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Repetitive sequence-derived PCR profiling using the BOX-A1R primer for rapid identification of the plant pathogen *Clavibacter michiganensis* subspecies *sepedonicus*

Neil C. Smith, Judy Hennessy and David E. Stead
Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK (Fax: +44 1904 462111; E-mail: n.smith@csl.gov.uk)

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Abstract

Repetitive sequence-derived PCR using the BOX-A1R primer was used to generate genomic fingerprints of *Clavibacter michiganensis* subspecies *sepedonicus,* the causal agent of bacterial ring rot disease of potato. A total of 35 *C. michiganensis* subsp. *sepedonicus* strains were selected for study in order to represent the widest possible historical, morphological and geographical diversity of the organism. Comparison was made with genomic fingerprints of *C. michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *insidiosus*, *C. michiganensis* subsp. *tessellarius*, *C. michiganensis* subsp. *nebraskensis* as well as other related Gram positive plant pathogens. The resultant genomic fingerprints and subsequent cluster analysis show *C. michiganensis* subsp. *sepedonicus* to form a remarkably homogeneous group with approximately 84% similarity between all of the strains tested. There was no evidence to suggest that fingerprints varied with historic, morphological or geographic diversity. In addition, *C. michiganensis* subsp. *sepedonicus* isolated from asymptomatic sugar beet had the same fingerprint as those which were isolated as potato pathogens. This group was easily distinguished from the clusters formed by the other subspecies of *C. michiganensis* and Gram positive plant pathogens. The potential for this technique to be used as a relatively rapid method to replace the time consuming and sometimes inconclusive eggplant bioassay test is discussed.

Introduction

Clavibacter michiganensis subspecies *sepedonicus* (CMS) is the causal agent of bacterial ring rot disease of potato (*Solanum tuberosum* L). It is one of five subspecies of the Gram positive coryneform bacterium *C. michiganensis* which is characterized by the ability to cause disease in plants and by the presence of 2,4-diaminobutyric acid in the peptidoglycan of their cell walls (Davis et al., 1985). Ring rot is considered to be one of the most internationally important seed potato diseases and in North America it is responsible for about 60% of annual seed potato acreage rejected for certification (de la Cruz et al., 1992). In Europe, outbreaks have been reported in a number of European Union member states, and an EU Directive aimed at controlling the spread of the pathogen throughout member states has been published (Anonymous, 1993). Ring rot can thus act as a barrier to trade in seed potatoes between the European Union and other parts of the world (Stead, 1993). Both North America and the European Union have zero tolerance programmes for the pathogen in an attempt to eradicate the disease from seed potato stocks.

Clavibacter michiganensis subspecies *sepedonicus* causes a disease that appears to be specific for potatoes but the organism has been found in asymptomatic association with sugarbeet (Bugbee et al., 1987). Tomato (*Lycopersicon esculentum*) and eggplant (*Solanum melongena*) can be artificially infected with the pathogen to produce wilting in the laboratory. Symptoms of the disease in potato include wilting of

the stem and a characteristic browning and rotting of the tubers, although often the disease may remain latent or symptomless in plants or tubers for several generations (Nelson, 1980). Although primary isolation of CMS from infected tubers showing typical symptoms is reasonably straightforward, isolation of the pathogen from advanced rots is made difficult because the organism grows very slowly and is easily overgrown by saprophytic bacteria. In addition, colony morphology can be variable (Lelliott and Stead, 1987). Protocols describing media semi-selective for CMS which may make the isolation of CMS from infected plant tissue easier have been published (de la Cruz et al., 1992; Jansing and Rudolph, 1998). New methods to confirm the identity of a suspected CMS isolate are needed because bioassay tests, which rely on the pathogenicity of CMS to eggplants, can be unsatisfactory. It is expensive and time consuming to set up and can take up to six weeks to confirm a suspected incidence of ring rot (Janse and van Vaerenbergh, 1987). Symptom development may also be affected by many factors including the strain of CMS chosen (Bishop and Slack, 1987). Identification using serological methods such as immunofluorescence (IF) and ELISA can be unreliable due to cross-reactions, as few of the antisera in current use have good specificity (Mills et al., 1997). Identification using traditional nutritional and physiological tests (Lelliott and Stead, 1987) are slow and not always conclusive. Methods such as fatty acid methyl ester (FAME) analysis, widely used to determine the identity of many plant pathogens, provide excellent identification of the species *C. michiganensis.* However, identification of *C. michiganensis* to below the species level is difficult because fatty acid profiles within the species have been found to overlap (Henningson and Gudmestad, 1991). Protein profiles are useful but labour intensive (Stead et al., 1998).

In recent years, genomic fingerprinting methods based on the polymerase chain reaction (PCR) have been developed for typing the genomes of bacteria both for taxonomic information (Louws et al., 1995) and to provide powerful new diagnostic procedures capable of identifying plant-associated bacteria to the subspecies level (Louws et al., 1998). One such method, called randomly amplified polymorphic DNA (RAPD), uses arbitrary primers to generate DNA fragments from a wide range of genomes (Welsh and McClelland, 1990). However, this technique requires extremely careful optimization to be certain of reproducibility and the DNA template preparation is laborious and time consuming. More recently, a number of bacterial fingerprinting techniques have been developed which are based on repeated DNA sequences which are known to occur throughout bacterial genomes. These techniques are collectively known as repetitive sequence-based PCR (Rep-PCR). Currently, there are three commonly used Rep-PCR techniques, each based on different repeated elements within bacterial chromosomes. These are the repetitive extragenic palindromic elements (REP-PCR), enterobacterial repetitive intergenic consensus sequence (ERIC-PCR) and the BOX-A1R element (BOX-PCR) (Versalovic et al., 1994). Their value lies in the fact that prior knowledge of target sequence DNA is not necessary, although empirical screening of a number of Rep-PCR techniques may be required to obtain the desired fingerprints (Louws et al., 1999).

In this paper, we report the development of a relatively simple method which uses the BOX-PCR technique to generate genomic fingerprints which allow the identification of CMS. It also allows the differentiation of CMS from other members of the genus *Clavibacter,* other Gram positive phytopathogens, and coryneform bacteria commonly encountered as potato endophytes.

Materials and methods

Bacterial cultures

Cultures were obtained from a broad range of morphological, geographic, historic and host diversity in an attempt to represent as much of the potential strain variation within the organisms as possible. A list of the strains used in this study is shown in Table 1. All strains were raised on yeast glucose mineral salts agar (YGM) and incubated at 21 °C for between three and five days, with the exception of *Clavibacter toxicus* which was grown at 28 °C. *C. xyli* subsp. *cynodontis* was grown on modified ratoon stunt disease (RSD) agar according to the method of Metzler et al. (1992).

Colony characteristics

For each of the microorganisms used, pigment production and colony morphology of colonies growing on YGM was recorded. Colony morphology was subdivided into three categories: *fluidal* where the colony material will flow if the plate is tipped at an angle of 45°,

Table 1. List of strains included in this study

Strain	Source and year of isolation (if known)	Pigment on YGM	Colony morphology on YGM	Host (if known)
Clavibacter michiganensis subsp. *sepedonicus*				
NCPPB 299	Canada 1951	Pale yellow	Dry	*S. tuberosum*
NCPPB 2140	Unknown 1942	Pale yellow	Dry	*S. tuberosum*
NCPPB 2137 (T)	Unknown	Cream	Mucoid	*S. tuberosum*
NCPPB 3384	Norway 1983	Cream	Fluidal	*S. tuberosum*
NCPPB 3898	Ukraine 1996	Cream	Dry	*S. tuberosum*
NCPPB 4053	Sweden 1994	Cream	Fluidal	*S. tuberosum*
NCPPB 3467	Poland 1985	Pale yellow	Mucoid	*S. tuberosum*
20.18[a]	Denmark 1991	Pale yellow	Dry	*S. tuberosum*
20.20[a]	Germany 1997	Pale yellow	Mucoid	*S. tuberosum*
20.21[a]	Germany 1997	Pale yellow	Mucoid	*S. tuberosum*
20.22[a]	Germany 1997	Cream	Fluidal	*S. tuberosum*
ND 9[b]	USA 1988	Pale yellow	Mucoid	*S. tuberosum*
CMS 729[c]	Sweden 1994	Cream	Fluidal	*S. tuberosum*
CMS 731[c]	Sweden 1994	Cream	Fluidal	*S. tuberosum*
UKRA[d]	Canada	Cream	Mucoid	*S. tuberosum*
R 14[d]	Canada	Cream	Mucoid	*S. tuberosum*
Eggplant[d]	Canada	Cream	Mucoid	*S. tuberosum*
1437[e]	Finland	Cream	Dry	*S. tuberosum*
1438[e]	Finland	Cream	Mucoid	*S. tuberosum*
ICMP 9731	USA 1984	Cream	Fluidal	*B. vulgaris* root
ICMP 9732	USA 1985	Cream	Fluidal	*B. vulgaris* seed
Monorica[b]	USA 1985-87	Cream	Mucoid	*B. vulgaris* seed
Dippe 2[b]	USA 1985	Cream	Fluidal	*B. vulgaris* seed
SB 109[b]	USA 1985	Cream	Fluidal	*B. vulgaris* seed
OLM 17[b]	USA 1985	Pale yellow	Fluidal	*B. vulgaris* seed
SP 301[f]	Denmark 1995	Cream	Mucoid	*S. tuberosum*
SP 303[f]	Netherlands 1995	Cream	Fluidal	*S. tuberosum*
SP 357[f]	Denmark 1994	Cream	Mucoid	*S. tuberosum*
SP 371[f]	Denmark 1996	Pale yellow	Mucoid	*S. tuberosum*
SP 375[f]	Denmark 1996	Cream	Mucoid	*S. tuberosum*
BJ 19[b]	USA 1985	Cream	Mucoid	*B. vulgaris* seed
ULTRA[b]	USA 1985	Cream	Mucoid	*B. vulgaris* seed
PD 60	Argentina 1977	Pale yellow	Fluidal	*S. tuberosum*
PD 6	USA 1976	Cream	Fluidal	*S. tuberosum*
PD 263	Canada	Cream	Mucoid	*S. tuberosum*
Clavibacter michiganensis subsp. *insidiosus*				
NCPPB 83	USA 1933	Cream/indigo	Fluidal	*M. sativa*
NCPPB 852	USA pre-1960	Cream/indigo	Dry	*M. sativa*
NCPPB 1020	Canada pre-1960	Yellow/indigo	Fluidal	*M. sativa*
NCPPB 1109 (T)	USA 1955	Yellow	Very fluidal	*M. sativa*
NCPPB 2415	New Zealand 1970	Yellow/indigo	Fluidal	*M. sativa*
NCPPB 2900	UK 1976	Cream/indigo	Fluidal	*M. sativa*
NCPPB 3032	UK 1975	Cream/indigo	Fluidal	*M. sativa*
LMG 3676	1968	Yellow/indigo	Mucoid	Unknown
LMG 7323	USA 1939	Yellow/indigo	Mucoid	*M. sativa*
Clavibacter michiganensis subsp. *nebraskensis*				
NCPPB 2578	USA 1971	Orange	Mucoid	*Z. mays*
NCPPB 2579	USA 1971	Orange	Fluidal	*Z. mays*
NCPPB 2580	USA 1971	Orange	Fluidal	*Z. mays*
NCPPB 2581 (T)	USA 1971	Orange	Fluidal	*Z. mays*
NCPPB 2582	USA 1974	Orange	Fluidal	*Z. mays*

Table 1. Continued

Strain	Source and year of isolation (if known)	Pigment on YGM	Colony morphology on YGM	Host (if known)
Clavibacter michiganensis subsp. *tessellarius*				
NCPPB 3664 (T)	USA 1978	Orange	Fluidal	*T. aestivum*
NCPPB 3665	USA 1978	Orange	Fluidal	*T. aestivum*
NCPPB 3666	USA 1978	Orange	Fluidal	*T. aestivum*
NCPPB 3667	USA 1978	Orange	Fluidal	*T. aestivum*
NCPPB 3669	USA 1978	Orange	Fluidal	*T. aestivum*
Clavibacter michiganensis subsp. *michiganensis*				
NCPPB 870	USA 1939	Yellow	Mucoid	*L. esculentum*
NCPPB 886	Zimbabwe 1960	Yellow	Fluidal	*L. esculentum*
NCPPB 1468	Channel Islands 1962	Yellow	Fluidal	*L. esculentum*
NCPPB 1574	Hungary 1963	Yellow	Fluidal	*L. esculentum*
NCPPB 2943	USA 1974	Pale yellow	Mucoid	*C. frutescens*
NCPPB 2979 (T)	Hungary 1957	Yellow	Fluidal	*L. esculentum*
NCPPB 3123	USA 1972	Pale yellow	Mucoid	*L. esculentum*
NCPPB 3225	UK 1981	Yellow	Fluidal	*L. esculentum*
NCPPB 3226	UK 1981	Yellow	Fluidal	*L. esculentum*
NCPPB 3227	UK 1981	Yellow	Fluidal	*L. esculentum*
NCPPB 3285	UK 1983	Cream	Fluidal	*L. esculentum*
NCPPB 3455	Hungary 1982	Yellow	Fluidal	*L. esculentum*
RPZBC 1680[g]	Unknown	Yellow	Fluidal	Unknown
RPZBC 1910[g]	Unknown	Yellow	Fluidal	Unknown
RPZBC 2405[g]	Unknown	Yellow	Mucoid	Unknown
LMG 3694	South Africa 1967	Yellow	Fluidal	*L. esculentum*
LMG 5610	Unknown	Yellow	Fluidal	*L. esculentum*
LMG 5724	Bulgaria	Yellow	Mucoid	*L. esculentum*
Clavibacter toxicus				
NCPPB 3552	Australia 1987	Yellow	Dry	*L. rigidum*
NCPPB 3553	Australia 1987	Yellow	Dry	*L. rigidum*
NCPPB 3810	Australia	Yellow	Dry	*A. sativa*
NCPPB 3811	Australia	Yellow	Dry	*Pharis* sp.
Clavibacter xyli subsp. *cynodontis*				
CXC 7[h]	USA 1980s	Yellow	Mucoid	*C. dactylon*
CXC 18[h]	USA 1980s	Yellow	Mucoid	*C. dactylon*
Rathayibacter rathayi				
NCPPB 80	UK 1934	Yellow	Mucoid	*D. glomerata*
NCPPB 797	UK 1960	Yellow	Dry	*D. glomerata*
NCPPB 2980 (T)	New Zealand 1969	Yellow	Dry	*D. glomerata*
Rathayibacter iranicus				
NCPPB 2253 (T)	Iran 1966	Yellow	Dry	*T. aestivum*
PD 164	Iran 1966	Yellow	Dry	*T. aestivum*
Rathayibacter tritici				
NCPPB 1857 (T)	Egypt 1966	Yellow	Mucoid	*T. aestivum*
Curtobacterium flaccumfaciens pv. *flaccumfaciens*				
NCPPB 1446 (T)	Hungary 1957	Yellow	Dry	*P. vulgaris*
Curtobacterium flaccumfaciens pv. *betae*				
NCPPB 374 (T)	UK 1955	Pale yellow	Dry	*B. vulgaris*
Curtobacterium flaccumfaciens pv. *oortii*				
NCPPB 2113 (T)	Netherlands 1967	Yellow	Dry	*T. gesneriana*
Curtobacterium flaccumfaciens pv. *poinsettiae*				
NCPPB 854 (T)	USA 1960	Orange/pink	Dry	*E. pulcherrima*

Table 1. Continued

Strain	Source and year of isolation (if known)	Pigment on YGM	Colony morphology on YGM	Host (if known)
Arthrobacter ilicis NCPPB 1228 (T)	USA 1960	White	Dry	*I. opaca*

(T): Type strain; NCPPB: National Collection of Plant Pathogenic Bacteria, York, UK; PD: Plantenziektenkundige Dienst, Wageningen, The Netherlands; ICMP: International Collection of Microorganisms from Plants, DSIR, Auckland, New Zealand; LMG: Collection Laboratorium Microbiologie, Universiteit Gent, Belgium.
[a]Kindly donated by D. Caffier, [b]N. Gudmestad, [c]P. Persson, [d]S.de Boer, [e]J. Tegel, [f]Keld Mansfield, [g]J. van Vaerenberg, [h]M. Metzler.

mucoid where the colony has a gummy consistency through the production of polysaccharide (Buchanan and Gibbons, 1974) and *dry* where little or no polysaccharide material was produced. Colony morphology of *C. xyli* subsp. *cynodontis* was recorded on modified RSD agar. The descriptions of all strains are shown in Table 1.

Verification of strains

The identity of CMS strains used in the study was confirmed by an immunofluorescent assay with the monoclonal antibody MAb 9A1 (Agdia, Indiana USA) according to the manufacturer's instructions. All other microorganisms used in this study were well-characterized strains from international collections of plant pathogens.

DNA template preparation

In order that the procedure be kept as simple as possible, template DNA for Rep-PCR fingerprinting was prepared by simple alkaline lysis. This involved boiling a 1 µl loopful of colony for 15 min in 100 µl of 50 mM NaOH prepared in molecular-biology grade water (BDH Merck Ltd, Lutterworth, UK). After boiling, the lysed cellular material and released DNA was rapidly cooled on ice, and the volume made up to 1 ml using molecular-biology grade water. These samples were stored at −80 °C until use. No samples were stored for more than one week before PCR analysis.

CMS DNA purification

Purified DNA was extracted from a selection of CMS strains, and partially purified using a Wizard genomic DNA purification kit protocol (Promega Corporation, Madison, USA) according to the manufacturer's instructions. This DNA was diluted tenfold for use in BOX-PCR.

PCR analysis

BOX, ERIC and REP primers (see Murry et al. (1995) for primer sequences) were synthesized by Genosys biotechnologies (Cambridge, UK) and stored at −20 °C until use. PCR was performed using a Perkin Elmer 9600 thermal cycler using the following programmes: BOX-PCR – 95 °C for 7 min, then 30 cycles of 94 °C for 1 min, 53 °C for 1 min, 65 °C for 8 min, with a final 16 min elongation step of 65 °C; ERIC-PCR – 95 °C for 7 min followed by 30 cycles of 94 °C for 1 min, 52 °C for 1 min, 65 °C for 8 min with a 16 min final elongation step of 65 °C; REP-PCR – 95 °C for 7 min, followed by 35 cycles of 94 °C for 1 min, 40 °C for 1 min, 65 °C for 8 min with a final 16 min extension step of 65 °C. The PCR reaction mixes for each of the three PCR procedures were those of Versalovic et al. (1994). Taq polymerase was supplied by Perkin Elmer (New Jersey, USA) and nucleotides were obtained from Pharmacia (St Albans, UK). Two microlitres of boiled crude extracted material was used in a 25 µl PCR reaction.

After PCR, 12 µl of PCR product containing the amplified fragments were run on a 20 cm 2% agarose gel (Sigma). A maximum of 20 tracks was run on each gel. The gels were run in TAE buffer at 105 V at room temperature for exactly 2 h. Electrophoresis gels were stained for exactly 40 min in 400 ml of 20 µg/ml ethidium bromide. The gel image was captured using a BioRad Fluor-S multi-imager gel documentation system (BioRad Laboratories, California, USA), and the digitized image converted into an 8-bit TIFF file for subsequent analysis of the fingerprint patterns. Three lanes of molecular weight standard (Marker VI, Roche Diagnostics, Lewes, UK) were included on each gel at positions 1, 10 or 11 and

20 to allow inter-gel comparative analysis of genetic fingerprints.

Fingerprint analysis

Analysis of the genetic fingerprint patterns generated by BOX-PCR was carried out using GelCompar Version 4.1 (Applied Maths, Kortrijk, Belgium). Eight-bit TIFF files of REP-PCR fingerprints were converted into GelCompar format normalised and the similarity of banding patterns calculated by applying the Pearson product-moment correlation coefficient. Cluster analysis was performed by using the unweighted pair group method using arithmetic averages algorithm (UPMGA), and the results presented as a dendrogram.

Results

Selection of fingerprinting method

A preliminary experiment was performed on a small panel of CMS and other *C. michiganensis* strains to determine whether BOX-PCR, ERIC-PCR or REP-PCR produced the clearest, most differential and most reproducible genetic fingerprints (data not shown). BOX-PCR produced fingerprints with 10–15 well spaced bands in the approximate size region of 200–2000 base pairs, and the most suitable protocol for fingerprint analysis (GelCompar Version 4.1 reference manual). BOX-PCR fingerprints were thus prepared for all strains in Table 1 to test its reliability for identification of CMS including a range isolated from a wide geographical area and displaying atypical phenotypic characteristics, such as differences in pigment production and colony fluidity. Strains from asymptomatic infections in sugar beet were also included. Strains from potato were isolated between the 1940s and the present.

Reproducibility of BOX-PCR fingerprints

Ten colonies of CMS type strain NCPPB 2137 were picked randomly from plates and prepared by alkaline lysis. The resultant samples were used to generate BOX-PCR fingerprints on different days over the course of a working week to test the reproducibility of CMS fingerprints using this technique. These resultant fingerprints show a correlation of 94.7%, strongly suggesting that the technique is reproducible between different PCR reactions. These results are presented as a dendrogram in Figure 1.

Genetic fingerprint analysis

A dendrogram of all BOX-PCR fingerprints is shown in Figure 2. The cophenic correlation, which is a measure of the faithfulness of the data within the dendrogram

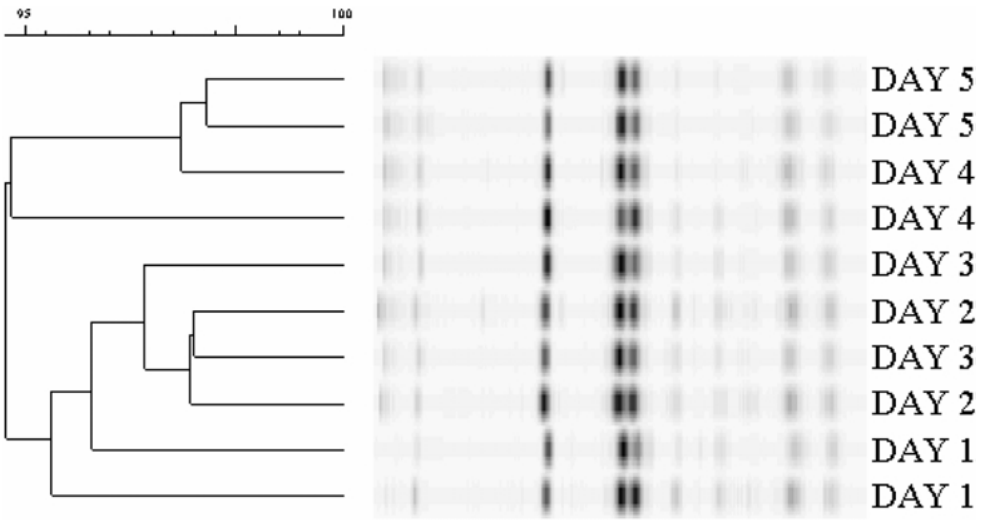

Figure 1. Reproducibility of *C. michiganensis* subsp. *sepedonicus* strain NCPPB 2137 BOX-PCR fingerprint. Key: Dendrogram of CMS strain NCPPB 2137 BOX fingerprint variability over five separate PCR reactions. DAY 1 to DAY 5 refers to replicated PCR reactions carried out on different days.

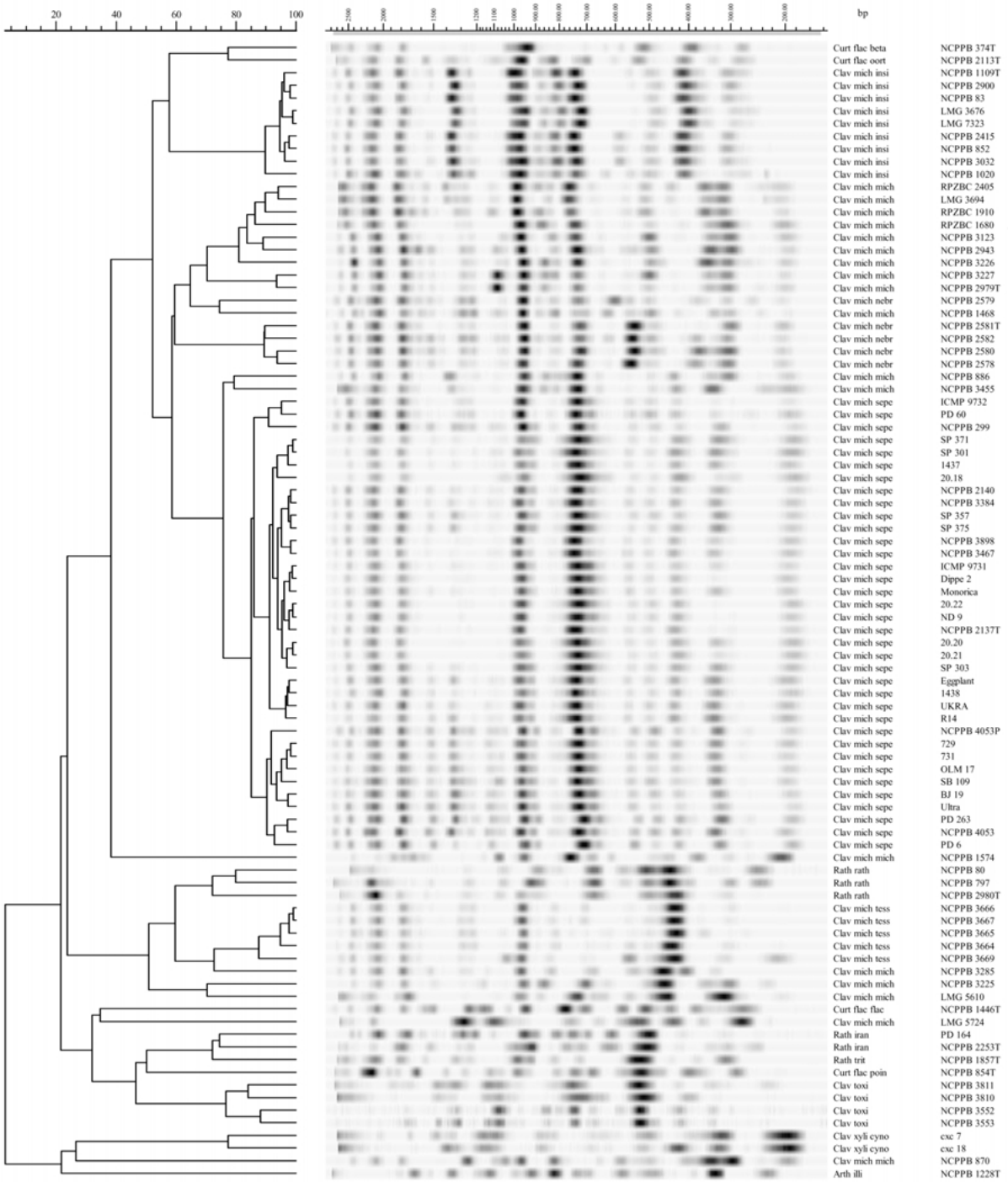

Figure 2. Dendrogram of BOX-PCR fingerprints for all strains included in this study. Key: T – type strain; P – DNA purified using Wizard DNA extraction kit. Clav mich sepe – *C. michiganensis* subsp. *sepedonicus*; Clav mich insi – *C. michiganensis* subsp. *insidiosus*; Clav mich mich – *C. michiganensis* subsp. *michiganensis*; Clav mich nebr – *C. michiganensis* subsp. *nebraskensis*; Clav mich tess – *C. michiganensis* subsp. *tessellarius*; Rath iran – *R. iranicus*; Rath trit – *R. tritici*; Curt flac poin – *C. flaccumfaciens* pv. *poinsettiae*; Curt flac beta – *C. flaccumfaciens* pv. *betae*; Curt flac oort – *C. flaccumfaciens* pv. *oortii*; Curt flac flac – *C. flaccumfaciens* pv. *flaccumfaciens*; Rath rath – *R. rathayi*; Arth illi – *A. ilicis*; Clav toxi – *C. toxicus*; Clav xyli cyno – *C. xyli* subsp. *cynodontis*.

(Anonymous GelCompar Version 4.1 manual), was 93.7% , thus demonstrating that the UPGMA method was appropriate generation of a dendrogram from these fingerprints. The dendrogram shows that all CMS strains form a tight group with 83.8 ± 5.9% similarity which is easily distinguishable not only from other Gram positive plant pathogens, but also from the other subspecies of *C. michiganensis*. A further 21 strains of CMS from the NCPPB all had similar profiles (data not shown). The most prominent feature in the fingerprint of CMS is a band sized between 700–750 base pairs. It is present in all strains of CMS included in this study and a similar sized band is also found in all *C. michiganensis* subsp. *insidiosus* (CMI) strains examined. There were no discernible differences between those strains that were isolated as potato pathogens and those which were isolated from asymptomatic sugar beet. Nor was there any evidence from these data that CMS demonstrates fingerprint variation based on geographical or historical distribution. Two strains of *C. michiganensis* subsp. *michiganensis* (CMM), NCPPB 886 and NCPPB 3455 which are both from tomato (*Lycopersicon esculentum*), are related to this group with a correlation of 76.0 ± 5.2%. *C. michiganensis* subsp. *nebraskensis*, *C. michiganensis* subsp. *tessellarius* and CMI, also group into distinct clusters, although all show more variability in their fingerprint patterns than CMS. CMM showed the most polymorphisms within its BOX-PCR fingerprints. There was also a single band of approximately 1000 base pairs in size, which was present in every strain of every subspecies of *C. michiganensis* in this study including the more variable CMM. It was also present in all of the *Curtobacterium* spp. and most of the *Rathayibacter* spp. type-strains fingerprinted. It was not present in the *C. toxicus* and *C. xyli* subsp. *cynodontis* used in this study or the type strains of *Arthrobacter ilicis*. Another common feature was the presence of two adjacent bands in the region corresponding to approximately 1750–2100 base pairs. These were present in virtually all strains throughout the dendrogram, and in only a few cases were there polymorphisms at this position, notably CMM strains LMG 5724 and LMG 5610, *A. ilicis* NCPPB 1228 and *C. toxicus* and *C. xyli* subsp. *cynodontis* strains.

Discussion

These data show that the use of the BOX-PCR offers a valuable addition to the sometimes inconclusive FAME analysis technique and the lengthy and expensive procedure of the eggplant bioassay to identify CMS from whole cells or colonies.The method is accurate for all CMS strains fingerprinted to date, relatively rapid and always allows differentiation from other taxa within *Clavibacter* and *Rathayibacter*. It also offers the advantage over immunofluorescence and PCR tests based on the detection of one single sequence because cross-reactions and therefore false positive results are virtually impossible. A suspect colony can be confirmed within two working days and the technique is not labour intensive. Another advantage of this technique is that, unlike FAME analysis, the accuracy of the diagnosis is not dependent on the method of culturing the organism. Since the BOX element is considered to be virtually ubiquitous on bacterial chromosomes (Versalovic et al., 1991), the age of a colony or the medium on which it was cultured is unlikely to affect the appearance of the fingerprint. Schneider and de Bruijn (1996) showed that the genetic fingerprints of *Azorhizobium caulinodans* were not affected even when 42-day-old colonies were used, although a background smear on the gel increased with age.

This study was concerned with development of a simple method to identify CMS strains once isolated from infected plant tissues by selective plating. Louws et al. (1995) showed that it is possible to use Rep-PCR to detect *Xanthomonas* and *Pseudomonas* pathogens directly from plant lesions. Although such an approach was not explored during this study, such a method, if successful, may be useful as a primary screen and may have value in the detection of symptomatic and latent CMS infections in potato tubers.

It is clear from the fingerprints that there is a great deal of homology between the bands in the profiles of the subspecies of *C. michiganensis.* While this paper does not intend to address the question of *Clavibacter* taxonomy, nevertheless the information contained within it supports the conclusion that the species *C. michiganensis* is generally well separated into subspecies. The main exception to this is CMM, whose fingerprints cluster into a number of groups. Individual CMM strains within each of these groups share high homology, thus agreeing with results obtained by Louws et al. (1998), who showed that, based on limited polymorphisms, the BOX-PCR fingerprints of a large study of CMM fell into at least four groups.

It is hoped that the development of this genetic fingerprinting technique, coupled with improvements

in media allowing the selective enrichment of CMS from symptomatic plant tissues, can be used to confirm suspect cases of bacterial ring rot. By reducing the reliance on the eggplant host test for isolation and particularly confirmation of CMS, this method has the potential to reduce the time required to make a diagnosis from the existing time under the EU Ring Rot Directive of up to seven weeks to the more practical time of approximately five days. Whereas eggplant tests may be required to obtain colonies of CMS from latently infected potato tuber lots, rapid identification of colonies by BOX-PCR should increase the confidence with which a presumptive diagnosis is made.

Confirmation of diagnosis of plant pathogenic bacteria has always relied on host tests, especially for bacteria of quarantine significance. The diagnostic information provided by a reliable, reproducible genetic fingerprint might also be appropriate for confirmation.

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