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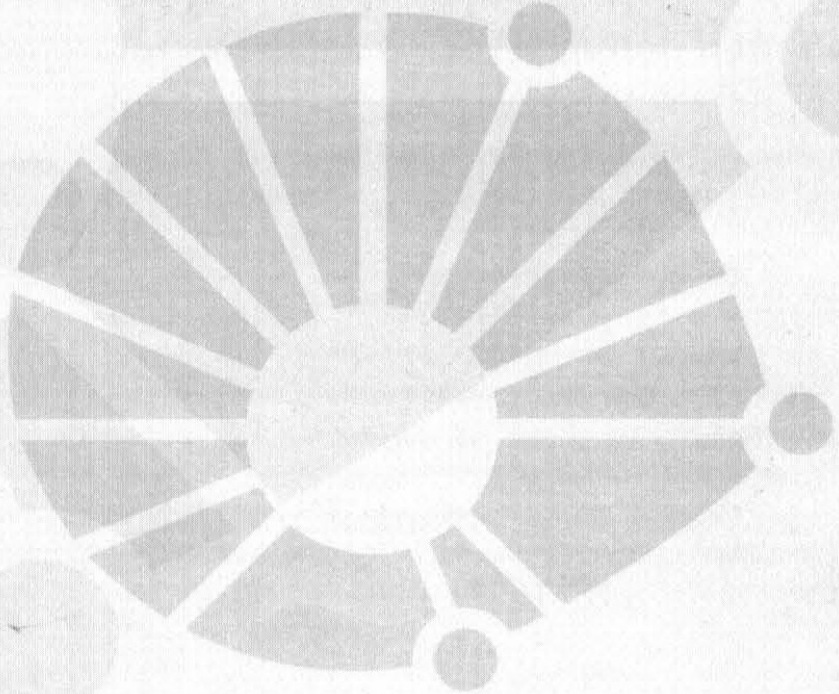

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## INSTITUTO DE BIOLOGIA

UNIVERSIDADE ESTADUAL DE CAMPINAS

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# DIVERSIDADE GENÉTICA EM LINHAGENS DE *Xylella fastidiosa* ISOLADAS DE CITROS



Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas, para obtenção do título de Mestre em Genética e Biologia Molecular – Área de Concentração Genética de Microrganismos

Orientadora: Profa. Dra. Yoko Bomura Rosato

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**RESUMO**

*Xylella fastidiosa* é uma bactéria Gram-negativa associada a doenças em culturas de grande importância econômica como videira, citros, café e ameixa, entre outras. Neste estudo, as relações genéticas de linhagens de *X. fastidiosa* isoladas de citros de diferentes regiões geográficas no Brasil foram investigadas. Linhagens de café, videira, ameixa e pêra foram também incluídas para comparação. A diversidade genética foi determinada através de técnicas moleculares, incluindo amplificação de DNA utilizando os *primers* ERIC, REP e BOX (rep-PCR), RFLP do gene RNAr 16S e da região espaçadora DNAr 16S-23S, RAPD e SDS-PAGE de proteínas. Na análise RFLP de DNAr 16S e região espaçadora DNAr 16S-23S, um baixo nível de polimorfismo foi observado. As técnicas de rep-PCR e RAPD revelaram um maior nível de polimorfismo e, além da relação com os hospedeiros, variação entre as linhagens de citros também foi encontrada. Linhagens de citros isoladas da região sul, compreendendo os estados do Rio Grande do Sul, Santa Catarina e Paraná formaram um grupo, enquanto que as linhagens isoladas de São Paulo e Sergipe formaram outro grupo. Na análise do perfil de proteínas obtido por SDS-PAGE, uma relação com os hospedeiros também foi revelada. A linhagem de pêra permaneceu distantemente relacionada às demais linhagens de *Xylella* em todas as análises. A hibridização DNA:DNA revelou uma homologia acima de 80% para as linhagens de videira, ameixa, citros e café, indicando que essas linhagens pertencem a uma mesma espécie genômica. A linhagem de pêra apresentou uma homologia abaixo de 20%, indicando que esta linhagem não pertence à espécie *X. fastidiosa*. As relações filogenéticas foram determinadas através do sequenciamento de DNAr 16S e região espaçadora DNAr 16S-23S de linhagens de diferentes hospedeiros. As árvores filogenéticas obtidas revelaram dois grupos principais, sendo que as linhagens de citros, café e ameixa formaram um grupo separado das linhagens de videira.

## INTRODUÇÃO

*Xylella fastidiosa* é uma bactéria Gram-negativa, limitada ao xilema das plantas, responsável por doenças em culturas de grande importância econômica como o mal de Pierce em videira, escaldadura da folha da ameixeira (EFA), clorose variegada dos citros (CVC) e escaldadura da folha do cafeeiro (EFC). A bactéria possui uma ampla gama de hospedeiros que incluem plantas mono e dicotiledôneas e é transmitida por enxerto de material contaminado e/ou cigarrinhas (Brlansky *et al.*, 1983).

Atualmente, todas as linhagens de *Xylella* pertencem a uma única espécie, entretanto, há diferenças em relação aos hospedeiros (Hopkins, 1989), requerimentos nutricionais (Hopkins, 1988), patogenicidade (Hopkins, 1984), e homologia de DNA (Chen *et al.*, 1992; 1995; Kamper *et al.*, 1985). Estudos de homologia revelaram que linhagens de *X. fastidiosa* obtidas de videira com o mal de Pierce são geneticamente uniformes (Leite *et al.*, 1993; Minsavage *et al.*, 1994) e constituem um grupo distinto daquele formado por linhagens associadas à redução de porte do pessegueiro, EFA e definhamento da vinca (Kamper *et al.*, 1985). A análise genética de algumas linhagens de *X. fastidiosa* isoladas de CVC e EFC revelou que linhagens associadas a essas doenças aparentemente estão mais relacionadas às do mal de Pierce da videira (Leite *et al.*, 1993; Minsavage *et al.*, 1994). Um estudo foi realizado com linhagens isoladas de citros do estado de São Paulo e dois haplótipos foram obtidos. Entretanto, não há uma caracterização extensa das linhagens de *X. fastidiosa* que ocorrem em diferentes regiões geográficas no Brasil.

Poucos estudos têm sido efetuados com a *X. fastidiosa* desde 1973, quando foi descrita pela primeira vez, em videira. Esta bactéria foi inicialmente confundida com um tipo de rickettsia e apenas em 1987 foi identificada como uma nova espécie (Wells *et al.*, 1987). Cinco linhagens

foram analisadas com relação à homologia de DNA e foram detectados valores entre 75 a 100%, indicando uma uniformidade dentro da espécie, mesmo quando proveniente de hospedeiros distintos como a videira e ameixeira (Kamper *et al.*, 1985; Wells *et al.*, 1987). Outros dados, de sorologia e ácidos graxos, confirmaram a similaridade entre as linhagens, entretanto, o número de linhagens analisadas foi relativamente baixo.

*X. fastidiosa* apresenta especificidade patogênica e grupos distintos têm sido delineados para a bactéria utilizando técnicas moleculares como RAPD (*random amplified polymorphic DNA*), RFLP (*restriction fragment length polymorphism*) e hibridização DNA:DNA (Chen *et al.*, 1992, 1995, Kamper *et al.*, 1985, Pooler & Hartung, 1995). Entretanto, as relações genéticas, sorológicas e de patogenicidade entre os diferentes patotipos de *X. fastidiosa* ainda não estão bem estabelecidas.

## OBJETIVOS

A escassez de informações sobre as relações genéticas e filogenéticas entre linhagens de *X. fastidiosa* que ocorrem no Brasil levou à realização de estudos para uma maior compreensão da estrutura das populações da bactéria. O presente trabalho teve por objetivo:

1. Caracterizar linhagens de *X. fastidiosa* através da análise da diversidade genética utilizando as técnicas de RFLP de rDNA 16S e região espaçadora 16S-23S, rep-PCR (PCR utilizando *primers* para seqüências repetitivo), RAPD, e SDS-PAGE (*sodium dodecyl sulfate polyacrylamide gel electrophoresis*) de proteínas totais

2. Estabelecer as relações filogenéticas entre linhagens de *X. fastidiosa* de diferentes hospedeiros através da análise de seqüências de rDNA 16S e região espaçadora 16S-23S

3. Investigar as relações taxonômicas de linhagens de *X. fastidiosa* de diferentes hospedeiros através de hibridização DNA:DNA

## REVISÃO DE LITERATURA

### *Xylella fastidiosa:* características gerais

Bactérias da espécie *X. fastidiosa* foram inicialmente associadas à membros da família Rickettsiaceae devido às similaridades na morfologia e ultra-estrutura da célula bacteriana e requerimentos nutricionais (Hopkins, 1989). Entretanto, foi verificado que o DNA de *X. fastidiosa* apresenta conteúdo de G+C entre 49,5 e 53,1 mol% (Wells *et al.*, 1987), enquanto que para a rickettsia *Rochalimaea quintana* esses valores estão em torno de 38,5 mol% (Davis *et al.*, 1980). Além disso, em estudos de homologia de DNA, a relação entre *X. fastidiosa* e espécies da família Rickettsiaceae foi de somente 2% e portanto a bactéria *X. fastidiosa* foi classificada como uma nova espécie (Wells *et al.*, 1987). *X. fastidiosa* foi caracterizada como sendo aflagelada, oxidase negativa e catalase positiva. As células possuem a forma de bastonetes medindo 0,25-0,35 x 0,9-3,5 μm, são aeróbicas, não-fermentativas e tem como temperatura ótima para crescimento ao redor de 28 °C.

### CVC e outras doenças causadas por *X. fastidiosa*

A clorose variegada dos citros (CVC), causada por *X. fastidiosa*, é hoje uma das doenças mais graves da citricultura no Brasil. A CVC foi inicialmente constatada na região noroeste do estado de São Paulo em 1987 (Rossetti *et al.*, 1990) e desde então atingiu todas as regiões citrícolas do estado. Atualmente a CVC encontra-se disseminada em praticamente todas as regiões produtoras de citros no país, incluindo os estados de Minas Gerais, Paraná, Rio Grande do Sul e Sergipe, entre outros (Tubelis *et al.*, 1993). A CVC atinge todas as variedades comerciais de

laranja doce, afetando principalmente a qualidade dos frutos, tornando-os impróprios para a comercialização. A CVC é também conhecida como "amarelinho" devido ao aspecto amarelado das folhas afetadas e dos frutos precocemente amadurecidos.

O patógeno é transmitido por insetos vetores da família Cicadellidae (de Negri & Garcia Jr., 1993) sendo as espécies de cigarrinhas mais comumente envolvidas na transmissão *Acrogonia terminalis, Dilobopterus costalimai* e *Oncometopia fascialis* (Roberto *et al.*, 1996). Recentemente, as espécies *Acrogonia virescens, Bucephalogonia xanthophis, Ferrariana trivittata, Homalodisca ignorata, Macugonalia leucomelas, Parathona gratiosa, Plesiommata corniculata* e *Sonesimia grossa* também foram reportadas como vetores, totalizando 11 espécies transmissoras da CVC (Revista do Fundecitrus, 1999).

*X. fastidiosa* também está associada ao mal de Pierce da videira, atualmente responsável por prejuízos na produção comercial de uvas no sudeste dos Estados Unidos (Hopkins, 1977). Os sintomas das plantas infectadas incluem necrose na borda das folhas, declínio do vigor da planta e baixa produção (Raju & Wells, 1986). Outra doença causada por *X. fastidiosa* é a escaldadura da folha da ameixeira (EFA) reportada inicialmente nos Estados Unidos em 1977, encontrada também na Argentina, Brasil e Paraguai (Raju & Wells, ,1986). Plantas com a doença apresentam sintomas semelhantes aos de videira com o mal de Pierce (Raju & Wells, 1986). No Brasil, a EFA foi constatada na década de 70 e foi a principal responsável pelo declínio da cultura de ameixa nos estados das regiões sul e sudeste do país (French & Kitajima, 1978; Kitajima *et al.*, 1981; Mohan *et al.*, 1980). A escaldadura da folha da pereira foi reportada por Leu & Su (1993) em Taiwan, e representa a segunda constatação de doença causada por *X. fastidiosa* no hemisfério oriental. O sintoma mais característico de plantas infectadas é a morte dos ramos (Leu & Su,

1993). Mais recentemente, plantas de cafeeiro têm sido reportadas como hospedeiro para *X. fastidiosa,* causando o depauperamento da planta (Beretta *et al.*, 1996; Paradela *et al.*, 1997). Plantas infectadas apresentam internódios curtos e tufos de folhas nas pontas dos ramos, podendo ou não apresentar deficiências de minerais, principalmente de zinco (Paradela *et al.*, 1997).

Os sintomas de doenças causadas por *X. fastidiosa* incluem a requeima do bordo de folhas, queda de folhas, morte de ponteiros, redução no tamanho de frutos e declínio no vigor das plantas. Esses sintomas sugerem que o mecanismo envolvido nessas doenças está relacionado com a disfunção do sistema de transporte de água da planta. De fato, células bacterianas, tiloses e goma têm sido encontradas bloqueando os vasos do xilema de plantas infectadas por *X. fastidiosa* (Mircetich *et al.*, 1976; Mollehauer e Hopkins., 1974, 1976). Entretanto, a presença de fitotoxinas (Lee *et al.*, 1992; Mircetich *et al.*, 1976) e o desbalanço hormonal (French & Stassi, 1978) também têm sido considerados.

**Métodos para detecção de variabilidade genética**

Os métodos tradicionais para caracterização de bactérias fitopatogênicas envolvem a realização de testes bioquímicos, sorológicos e de patogenicidade (Schaad, 1988). Recentemente, métodos moleculares baseados na análise de proteínas e principalmente ácidos nucléicos têm sido predominantemente utilizados para a caracterização bacteriana (Gabriel *et al.*, 1989; Hildebrand *et al.*, 1990; Palleroni *et al.*, 1993; Vauterin *et al.*, 1991, 1993). Neste estudo foram utilizadas técnicas que possuem diferentes níveis de resolução para detectar variabilidade entre as linhagens no nível específico e/ou infra-específico.

## RAPD

A técnica de RAPD é uma das mais utilizadas para a detecção de variabilidade genética. Este método foi introduzido em 1990 (Welsh & McClelland, 1990; Williams *et al.*, 1990) e tem sido aplicado na diferenciação de linhagens de diferentes grupos de procariotos, incluindo bactérias Gram-positivas e -negativas (Lawrence *et al.*, 1993; Grajal-Martin *et al.*, 1993; Cave *et al.*, 1994). Nessa abordagem, *primers* de seqüência arbitrária anelam ao DNA molde em baixa estringência produzindo um perfil de fragmentos típico para a linhagem, possibilitando a tipagem de organismos. Além da diferenciação entre indivíduos, é possível obter informações genéticas das populações, comparáveis àquelas obtidas com análises de RFLP e hibridização (Harrison *et al.*, 1992).

RAPD é um método sensível para determinar as relações genéticas entre organismos patogênicos (Pooler *et al.*, 1996) e tem como vantagem principal o fato de não requerer conhecimento prévio da estrutura genômica do organismo em análise (Cave et al, 1994: Grajal-Martin *et al.*, 1993; Lawrence *et al.*, 1993). A obtenção do perfil de RAPD demanda pequenas quantidades de DNA, o que torna o método especialmente útil para organismos que crescem lentamente, como a *X. fastidiosa*.

## Seqüências REP, ERIC e BOX

Uma abordagem relativamente nova introduzida no estudo da taxonomia de bactérias envolve uma técnica rápida e altamente reproduzível baseada em amplificação de PCR. Esta técnica, denominada rep-PCR, utiliza *primers* baseados em seqüências de DNA repetitivas (rep) dispersas nos genomas de diversas espécies bacterianas (Lows *et al.*, 1994). As seqüências rep

compreendem os elementos REP (*repetitive extragenic palindromes;* Stern *et al.*, 1984), ERIC (*enterobacterial repetitive intergenic consensus*; Sharples & Lloyd, 1990) e BOX (*Box element*; Martin *et al.*, 1992).

As seqüências REP consistem de repetições invertidas de 35-40 pb e são encontradas em *clusters*, onde cópias sucessivas são organizadas em orientação alternada (Martin *et al.*, 1992). O número de cópias varia entre 500 a 1000 no cromossomo de *Escherichia coli* e *Salmonella typhimurium* (Gilson *et al.*, 1984; Stern *et al.*, 1984). As seqüências ERIC têm o tamanho de 124-127 pb e o número de cópias é de 30-50 em *E. coli* e 150 em *S. typhimurium* (Sharples & Lloyd, 1990; Hulton *et al.*, 1991). Tanto as seqüências ERIC como os elementos REP estão localizados em regiões não-codificadoras, mas são provavelmente transcritos e possuem um potencial para formar estruturas secundárias (Martin *et al.*, 1992). As seqüências BOX, com relação ao tamanho e número de cópias, são mais semelhantes às seqüências ERIC (Martin *et al.*, 1992). Acredita-se que essas seqüências tenham um papel na replicação, estando envolvidas na ligação da DNA girase e terminação da transcrição, entre outras funções (Versalovic *et al.*, 1991).

A utilização dos *primers* rep tem permitido a caracterização de bactérias Gram-negativas e -positivas devido a complexidade dos produtos gerados pela amplificação por PCR (de Bruijn, 1992; Louws *et al.*, 1995). A técnica de rep-PCR tem sido considerada particularmente vantajosa para a análise de grandes populações de bactérias por ser um método simples e rápido, podendo ser utilizada na caracterização de bactérias em diferentes níveis taxonômicos (Vera Cruz *et al.*, 1996; Lows *et al.*, 1995).

**SDS-PAGE de proteínas**

Outra abordagem muito utilizada na classificação bacteriana é a separação de proteínas totais em SDS-PAGE (Kersters, 1985). A eletroforese de proteínas é uma técnica rápida para caracterizar e comparar um grande número de linhagens e fornece informações a respeito da similaridade entre linhagens dentro de uma mesma espécie (Kersters, 1985). SDS-PAGE de proteínas tem sido utilizada com eficiência na diferenciação de grupos e patovares em espécies como *Xanthomonas campestris*, uma bactéria filogeneticamente relacionada a *X. fastidiosa* (Vauterin *et al.*, 1991; Bouzar *et al.*, 1994). Além de ser utilizada na detecção de diversidade genética e classificação bacteriana, esta técnica também pode ser utilizada para caracterizar novas linhagens (Vauterin *et al.*, 1991). Análises de SDS-PAGE têm demonstrado correlação com dados obtidos por outras técnicas, como cromatografia de ácidos graxos celulares e hibridização DNA:DNA (Stead, 1989; Vauterin *et al.*, 1990).

**Análise de rDNA 16S**

A maior parte dos estudos das relações filogenéticas de bactérias é baseada na análise dos genes do rRNA (Woese, 1987), que incluem os genes 16S, 5S e 23S. O rDNA 16S tem sido o mais utilizado para delinear as relações entre diversos grupos no nível de família, gênero e espécie (Yabuuchi *et al.*, 1990; Busse *et al.*, 1992). A comparação das seqüências de nucleotídeos desse gene em linhagens filogeneticamente próximas mostra que, além das regiões conservadas, que são importantes para investigações filogenéticas mais distantes, fragmentos altamente variáveis também estão presentes nessas moléculas, os quais podem ser utilizados para diferenciação no nível específico e infra-específico (Busse *et al.*, 1996).

O uso de rDNA 16S na filogenia bacteriana tem aumentado consideravelmente e atualmente um número crescente de espécies tem sido descrito sem os outrora requeridos exames de reassociação de DNA (Vandamme *et. al.*, 1996). Esta posição tem gerado controvérsias devido ao pequeno número de diferenças detectadas em alguns gêneros como *Bacillus* (Fox *et al.*, 1992). Stackebrandt e Goebel (1994) propõem que os estudos da seqüência de rDNA 16S possam substituir os estudos de hibridização de DNA como parte da descrição de uma nova espécie. Neste caso, o nível de similaridade deve estar abaixo de 97% e as seqüências de rDNA 16S de todos os taxa relevantes devem estar disponíveis para comparação.

**Análise da região espaçadora rDNA 16S-23S**

A análise da região espaçadora rDNA 16S-23S tem se mostrado uma abordagem promissora para a caracterização de bactérias, explorando a variação genética presente nessa região (Barry *et al.*, 1991). Os genes que codificam os rRNAs de bactérias são separados por regiões espaçadoras internas, que apresentam variações na seqüência e no tamanho (Barry *et al.*, 1991, Navarro *et al.*, 1992, Leblond-Bourget *et al.*, 1996). Na região espaçadora, podem ser encontrados genes que codificam para tRNAs, que na maioria das bactérias são encontrados na forma de *clusters*, separadas por uma curta região espaçadora. As variações encontradas na região intergênica são devido em parte ao número e tipo de genes de tRNA presentes nessa região (Gürtler & Stanisich, 1996).

Ao contrário dos genes de rRNA, que são altamente conservados, a região espaçadora 16S-23S é mais variável em tamanho e seqüência de nucleotídeos (Dolzani *et al.*, 1995).

Dependendo do grau de variação encontrado nessa região, o polimorfismo pode ser utilizado para distinção no nível de gênero, espécie (Barry *et al.*, 1991) ou linhagem (Dolzani *et al.*, 1994).

**Hibridização DNA:DNA**

A hibridização DNA:DNA tem se constituído na base para determinar a identidade de isolados bacterianos (Wayne *et al.*, 1987). Esta técnica é considerada o padrão de referência para a taxonomia de bactérias, e é recomendada para a caracterização de novas linhagens no nível de espécie (Stackebrandt & Goebel, 1994). Um comitê *ad hoc* do Comitê Internacional de Sistemática de Bactérias estipulou que a hibridização DNA:DNA representa a melhor técnica para identificar linhagens no nível de espécie (Wayne *et al.*, 1987). Segundo estes autores, uma espécie genômica inclui linhagens que apresentam uma homologia igual ou acima de 70%, com Δtm de 5°C ou menor.

Esse método tem sido gradualmente estimulado para a caracterização taxonômica e como exemplo pode-se citar a recente proposta de reclassificação geral do gênero *Xanthomonas*, onde foram sugeridas 20 novas espécies, baseada principalmente nos dados de hibridização DNA:DNA (Vauterin *et al.*, 1995). O estabelecimento dos grupos genômicos é portanto uma característica que vem sendo adotada para o estabelecimento correto dos grupos taxonômicos. Até o momento, a reassociação de DNA é o método mais importante para o delineamento do conceito de espécie e apresenta um alto grau de correlação com as análises sorológicas e fenéticas (Stackebrand & Goebel, 1994).

# Artigo I

## POLYMORPHISM OF *Xylella fastidiosa* STRAINS BY RAPD-PCR AND SDS-PAGE OF PROTEINS

ANGELA MEHTA, RUI P. LEITE JR. & YOKO B. ROSATO

**Submetido à Fitopatologia Brasileira**

# POLYMORPHISM OF *Xylella fastidiosa* STRAINS BY RAPD-PCR AND SDS-PAGE OF PROTEINS

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## ABSTRACT

Genetic diversity among strains of *Xylella fastidiosa* was assessed by RAPD-PCR and SDS-PAGE of whole-cell proteins. For RAPD-PCR, six primers were used to analyze twenty three stains of *X. fastidiosa* isolated from citrus. Strains isolated from coffee, grapevine, plum and

pear were also included in this study for comparison. The analysis of the PCR products revealed five major clusters at 65% of similarity: grapevine, pear, citrus-coffee, citrus and plum. The coffee strains were closely related to the citrus strains with a similarity of approximately 65%. On the other hand, the grapevine, plum and pear strains showed to be distantly related to citrus with a similarity index below 30%. The grouping of the citrus strains showed a relationship to the geographic location where they were isolated. Most strains isolated from the Southern States of Brazil formed one group whereas strains from the States of São Paulo and Sergipe formed another group. Ten strains representing the five major clusters obtained by RAPD-PCR were analyzed by SDS-PAGE of proteins. The whole cell protein profiles obtained showed a low level of polymorphism, however a relationship to the hosts was obtained.

Keywords: genetic diversity, citrus variegated chlorosis.

## RESUMO

### Polimorfismo de linhagens de *Xylella fastidiosa* através de RAPD-PCR e SDS-PAGE de proteínas

A diversidade genética de linhagens de *Xylella fastidiosa* foi avaliada por RAPD-PCR e SDS-PAGE de proteínas totais. Em RAPD-PCR, seis primers foram utilizados para analisar vinte e três linhagens de *X. fastidiosa* isoladas de citros. Linhagens isoladas de cafeeiro, videira, ameixeira e pereira também foram incluídas neste estudo para comparação. A análise dos produtos de RAPD-PCR revelou cinco grupos principais a 65% de similaridade: videira, pêra, citros-café, citros e ameixa. As linhagens de café apresentaram uma alta similaridade com citros

Entretanto, as linhagens de videira, ameixa e pêra permaneceram distantemente relacionadas em relação a citros com uma similaridade abaixo de 30%. O agrupamento das linhagens de citros mostrou uma relacão com a região geográfica onde foram isoladas. A maioria das linhagens isoladas dos estados do sul do Brasil formaram um grupo enquanto que as linhagens dos estados de São Paulo e Sergipe formaram outro grupo. Dez linhagens representando os cinco grupos principais obtidas por RAPD-PCR foram analisadas por SDS-PAGE de proteínas totais. Os perfis de proteína obtidos revelaram um baixo nível de polimorfismo, porém uma relação com os hospedeiros foi obtida.

## INTRODUCTION

*Xylella fastidiosa* (Wells *et al.*,1987) is a Gram-negative bacterium, responsible for diseases in many important crops including grapevine, plum, peach, and citrus (Hopkins, 1989; Chang *et al.*,1993). In Brazil, *X. fastidiosa* is associated with plum leaf scald which has affected the production of this crop in the Southern States since it's detection in the 1970s (French & Kitajima, 1977). Recently, coffee trees have also been reported as a host for *X. fastidiosa* and the most characteristic symptom of infected plants is the presence of short internodes (Paradela *et al.*,1997). The economically most important disease caused by *X. fastidiosa* in Brazil is citrus variegated chlorosis (CVC) (Chang *et al.*,1993). CVC was firstly reported in the Northwest region of São Paulo State (Rossetti *et al.*,1990) but is now spread in several states throughout the country causing heavy losses in the citrus industry.

Genetic characterization studies of *X. fastidiosa* have been mostly limited to the investigation of the relationships between strains from different hosts by using molecular

techniques such as RAPD, RFLP and DNA hybridization (Kamper *et al.*,1985; Chen *et al.*,1995; Pooler & Hartung, 1995). A previous study was conducted to analyze the genetic diversity among 36 strains of *Xylella* isolated from citrus from the State of São Paulo and two major haplotypes were distinguished within the population (Rosato *et al.*,1998). To date, the genetic diversity among the citrus strains occurring in different regions of Brazil remains unknown.

The use of RAPD to differentiate closely related strains has been applied to different groups of procaryotes (Lawrence *et al.*, 1993). RAPD is a fast and sensitive method for determining genetic relationships among pathogenic organisms and is especially advantageous when compared to other methods because no preknown sequences are required (Lawrence *et al.*,1993). Another approach widely used in bacterial classification is the separation of cellular proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Kersters, 1985). Protein electrophoresis is a rapid fingerprinting technique to characterize and compare large numbers of strains at the infraspecific level (Kersters, 1985) and has been used successfully for grouping different pathovars of plant pathogenic bacteria such as *Xanthomonas* spp. (Vauterin *et al.*,1991).

In this study, we analyzed the relationships among strains of *X. fastidiosa* isolated from citrus from different geographic regions by using RAPD-PCR and SDS-PAGE of whole-cell proteins. Strains isolated from grapevine, coffee, plum and pear were also included to compare the relationships of the citrus strains with strains from other hosts.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions:** Twenty three strains of *X. fastidiosa* isolated from citrus were analyzed in this study (Table 1). Strains isolated from grapevine, coffee, plum and pear were also included for comparative purposes. The strains are identified by a code number and the two letters preceding the numbers are derived from the host (CI: citrus, CO: coffee, GR: grape, PL: plum and PE: pear). Strains isolated from citrus showing CVC symptoms were collected in different states of Brazil, including Rio Grande do Sul (RS), Santa Catarina (SC), Paraná (PR), São Paulo (SP) and Sergipe (SE). The strains were cultivated on solid BCYE medium (Wells *et al.*,1981) for a period of approximately 10 days at 28°C. For long-term storage, bacterial cells were harvested from Petri plates and maintained in PD3 medium (Davis *et al.*,1980) containing 30% glycerol, at -70°C.

**DNA extraction:** DNA was extracted according to Goss *et al.* (1990) with some modifications. Cells were scraped from the BCYE plates, washed with TAS buffer (50 mM Tris-HCl, 50 mM EDTA, 150 mM NaCl, pH 8.0) and resuspended in 450 µL of the same buffer. SDS (final concentration of 1%) and proteinase K (150 µg/mL) were added and the tubes were incubated for 1 h at 50°C. Cell debris were removed by using phenol and chloroform extraction. The suspension was dialyzed in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.8) for 48 h.

**PCR conditions:** The primers OPR1, OPR2, OPR4, OPR5, OPR6, OPR7, OPR8, OPQ2, OPQ3, OPQ4 and OPP1 (Operon Technologies, USA) were used to amplify the DNA of *X. fastidiosa*

strains. PCR reactions were carried out in a final volume of 25 µL containing 100 ng of DNA, 2 mM $MgCl_2$, 100µM dNTP, 15 pmoles of primer and 0.5 U of *Taq* DNA polymerase (Amersham-Pharmacia, Sweeden). Reactions were performed in a Thermal Cycler 4800 (Perkin Elmer, USA) with an initial denaturation of 4 min at 94°C followed by 45 cycles of 1min at 94°C, 1 min at 40°C and 2 min at 72°C. The RAPD reactions were repeated at least twice for all strains. The amplification products were separated in 1% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide. Fragments with molecular weight higher than 4 kb and lower than 0.4 kb were not considered in the analysis.

**SDS-PAGE**: Strains representing the five major clusters obtained by RAPD-PCR were analyzed by SDS-PAGE of proteins. Whole cell protein profiles of ten *X. fastidiosa* strains were determined by using the method described by Jackman (1985). Proteins were extracted by the Laemmli method (Laemmli, 1970), submitted to SDS-PAGE and visualized after silver staining (Blum *et al.*,1987). Protein extractions were repeated at least twice for all the strains analyzed. Bands with molecular weight higher than 130 kDa and lower than 10 kDa were not considered in the analysis.

**Data analysis:** The profiles obtained by RAPD-PCR and SDS-PAGE were analyzed in a binary form. Bands were scored as present (1) or absent (0) for each strain. Similarity matrices were constructed by using the program similarity for qualitative data (SIMQUAL), with the Jaccard coefficient ($S_J$). Dendrograms were constructed by using the UPGMA (unweighted pair group method with arithmetic mean) algorithm, by using the program NTSYS-PC (Rohlf, 1989).

Clustering analysis was performed on the data obtained by the SDS-PAGE of proteins and the combined data sets generated by the six primers used in RAPD-PCR.

## RESULTS AND DISCUSSION

**RAPD-PCR Fingerprinting:** Out of the eleven primers tested, only the primers OPR1, OPR2, OPR8, OPQ3, OPQ4 and OPP1 revealed clear and reproducible amplification products and thus were used to assess the genetic diversity of thirty strains of *X. fastidiosa*. The fingerprint patterns obtained by using the primers OPR8 and OPQ3 are shown in Figure 1 and Figure 2, respectively. The combined data sets yielded 54 scorable bands which were used in the cluster analysis (Figure 3). PCR products ranged from one to seven bands per strain and the sizes from 0.5 to 4.0 kb.

RAPD-PCR distinguished five major clusters at 65% of similarity: citrus-coffee, citrus, grapevine, pear, and plum. The two coffee and twenty two citrus strains formed the citrus-coffee cluster, and within this cluster, two major citrus subgroups were obtained which showed a relationship to the geographic region of isolation. Most of the citrus strains isolated from the Southern states belonged to the same group whereas the two strains from the State of Sergipe and most strains from São Paulo State formed another group. Although the CVC was reported recently (Rossetti *et al.*,1990), genetic diversity already occurs within the *X. fastidiosa* strains isolated from citrus. This variability may be due to the accumulation of neutral mutations or to adaptation of a predominant strain to environmental changes as it may have occurred in the establishment of the two distinct citrus populations of *Xylella*.

Unexpectedly, strain CI X0 appeared separated from all the other citrus strains with a

similarity of 55% and formed a distinct cluster. Hopkins (1984; 1989) has reported that *X. fastidiosa* can undergo phenotypic changes in culture, with loss of bacterial aggregation properties and pathogenicity. It is possible that constant manipulation of this bacterium could have led to changes in some genetic characteristics resulting in the differentiation of strain CI X0 from the other citrus strains.

The two coffee strains CO 11752 and CO 12288, isolated from different geographic regions, presented a similarity of 75% between them. To date, few strains are available for studies and effort has been made to isolate *X. fastidiosa* from coffee trees, but with limited success. Nonetheless, coffee strains have been reported to have genetic diversity and have been grouped based on the geographic region where they were isolated (Leite *et al.*, 1999). The coffee strains, though different between them, were closely related to the citrus strains (similarity of 60%). The similarity between coffee and citrus strains observed in this study was previously reported (Leite *et al.*, 1999; Rosato *et al.*, 1998). The similarities between the populations of citrus and coffee may indicate a common origin of the bacterium to both hosts. Paradela *et al.* (1995) suggested that *X. fastidiosa* may have disseminated from coffee plants to citrus based on the fact that the region where CVC first appeared was previously cultivated with coffee and the trees that still remain, show a high incidence of coffee leaf scorch.

The grapevine and plum clusters showed similarities of approximately 35 and 20%, respectively, with the citrus-coffee cluster. The existence of different groups of *X. fastidiosa* has been suggested by Pooler & Hartung (1995) based on the RAPD analysis of eleven strains which were clustered into five groups: citrus, plum-elm, grape-ragweed, almond and mulberry. Chen *et al.* (1995) also reported differences in seventeen strains of *X. fastidiosa* from four hosts and

observed a low similarity among strains from grapevine and plum. *X. fastidiosa* was first reported as a rickettsia-like bacteria in 1973 causing Pierce´s disease of grapevines and since then has been described causing diseases in several host plants. The extensive genetic diversity observed among *X. fastidiosa* populations may have facilitate the adaptation to new environments and different hosts.

The pear strain was distantly related to the other strains with a similarity coefficient lower than 15%. The pear leaf scorch was reported in Taiwan in 1993 (Leu & Su, 1993) and only one strain was available for our study. Although the separation of *Xylella* isolated from pear is appealing, a higher number of strains would be necessary to confirm the relationship between pear strains and strains from other hosts.

**SDS-PAGE:** SDS-PAGE analysis of whole-cell proteins was performed for ten strains of *X. fastidiosa* representing the major clusters obtained by the RAPD-PCR technique. The profiles were reproducible and the molecular weight of the protein bands ranged from 10 to 130 kDa (Figure 4).

The protein profiles yielded a total of 39 scorable bands, many of which were common to all strains. Bands of approximately 60, 50 and 20 kDa were evident and easily distinguished from the others due to their intensity and broadness and were present in all the strains analyzed. The citrus strains, except strain CI X0, showed identical patterns of 26 bands. Strain CI X0 was differentiated from the other citrus strains due to differences in two bands. The profile of the coffee strain CO 12288 was very similar to that presented by the citrus strains, however, differences in two bands were observed. The grape strain GR 8935 also showed the presence of

two differential bands when compared to citrus. The two plum strains presented identical profiles and showed differences in six bands two of which were not present in any other strain. The pear strain showed a very distinct protein fingerprint pattern from that of citrus with eight differential bands.

The dendrogram obtained (Figure 5) revealed a low level of polymorphism and six groups were distinguished. Most strains presented a similarity above 75% except the pear strain which showed to be distantly related to the other strains of *X. fastidiosa* with a similarity below 50%. The citrus strains isolated from different geographic regions could not be distinguished by this method, however, strain CI X0, as in RAPD-PCR, remained apart from the other citrus strains. The grapevine and coffee strains formed different groups with a similarity of 85 and 90%, respectively, with the citrus group. The two plum strains which were differentiated by the RAPD-PCR technique could not be distinguished by the protein profile analysis.

The analysis of whole-cell proteins shows phenotypic features which are in general more conserved than some genetic characteristics. This can be observed when comparing the high level of genetic diversity detected by the RAPD-PCR analysis with the conserved protein profiles revealed by the SDS-PAGE of *X. fastidiosa* strains. Although changes may have occurred at the genetic level, they do not seem to interfere in the expression of the final gene product. In spite of the similarity observed in the protein patterns of the strains analyzed in this study, the diversity encountered was sufficient to distinguish strains from different hosts. SDS-PAGE of proteins has been used efficiently to distinguish phenotipically distinct groups and pathovars of phytopathogenic bacteria such as *Xanthomonas* spp. (Vauterin *et al.*,1991). Using the same approach, Vandamme *et al.*(1998) also reported different genomovars of *Pelistega europeae.*

Although a small number of strains were analyzed by the SDS-PAGE of proteins, the results obtained in this study suggest that the protein profile analysis could be a potential method to differentiate pathotypes of *X. fastidiosa.*

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**Table 1. Bacterial strains of *Xylella fastidiosa***

| [1] Strain | Host | Origin | Source or reference |
|---|---|---|---|
| GR 8935, GR 9713 | *Vitis vinifera* | United States | [2] IAPAR (8935=*ATCC 35879) |
| CO 11752 | *Coffea arabica* | Brazil, São Paulo (SP) | IAPAR |
| CO 12288 | *Coffea arabica* | Brazil, Paraná (PR) | IAPAR |
| PE PLS | *Pyrus pyrifolia* | Taiwan | [3] IBSBF (Leu & Su, 1993) |
| PL 9746 | *Prunus salicina* | Brazil, Paraná (PR) | IAPAR |
| PL 788 | *Prunus salicina* | United States | IBSBF (788=ATCC 35871) |
| CI 12290, CI 11834 CI 11347, CI 11066 CI 11067 | *Citrus sinensis* | Brazil, Paraná (PR) | IAPAR |
| CI 12291, CI 11380 CI 11779 | *Citrus sinensis* | Brazil, Santa Catarina (SC) | IAPAR |
| CI 11775, CI 11780 | *Citrus sinensis* | Brazil, Rio Grande do Sul (RS) | IAPAR |
| CI 11399, CI 11400 | *Citrus sinensis* | Brazil, Sergipe (SE) | IAPAR |
| CI 10438, CI 11036 CI 11038, CI 9712 CI 11039, CI 10414 CI 11037, CI 12302 | *Citrus sinensis* | Brazil, São Paulo (SP) | IAPAR |
| CI E1, CI 4A | *Citrus sinensis* | Brazil, São Paulo (SP) | [4] Fundecitrus |
| CI X0 | *Citrus sinensis* | Brazil, São Paulo (SP) | (Chang *et al*., 1993) |

[1] GR:grape, CO: coffee, PE:pear, PL: plum, CI:citrus

[2] IAPAR - Instituto Agronômico do Paraná, Londrina, PR, Brazil.

[3] IBSBF- Instituto Biológico, Seção de Bacteriologia Fitopatológica, Campinas, SP, Brazil.

[4] Fundecitrus - Fundo Paulista de Defesa da Citricultura, Araraquara, SP, Brazil.

*ATCC = American Type Culture Collection, Rockville, Maryland, USA

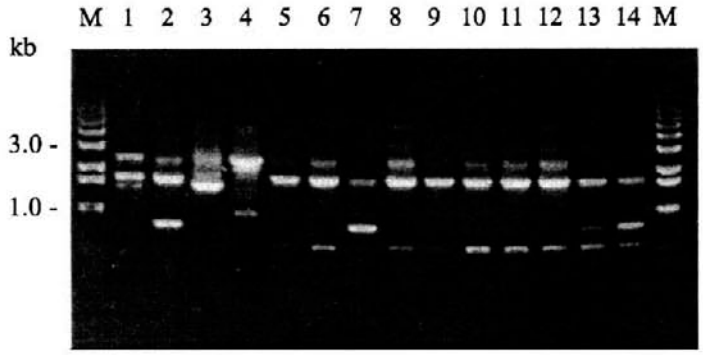


Figure 1. RAPD-PCR fingerprint patterns of some *X. fastidiosa* strains using the primer OPR8. M, ladder 1 kb; 1, 8935; 2, 11752; 3, 788; 4, PLS; 5, 11067; 6, 11347; 7, 12291; 8, 11779; 9, 11775; 10, 11780; 11, 10438; 12, 11036; 13, 11399; 14, 11400.

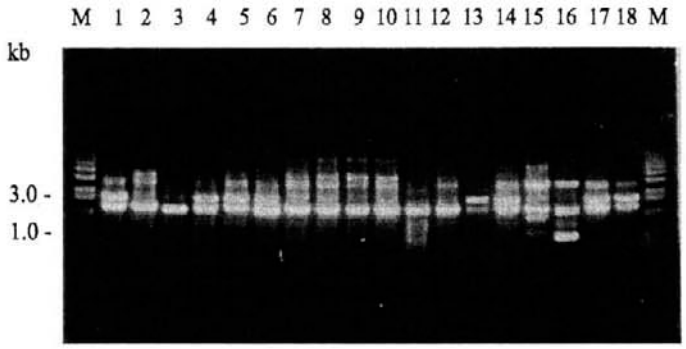


Figure 2. RAPD-PCR fingerprint patterns of some *X. fastidiosa* strains using the primer OPQ3. M, ladder 1 kb; 1, 11037; 2, X0; 3, 11399; 4, 11039; 5, E1; 6, 12290; 7, 11834; 8, 11347; 9, 11066; 10, 11380; 11, 11779; 12, 11775; 13, 12302. 14, 10414; 15, PLS; 16, 9746; 17, 11400; 18, 4 A.

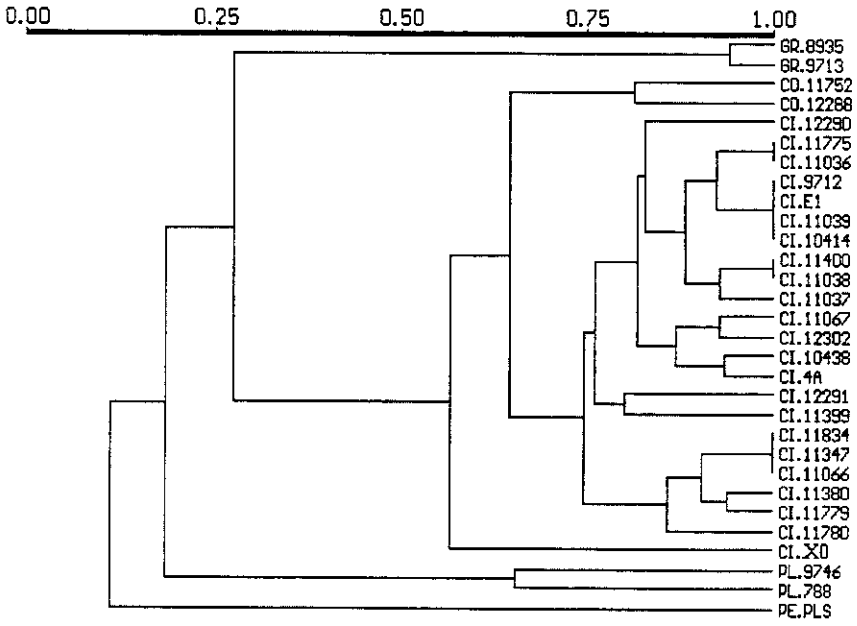


Figure 3. $S_J$/UPGMA cluster analysis of *X. fastidiosa* strains based on the RAPD-PCR data obtained using the primers OPR1, OPR2, OPR8, OPQ3, OPQ4 and OPP1.

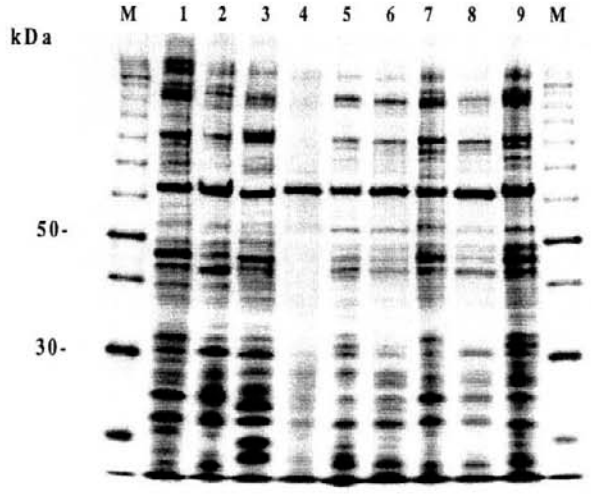


Figure 4. SDS-PAGE of whole-cell proteins of *X. fastidiosa* strains. M, ladder 10 kDa; 1, 8935; 2, 788; 3, PLS; 4, 12288; 5, 11066; 6, 11775; 7, 10438; 8, 11779; 9, X0.

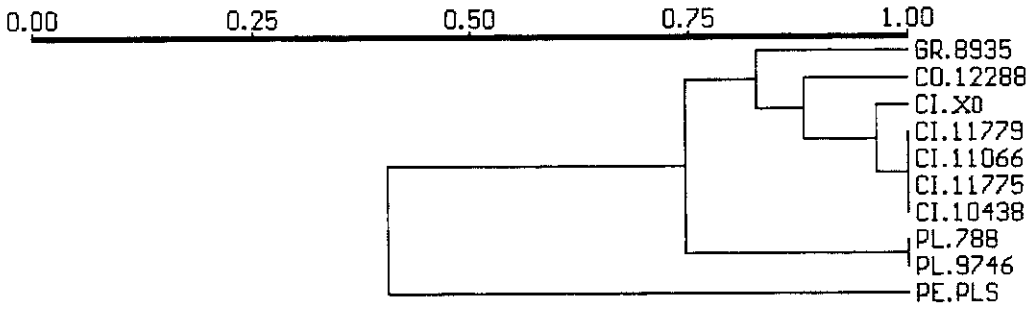


Figure 5. $S_J$/UPGMA cluster analysis based on the data obtained by the analysis of the protein profiles of *X. fastidisoa* strains by SDS-PAGE.

# Artigo II

**ASSESSMENT OF THE GENETIC DIVERSITY OF *Xylella fastidiosa* ISOLATED FROM CITRUS IN BRAZIL BY PCR-RFLP OF THE 16S rDNA AND 16S-23S INTERGENIC SPACER AND rep-PCR FINGERPRINTING**

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**Submetido à Antonie van Leeuwenhoek Journal of Microbiology**

# Assessment of the genetic diversity of *Xylella fastidiosa* isolated from citrus in Brazil by PCR-RFLP of the 16S rDNA and 16S-23S intergenic spacer and rep-PCR fingerprinting

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**Running title: Genetic diversity in *Xylella fastidiosa***

**Key words: *Xylella fastidiosa*, RFLP-16S, RFLP-IGS, ERIC, REP, BOX**

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# Assessment of the genetic diversity of *Xylella fastidiosa* isolated from citrus in Brazil by PCR-RFLP of the 16S rDNA and 16S-23S intergenic spacer and rep-PCR fingerprinting

The genetic diversity among twenty three strains of *Xylella fastidiosa*, isolated from sweet orange citrus, was assessed by RFLP analysis of the 16S rDNA and 16S-23S intergenic spacer and rep-PCR fingerprinting. Strains that were isolated from coffee, grapevine, plum and pear were also included in this study. The PCR products obtained by the amplification of the 16S rDNA and 16S-23S spacer were digested with restriction enzymes and the dendrogram constructed showed a low level of polymorphism. However, a relationship to some hosts was revealed. In rep-PCR fingerprinting, a clustering according to the host was also observed by using the BOX, ERIC and REP primers. Two major groups were detected within the citrus cluster and relationships to the geographic origin of the strains were revealed. Citrus strains isolated from the States of São Paulo and Sergipe formed one group whereas strains from the Southern States formed another group. A distinct origin of *X. fastidiosa* in the Southern and Southeastern States is postulated. The pear strain used in the present work was distantly related to all the other *X. fastidiosa* strains analyzed.

## Introduction

*Xylella fastidiosa* (Wells et al., 1987) is a nutritionally fastidious, Gram-negative

bacterium, limited to the xylem of plants and responsible for many diseases in important crops. The bacterium has a wide host range, including mono- and dicotyledonous plants, and is transmitted by grafting and leafhopper vectors (Purcell, 1990). On citrus, *X. fastidiosa* causes citrus variegated chlorosis (CVC) (Chang et al., 1993) which has become a serious problem in Brazil, causing losses of more than 100 million dollars each year (Laranjeira et al., 1998). CVC was first observed on sweet orange trees in the Northwest region of the State of São Paulo in 1987 (Rossetti et al., 1990) however, the disease has now been identified in many other States in the country. *X. fastidiosa* is also associated with Pierce's disease of grapevine and plum leaf scald, causing heavy losses in these crops (Hopkins, 1977). Pierce's disease is the limiting factor for the production of grapes in Southeastern United States and the disease incidence has been increasing in California (Chen et al., 1995). Recently, coffee plants have also been reported as an alternative host for *X. fastidiosa* and the most characteristic symptoms of infected plants have been described to be leaf scald and short internodes (de Lima et al., 1998; Paradela et al., 1997). Another disease associated with *X. fastidiosa* is pear leaf scorch (PLS) reported in Taiwan in 1993 (Leu & Su, 1993).

In spite of the variability in pathogenicity, all strains of *Xylella* belong to the same species (Chen et al., 1995). Although *X. fastidiosa* is well characterized, the genetic, serological and pathogenic relationships among the pathotypes are still uncertain (Sherman et al., 1989). In order to elucidate the relationships between strains of *X. fastidiosa*, many molecular techniques such as RAPD, RFLP and DNA hybridization have been used and different groups were distinguished (Chen et al., 1992; Chen et al., 1995; Kamper et al., 1985; Pooler & Hartung, 1995; Rosato et al., 1998).

The rRNA genes are fundamental for all organisms and the 16S rDNA is the target selected by most studies to infer the phylogenetic relationships and characterization of many bacterial species. The 16S genes are similar in length and contain conserved regions that permit analysis of distantly related organisms, and variable regions, which are used to define the relationships at a lower taxa level. A large amount of sequence information is now available for this gene in different species (Olsen et al., 1991).

Analysis of the ribosomal 16S-23S intergenic spacer (IGS) has become another promising approach for bacterial identification by exploiting the genetic variation within this region at the species level or among strains of the same species (Gürtler & Stanisich, 1996). Unlike rDNA genes, which are highly conserved in most bacterial species, the IGS region is more variable in its length and sequence. Depending on the degree of variation within the spacer, the polymorphisms found can be used for differentiation at the genus, species or strain levels.

A third approach, based upon PCR amplification using primers corresponding to repetitive sequences have been used to analyze the genetic diversity among related strains. These sequences include: repetitive extragenic palindromes (REP) (Stern et al., 1984), enterobacterial repetitive intergenic consensus (ERIC) (Hulton et al., 1991) and BOX element (BOX 1 A) (Martin et al., 1992). REP sequences are the best characterized family of bacterial repetitive sequences, and consist of inverted repeats found in clusters with successive copies arranged in alternate orientation. ERIC sequences resemble REP elements, although the nucleotide sequence is entirely different. Both elements are located in non-coding regions, which are probably transcribed and include a potential stem-loop structure. BOX sequences were firstly described in the Gram-positive bacterium *Streptococcus pneumoniae* and are more similar to ERIC sequences than to

REP with respect to their size and number of copies (Martin et al., 1992). It has been suggested that BOX sequences may represent regulatory elements of genes including competence-specific and virulence-related genes (Martin et al., 1992).

In a previous study, a population of *X. fastidiosa* isolated from citrus in the State of São Paulo was analyzed and showed three major haplotypes by RAPD clustering (Rosato et al., 1998). In this study, the relationships among twenty three other strains of *X. fastidiosa*, isolated from diseased sweet orange, were analyzed in an attempt to verify the genetic diversity of *X. fastidiosa* populations from different geographic regions, and to assess the relationships between strains and different hosts. Restriction fragment length polymorphism (RFLP) of the 16S rDNA and IGS region and repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprinting were used.

## Materials and Methods

### Bacterial strains and culture conditions

Twenty three strains of *X. fastidiosa* isolated from citrus showing symptoms of CVC were used in this study (Table 1). Other strains isolated from different hosts (coffee, plum, pear and grapevine) were included for comparative purposes. The twenty three strains isolated from citrus were collected in different geographic regions of Brazil. The Southern region included the States of Rio Grande do Sul (RS), Santa Catarina (SC) and Paraná (PR); the Southeastern region included the State of São Paulo (SP) and the Northeastern region, the State of Sergipe (SE), at least 2500 km distant from SP. The strains were cultivated on solid BCYE (Buffered Cysteine

Yeast Extract) medium (Wells et al., 1981) for a period of approximately 10 days at 28°C. For long-term storage, bacterial cells were harvested from Petri dishes and maintained in Pierce's Disease medium # 3 (PD3) (Davis et al., 1980) containing 30% glycerol, at -70°C.

**DNA extraction**

Cells were scraped from the BCYE plates, washed with TAS buffer (50 mM Tris-HCl, 50 mM EDTA 150 mM NaCl, pH 8.0) and resuspended in 450 µL of the same buffer. SDS (final concentration of 1%) and proteinase K (150 µg/mL) were added to the cell suspension and the tubes were incubated for 1 h at 50°C. Cell debris were removed by adding 500 µL of phenol to the suspension and centrifuging for 5 min. The supernatant was transfered to another tube and 500 µL of chloroform were added. The tubes were centrifuged for 5 min and the supernatant was transfered to a fresh tube. The suspension was dialyzed in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.8) for 48 h.

**RFLP of the 16S rDNA and IGS region**

The genetic diversity among thirty strains of *X. fastidiosa* was investigated by PCR-RFLP of the 16S rDNA and the 16S-23S spacer region. The 16S rDNA was amplified using the primers: 27f 5'-AGAGTTTGATCMTGGCTCAG-3' and 1525r 5'-AAGGA-GGTGWTCCARCC-3' (Lane, 1991). The primers 16S uni1330 5'-GTTCCCGGGCCTTGTACACAC-3' and 23S uni322 anti 5'-GGTTCTTTTCGCCTTTCCCTC-3' from conserved regions of *Xanthomonas* spp. (Honeycutt et al., 1995) which are phylogenetically related to *X. fastidiosa* (Wells et al., 1987), were used to amplify the IGS region.

PCR amplification was performed in a total volume of 25 µL containing 30-50 ng of DNA, 0.5 µM of each primer, 100 µM dNTP, 2.5 mM $MgCl_2$ and 0.5 units of *Taq* DNA polymerase (Amersham-Pharmacia). Amplification was performed in a Thermal Cycler 4800 (Perkin Elmer) and the conditions used for the 16S rDNA were: one cycle of 1 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 65°C and 2 min at 72°C. A 5 µL aliquot of the PCR products was digested separately with the restriction enzymes *Hin*fI, *Taq*I, *Alu*I, *Hae*III and *Sau*3A, separated by gel electrophoresis in 3% agarose gels and visualized after staining with ethidium bromide. The cycling conditions for amplification of the IGS region were: one cycle of 4 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 65°C and 1 min at 72°C. The PCR products were digested with *Hin*fI, *Dde*I and *Sau*3A, and the bands were resolved as described for 16S rDNA.

**rep-PCR conditions**

The rep-PCR reactions were carried out in a final volume of 25 µL containing 30-50 ng of DNA, 50 pmol of each primer, 300 µM dNTP, 2.5 mM $MgCl_2$ and 0.5 units of *Taq* DNA polymerase (Amersham-Pharmacia). The primer sequences were: BOX (BOXA1R [5'-CTACGGCAAGGCGACGCTGACG-3']), ERIC (ERIC1R [ATGTAAGCTCCTGG-GGATTCAC-3'] and ERIC2 [5'-AAGTAAGTGATGGGGTGAGCG-3']), and REP (REP1R-I [5'-IIICGICGICATCIGGC-3'] and REP2-I [5'-ICGICTTATCIGGCCTAC-3']). The following cycling conditions were used: BOX, one cycle of 7 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 53°C, 8 min at 65°C, and one cycle of 16 min at 65°C; ERIC, one cycle of 7

min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, 8 min at 65°C, and one cycle of 16 min at 65°C; REP, one cycle of 6 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 40°C, 4 min at 65°C, and one cycle of 16 min at 65°C. A 20 μL aliquot of each amplification reaction was loaded on a 1.8% agarose gel and submitted to electrophoresis. The PCR fragments were visualized after staining the gel with ethidium bromide and the amplification reactions were repeated at least twice for each strain.

**Data analysis**

DNA profiles obtained by using RFLP-16S, RFLP-IGS and rep-PCR were analyzed in a binary form. Bands were scored as present (1) or absent (0) for each strain. Faint bands and fragments with molecular weight lower than 200 bp in rep-PCR and 100 bp in PCR-RFLP were not considered in the analysis. Similarity matrices were constructed by using the program similarity for qualitative data (SIMQUAL), with the Jaccard coefficient ($S_J$). Dendrograms were constructed by using the UPGMA (unweighted pair group method with arithmetic mean) algorithm, with the NTSYS-PC program (Rohlf, 1989). Clustering analysis was performed on the data generated by each fingerprinting primer and on the combined data sets generated by rep-PCR fingerprinting and PCR-RFLP analysis.

**Results**

**RFLP of the 16S rDNA and IGS region**

The amplification of the 16S rDNA yielded a unique band of 1.4 kb for all the strains

analyzed indicating no variation in the length of this gene in *X. fastidiosa* (data not shown). The PCR products were digested with the restriction enzymes *Taq*I, *Alu*I, *Hin*fI, *Hae*III and *Sau*3A. Bands in the molecular weight range of 100 to 1000 bp were considered for analysis. *Hin*fI produced the same pattern for all thirty strains analyzed. The enzymes *Alu*I, *Taq*I and *Hae*III differentiated only the pear strain, and *Sau*3A differentiated the two plum strains PL 9746 and PL 788 (Table 2).

Using the IGS primers, a unique PCR product of 1.2 kb was generated for all thirty strains of *X. fastidiosa* (data not shown). The digestion with the restriction enzymes *Hin*fI, *Dde*I and *Sau*3A yielded bands ranging from 100 to 450 bp, which were considered in the analysis. The patterns generated by strains representing the different hosts are shown in Figure 1. The digestion with the three restriction enzymes differentiated only the pear strain. All the other strains presented identical RFLP patterns (Fig. 1).

The data obtained from the RFLP of the 16S and IGS were combined for cluster analysis and a total of 45 bands were scored. As the 16S and IGS region are contiguous in the genome and components of the rDNA operon, they have been placed together for analysis by Vinuesa *et al.* (1998). This strategy was also applied to our study and a good resolution was obtained. The dendrogram (Fig. 2) revealed a low level of genetic polymorphism. The citrus-coffee-grapevine cluster was distinguished from the plum and pear strains. The plum strains showed a similarity to the citrus-coffee-grapevine cluster of approximately 92% and a striking difference was found for the pear strain (45% of similarity).

**rep-PCR fingerprinting**

The genetic diversity of the thirty strains of *X. fastidiosa* was also assessed by rep-PCR fingerprinting using the primers BOX, ERIC and REP. PCR products ranged from 7 to 14 bands per strain and in size from 0.2 to 6.0 kb. Only bands occurring in two out of three repetitions were considered for analysis. Strains that exhibited very similar patterns with differences regarding the intensity of the bands were considered as having the same pattern. BOX and ERIC-PCR (Fig. 3 and Fig. 4, respectively) revealed similar results regarding the differentiation of strains. Most strains differed in 1 to 5 bands, except for the pear strain that presented the most dissimilar fingerprint pattern. Using the BOX primer, strain PL 9746 also showed a very distinct profile. REP-PCR produced nine different genotypes (Fig. 5) and yielded the most discriminative banding patterns for the citrus strains. The citrus strains from different geographic regions showed different REP patterns. The plum strains PL 9746 and PL 788 were differentiated, as well as the coffee strains CO 11752 and CO 12288. The pear strain presented a unique fingerprint profile.

The combined data sets yielded sixty five scorable bands which were used to construct a dendrogram (Fig. 6). Five clusters were distinguished at 70% of similarity: citrus-coffee, grapevine, pear and two plum clusters. The citrus-coffee cluster was formed by twenty three citrus and two coffee strains. Within this cluster, two major groups, at 85% of similarity, were obtained. Most of the citrus strains isolated from the Southern States (PR, SC and RS) belonged to the same group whereas the two strains from SE (Northeast), most of the strains (9) from SP (Southeast) and strain CO 12288 formed another group. The strain CI X0 was differentiated from the two major citrus subgroups. The strains GR 8935 and GR 9713 formed the grapevine cluster showing 60% similarity with the citrus-coffee cluster. The pear strain was distantly related to the

citrus-coffee cluster, showing a similarity lower than 20%. The two plum strains analyzed were quite distinct to each other with a similarity lower than 60% between them.

**Discussion**

In the present study, different PCR-based techniques were used to assess the genetic diversity of *X. fastidiosa* isolated from sweet orange citrus from different regions in Brazil. The study included strains mainly from the two major citrus-producing States (SP and PR) since strains from other regions are not frequently isolated.

The PCR was used to amplify the 16S rDNA gene and the 16S-23S intergenic spacer and revealed a unique PCR product in each case (data not shown). This result might indicate the existence of one copy of rDNA or, if different alleles are present, they are very similar in length. Using the RFLP of the 16S rDNA and IGS region, a low degree of genetic polymorphism was detected, however, a relationship to some hosts was observed. The clustering according to the hosts, although based on a small number of differences presented by the PCR-RFLP, was also obtained by other authors using different techniques and strains from hosts other than citrus (Chen et al., 1995; Pooler and Hartung 1995). Taken together, these results show that such correlation exists even though some strains from diverse hosts were not differentiated as it occurs for strains from citrus and coffee. The lack of differentiation in these strains may indicate the occurrence of a common origin and a recent divergence to different hosts. It would be interesting to exploit a more detailed analysis of the rDNA region by DNA sequencing to verify the extension and sites of mutation.

rep-PCR fingerprinting was shown to be more useful to detect variability in *X. fastidiosa*

strains. The complexity of the products generated by rep-PCR, mainly by using the REP primers, enabled the detection of a higher level of polymorphism among the citrus strains. Within the citrus-coffee cluster two major subgroups were revealed. These subgroups showed relationships to the geographic region where they were isolated. Most of the citrus strains isolated from the Southern States belonged to the same group whereas the two strains from SE and most strains from SP formed another group. The reasons for the maintainance of distinct populations are not clear. It is possible that a distinct introduction of *X. fastidiosa* occurred in these regions and time was not sufficient to permit exchange of genetic material between both pathogen populations. The Southern States of Brazil lie along the Argentina borders which could have facilitated the dissemination of the bacterium by insect vectors from citrus showing *pecosita* symptoms, caused by *X. fastidiosa* (Contreras, 1992). The primary origin of CVC in SP (Southeast region), on the other hand, has been tentatively attributed to common insect vectors feeding on coffee and citrus plants. The presence of the bacteria in coffee plants from regions where citrus plants are not present, and the fact that the region where CVC appeared in Brazil was previously a coffee field, support the hypotheses that *X. fastidiosa* was disseminated from coffee plants to citrus (Paradela et al., 1997). Indeed the two coffee strains analyzed presented very similar fingerprint profiles to the citrus strains. The similarity between coffee and citrus strains observed in this study was previously reported (de Lima et al., 1998; Leite et al., 1998; Rosato et al., 1998). The diseases caused by *X. fastidiosa* on both hosts were detected in Brazil recently and many studies related to these pathogens have been initiated. A few strains of coffee have been isolated and the specificity in the pathogenicity is still undetermined. The similarity between the citrus strains from the Southeast and Northeast of Brazil can be attributed to the transport of seedlings and grafting

material from SP to SE in the 1980's when the disease was still unknown (Laranjeira et al., 1996). This grafting material could have originated the disease, which is now spread in the State of SE.

An intriguing result obtained in this study was that strain CI X0 appeared separated from all the other citrus strains in rep-PCR fingerprinting. This strain was one of the first strains isolated from symptomatic citrus and it was the strain used to fulfill the Koch's postulates by Chang *et al.* (1993). Hopkins (1984, 1989) has reported that *X. fastidiosa* can undergo phenotypic changes in culture, with loss of bacterial aggregation properties and pathogenicity. It is possible that constant manipulation of this bacterium could have led to changes in some genetic characteristics resulting in the differentiation of strain CI X0 from the other citrus strains.

Another unexpected outcome was obtained with the strain isolated from pear. The pear strain was identified as *X. fastidiosa* by various traits (Leu & Su, 1993) however it was very distantly related to other *Xylella* strains. Unfortunately there was only one pear strain available for this study, therefore a higher number of strains would be necessary to confirm the relationship of pear strains to strains from other hosts.

Although the CVC has been reported recently (Rossetti et al., 1990), genetic diversity is already existent within the *Xylella* strains isolated from diseased citrus. Detection of different genotypes may indicate the possibility of diverse response in the host-pathogen interaction. Therefore the major genotypes present in the pathogen population should be taken into account in citrus breeding programs and testing of germplasm to search for CVC resistance.

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*Table* 1. **Bacterial strains of *Xylella fastidiosa***

| [1] Strain | Host | Origin | Source or reference |
|---|---|---|---|
| GR 8935, GR 9713 | *Vitis vinifera* | United States | [2] IAPAR (8935=*ATCC 35879) |
| CO 11752 | *Coffea arabica* | Brazil, São Paulo (SP) | IAPAR |
| CO 12288 | *Coffea arabica* | Brazil, Paraná (PR) | IAPAR |
| PE PLS | *Pyrus pyrifolia* | Taiwan | [3] IBSBF (Leu & Su, 1993) |
| PL 9746 | *Prunus salicina* | Brazil, Paraná (PR) | IAPAR |
| PL 788 | *Prunus salicina* | United States | IBSBF (788=ATCC 35871) |
| CI 12290, CI 11834 CI 11347, CI 11066 CI 11067 | *Citrus sinensis* | Brazil, Paraná (PR) | IAPAR |
| CI 12291, CI 11380 CI 11779 | *Citrus sinensis* | Brazil, Santa Catarina (SC) | IAPAR |
| CI 11775, CI 11780 | *Citrus sinensis* | Brazil, Rio Grande do Sul (RS) | IAPAR |
| CI 11399, CI 11400 | *Citrus sinensis* | Brazil, Sergipe (SE) | IAPAR |
| CI 10438, CI 11036 CI 11038, CI 9712 CI 11039, CI 10414 CI 11037, CI 12302 | *Citrus sinensis* | Brazil, São Paulo (SP) | IAPAR |
| CI E1, CI 4A | *Citrus sinensis* | Brazil, São Paulo (SP) | [4] Fundecitrus |
| CI X0 | *Citrus sinensis* | Brazil, São Paulo (SP) | (Chang *et al*., 1993) |

[1] GR:grape, CO: coffee, PE:pear, PL: plum, CI:citrus

[2] IAPAR - Instituto Agronômico do Paraná, Londrina, PR, Brazil.

[3] IBSBF- Instituto Biológico, Seção de Bacteriologia Fitopatológica, Campinas, SP, Brazil.

[4] Fundecitrus - Fundo Paulista de Defesa da Citricultura, Araraquara, SP, Brazil.

*ATCC = American Type Culture Collection, Rockville, Maryland, USA

*Table* **2. Observed fragments of the RFLP-16S rDNA and RFLP-IGS of *X. fastidiosa* strain**

| **ENZYME** | Fragment sizes (bp) | | |
|---|---|---|---|
| **RFLP-16S** | Citrus-coffee-grapevine | Plum | Pear |
| *Hin* fI | 1000, 200, 100 | as citrus | as citrus |
| *Taq* I | 880, 380, 230, 110 | as citrus | 800, 380, 230, 110 |
| *Alu* I | 250, 210, 180, 100 | as citrus | 380, 250, 200, 100 |
| *Hae* III | 550, 280, 180, 160 | as citrus | 500, 280, 180, 170 |
| *Sau* 3A | 700, 330, 270, 180 100 | 580, 320, 270, 180 100 | as citrus |
| **RFLP-16S-23S** | | | |
| *Hin* fI | 440, 420, 240 | as citrus | 400, 260, 240, 130 |
| *Dde* I | 450, 280, 250 | as citrus | 270, 260, 230 |
| *Sau* 3A | 420, 390, 180, 150 | as citrus | 390, 280, 170, 150 |

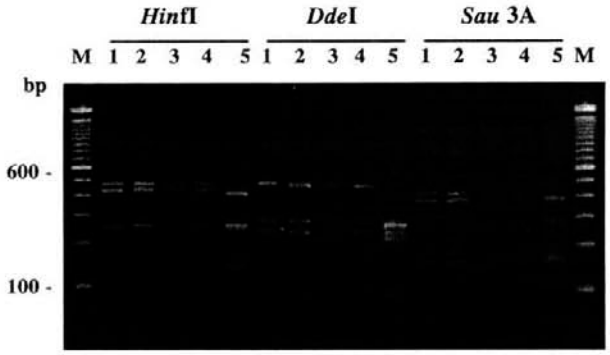


*Figure* 1. Restriction patterns of amplified 16S-23S rDNA intergenic spacer from *Xylella fastidiosa* strains. 1, GR 8935; 2, CO 12288; 3, CI 11038; 4, PL 788; 5, PE PLS. PCR products were digested with *Hin*fI, *Dde*I and *Sau*3A as indicated. M, DNA molecular weight size marker (100 bp ladder, Gibco-BRL).

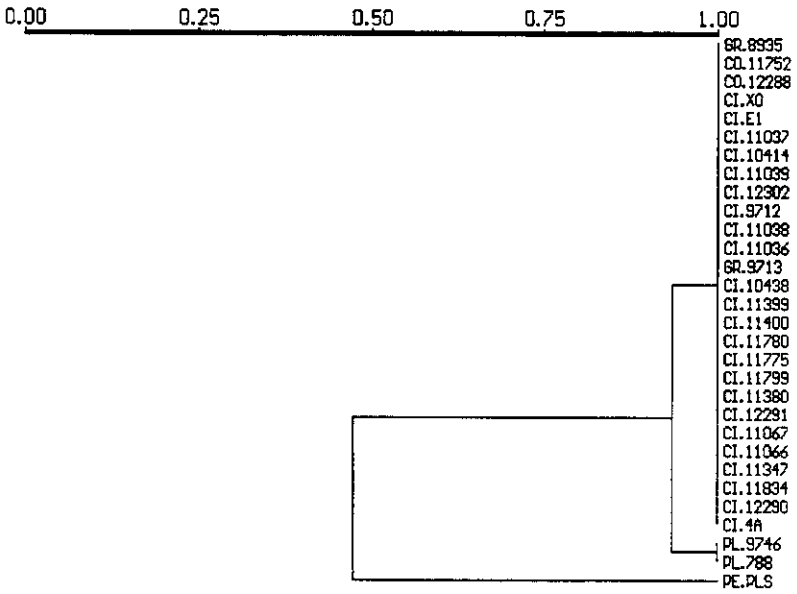


*Figure* 2. $S_J$/UPGMA cluster analysis of strains of *Xylella fastidiosa* based on the combined restriction pattern of amplified 16S rDNA and 16S-23S intergenic spacer.

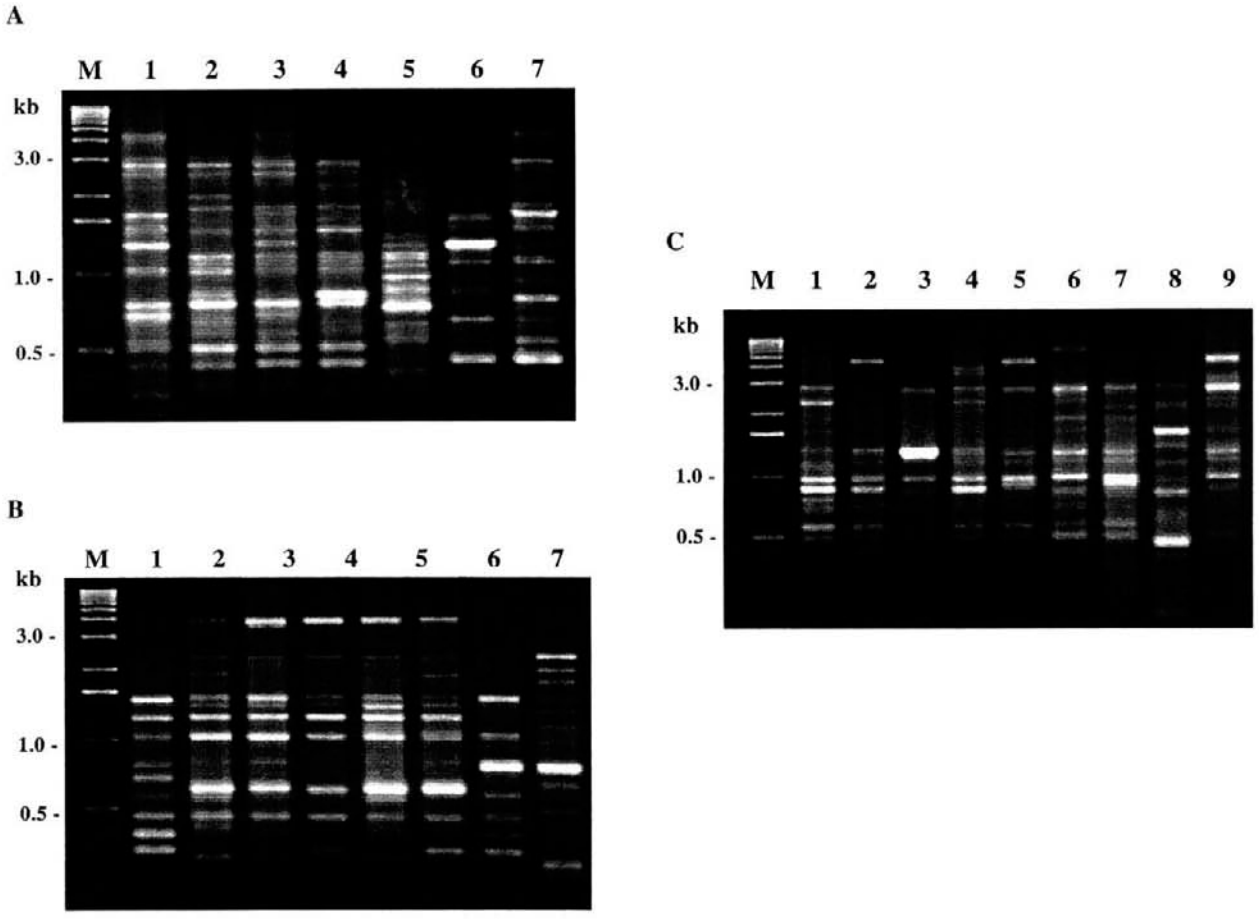


*Figure* 3. PCR fingerprint patterns distinguished by each primer. A, BOX, 1, GR 8935; 2, CI 4A; 3, CI11347; 4, CI 11400; 5, PL 788; 6, PE PLS; 7, PL9746. B, ERIC, 1, GR 8935; 2, CI E1; 3, CI 11036; 4, CI 11775; 5, CI 9712; 6, CI X0; 7, PL 788; 8, PE PLS. C, REP, 1, GR 8935; 2, PL 9746; 3, CI 10438,; 4, PL 788; 5, CI 10414; 6, CI X0; 7, CI E1; 8, PE PLS; 9, CO 11752. M, DNA molecular weight size marker (1 kb ladder, Gibco-BRL).

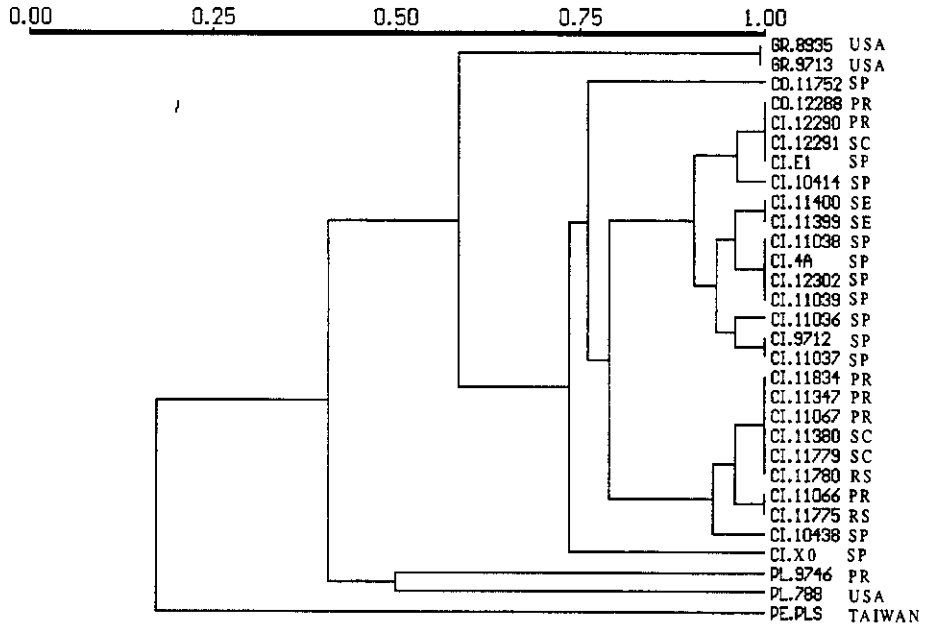


*Figure* 4. $S_J$/UPGMA cluster analysis of strains of *Xylella fastidiosa* based on the combined genomic fingerprints using primers corresponding to BOX1A element (BOX), enterobacterial repetitive intergenic consensus sequences (ERIC) and repetitive extragenic palindromic sequences (REP).

# Artigo III

**PHYLOGENETIC RELATIONSHIPS OF *Xylella fastidiosa* STRAINS FROM DIFFERENT HOSTS BASED ON 16S rDNA AND 16S-23S INTERGENIC SPACER SEQUENCES**

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# Phylogenetic relationships of *Xylella fastidiosa* strains from different hosts based on 16S rDNA and 16S-23S intergenic spacer sequences

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**Running title: Phylogenetic analysis of *Xylella fastidiosa***

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**Keywords: *Xylella fastidiosa*, rDNA, DNA:DNA hybridization**

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## SUMMARY

The phylogenetic relationships of *Xylella fastidiosa* strains isolated from different hosts was inferred by sequence analysis of the 16S rDNA and 16S-23S intergenic spacer region. A high level of similarity (97.1-100%) was found in the 16S rDNA of the *X. fastidiosa* strains. The 16S-23S region showed a higher level of variation, with similarity values ranging from 79.8-100%. The pear strain presented the most dissimilar sequences. The phylogenetic trees, constructed using the Neighbor-Joining method, showed that the citrus, coffee and plum strains were grouped into one cluster and strains from grapevine formed another cluster. The pear strain remained isolated from all the other *Xylella* strains in both analyses and presented DNA:DNA hybridization values below 20% with citrus. These results show that this strain does not belong to the *X. fastidiosa* genomic species.

## INTRODUCTION

*Xylella fastidiosa* is a xylem-limited bacterium, responsible for diseases in many

economically important crops such as grapevine, peach, plum, pear and more recently, citrus and coffee (for a revision, see Hopkins, 1977). This Gram-negative, slow growing bacterium has a wide host range and is transmitted by grafting and leafhopper vectors (Purcell, 1990). *X. fastidiosa* was first identified in 1973 causing Pierce's disease of grapevine, and is responsible for heavy losses in this crop (Chen *et al.*, 1995; Hopkins, 1977). Recently, citrus variegated chlorosis (CVC) (Chang *et al.*, 1993) and coffee leaf scorch (CLS) (Paradela Filho *et al.*, 1997), also caused by *X. fastidiosa,* have been reported in Brazil.

Many molecular techniques have been used to characterize *X. fastidiosa* strains and different groups have been distinguished (Chen *et al.*, 1992; 1995; Pooler & Hartung, 1995; Rosato *et al.*, 1998). However, data regarding the phylogenetic relationships and genetic relatedness of strains of *X. fastidiosa* from recently reported diseases remains limited.

Ribosomal DNA has been widely used to infer phylogenetic relationships in microorganisms (Woese, 1987). The sequence analyses of the small subunit, 16S rRNA, has been frequently used as a powerful and accurate method for determining inter- and intraspecific relationships (Leblond-Bourget *et al.*, 1996). However, as evolutionary distances decrease, insufficient diversity is often found in the 16S gene and thus genetic relationships of closely related species can not be well defined (Rogall *et al.*, 1990). It has been proposed that the spacer region 16S-23S could overcome this problem due to its higher variation in length and sequence. Indeed the analysis of this region has successfully differentiated strains of many groups of bacteria and sequences of the 16S-23S of many species have become available for comparison (Leblond-Bourget *et al.*, 1996; Yoon *et al.*, 1997).

In this study, we have sequenced the 16S rDNA and 16S-23S intergenic spacer of *X. fastidiosa* strains as an attempt to determine the phylogenetic relationships among strains from different hosts and between *X. fastidiosa* and related species. We have also performed DNA:DNA hybridization in order to determine the taxonomic level of the *X. fastidiosa* strains isolated from different hosts.

## METHODS

**Bacterial strains and culture conditions.** Strains of *X. fastidiosa* isolated from citrus (CI), coffee (CO), grapevine (GR), plum (PL) and pear (PE) were used for the sequencing and DNA:DNA hybridization methods. The strains used in this study were supplied by Dr. R. P. Leite Jr. or Dr. J. Rodrigues Neto. The strains were cultivated on solid Buffered Cystein Yeast Extract (BCYE) medium (Wells *et al.*, 1981a) for a period of approximately 10 days at 28°C. For long-term storage, bacterial cells were harvested from Petri plates and maintained in Pierce's disease medium # 3 (PD3) (Davis *et al.*, 1980a) containing 30% glycerol, at -70°C.

**DNA extraction.** Cells were scraped from the BCYE plates, washed with TAS buffer (50 mM Tris-HCl, 50 mM EDTA 150 mM NaCl, pH 8.0) and resuspended in 450 µL of the same buffer. SDS (final concentration of 1%) and proteinase K (150 µg $mL^{-1}$) were added and the tubes were incubated for 1 h at 50°C. Cell debris were removed by using phenol and chloroform extraction. The suspension was dialysed in TE buffer (40 mM Tris-HCl, 1 mM EDTA, pH 7.8) for 48 h.

**DNA:DNA hybridization.** The hybridization was performed using the Hybri slot blot manifold as described by the manufacturer (BioRad) with nylon membrane (Amersham). The probes were labeled with dygoxigenin using the DIG DNA labeling kit (Boeringer-Mannheim). The hybridization buffer containing 50% formamide, 5x SSC (0.15 M NaCitrat; 1.5 M NaCl, pH 7.0), N-laurilsarcosine (0.1%), SDS (0.02%), blocking reagent (2%) was used and the hybridization was performed at 42°C overnight. The membranes were washed at 68°C in a solution containing 0.1 SSC and 0.1% SDS. The detection of bands was carried out using the chemiluminescent substrate CSPD (Disodium 3- (4-methoxyspiro{1,2-dioxetane-3,2"-(5"-chloro) tricyclo [3.3.1.1$^{3,7}$] decan}-4-yl) phenyl phosphate) as recommended by the manufacturer (Boeringer-Mannheim). The signal, detected by exposure to an X-ray film (Kodak), was measured using the Ultrascan XL-Enhancer Laser Densitometer (LKB Bromn.).

**Amplification of the 16S rDNA and 16S-23S spacer.** The 16S gene was amplified using the primers: 27f 5'-AGAGTTTGATCMTGGCTCAG-3' and 1525r 5'-AAGGA-GGTGWTCCARCC-3' (Lane, 1991). The primers 16S uni1330 5'-GTTCCCGGGCCTTGTACACAC-3' and 23S uni322 anti 5'-GGTTCTTTTCGCCTTTCCCTC-3' from conserved regions of *Xanthomonas* spp. (Honeycutt *et al.*, 1995) which are phylogenetically related to *X. fastidiosa* (Wells *et al.*, 1987), were used to amplify the 16S-23S region.

Amplification of the 16S rDNA gene and 16S-23S spacer of *X. fastidiosa* strains from different hosts was performed in a total volume of 25 µL containing 30-50 ng of DNA, 0.5 µM of each primer, 100 µM dNTP, 2.5 mM $MgCl_2$ and 0.5 U of *Taq* DNA polymerase (Amersham-

Pharmacia), in a Thermal Cycler 4800 (Perkin Elmer). PCR reactions were performed in the following conditions: 16S rDNA, 1x (1 min at 94°C), 40x (1 min at 94°C, 1 min at 65°C, 2 min at 72°C) and 16S-23S region, 1x (4 min at 94°C), 40x (30 s at 94°C, 30 s at 65°C, 1 min at 72°C).

**Cloning and sequencing methods.** The amplified 16S and 16S-23S intergenic spacer of *X. fastidiosa* strains were cloned into pBluescript II KS+ (Stratagene) or pGEM (Promega) cloning vectors. Subclonings were performed when necessary. Plasmid DNA of the clones was extracted using the Nucleospin Nucleic Acid Purification Kit (Clontech) and used in the sequencing reactions. The PCR reactions for sequencing were performed in a total volume of 10 µL containing 300-500 ng of DNA, 5 pmoles of primer (M13 forward or M13 reverse), 3 µL of buffer (20 mM Tris HCl, pH 9.0, 5 mM $MgCl_2$) and 1 µL of ABI PRISM big dye terminator cycle sequencing ready reaction kit (Perkin Elmer). The reactions were conducted with an initial denaturation of 5 min at 95°C, followed by 25 cycles with a denaturation of 1 min at 95°C, annealing of 10 s at 55°C and extension of 4 min at 65°C. The reactions were precipitated in 80 µL of isopropanol 75% and washed with ethanol 70%. The sequencing was performed in an automatic sequencer (ABI PRISM$^{TM}$ 377, Perkin Elmer) and repeated twice for all the clones.

**Sequence analysis.** The 16S rDNA and 16S-23S spacer of *X. fastidiosa* strains sequenced in this study, as well as other *X. fastidiosa* sequences available in the GenBank, were used for comparison. Sequences of *Xanthomonas* species representing the different cores obtained in the analysis of the 16S (Hauben *et al.*, 1997) and 16S-23S spacer (Gonçalves & Rosato, unpublished)

were also included in the analysis. The accession numbers are given in Table 1. The sequences were aligned using the Clustal W program (Thompson *et al.* 1994) and the phylogenetic tree was constructed using the p-distance with the Neighbor-Joining method using the MEGA package (Kumar *et al.*, 1993). Bootstrap analysis with 1000 replications was performed to provide support for the branches of the trees obtained.

## RESULTS AND DISCUSSION

### DNA:DNA hybridization

Eleven *X. fastidiosa* strains (CI 11067, CI 11380, CI 11039, CI X0, CO 11752, CO 12288, GR 8935, GR 9713, PL 9746, PL 788 and PE PLS) isolated from five different hosts were investigated by DNA:DNA hybridization. An initial hybridization using the 16S rDNA of *Xanthomonas* as probe was performed (Fig. 1a) to correct eventual errors in the DNA quantification. The bands obtained were read in a Densitometer and a band was considered as control with a defined DNA concentration. The other readings were corrected accordingly. The membranes were washed and submitted to another hybridization using DNA of the citrus strain CI 11067 as probe (Fig. 1b).

The level of homology presented by strains from citrus, coffee, grapevine and plum were considered high, ranging from 85 to 140%. These results show that *X. fastidiosa* strains isolated from these hosts, belong to the same DNA homology group and therefore to the same species. The present findings confirm the homology levels (above 85%) obtained by Wells *et al.* (1987) in the analysis of *X. fastidiosa* strains isolated from different hosts (grapevine, peach, periwinkle,

almond, plum, elm, sycamore, oak, ragweed and mulberry). Using the same approach, Kamper *et al.* (1985) analysed five strains of *X. fastidiosa* from plum, peach, periwinkle and grapevine, and the level of DNA homology ranged from 75-100%.

A striking result was however obtained for the pear strain PE PLS, which presented a low homology with citrus (below 20%). This result was consistent in all repetitions, even when DNA of a grapevine strain was used as a probe (data not shown). Pear strains were identified as *X. fastidiosa* by several characteristics such as the presence of rippled cell wall, rod shaped cells and growth fastidiousness (Leu & Su, 1993), however, the homology results strongly suggest that strain PE PLS does not belong to the species *X. fastidiosa*. Previous reports also described a low similarity between strain PE PLS and other *X. fastidiosa* strains in genetic diversity studies using rep-PCR and RAPD fingeprinting (Mehta *et al.*, 1999a; 1999b). Pear leaf scorch is the second disease caused by *X. fastidiosa* reported outside America, following the report of almond leaf scorch in India (Jindal & Sharma, 1987), and strains from this disease have not been isolated elsewhere. Further studies need to be performed with a higher number of strains in order to confirm the existence of a second homology group within the *Xylella* genus and confirm the relationships between strains isolated from pear and *X. fastidiosa* strains from other hosts.

**Comparison of the 16S sequences**

The 16S rDNA gene of five *X. fastidiosa* strains isolated from citrus (CI 52), coffee (CO 01), plum (PL 788), grapevine (GR 8935) and pear (PE PLS) was sequenced and all the strains yielded sequences comprising 1536 bases. The citrus and coffee strains showed identical sequences, whereas a small number of nucleotide substitutions, 2 and 4, in the plum and

grapevine sequences, respectively, were detected in relation to the citrus sequence. A higher number of alterations, 20, were however revealed in the pear sequence. Most of these alterations consisted of base substitutions (13 transitions and 6 transversions).

The five *X. fastidiosa* 16S sequences were also aligned with nine other *X. fastidiosa* sequences available in the GenBank (Table 1). Signature sequences corresponding to the *E. coli* positions 170 (CTAATACCG), 315 (YCACAYYG), 510 (CTAACTYYG) and 1410 (TCACACCATG) (Stackebrandt *et al.*, 1988) were found in all *X. fastidiosa* 16S sequences analysed, which confirms that *X. fastidiosa* belongs to the gamma subdivision of *Proteobacteria* (Wells *et al.*, 1987).

Similarity percentages within *X. fastidiosa* species ranged from 97.1-100% (Table 2), which revealed the high degree of conservation in this sequence. The lowest level of similarity was found between the pear strain (PE PLS) and the oak strain (OLS92-3). The 16S rDNA gene has been reported to present a low level of divergence even among distinct species. Leblond-Bourget *et al.* (1996) analysed the 16S sequence of 18 different species of *Bifidobacterium* and encountered a similarity level of 92-99%. Similarly, a mean similarity value of 98.2% was found among *Xanthomonas* spp. (Hauben *et al.*, 1997).

The high levels of homology of the 16S rDNA sequence obtained for the pear strain (97.1-98.5%) contrasts with the low DNA:DNA homology (below 20%) found between the pear strain and the citrus strain. The findings obtained in this study resemble those found for the genus *Xanthomonas* where some strains showing extremely low levels of DNA:DNA homology did not necessarily exhibit a low level of 16S rDNA similarity (Hauben *et al.*, 1997).

A striking similarity of 95% was found between the *X. fastidiosa* 16S sequence and that of *Pseudomonas boreopolis*, a bacterium isolated from the soil. There is however some uncertainty about the taxonomic position of *P. boreopolis* which could be assigned as a *Xanthomonas* species based on DNA:rDNA hybridization (de Vos *et al.*, 1989). The similarity levels between *Xanthomonas* species and *X. fastidiosa* strains ranged from 94.3 to 96.1% (data not shown) and the highest similarity value was found between *X. campestris* and strain PE PLS (Table 2).

A phylogenetic tree was constructed using the 16S rDNA of the *X. fastidiosa* strains sequenced in this study and those available in the GenBank as well as the 16S sequence of *P. boreopolis*. *X. campestris* was used as the outgroup strain. The tree obtained revealed two major clusters. Cluster I comprised the grapevine strains (GR 8935, PCE-FG and r116v11) and the mulberry strain (Mul-2), and cluster II included strains isolated from citrus (CI 52, CVC93-2), coffee (CO 01), plum (PL 788 and PLS2-9), periwinkle (PWT-22), and strain PP4-5. Two other minor clusters were formed by single strains, the oak (OSL92-3) and pear (PE PLS) strains. The *X. fastidiosa* group was close to *P. boreopolis*, possibly a *Xanthomonas* species, and to *X. campestris*, confirming earlier results (Wells *et al.*, 1987) that showed that *Xanthomonas* is the closest genus to *Xylella*.

**Analysis of the 16S-23S spacer region**

The sequence of the 16S-23S intergenic spacer region of the strains CI 52, CI X0, CI 11067, CO 01, GR 8935, PL 788 and PE PLS of *X. fastidiosa* was also determined using the 1.1 kb fragment yielded in the PCR reaction. The entire fragment was sequenced in both directions and the ends corresponding to part of the 16S and 23S gene were eliminated according to other

similar 16S-23S sequences deposited in the GenBank. After the exclusion of the flanking ends, a stretch of 511 to 523 bases was obtained. Comparisons of the seven spacer sequences obtained in this study to other *X. fastidiosa* sequences deposited in the GenBank revealed similarity levels ranging from 79.8 to 100% (Table 3). Most strains showed similarities from 97 to 100%, however the values were highly decreased when the pear sequence was compared (81.3-79.8%). The lowest similarity was observed between the pear (PE PLS) and oak (88.9) strains. Analysis of most *Xylella* sequences revealed some variable sites, 17 in the entire sequence, which included addition/deletion or substitutions. The most striking differences were observed in relation to the pear sequence: 29 base additions and 18 deletions, both occurring in blocks of nucleotides or as single alterations. Also 42 substitutions exclusives to this sequence were found.

A high level of variation has been reported in the 16S-23S spacers of closely related taxa (Normand *et al.*, 1996). However, the results obtained in the sequence analysis of *X. fastidiosa* showed that for most strains, the number of isolated substitutions found in the 16S gene did not differ considerably from that found in the 16S-23S spacer, as would be expected. Similar results were obtained by Luz *et al.* (1998) while analysing *Salmonela enterica* subspecies. The same authors report that the main differences found consisted of large insertions and/or deletions, as detected for the pear sequence in this study. All these alterations show the divergence of the pear strain to the *Xylella* strains from other hosts and thus support the classification of this strain into a different species.

When comparing *X. fastidiosa* sequences with those from *Xanthomonas* spp., similarity levels ranging from 59.3 to 70.6% were obtained (data not shown). *X. campestris* was again the closest species to *X. fastidiosa*. Regions exclusive to *Xylella* were revealed along the sequence at

the positions 47-50; 121-123, 137 and 304-318 of the citrus sequence. These sequences were present in all the *Xylella* strains and absent in the *Xanthomonas* strains used, showing the specificity of these sequences within the genus.

Figure 2 shows the two tRNA ($tRNA^{ala}$ and $tRNA^{ile}$) locations in the *Xylella* sequences, similarly found in most spacer regions in Gram-negative bacteria (Gurtler & Stanisich, 1996). The $tRNA^{ala}$ and $tRNA^{ile}$ were 76 and 77 bp long (76 bp for strain GR 8935) and use the UGC and GAU anticodon, respectively. Except for the pear sequence, $tRNA^{ala}$ is highly conserved in most *Xylella* sequences whereas a few changes were detected in the $tRNA^{ile}$, including one deletion in the grapevine sequence and one substitution in the coffee sequence.

The 16S-23S intergenic spacer of the seven *X. fastidiosa* strains sequenced and those available in the GenBank were used to construct a phylogenetic tree. *X. campestris* was used as the outgroup strain. The *X. fastidiosa* strains were grouped into two major clusters. Cluster I comprised strains from citrus (CI 52, CI 11066 and CI X0), coffee (CO 01), plum (PL 788), peach (5S2) and oak (88.9). Strains from *Acer macrophila* (am), oleander (ann1) and grapevine (GR 8935) formed cluster II. The pear strain remained apart from the other *Xylella* strains. The topology of the tree obtained with the 16S-23S spacer is comparable to that obtained using the 16S data, although different strains were used in each case. The citrus, coffee and plum strains were clustered together and the pear strain was clearly separated from other *Xylella* in both trees, however, an inconsistency was found for the oak strains. In the 16S tree the oak strain OSL92-3 was well separated from the other strains (bootstrap value of 98%) whereas in the 16S-23S spacer tree, the oak strain 88.9 was included in cluster I among strains from other hosts. At this stage we

do not have sufficient information about these strains to confirm the degree of divergence among oak strains and *X. fastidiosa* from other hosts.

It has been suggested that enough variability exists among strains of *X. fastidiosa* to justify a taxonomic separation at the subspecies or pathovar level (Hopkins, 1989). In this study, we have shown that such variation exists even when comparing highly conserved genes such as the 16S rDNA. The distinction between plum and peach strains from grapevine strains by 16S and 16S-23S data seems coherent with other studies using growth characteristics (Wells *et al.*, 1987) and pathogenicity (Hopkins, 1989). Also, Kamper *et al.* (1985), based on DNA hybridization studies, considered that the level of homology of 75% found between strains of PD and plum leaf scald was low enough to support distinction at the varietal and, possibly at the subspecies level. The relatedness of the plum strain to citrus and coffee strains found in both trees was however unknown. Plum leaf scald (PLS) was the first disease caused by *X. fastidiosa* reported in the Southern part of Brazil (French & Kitajima, 1978). The similarity between the plum strains with citrus and coffee strains may indicate a common origin for the CVC, CLS and PLS bacteria. Although this hypothesis is speculative, this possibility can not be ruled out, since single strains of *X. fastidiosa* have been reported to cause diseases in various hosts (Davis *et al.*, 1980b; Wells *et al.*, 1981b). Cross inoculation tests need to be performed in order to obtain more information regarding the specificity in pathogenicity between the plum, citrus and coffee strains and confirm the relatedness among these strains.

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Table 1. Bacterial strains and their 16S rDNA and/or 16S-23S GenBank accession numbers

| Organism | Strain | Host | GenBank Accession # | |
|---|---|---|---|---|
| | | | 16S | 16S-23S |
| *Xylella fastidiosa* | PL.788* | Plum | AF203388 | AF203395 |
| *Xylella fastidiosa* | CO.01* | Coffee | AF203390 | AF203394 |
| *Xylella fastidiosa* | GR.8935* | Grapevine | AF203391 | AF203397 |
| *Xylella fastidiosa* | PE.PLS* | Pear | AF203392 | AF203396 |
| *Xylella fastidiosa* | CI.52* | Citrus | AF203389 | AF203393 |
| *Xylella fastidiosa* | CI.11067* | Citrus | | AF237650 |
| *Xylella fastidiosa* | CI.X0* | Citrus | | AF237651 |
| *Xylella fastidiosa* | PP4-5 | Unknown | AF159580 | |
| *Xylella fastidiosa* | CVC93-2 | Citrus | AF159575 | |
| *Xylella fastidiosa* | PD28-5 | Grapevine | AF159574 | |
| *Xylella fastidiosa* | PWT-22 | Periwinkle | AF159578 | |
| *Xylella fastidiosa* | PLS2-9 | Plum | AF159579 | |
| *Xylella fastidiosa* | Mul-2 | Mulberry | AF159576 | |
| *Xylella fastidiosa* | PCE-FG | Grapevine | AF159572 | |
| *Xylella fastidiosa* | r116v11 | Grapevine | AF159573 | |
| *Xylella fastidiosa* | OSL92-3 | Oak | AF159577 | |
| *Xylella fastidiosa* | 5s2 | Peach | | AF073206 |
| *Xylella fastidiosa* | 88.9 | Oak | | AF073210 |
| *Xylella fastidiosa* | am | Acer macrophila | | AF073219 |
| *Xylella fastidiosa* | ann1 | Oleander | | AF073215 |
| *Pseudomonas boreopolis* | | | AB021391 | |
| *Xanthomonas campestris* | | | AF123092 | AF209755 |
| *Xanthomonas axonopodis* | | | AF123090 | AF209753 |
| *Xanthomonas vesicatoria* | | | AF123089 | AF123088 |
| *Xanthomonas pisi* | | | Y10758 | AF209761 |
| *Xanthomonas hyacinthi* | | | Y10754 | AF209759 |
| *Xanthomonas sacchari* | | | Y10766 | AF209762 |

* Strains sequenced in this study

Table 2. Similarity levels (%) of the 16S of *X. fastidiosa* strains and related species, including gaps

| | % Sequence similarity | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | CO.01 | CI.52 | PD28 | r116v11 | PCE-FG | Mul-2 | GR.8935 | PP4-5 | PLS2-9 | PL.788 | PWT-22 | OSL92-3 | PE.PLS | CVC93-2 | *P. boreopolis* |
| CI.52 | 100 | | | | | | | | | | | | | | |
| PD28 | 99.4 | 99.4 | | | | | | | | | | | | | |
| r116v11 | 99.0 | 99.0 | 99.3 | | | | | | | | | | | | |
| PCE-FG | 99.2 | 99.2 | 99.4 | 99.1 | | | | | | | | | | | |
| Mul-2 | 99.3 | 99.3 | 99.4 | 99.0 | 99.2 | | | | | | | | | | |
| GR.8935 | 99.7 | 99.7 | 99.7 | 99.3 | 99.5 | 99.6 | | | | | | | | | |
| PP4-5 | 99.6 | 99.6 | 99.5 | 99.1 | 99.5 | 99.4 | 99.6 | | | | | | | | |
| PLS2-9 | 99.3 | 99.3 | 99.1 | 98.7 | 98.9 | 99.0 | 99.3 | 99.5 | | | | | | | |
| PL.788 | 99.8 | 99.8 | 99.5 | 99.1 | 99.3 | 99.4 | 99.8 | 99.8 | 99.5 | | | | | | |
| PWT-22 | 99.4 | 99.4 | 99.2 | 99.1 | 99.1 | 99.1 | 99.4 | 99.6 | 99.2 | 99.6 | | | | | |
| OSL92-3 | 98.3 | 98.3 | 98.3 | 98.2 | 98.1 | 98.0 | 98.3 | 98.3 | 99.0 | 98.3 | 98.3 | | | | |
| PE.PLS | 98.5 | 98.5 | 98.2 | 97.8 | 98.0 | 98.1 | 98.5 | 98.4 | 98.1 | 98.5 | 98.1 | 97.1 | | | |
| CVC93-2 | 99.5 | 99.5 | 99.1 | 98.7 | 98.9 | 99.0 | 99.2 | 99.3 | 98.9 | 99.3 | 99.0 | 97.9 | 98.0 | | |
| *P. boreopolis* | 95.7 | 95.7 | 95.4 | 95.1 | 95.2 | 95.4 | 95.5 | 95.6 | 95.4 | 95.7 | 95.5 | 95.1 | 96.3 | 95.2 | |
| *X. campestris* | 95.5 | 95.5 | 95.3 | 94.9 | 95.1 | 95.3 | 95.5 | 95.5 | 95.4 | 95.6 | 95.2 | 95.17 | 96.1 | 95.1 | 97.1 |

Table 3. Similarity levels (%) of the 16S-23S spacer of *X. fastidiosa* strains and *X. campestris*, including gaps

| | % Sequence similarity | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | CI.52 | CO.01 | CI.11067 | CI.X0 | PL.788 | 5s2 | 88.9 | am | ann1 | GR.8935 | PE.PLS |
| CO.01 | 99.8 | | | | | | | | | | |
| CI.11067 | 99.8 | 99.6 | | | | | | | | | |
| CI.X0 | 99.8 | 99.6 | 100 | | | | | | | | |
| PL.788 | 99.4 | 99.2 | 99.2 | 99.2 | | | | | | | |
| 5s2 | 98.2 | 98.0 | 98.4 | 98.4 | 98.8 | | | | | | |
| 88.9 | 98.0 | 97.8 | 98.2 | 98.2 | 98.6 | 99.8 | | | | | |
| am | 97.1 | 96.9 | 97.0 | 97.0 | 97.8 | 98.5 | 98.3 | | | | |
| ann1 | 97.1 | 96.9 | 97.0 | 97.0 | 97.8 | 98.5 | 98.3 | 99.2 | | | |
| GR.8935 | 97.6 | 97.3 | 97.4 | 97.4 | 98.2 | 97.2 | 97.0 | 98.2 | 97.8 | | |
| PE.PLS | 81.1 | 80.9 | 81.3 | 81.3 | 81.1 | 80.1 | 80.0 | 79.8 | 79.8 | 80.6 | |
| *X. campestris* | 70.0 | 69.8 | 70.0 | 70.0 | 69.8 | 70.1 | 69.7 | 70.0 | 69.6 | 69.1 | 70.6 |

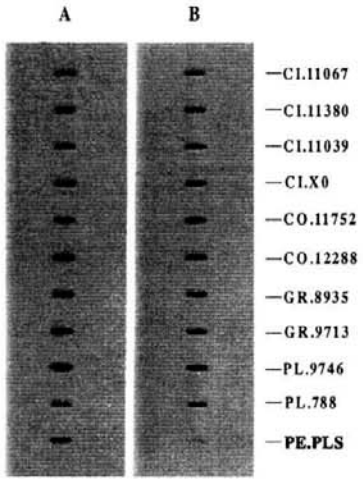


Fig.1. DNA:DNA hybridization using *X. fastidiosa* strains from different hosts and *Xanthomonas* 16S gene (A) and DNA of the *X. fastidiosa* citrus strain CI 11067 (B) as probe.

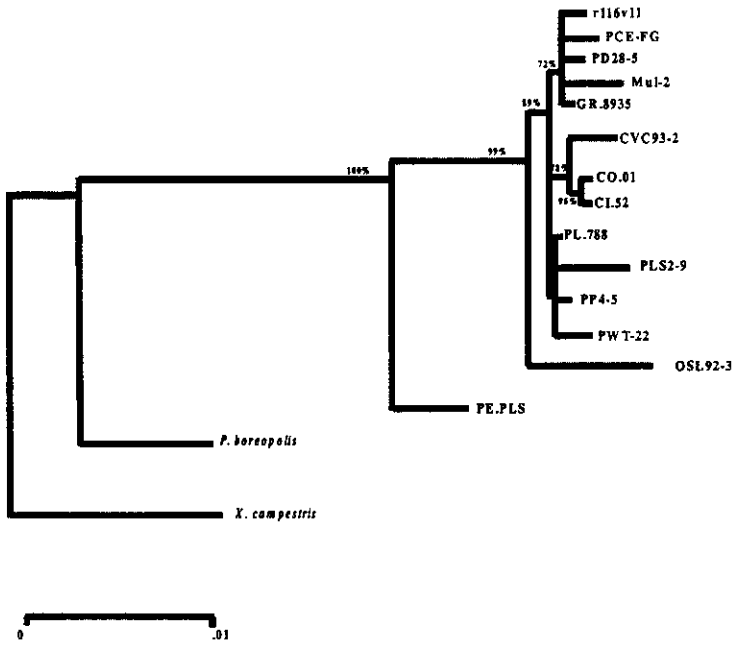


Fig. 2. Phylogenetic tree constructed by the Neighbor-Joining method, based on the 16S sequence data of *X. fastidiosa* and *P. boreopolis,* with *X. campestris* as the outgroup. Gaps and missing information were excluded from the analysis. The bar indicates 1% sequence divergence and the numbers above the branches are bootstrap values obtained for 1000 replications (only values higher than 70% are shown).

```
am              TATCGGAAG-TGCGGCTGGATCAC-TCCTTTTGAGTATGGTGA-ATATAATTGTCTTACC 57
ann1            TATCGGAAG-TGCGGCTGGATCAC-TCCTTTTGAGTATGGTGA-ATATAATTGTCTTATC 57
GR.8935         TATCGGAAGGTGCGGCTGGATCACCTCCTTTTGAGTATGGTGA-ATATAATTGTCTTACC 59
5s2             TATCGGAAG-TGCGGCTGGATCAC-TCCTTTTGAGTATGGTGA-ATATAATTGTCTTATC 57
88.9            TATCGGAAG-TGCGGCTGGATCAC-TCCTTTTGAGTATGGTGA-ATATAATTGTCTTATC 57
PL.788          TATCGGAAGGTGCGGCTGGATCACCTCCTTTTGAGTATGGTGA-ATATAATTGTCTTATC 59
CI.52           TATCGGAAGGTGCGGCTGGATCACCTCCTTTTGAGTATGGTGA-ATATAATTGTCTTATC 59
CO.01           TATCGGAAGGTGCGGCTGGATCACCTCCTTTTGAGTATGGTGA-ATATAATTGTCTTATC 59
CI.11067        TATCGGAAGGTGCGGCTGGATCACCTCCTTTTGAGTATGGTGA-ATATAATTGTCTTATC 59
CI.X0           TATCGGAAGGTGCGGCTGGATCACCTCCTTTTGAGTATGGTGA-ATATAATTGTCTTATC 59
PE.PLS          TATCGGAAGGTGCGGCTGGATCACCTCCTTTTGAGTATGGCAGCATATCATCGTCCTGTT 60
                ********* ************** ***************    **** ** *** *

am              AGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATG 117
ann1            AGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATG 117
GR.8935         AGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATG 119
5s2             AGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATG 117
88.9            AGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATG 117
PL.788          AGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATG 119
CI.52           AGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATG 119
CO.01           AGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATG 119
CI.11067        AGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATG 119
CI.X0           AGGCGTCCTCACAAGTTACTTGCATTCAGGGTTTGATGTTGGCATAGGTTTGGGTTTATG 119
PE.PLS          AGGCGTCCTCACAAGTTACCTGCATTCAGAGTTTTATGTTGGCAT-----TGGGTT---- 111
                ****************** ********* **** **********     ******

am              TTGGCGATTTTTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAG 177
ann1            TTGGCGATTTTTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAG 177
GR.8935         TTGGCGATTTTTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAG 179
5s2             TTGGCGATTTTTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAG 177
88.9            TTGGCGATTTTTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAG 177
PL.788          TTGGCGATTTTTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAG 179
CI.52           TTGGCGATTTTTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAG 179
CO.01           TTGGCGATTTTTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAG 179
CI.11067        TTGGCGATTTTTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAG 179
CI.X0           TTGGCGATTTTTGTTCTGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAG 179
PE.PLS          ----CGAGTTCAGTTCGGGGGGCCTTAGCTCAGCTGGTAGAGCACCTGCTTTGCAAGCAG 167
                    *** **  **** ****************************************

am              GGGGTCGTCGGTTCGATCCCGACAGGCTCCACCATGAAAGTAT-TTATGGGTCTGTAGCT 236
ann1            GGGGTCGTCGGTTCGATCCCGACAGGCTCCACCATCAAAGTAT-TTATGGGTCTGTAGCT 236
GR.8935         GGGGTCGTCGGTTCGATCCCGACAGGCTCCACCATGAAAGTAT-TTATGGGTCTGTAGCT 238
5s2             GGGGTCGTCGGTTCGATCCCGACAGGCTCCACCATGAAAGTAT-TTATGGGTCTGTAGCT 236
88.9            GGGGTCGTCGGTTCGATCCCGACAGGCTCCACCATGAAAGTAT-TTATGGGTCTGTAGCT 236
PL.788          GGGGTCGTCGGTTCGATCCCGACAGGCTCCACCATGAAAGTAT-TTATGGGTCTGTAGCT 238
CI.52           GGGGTCGTCGGTTCGATCCCGACAGGCTCCACCATGAAAGTAT-TTATGGGTCTGTAGCT 238
CO.01           GGGGTCGTCGGTTCGATCCCGACAGGCTCCACCATGAAAGTAT-TTATGGGTCTGTAGCT 238
CI.11067        GGGGTCGTCGGTTCGATCCCGACAGGCTCCACCATGAAAGTAT-TTATGGGTCTGTAGCT 238
CI.X0           GGGGTCGTCGGTTCGATCCCGACAGGCTCCACCATGAAAGTAT-TTATGGGTCTGTAGCT 238
PE.PLS          GGTGTCGTCGGTTCGATCCCGGCAGGCTCCACCA---GGGTGTCTTATGGGTCTGTAGCT 224
                ** ****************** ************     ** * ****************

am              CAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACC 296
ann1            CAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACC 296
GR.8935         CAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCG-TGGTTCGAGTCCTCCCAGACC 297
5s2             CAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACC 296
88.9            CAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACC 296
PL.788          CAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACC 298
CI.52           CAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACC 298
CO.01           CAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCAAGTCCTCCCAGACC 298
CI.11067        CAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACC 298
CI.X0           CAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACC 298
PE.PLS          CAGGTGGTTAGAGCGCACCCCTGATAAGGGTAAGGTCGGTGGTTCGAGTCCTCCCAGACC 284
                ******************************* ****** ****** **************
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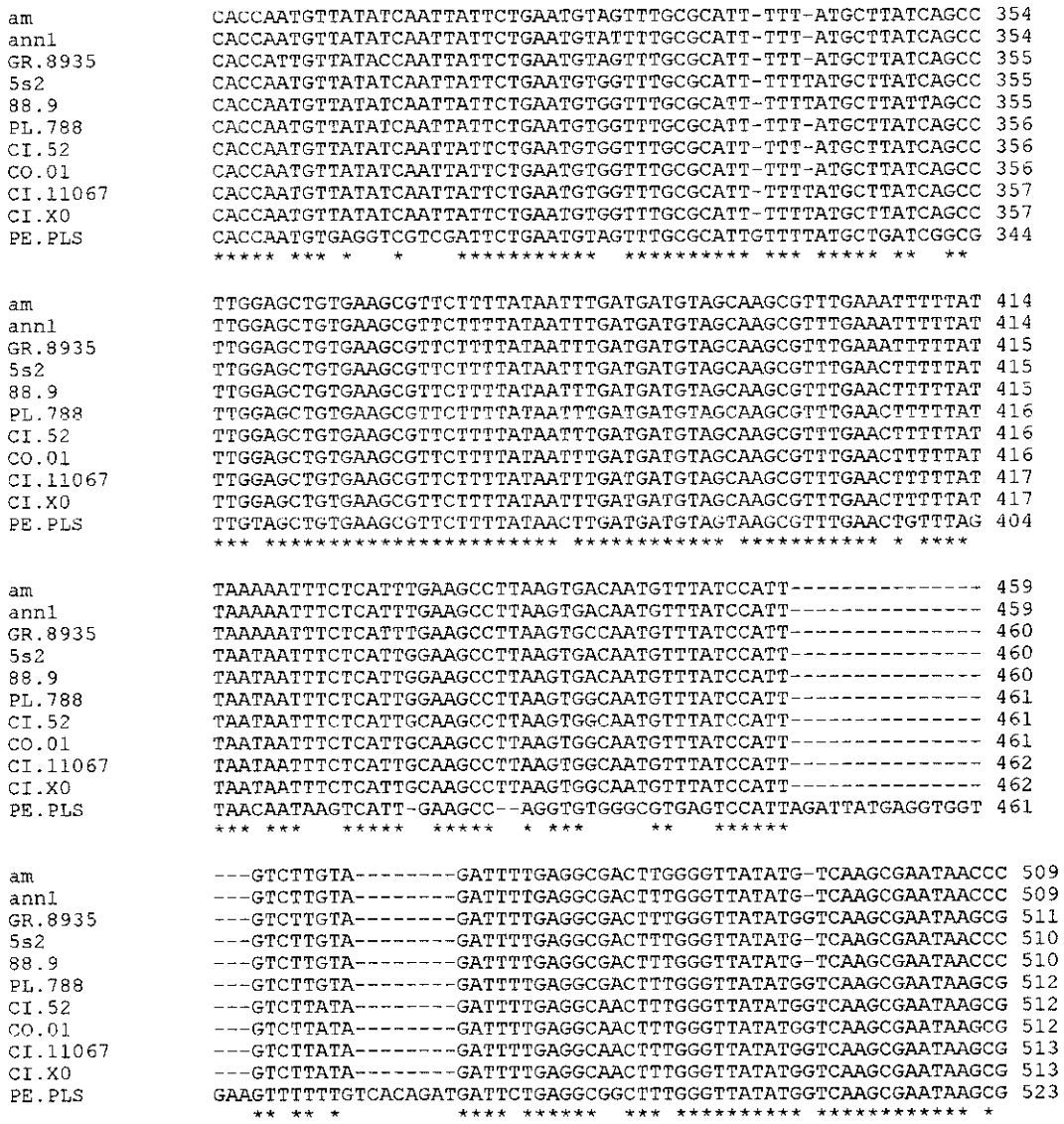


Fig. 3. Alignment of the 16S-23S spacer sequences of *X. fastidiosa*. The sequences of strains 88.9, am, ann1 and 5s2 were obtained from the GenBank. Sequences in red indicate the $tRNA^{ala}$ and in blue $tRNA^{ile}$. The symbol * indicates the occurence of identical bases for all sequences, and – indicates a gap.

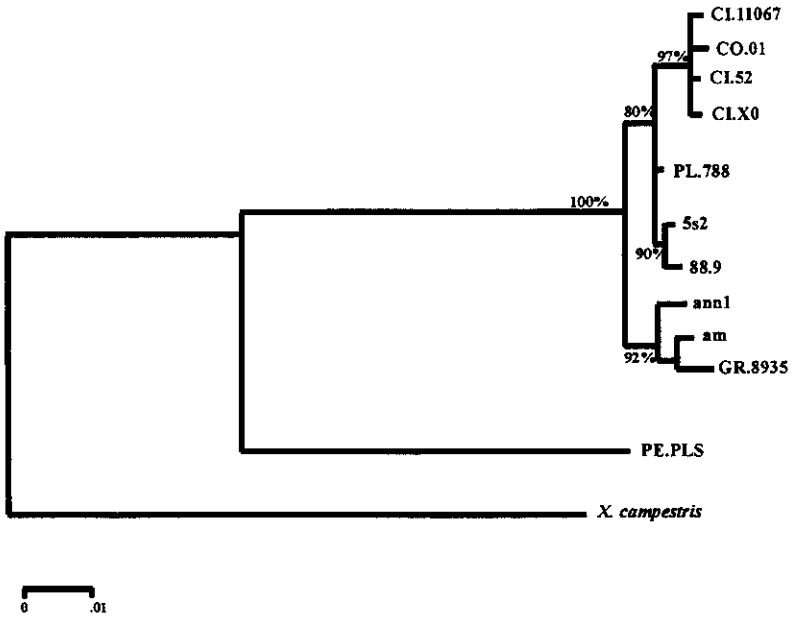


Fig. 4. Phylogenetic tree constructed by the Neighbor-Joining method, based on the 16S-23S spacer sequence data of *X. fastidiosa,* with *X. campestris* as the outgroup. Gaps and missing information were excluded from the analysis. The bar indicates 1% sequence divergence and the numbers above the branches are bootstrap values obtained for 1000 replications (only values higher than 70% are shown).

## CONCLUSÕES

A análise da diversidade genética de *X. fastidiosa* revelou que as técnicas utilizadas apresentaram níveis de resolução distintos na detecção de variabilidade genética entre linhagens de diferentes hospedeiros e entre linhagens de mesmo hospedeiro. A técnica de RFLP de rDNA 16S e região espaçadora 16S-23S revelou um baixo nível de polimorfismo, entretanto a diferenciação de linhagens de alguns hospedeiros foi obtida. As técnicas de SDS-PAGE, rep-PCR e RAPD revelaram um maior nível de polimorfismo e além da relação com os hospedeiros, variação entre as linhagens de citros foi obtida. O sequenciamento de rDNA 16S e região espaçadora 16S-23S revelou poucas variações nas seqüências para a maioria das linhagens, entretanto as linhagens formaram grupos distintos nas árvores filogenéticas.

Os resultados obtidos neste estudo permitiram as seguintes conclusões:

1. A análise da diversidade genética de linhagens de *X. fastidiosa* isoladas de citros mostrou que embora a CVC tenha surgido recentemente, já existem variações entre as linhagens. Duas populações principais foram diferenciadas: uma formada por linhagens isoladas da região sul e outra por linhagens dos estados de SP e SE. Diversidade dentro dessas populações foi também encontrada, indicando que uma alta taxa de recombinação entre as linhagens de citros possa estar ocorrendo.

2. Embora um número limitado de linhagens de diferentes hospedeiros tenha sido utilizado, uma evidente relação com os hospedeiros foi observada utilizando-se as técnicas de

RAPD, SDS-PAGE de proteínas e rep-PCR. Esses resultados reforçam a hipótese da existência de diferentes patotipos de *X. fastidiosa*.

3. A linhagem de pêra (PLS) apresentou uma baixa similaridade com as demais linhagens, e uma baixa homologia com citros na análise de hibridização DNA:DNA, indicando que esta linhagem não pertence à espécie genômica *X. fastidiosa*. Na análise filogenética, esta linhagem também permaneceu isoladas de todas as outras linhagens de *X. fastidiosa*, reforçando a hipótese de que ela represente uma nova espécie de *Xylella*.

4. As árvores filogenéticas obtidas com as seqüências de rDNA 16S e região 16S-23S revelaram que as linhagens de citros, café e possivelmente as linhagens de ameixa formaram um grupo e linhagens de videira formaram outro grupo. Estes resultados confirmam a distinção entre as linhagens de ameixa e videira baseada em característica de patogenicidade e taxa de crescimento fornecendo suporte para a separação de linhagens de *X. fastidiosa* destes hospedeiros no nível subespecífico.

**SUMMARY**

*Xylella fastidiosa* is a Gram-negative bacterium associated to diseases in many economically important crops such as grapevine, citrus, coffee and plum. In this study, the genetic relationships among *X. fastidiosa* strains isolated from citrus in different geographic regions of Brazil was investigated. Strains isolated from coffee, grapevine, plum and pear were also included for comparison. The genetic diversity was investigated using molecular techniques including amplification of DNA using the primers ERIC, REP and BOX (rep-PCR), RFLP of the 16S rDNA and 16S-23S intergenic spacer, RAPD and SDS-PAGE of proteins. RFLP of the 16S rDNA and 16S-23S intergenic spacer revealed a low level of polymorphism. The RAPD and rep-PCR techniques showed a higher level of polymorphism, demonstrating the relationships of strains with the hosts and the variation among the citrus strains. Citrus strains isolated from the Southern States including Rio Grande do Sul, Santa Catarina and Paraná, formed one group, whereas strains isolated from the States of São Paulo and Sergipe formed another group. The analysis of the protein profiles obtained by SDS-PAGE also showed a relationship between strains and their hosts. The pear strain was distantly related to the other strains of *X. fastidiosa* in all the techniques used. DNA:DNA hybridization revealed a homology above 80% for the strains isolated from grapevine, plum, citrus and coffee, showing that these strains belong to the same genomic species. The pear strain presented a homology level below 20%, indicating that this strain does not belong to the genomic species *X. fastidiosa*. The phylogenetic relationships among strains from different hosts was assessed by sequence analysis of the 16S rDNA and 16S-23S intergenic spacer. The phylogenetic trees obtained revealed two major clusters: the citrus, coffee and plum strains formed one cluster, distinct from that formed by the grapevine strains.

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