however, is hampered by the lack of fast, easy and sensitive detection methods. So far, the most commonly used method for detecting *X. fastidiosa* in suspected insect vectors is enzyme-linked immunosorbent assay (ELISA), which has a limitation for detecting low numbers of bacterial cells. Use of polymerase chain reaction (PCR) to detect *X. fastidiosa* in insect vectors has been complicated by the lack of quick and easy DNA extraction protocols and by the inhibition of PCR by components in insect extracts (Vega *et al.*, 1993). An immunomagnetic separation and nested PCR method has been developed that detects low levels of bacteria in leafhoppers such as *Graphocephala versuta*, *Graphocephala coccinea*, *Erythroneura* and *Typhlocybia* species (Pooler et al., 1997). The procedure, however, requires the use of *X. fastidiosa*-specific antibody, specific IgG coated magnetic beads and a magnetic capture stand, which makes it very expensive to use. This procedure is also very time-consuming. It takes more than one day to complete since it requires time for insect preparation and bacterial separation plus time for two rounds of PCR. Although the use of insoluble acid-washed polyvinylpyrrolidone (PVPP) and Chelex 100 resin matrix for DNA extraction has been developed that allows sensitive detection of *X. fastidiosa* in citrus plants and sharpshooter leafhoppers by nested PCR (Cicapina *et al.*, 2004), it is laborious and time-consuming to prepare acid-washed PVPP and activated Chelex 100 resin, and to run two-rounds of PCR.

Recently, Rodrigues *et al.* (2003) developed a number of primer pairs, including a set C pair that targets part of the 16S rRNA gene of *X. fastidiosa* that has been used for specific detection of *X. fastidiosa* in field-collected plants, asymptomatic but infected plants, and in known insect vectors. In this study, we describe the development of a detection method that combines a commercially available DNA extraction kit with a one-step PCR using the 16S set C primers for a simple,

quick and efficient detection of *X. fastidiosa* in potential insect vectors.

MATERIALS AND METHODS

Insect samples. Insects were collected during July 22-24, 2004 from the National Park Service, Daingerfield Island Nursery in Alexandria, VA (USA). This nursery has a moderate incidence of bacterial leaf scorch in elm, mulberry, porcelain berry, and wild grape. Yellow sticky insect traps (97 cm^2, Gempler's, Belleville, WI, USA) were placed on the periphery of selected trees showing leaf scorch symptoms. Cicadellinid leafhoppers and the treehopper *Entilia concisa* caught on the traps were removed, identified to species, and placed into individual plastic storage cups based on location, trap number, and species. The insect samples were stored frozen at -20°C until used for DNA extraction.

DNA extraction from insect samples. Insect DNA from selected insect samples was extracted using a DNeasy tissue kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol "Purification of Total DNA from Animal Tissues", or using a FastDNA kit (Qbiogene, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions for DNA extraction from animal tissues, plant, bacteria and other samples, with a few modifications. For DNA extraction using the DNeasy tissue kit, whole insects were placed individually into 1.5 ml microcentrifuge tubes containing 180 µl of buffer ATL (a component of the kit), and ground using a disposable pestle. Then 20 µl of proteinase K was added to the tube, and mixed with a vortex mixer. The tube was incubated at 55°C for 1 h with occasional manual mixing to disperse the sample. Total insect DNA was eluted with 100 µl of elution buffer AE provided in the kit. For DNA extraction using the FastDNA kit, whole insects were placed individually into a FastDNA lysing matrix tube, and 1 ml of cell lysis solution TC was added to the tube. The insect tissue was homogenized in a FastPrep FP120 instrument (Qbiogene, Inc., Carlsbad, CA, USA) at speed 6 for 40 sec. Total insect DNA was eluted with 100 µl of distilled elution solution.

DNA extraction from plant samples. As a control to find out whether the DNeasy tissue and the FastDNA kits are suitable for preparing high-quality insect DNA free from substances inhibitory to PCR amplification, DNA from one healthy and two *X. fastidiosa*-infected oleander plants with leaf scorch symptoms (Huang *et al.*, 2004) was extracted individually using a DNeasy plant kit (Qiagen Inc., Valencia, CA, USA) and the same FastDNA kit (Qbiogene, Inc., Carlsbad, CA, USA). A total of 0.1 g of leaf midribs from each of the oleander plants was cut into small pieces. Half of the cut sample (0.05 g) was placed into a FastDNA lysing matrix tube containing 410 µl of buffer AP1 (for the DNeasy plant kit), and the other 0.05 g was placed into another lysing matrix tube containing 800 µl of CLS-VF, plus 200 µl of PPS (for the FastDNA kit). The tubes were put into a FastPrep FP120 instrument. The plant tissue was homogenized at speed 4 for 40 sec, followed by DNA extraction using the DNeasy plant or FastDNA kit. The plant DNA was eluted with 100 µl of the respective elution buffer provided in the kit according to the manufacturers' instructions.

PCR amplification conditions. For the one-step PCR, the reaction was performed by adding 5 µl of insect or plant DNA to 15 µl of the PCR master mix containing a final concentration of 1x *Taq* buffer, 0.125 mM dNTP, 5 pmol each of *X. fastidiosa*-specific 16S set C primers A-19 (5'-CTCCTCGCGGTTAAGCTAC-3') and S-21 (5'-GCAAATTGGCACTCAGTATCG-3') (Rodrigues *et al.*, 2003), and 1 unit of *Taq* polymerase (Qiagen Inc., Valencia, CA, USA). To test PCR inhibition effect of the FastDNA samples, 5 µl of DNA prepared by the FastDNA kit from each of the two inoculated oleander plants was mixed with 5 µl of DNA prepared by the DNeasy kit from the same inoculated plant, and the mixture was added to 10 µl of the PCR master mix.

Amplification was performed using 1 cycle of 3 min at 94°C, 30 cycles of 1 min at 94°C, 0.5 min at 55°C, and 2 min at 72°C, and a final extension of 7 min at 72°C (Rodrigues *et al.*, 2003). Twelve ng of *X. fastidiosa* genomic DNA purified from a mulberry strain (Huang and Sherald, 2004) was used as a positive control and water as a negative control. The PCR product was analyzed by electrophoresis in 1.0% agarose gels stained with ethidium bromide.

To determine its efficiency in detecting *X. fastidiosa* in potential insect vectors, the one-step PCR using the 16S set C primers was compared with a nested PCR using primer set 272 (Pooler and Hartung, 1995) that detected *X. fastidiosa* in plants and insects before (Pooler *et al.*, 1997; McElrone *et al.*, 1999). The first round of the nested PCR was performed by adding 5 µl of the insect or plant DNA to 10 µl of the PCR master mix described above using external primers 272-1 and 272-2 (Pooler and Hartung, 1995). For the second-round PCR, 4 µl of the first round reaction mixture was added to 36 µl of the PCR master mix using the internal *X. fastidiosa*-specific primers 272-1-int and 272-1-int (Pooler and Hartung, 1995). Amplification for nested PCR was carried out in a Peltier Thermal Cycler PTC-210 (MJ Research Inc., Las Vegas, Nev, USA) using the conditions described by Pooler *et al.* (1997).

Purification, cloning and sequencing of PCR products. Selected PCR products obtained by nested PCR

and by one-step PCR were purified and cloned as described before (Huang *et al.*, 2003). Both strands of the inserts in the clones were sequenced using M13 forward (-21) and M13 reverse primers, respectively, at the Genomics and Sequencing Laboratory, Auburn University, AL, USA. The sequence of the PCR product was used as a query sequence for a GenBank database search by Blastn (Altschul *et al.*, 1997).

RESULTS

Five species of leafhoppers, including *G. versuta*, *G. coccinea*, *Aulacizes irrorata*, *Draeculacephala mollipes* and *Oncometopia undata*, and a treehopper species, *Entilia consisa*, were collected from the nursery. Since *G. versuta* was the most abundant, and has tested positive for *X. fastidiosa* before (Pooler *et al.*, 1995), it was selected to compare two commercially available, widely used DNA extraction kits that have similar costs, DNeasy tissue kit (Qiagen Inc., Valencia, CA, USA) and FastDNA kit (Qbiogene Inc., Carlsbad, CA, USA), and two PCR assays, the nested PCR using the primer set 272 (Pooler and Hartung, 1995) and the one-step PCR using the recently developed 16S set C primers (Rodrigues *et al.*, 2003), for the detection of *X. fastidiosa* in potential insect vectors. We randomly chose a total of 60 insects, 20 each time, from one trap that contained the most *G. versuta*. DNA from 30 of the insects was extracted with the DNeasy tissue kit and the other 30 with the FastDNA kit. The DNA was then subjected to nested PCR or one-step PCR as described in the Materials and Methods.

We found that 15 and 14 out of 30 *G. versuta* tested positive for *X. fastidiosa* when the insect DNA was extracted with the DNeasy tissue kit followed by the nested or one-step PCR, respectively (Table 1, Fig. 1). No *G. versuta*, however, tested positive when its DNA was extracted with the FastDNA kit followed by either PCR assay (Table 1, Fig. 1). We randomly chose nested and one-step PCR products from three *X. fastidiosa*-positive (DNeasy) DNA samples for cloning and sequencing. The sequences of the PCR products were most similar to a 472-bp sequence or a partial 16S DNA of *X. fastidiosa* in GenBank by Blastn search (data not shown).

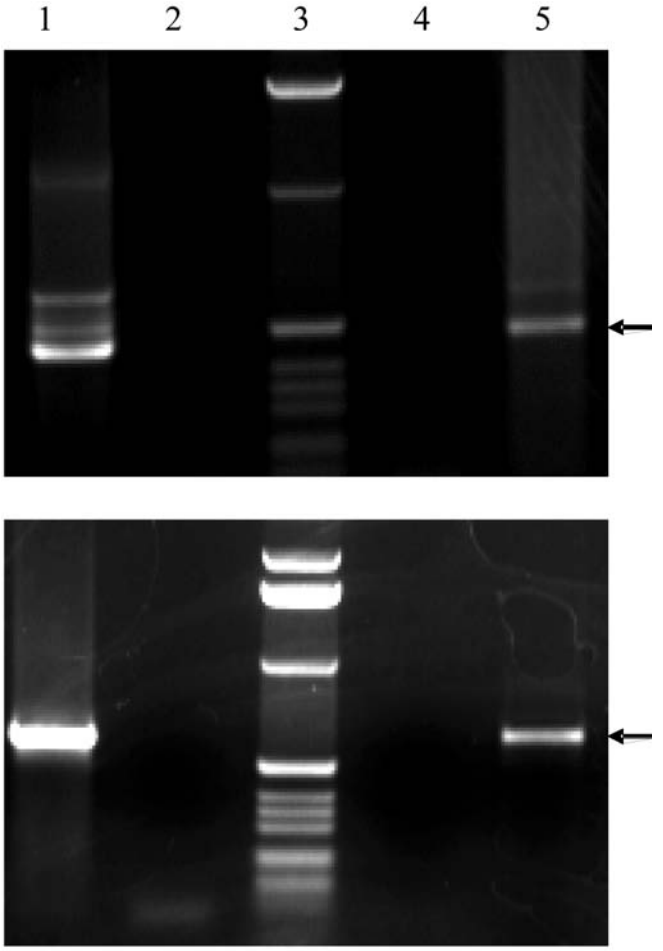

Fig. 1. Typical amplification of *X. fastidiosa* in *G. versuta* by nested PCR (Top) and one-step PCR (Bottom). Lanes: 1, positive control (*X. fastidiosa* genomic DNA); 2, water control; 3, 1-kb ladder; 4, DNA extract prepared with the FastDNA kit; 5, DNA extract prepared with the DNeasy kit. The arrows indicate the locations of the 472-bp and 620-bp PCR products at the top and bottom pictures, respectively.

To find out whether the negative PCR result of the FastDNA samples was due to the absence of *X. fastidiosa* in the insect samples or the presence of PCR inhibitors in the DNA samples prepared by the kit, we extracted leaf petiole DNA from two oleander plants previously inoculated with *X. fastidiosa* (Huang *et al.*, 2004),

Table 1. Comparison of DNA extraction and PCR methods for the detection of *X. fastidiosa* in the potential insect vector *G. versuta*.

PCR method	DNA extraction method	
	DNeasy tissue kit	FastDNA kit
Nested PCR using primer set 272	15/30 [a] (5.0+2.0) [b]	0/30 (0+0)
One-step PCR using 16S set C primers	14/30 (4.7 + 1.5)	0/30 (0+0)

[a] Number positive/number tested.
[b] Values are means of positive insect samples plus standard error of three tests, each containing ten insects.

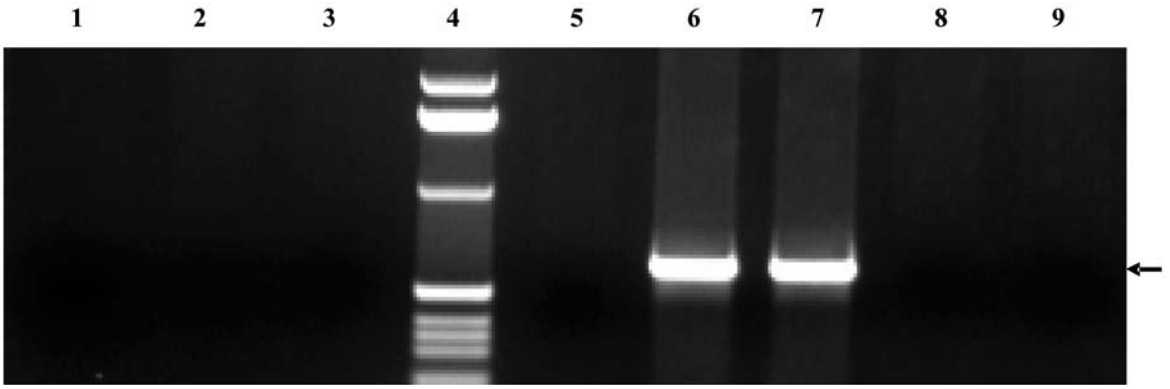

Fig. 2. Amplification of *X. fastidiosa* in oleander leaves by one-step PCR using the 16S set C primers. Lanes 1, extract prepared using the FastDNA kit with leaves from a healthy oleander plant; 2-3, extracts prepared using the FastDNA kit with each of two infected oleander plants; 4, 1-kb ladder; 5, extract prepared using the DNeasy kit with leaves from a healthy plant; 6-7, extracts prepared using the DNeasy kit with each of two infected oleander plants; 8-9, mixtures of the extract prepared by the DNeasy kit with the extract prepared by the FastDNA kit from each of the two inoculated plants. The arrow indicates the location of the 620-bp PCR product.

and from one control plant with the DNeasy plant kit and the same FastDNA kit separately. *X. fastidiosa* was detected in the two inoculated plants showing leaf scorch symptoms when the DNeasy kit but not when the FastDNA kit was used for DNA extraction, followed by PCR using the 16S set C primers (Fig. 2). The bacterium was not detected in the two inoculated plants, however, when total DNA extracted by the DNeasy kit from each of the two plant samples was mixed with the one extracted by the FastDNA kit from the same plant sample (Fig. 2). The control plant tested negative by PCR when either kit was used for DNA extraction (Fig. 2).

To determine if other insect species collected from the nursery carried *X. fastidiosa*, insect DNA from 13 adults of *E. concisa*, 5 *G. coccinea*, 4 *A. irrorata,* 3 *O. undata* and 1 *D. mollipes* was extracted individually with the DNeasy tissue kit, followed by one-step PCR using the 16S set C primers. We detected *X. fastidiosa* in 6 of the 13 *E. concisa* and 2 of the 5 *G. coccinea,* but not in any of the *A. irrorata, O. undata* or *D. mollipes* adults.

DISCUSSION

In this study, we sought to optimize conditions for DNA extraction and PCR amplification for detection of *X. fastidiosa* in potential insect vectors. Field collected adults of *G. versuta* were reported to carry *X. fastidiosa* previously by Pooler *et al.* (1997). We used *G. versuta* collected from the same nursery as Pooler *et al.* (1997) to compare the DNeasy tissue kit to the FastDNA kit for their usefulness in preparing insect DNA as a PCR amplification target. None of the *G. versuta* samples tested positive for *X. fastidiosa* by PCR when their DNA was prepared using the FastDNA kit; however, half of the 30 insect samples tested positive for the bacterium when their DNA was prepared using the DNeasy tissue kit. *X. fastidiosa* also could not be detected in leaves collected from the two leaf-scorched, inoculated oleander plants when the total DNA was extracted using the FastDNA kit, and when the FastDNA extract was added to the DNeasy extract from the same plant sample. This strongly suggests that we were unable to remove substances inhibitory to PCR amplification using the FastDNA kit.

We also tested a one-step PCR assay using the *X. fastidiosa*-specific 16S set C primers recently developed by Rodrigues *et al.* (2003) for detection of the bacterium in potential insect vectors. The set C primers target part of the 16S rRNA gene that has two copies in *X. fastidiosa* (Simpson *et al.*, 2000). This amplicon, therefore, may also be useful for phylogenetic analysis and tracking the mobility of *X. fastidiosa* isolates between different plants and insects in the environment. We also tested the nested PCR method using the primer set 272 that successfully detected *X. fastidiosa* in several insects (Pooler *et al.*, 1997) and plants (McElrone *et al.*, 1999) as a control, and for comparison with the one-step PCR. The 272 primers were designed based on randomly amplified polymorphic DNA that has only one copy present in a non-coding region of *X. fastidiosa*. We found that an almost comparable number of *G. versuta* were detected carrying *X. fastidiosa* by the one-step PCR and by the nested PCR, suggesting that efficient detection of *X. fastidiosa* can be obtained using the one-step PCR. DNA sequencing of selected PCR products cloned from the two PCR assays confirmed that they are most similar to a partial 16S DNA or a 472-bp sequence of *X. fastidiosa* in GenBank. Since degenerate primers for bacterial 16S rRNA genes are available (Weisburg *et al.*, 1991), the sensitivity of the 16S set C primers can be further improved, if needed, in a nested PCR using the

degenerate primers in the first round and the 16S set C primers in the second round. Nevertheless, efficient detection of *X. fastidiosa* in potential insect vectors by one-step PCR will not only save time and materials but also avoid contamination problems commonly associated with nested PCR.

We describe a rapid, technically easy and convenient method for efficient detection of *X. fastidiosa* in potential insect vectors. Our method combines the commercially-available DNeasy tissue kit for the preparation of insect DNA with a one-step PCR protocol using *X. fastidiosa*-specific 16S set C primers. Organic solvents, precipitation with ethanol, *X. fastidiosa*-specific antibody, specialized equipment, and nested PCR are not required. The entire protocol for detection of *X. fastidiosa* in insects, from DNA extraction and amplification to gel electrophoresis and visual documentation, can be performed in less than a day, and is well suited for testing large numbers of samples. The protocol may also be applied to plant samples by substituting the DNeasy tissue with the DNeasy plant kit as done for the oleander samples in this study, although a more economic tissue grinding method needs to be developed. By using this protocol, we also detected *X. fastidiosa* in the leafhopper *G. coccinea* and in the treehopper *Entilia concisa.* Of the five leafhopper species sampled, *G. versuta* and *G. coccinea* were found carrying *X. fastidiosa* previously as reported by Pooler *et al.* (1997) using immunomagnetic separation and nested PCR. This is the first report of *E. concisa* testing positive for *X. fastidiosa*. The role and importance of *G. versuta*, *G. coccinea* and *E. concisa* in transmitting the bacterium and causing bacterial leaf scorch diseases in landscape trees remains to be determined.

ACKNOWLEDGMENTS

We thank John Hartung and Ing-Ming Lee, USDA-ARS, and John Hartman, University of Kentucky, for critical review of this manuscript. We also thank Jeffrey Rex and April Stehr for technical assistance.

REFERENCES

Altschul S.F., Madden T.L., Schaffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Ciapina L.P., Carareto Alves L.M., Lemos E.G.M., 2004. A nested-PCR assay for detection of *Xylella fastidiosa* in citrus plants and sharpshooter leafhoppers. *Journal of Applied Microbiology* **96**: 546-551.

DeLong D.M., Severin H.H.P., 1949. Characters, distribution, and food plants of leafhopper vectors of virus causing Pierce's disease of grapevines. *Hilgardia* **19**: 71-186.

DeLong D.M., Severin H.H.P., 1950. Spittle-insect vectors of Pierce's disease virus I. Characters, distribution, and food plants. *Hilgardia* **19**: 339-355.

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

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

Huang Q., Brlansky R.H., Barnes L., Li W., Hartung J.S., 2004. First report of oleander leaf scorch caused by *Xylella fastidiosa* in Texas. *Plant Disease* **88**: 1049.

Huang Q., Li W., Hartung, H.S., 2003. Association of *Xylella fastidiosa* with leaf scorch in Japanese beech bonsai. *Canadian Journal of Plant Pathology* **25**: 401-405.

Huang Q., Sherald J.L., 2004. Isolation and phylogenetic analysis of *Xylella fastidiosa* from its invasive alternative host, porcelain berry. *Current Microbiology* **48**: 73-76.

McElrone A.J., Sherald J.L., Pooler M.R., 1999. Identification of alternative hosts of *Xylella fastidiosa* in the Washington, D.C., area using nested polymerase chain reaction (PCR). *Journal of Arboriculture* **25**: 258-263.

Pooler M.R., Hartung 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.L., 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.

Purcell A.H., Finley A.H., 1979. Evidence for noncirculative transmission of Pierce's disease bacterium by sharpshooter leafhoppers. *Phytopathology* **69**: 393-395.

Rodrigues J.L.M., Silva-Stenico M.E., Gomes J.E., Lopes J.R.S., Tsai S.M., 2003. Detection and diversity assessment of *Xylella fastidiosa* in field-collected plant and insect samples by using 16S rRNA and *gyrB* sequences. *Applied and Environmental Microbiology* **69**: 4249-4255.

Sherald J.L., 2001. *Xylella fastidiosa*, a bacterial pathogen of landscape trees. In: Ash C.L. (ed.). Shade tree wilt diseases, pp. 191-202. APS Press, St. Paul, USA.

Simpsons A.J.G., Reinach F.C., Arruda P. *et al.*, 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* **406**: 151-157.

Vega F.E., Davis R.E., Barbosa P., Dally E.L., Purcell A.H., Lee I.M., 1993. Detection of a plant pathogen in a nonvector insect species by the polymerase chain reaction. *Phytopathology* **83**: 621-624.

Weisburg W.G., Barns S.M., Pelletier D.A., Lane D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**: 697-703.

Received 24 May 2005
Accepted 26 October 2005