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# Comparative study of genetic diversity of *Clavibacter michiganensis* subsp. *michiganensis* isolates from the Canary Islands by RAPD-PCR, BOX-PCR and AFLP

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Molecular characterization of seedborne pathogens is an important issue when discerning their origin and tracking the spread of a disease. In the Canary Islands (Spain), *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) was first detected in 2002, causing severe losses in many tomato-growing areas. Fifty four strains of this bacterium isolated from 2002 to 2007 and 19 strains from different countries were characterized for genetic diversity. RAPD-PCR, BOX-PCR and AFLP provided differentiation among Cmm strains whereas no differences were observed with ERIC-PCR, REP-PCR and 16S-23S ITS PCR-RFLP. RAPD-PCR and BOX-PCR revealed high homogeneity among the Canary Island strains (>80 and >75% of similarity, respectively) which could not be grouped based on tomato cultivar, location or year of isolation. By contrast, strains of Cmm from other countries displayed high diversity, providing several clusters, most of which were composed of a single strain. Similarly, AFLP analysis of 29 selected strains of Cmm gave the same profile for the Canarian ones (>90% of similarity) whereas high polymorphism was obtained with strains from different countries. Moreover, two strains, one from the USA and another from Spain, were related to the Canarian strains, according to RAPD-PCR (>60% of similarity), BOX-PCR (>75%) and AFLP analysis (>90%), suggesting a common origin. The circumstances under which the Cmm outbreaks occurred in the Canary Islands and the high homogeneity observed among the Canarian strains would suggest that the bacterium was introduced into the region from only one origin.

*Keywords*: cluster analysis, quarantine bacteria, Rep-PCR, PCR-RFLP, *Solanum lycopersicum*, tomato bacterial canker

## Introduction

*Clavibacter michiganensis* subsp. *michiganensis* (Cmm) causes the disease known as bacterial canker of tomato. At present, this bacterium is a quarantine organism in the European Union (Anonymous, 2000) and is considered the most internationally important tomato seedborne pathogen, producing major economic losses both in greenhouse and field tomato crops worldwide (Gleason *et al.*, 1993). Since it was first described in Michigan, USA in 1909 (Smith, 1914), this disease has been characterized by start-and-stop patterns of occurrence (Strider, 1969) causing severe outbreaks in the past decades in many countries, as reported in the USA, Canada and Kenya (Strider, 1969; McKeen, 1973; Sherf & Macnab, 1986) and more recently in Chile, Turkey and Israel (Vega *et al.*, 2001; Sahin *et al.*, 2002; Kleitman *et al.*, 2008).

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The first report of Cmm isolation from tomato in Spain was in 1978 when the pathogen was also found in the Murcia region (southeast Spain), and in the following years the bacterium was found in other Spanish locations along the Mediterranean coast (López *et al.*, 1985). In the Canary Islands the disease was first detected on the two main islands of the archipelago (Tenerife and Gran Canaria) in 2002 and on another island (La Palma) two years later. In the Canaries, importing tomato seed lots from other countries is a common practice at the beginning of each growing season. Several nurseries, located principally on the islands of Tenerife and Gran Canaria, import seed lots producing millions of tomato seedlings that are planted in the fields.

Information regarding the introduction and subsequent spread of bacterial canker is essential to understanding its dynamics and designing successful management strategies. Epidemiological studies in which this pathogen disseminated under different cropping systems (Chang *et al.*, 1991, 1992; Gleason *et al.*, 1991) have

shed light on the local spread of the pathogen under field conditions. However, information about the structure and diversity of natural Cmm populations is lacking (Eichenlaub *et al.*, 2006) and few studies have been reported on comparative analysis of genetic variability and population diversity of this pathogen (Nazari *et al.*, 2007; Kleitman *et al.*, 2008). Acquiring knowledge of the genetic structure of Cmm populations within each country will help define quarantine risks posed by exotic strains. Moreover, it will provide valuable information which can help establish efficient strategies for bacterial canker control.

The objectives of this study were: i) to characterize the population of Cmm from the Canary Islands by several molecular methods; ii) to compare its genetic variability with some strains of Cmm isolated in other countries; and iii) clarify whether the strains responsible for bacterial canker outbreaks in the Canary Islands have one or several inoculum sources.

## Materials and methods

### Bacterial strains and culture conditions

A total of 73 Cmm strains were used in this study (Table 1). Fifty-four of these strains isolated in the Canaries (from 2002, date of the first isolations of this bacterium in the islands, to 2007) were obtained from 47 fields of five tomato producing areas of Tenerife (Adeje, Arico, Guía de Isora, Granadilla and Santiago del Teide) and three on Gran Canaria (Agüimes, Santa Lucía and San Nicolás de Tolentino) (Fig. 1). Twenty of them were isolated from September 2002 to May 2003 from different areas, greenhouses, tomato cultivars or nurseries to represent the variability among the strains. Fourteen were seven pairs of strains obtained from the same greenhouse but in different years and the remaining 20 strains were isolated after 2002 and included twelve from fields not affected in 2002. The strains were isolated on semiselective medium mSCM (Waters & Bolkan, 1992) or on general medium YPGA (yeast extract, 5 g; bactopeptone, 5 g; glucose, 10 g; agar, 15 g; in 1 L of distilled water) from tomato plants showing disease symptoms. The identity of Cmm strains was confirmed by conventional characterization, according to Davis & Vidaver (2001) and by nitrocellulose membrane ELISA (NCM-ELISA) (De León *et al.*, 2006), immunofluorescence using anti-Cmm polyclonal antibodies (Plant Research International, Wageningen, The Netherlands) and by PCR using the primers CMM5 and CMM6 (Table 2) developed by Dreier *et al.* (1995), following the methodology described in EPPO protocols (EPPO, 2005).

The remaining 19 strains of Cmm from other countries were obtained from different collections. In addition, strain Cms-NCPPB 2140.3 of *C. michiganensis* subsp. *sepedonicus* was included as out-group in this study. The strains were stored at −80°C in 25% glycerol. They were grown on YPGA plates at 28°C for 72 h before DNA isolation.

Table 1 Bacterial strains of *Clavibacter michiganensis* subsp. *michiganensis* used in this study

| Code[a] | Origin[b] | Season[c] | Source[d] |
|---|---|---|---|
| CI-01TF-02[f] | Canary Islands (Tenerife – GI) | 2002–03 | SLSV |
| CI-02GC-02 | Canary Islands (Gran Canaria – SL) | 2002–03 | " |
| CI-03TF-03 | Canary Islands (Tenerife – ST) | 2002–03 | " |
| CI-04GC-02 | Canary Islands (Gran Canaria – SL) | 2002–03 | " |
| CI-05TF-02 | Canary Islands (Tenerife – GI) | 2002–03 | " |
| CI-06GC-02[e,f] | Canary Islands (Gran Canaria – AG) | 2002–03 | " |
| CI-07GC-02 | Canary Islands (Gran Canaria – SN) | 2002–03 | " |
| CI-08GC-02 | Canary Islands (Gran Canaria – SN) | 2002–03 | " |
| CI-09TF-03 | Canary Islands (Tenerife – GI) | 2002–03 | " |
| CI-10TF-03 | Canary Islands (Tenerife – GI) | 2002–03 | " |
| CI-11TF-03[e] | Canary Islands (Tenerife – AR) | 2002–03 | " |
| CI-12TF-03 | Canary Islands (Tenerife – AR) | 2002–03 | " |
| CI-13TF-02 | Canary Islands (Tenerife – GI) | 2002–03 | " |
| CI-14TF-02 | Canary Islands (Tenerife – AR) | 2002–03 | " |
| CI-15TF-02 | Canary Islands (Tenerife – AR) | 2002–03 | " |
| CI-16TF-03 | Canary Islands (Tenerife – AR) | 2002–03 | " |
| CI-17TF-03 | Canary Islands (Tenerife – AR) | 2002–03 | " |
| CI-46TF-02[e] | Canary Islands (Tenerife) | 2002–03 | " |
| CI-18GC-02 | Canary Islands (Gran Canaria – SN) | 2002–03 | " |
| CI-19TF-03 | Canary Islands (Tenerife - GR) | 2002–03 | " |
| CI-20TF-03[e,f] | Canary Islands (Tenerife – GI) | 2002–03(1) | " |
| CI-21TF-05[f] | Canary Islands (Tenerife – GI) | 2005–06(1) | " |
| CI-22TF-03[f] | Canary Islands (Tenerife – GI) | 2002–03(2) | " |
| CI-23TF-06[f] | Canary Islands (Tenerife – GI) | 2005–06(2) | " |
| CI-24TF-03[f] | Canary Islands (Tenerife – ST) | 2002–03(3) | " |
| CI-25TF-05[e,f] | Canary Islands (Tenerife – ST) | 2004–05(3) | ICIA |
| CI-26TF-03 | Canary Islands (Tenerife – GR) | 2002–03(4) | SLSV |
| CI-27TF-04 | Canary Islands (Tenerife – GR) | 2003–04(4) | " |
| CI-28TF-04[f] | Canary Islands (Tenerife – GI) | 2003–04(5) | " |
| CI-29TF-06[f] | Canary Islands (Tenerife – GI) | 2005–06(5) | " |
| CI-30TF-03 | Canary Islands (Tenerife – GR) | 2002–03(6) | " |
| CI-31TF-05 | Canary Islands (Tenerife – GR) | 2004–05(6) | ICIA |
| CI-32TF-03[f] | Canary Islands (Tenerife – GI) | 2002–03(7) | SLSV |
| CI-33TF-05[f] | Canary Islands (Tenerife – GI) | 2004–05(7) | ICIA |
| CI-41TF-05 | Canary Islands (Tenerife – GI) | 2004–05 | " |
| CI-42TF-05[f] | Canary Islands (Tenerife – GI) | 2004–05 | " |
| CI-43TF-05 | Canary Islands (Tenerife – GI) | 2004–05 | SLSV |
| CI-44TF-04 | Canary Islands (Tenerife – GI) | 2004–05 | " |
| CI-45TF-05[f] | Canary Islands (Tenerife) | 2004–05 | " |
| CI-34TF-06[f] | Canary Islands (Tenerife – GI) | 2005–06 | " |
| CI-35TF-05 | Canary Islands (Tenerife – GI) | 2005–06 | " |
| CI-36TF-06 | Canary Islands (Tenerife – AR) | 2005–06 | " |
| CI-37TF-06 | Canary Islands (Tenerife – GI) | 2005–06 | " |
| CI-38TF-06[f] | Canary Islands (Tenerife – GI) | 2005–06 | " |
| CI-39TF-06 | Canary Islands (Tenerife – GI) | 2005–06 | " |
| CI-40TF-06 | Canary Islands (Tenerife – GR) | 2005–06 | " |
| CI-47TF-06[f] | Canary Islands (Tenerife – GI) | 2006–07 | " |
| CI-48TF-06 | Canary Islands (Tenerife – GI) | 2006–07 | " |
| CI-49GC-06[f] | Canary Islands (Gran Canaria – SN) | 2006–07 | " |
| CI-50GC-06[f] | Canary Islands (Gran Canaria – SN) | 2006–07 | " |
| CI-51TF-06[f] | Canary Islands (Tenerife – AD) | 2006–07 | " |
| CI-52TF-07[f] | Canary Islands (Tenerife – GI) | 2006–07 | " |
| CI-53TF-07 | Canary Islands (Tenerife – GI) | 2006–07 | " |
| CI-54TF-07[f] | Canary Islands (Tenerife – GI) | 2006–07 | " |
| ES-IVIA613 | Spain | – | IVIA |
| ES-2637[f] | Spain (Badajoz) | 2002 | " |
| ES-2728·2 | Spain (Barcelona) | 2003 | " |
| ES-2686·1[f] | Spain (Granada) | 2002 | " |
| CL-2819·5 | Chile | – | " |
| CL-2819·4 | Chile | – | " |

Table 1 *Continued*

| Code[a] | Origin[b] | Season[c] | Source[d] |
|---|---|---|---|
| CL-2819.3[f] | Chile | – | '' |
| IT-A2Cm2[e] | Italy | 1988 | A. Zoina |
| IT-A2Cmm.mell | Italy | 2002 | '' |
| NL-PRI.B8[e] | The Netherlands | – | J. van der Wolf |
| NL-PRI.183 | The Netherlands | – | '' |
| NL-PD201[f] | The Netherlands | 1979 | J. D. Janse |
| NZ-542 [e] | New Zealand | – | N. Schaad |
| PT-DGPCPO-103[e,f] | Portugal | 2003 | M. Eloy |
| PT-DGPCPO-87 | Portugal | 2003 | '' |
| US-RG97·1 | USA | – | R. D. Gitaitis |
| US-DR.59 | USA | – | R. Braun |
| US-BR·4R1[e,f] | USA | – | '' |
| US-Cmm1·R2 | USA | – | '' |
| Cms-NCPPB-2140[f] | USA | 1942 | NCPPB collection |

[a]Strain codes are prefixed by abbreviations for the location of origin.
[b]The island and the area in which the Canary Islands strains were obtained is indicated. **AD**: Adeje; AG: Agüimes; AR: Arico; GI: Guía de Isora; GR: Granadilla; SL: Santa Lucía; SN: San Nicolás de Tolentino; ST: Santiago del Teide. (See Fig. 1).
[c]Each pair of strains marked with the same number (in brackets) was isolated in the same greenhouse but in a different year.
[d]SLSV: Sección de Laboratorio de Sanidad Vegetal de la Consejería de Agricultura Ganadería y Pesca del Gobierno de Canarias (Canary Islands, Spain). ICIA: Instituto Canario de Investigaciones Agrarias (Canary Islands, Spain). IVIA: Instituto Valenciano de Investigaciones Agrarias (Valencia, Spain).
[e]Strains of *C. michiganensis* subsp. *michiganensis* used in the preliminary evaluation of molecular characterization methods: RAPDs, rep-PCR and RFLP.
[f]Strains used for AFLP analysis.

## DNA extraction for PCR

Bacterial DNA was extracted using the Easy-DNA kit, protocol 3 (Invitrogen, Inc.). The quantity and purity of extracted DNA were assessed by the ratio (pure DNA $OD_{260}/OD_{280}$ ranging from 1·8 to 2·0) and final DNA concentrations were adjusted to 35 ng $\mu L^{-1}$ and stored at −20°C before use.

## RAPD-PCR analysis

In preliminary assays, 96 random primers from the primer kits OP (Operon Technologies, Inc.) and primers 180–4 and 180–8, previously used by Pastrik & Rainey (1999) to identify and differentiate *C. michiganensis* subspecies, were evaluated for their ability to differentiate 10 strains of Cmm from different origins (Table 1). Four random primers that provided reproducible distinctive patterns of amplified DNA fragments were selected for further analysis with all the strains (Table 2). Amplifications were carried out in a 25-μL reaction volume containing 75 ng of genomic DNA, 0·4 μM of a single primer, 2 mM $MgCl_2$, 100 μM dNTP mixture, 2·5 μL of 10 × reaction buffer, and 1·5 U of BioTaq DNA-polymerase (Bioline). The reaction mixtures were initially denatured for 3 min at 94°C, and then subjected to 35 cycles of 94°C for 30 s, 36°C for 25 s and 72°C for 1 min (rate of heating from 36°C to 72°C was regulated to 0·3°C $s^{-1}$), and a final extension for 10 min at 72°C using an Eppendorf Mastercycler Gradient. PCR amplification products were run in 1·5% (w/v) agarose gel electrophoresis in 0·5 × TAE (Tris-acetic-EDTA buffer) at 100 V, stained with ethidium bromide (0·5 μg $mL^{-1}$), and visualized on a UV transilluminator and photographed. On each gel, a 100 bp ladder was used for reference (Invitrogen, Inc.). The RAPD patterns were repeated at least once under the same conditions and only reproducible patterns were analysed.

## Rep-PCR analysis

Primer sets of repetitive sequence-based PCR (rep-PCR), REP (primers REP1R-I and REP2-I), ERIC (primers ERIC1R and ERIC2) and BOX (primer BOXA1R) were used (Table 2) with the same 10 selected strains as above. PCR amplifications were performed in 25-μL reaction volumes with 15 ng of genomic DNA, 80 pmol of each primer, 2 mM $MgCl_2$, 200 μM dNTP mixture, 2·5 μL of 10 × reaction buffer, and 1 U of BioTaq DNA polymerase (Bioline). Amplifications were performed with an initial denaturalization step of 95°C for 5 min, followed by 40 cycles of 93°C for 45 s, 44°C (REP) or 50°C (ERIC, BOX) for 1 min, and 72°C for 1 min with a final extension step of 72°C for 6 min using a Tprofessional Thermocycler (Biometra). Amplified DNA fragments were separated by electrophoresis in 1·2% (w/v) agarose gels in 0·5 × TBE (Trisborate-EDTA buffer) at 90 V, stained with ethidium bromide (0·5 μg $mL^{-1}$), visualized on a UV transilluminator and photographed. On each gel, 1 Kb plus DNA ladder was used for reference (Invitrogen Inc.).

After analysing the results obtained with the 10 selected strains (Table 1) the BOX primer was chosen to perform the analysis with all isolates used in this study and the amplifications were repeated at least once with all the strains.

## PCR restriction fragment length polymorphism (PCR-RFLP) analysis of 16S-23S intergenic spacer region

The same 10 selected strains of Cmm were subjected to PCR-RFLP analysis (Table 1). A fragment of approximately 2300 bp of the 16S-23S intergenic spacer region was amplified using the primer pair FGPS6 from the 16S rDNA gene, and FGPL132' from the 23S rDNA gene (Ponsonnet & Nesme, 1994) (Table 2). Amplification by PCR was performed in a total volume of 50 μL containing 75 ng of genomic DNA, 200 μM of both primers, 3 mM $MgCl_2$, 200 μM dNTP mixture, 5 μL of 10 × reaction buffer, and 1·5 U of DNA polymerase (Invitrogen Inc.). The reaction mixtures were initially denatured for 3 min at 94°C, and then subjected to 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, and a final extension for 10 min at 72°C using a 9600 Perkin Elmer

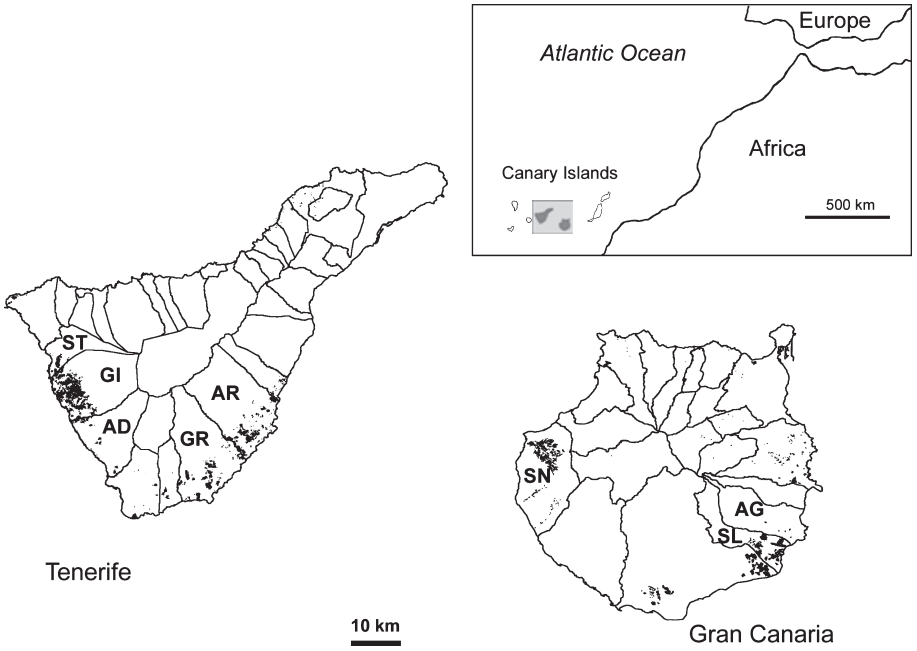


Figure 1 Schematic map showing location of the Canary Islands (upper square) and enlargement of Tenerife and Gran Canaria (from the shaded square) displaying the eight areas in which a total of 47 greenhouses were sampled to obtain the *Clavibacter michiganensis* subsp. *michiganensis* strains used in this study. Adeje (AD); Agüimes (AG); Arico (AR); Guía de Isora (GI); Granadilla (GR); Santa Lucía (SL); San Nicolás de Tolentino (SN); Santiago del Teide (ST). Tomato plantations on both islands are highlighted in black.

DNA thermal cycler. Amplified DNA was examined by electrophoresis in 0·8% agarose with 5 μL aliquots of PCR products. Ten microlitres of each amplification product were digested for 3 h at 37°C with the restriction endonucleases *Hae*III, or *Nde*II supplemented with 1·5 μL of dithiothreitol (DTT) and *Cfo*I (New England Biolabs), added at 5 U of enzyme per reaction. The restriction fragments were separated by electrophoresis in 1·5% agarose gel in 0·5 × TAE buffer at 100 V, stained with ethidium bromide (0·5 μg $mL^{-1}$), and visualized on a UV transilluminator. The restriction patterns were repeated at least once.

Table 2 Primers used for detection or genotyping of *Clavibacter michiganensis* subsp. *michiganensis* strains

| Method | Primers | Sequence |
|---|---|---|
| Detection | CMM5 | 5′-GCGAATAAGCCCATATCAA-3′ |
| | CMM6 | 5′-CGTCAGGAGGTCGCTAATA-3′ |
| RAPDs | OP-D8 | 5′-GTGTGCCCCA-3′ |
| | OP-E20 | 5′-AACGGTGACC-3′ |
| | OP-R12 | 5′-ACAGGTGCGT-3′ |
| | 180-4 | 5′-CGCCCGATCC-3′ |
| Rep-PCR | REP1R-I | 5′-IIIICGICGICATCIGGC-3′ |
| | REP2-I | 5′-ICGICTTATCIGGCCTAC-3′ |
| | ERIC1R | 5′-ATGTAAGCTCCT GGGGATTCAC-3′ |
| | ERIC2 | 5′-AAGTAAGTGACTGGGGTGAGCG-3′ |
| | BOXA1R | 5′-CTACGGCAAGGCGACGCTGACG-3′ |
| RFLP | PGPS6 | 5′-GGAGAGTTAGATCTTGGCTCAG-3′ |
| | FGPL132′ | 5′-CCGGGTTTCCCCATTCGG-3′ |
| AFLP | EcoR1 | 5′-GACTGCGTACCAATTC-3′ |
| | MseI-G | 5′-GATGAGTCCTGAGTAAG-3′ |

## AFLP analysis

A subset of 30 strains (29 of Cmm and one of *C. michiganensis* subsp. *sepedonicus*) were subjected to AFLP-PCR analysis (Table 1). The AFLP procedure was performed as previously described by Vos *et al.* (1995), with slight modifications using an AFLP Core Reagent commercial kit (Gibco BRL) and following the manufacturer's instructions. DNA (200 ng) was digested with *Eco*R1 and *Mse*I enzymes and then ligated to the respective adapters (1 h at 30°C plus 1 h at 25°C). Selective amplification was carried out with *Eco*RI and *Mse*I primers (Table 2) complementary to the adapter sequences, and the *Eco*RI and *Mse*I restriction sites respectively, with additional selective nucleotide (G) at the 3′ end of the *Mse*I primer. The samples were analyzed in a Beckman-Coulter CEQ$^{TM}$ 8000 Genetic Analysis System, with an internal DNA size standard for analysis of fragments up to 600 nucleotides.

## Data analysis

Reproducible pattern bands obtained by RAPD-PCR, rep-PCR, PCR-RFLP and AFLP were analysed using the Quantity One 4·0 software (Bio-Rad) after manual correction for faint bands and mismatches. Data were normalized and bands were scored as present (1) or absent (0) and the readings were entered in a computer file as a binary matrix. Pairwise comparisons of band patterns were estimated using Dice coefficient to obtain genetic similarity. A cluster analysis was carried out with similar-

ity estimates by the unweighted pair-group method with arithmetic average (UPGMA) using TREECON software for Windows (Van de Peer & De Wachter, 1994). Four series of RAPD-PCR profiles were combined to obtain a unique dendrogram. Robustness of the internal branches was assayed by bootstrap analysis (1000 runs).

## Results

### Identification of the Canary Islands strains

Strains of Cmm from the Canary Islands were obtained from tomato plants showing symptoms of bacterial canker. Typical mucoid and yellow colonies were obtained on YPGA plates and colonies were grey and mucoid after 10–12 days of incubation on mSCM semiselective medium with distinguishable internal flecks (Waters & Bolkan, 1992). All the strains showed the general characteristics of the species and a positive reaction was obtained in NCM-ELISA or immunofluorescence with the specific polyclonal antibody recommended for *C. michiganensis* subsp. *michiganensis* identification (EPPO, 2005). They also generated the expected PCR product of 614 bp with CMM5 and CMM6 primers (Dreier *et al.*, 1995)

### Preliminary RAPD-PCR, rep-PCR and PCR-RFLP analyses on *C. michiganensis* subsp. *michiganensis* strains

Ten Cmm strains from different origins: Canary Islands (CI-06GC-02, CI-11TF-03, CI-20TF-03, CI-25TF-05 and CI-46TF-02), Italy (IT-A2Cm2), The Netherlands (NL-PRI.B8), New Zealand (NZ-542), Portugal (PT-DGPCPO-103) and the USA (US-BR.4R1), were used to determine whether RAPD-PCR, rep-PCR (BOX-PCR, ERIC-PCR or REP-PCR) or RFLP produced clear, reproducible and differential genetic fingerprints. After screening 98 RAPD primers, only four (Table 2) were chosen for further analysis with all the strains. All the other primers were discarded. This panel of Cmm strains showed similar ERIC-PCR and REP-PCR band profiles (data not shown). However, BOX-PCR amplification generated well differentiated and distinctive band patterns among the strains tested.

RFLP analysis with primers from the 16S-23S intergenic region provided a single band of about 2300 bp. After digestion with *Hae*III, *Nde*II and *Cfo*I restriction enzymes, six, five and seven fragments per profile were obtained, respectively. The three restriction enzymes gave similar band profiles with the 10 Cmm strains evaluated (data not shown).

### Genetic diversity of *C. michiganensis* subsp. *michiganensis* by RAPD, BOX and AFLP

Four selected 10-base primers (180–4, OP-D8, OP-E20 and OP-R12) yielded reproducible RAPD fingerprints, which enabled differentiation between strains of Cmm. Data from RAPD fingerprints, providing a total of 70 reproducible bands between 200 and1500 bp, were combined to generate a single dendrogram (Fig. 2). In spite of the high number of bands obtained, the analysis based on the RAPD polymorphism grouped the Canary Island strains in a unique cluster with 80% or higher similarity. By contrast, although strains from different countries provided profiles with common bands, they showed a high level of heterogeneity generating 18 different patterns, most of which were represented by a single strain. The strain with the closest similarity value (75%) to the Canary Islands strains was ES-2686.1 from Granada (Spain). This strain was isolated in 2002, but from the south of Spain.

A dendrogram of all BOX-PCR fingerprints is shown in Fig. 3. This method also leads to the differentiation of strains at the subspecies level. Although diversity structure tested by bootstrap analysis showed low values, these data are in agreement with the results obtained by RAPD-PCR because the analysis of BOX-PCR patterns also grouped the Canary Island strains in a unique cluster (>75% similarity). Furthermore, strains US-BR-4R1 (USA) and ES-2686.1 (Granada, Spain) were also grouped with the Canary Island isolates. As observed with RAPD-PCR, BOX-PCR analysis showed high heterogeneity with the remaining strains obtained from geographically distinct locations, and 12 different profiles were found in 17 strains. Neither RAPD-PCR nor BOX-PCR gave sufficient genetic differentiation to clearly discriminate among the Canary Island strains from different islands, production areas, cultivars, greenhouses or cropping season.

A subset of 29 strains of Cmm (and one strain of *C. michiganensis* subsp. *sepedonicus*) was selected to cover the genetic diversity indicated by RAPD-PCR and BOX-PCR data, to be analyzed by AFLP. A total of 170 scoreable fragments were generated, and five AFLP types for Cmm were identified (Fig. 4). The resulting dendrogram confirmed the genetic homogeneity of the 23 Canary Island strains grouped in the main cluster, with over 90% similarity. Again, they could not be separated based on geographical location, tomato cultivar, greenhouse or cropping season. Once more, the two strains of Cmm (ES-2686.1 and US-BR4R) from Spain and the USA were also related to this group (90% similarity). Fingerprint patterns of the remaining Cmm strains from different origins showed high levels of heterogeneity giving four AFLP types, each composed of a single strain.

## Discussion

Genetic diversity of Cmm strains isolated from the Canary Islands and from different countries was evaluated by RAPD-PCR, rep-PCR (ERIC, REP and BOX), PCR-RFLP and AFLP. To the authors' knowledge, this is the first work carried out in Europe to focus on the genetic diversity within strains of this regulated pathogen.

A preliminary evaluation of 10 strains of Cmm from different origins showed that RAPD-PCR (using four out

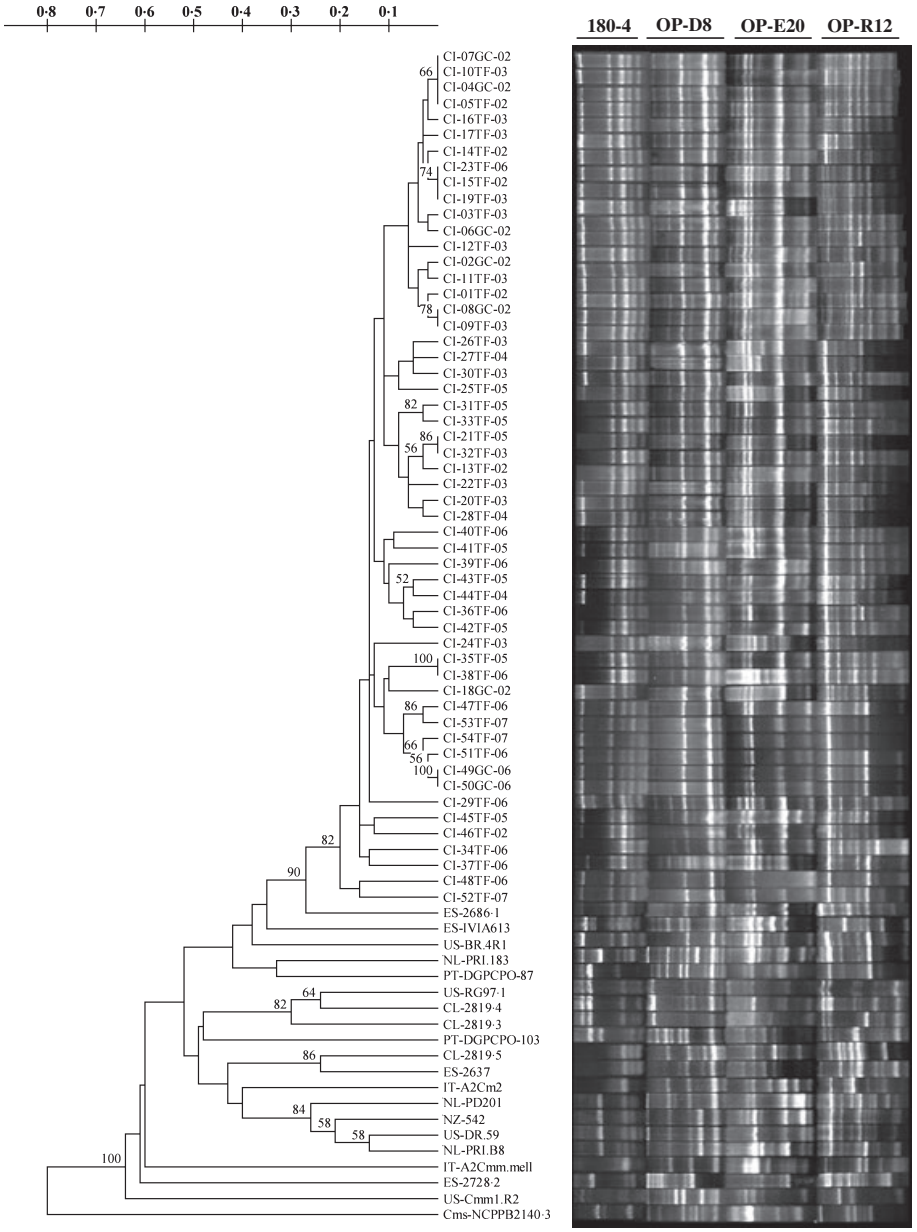


Figure 2 Dendrogram of genetic similarity generated by 73 *Clavibacter michiganensis* subsp. *michiganensis* strains. The similarity is the result of the combined data set of four RAPD primers (180–4, OP-D8, OP-E20 and OP-R12) using UPGMA analysis and Dice coefficient. A strain of *C. michiganensis* subsp. *sepedonicus* was included as out-group. The scale at the top indicates the degree of genetic relatedness between strains. Numbers at the nodes of clusters represent bootstrap values generated from 1000 replications (only values greater than 50% are shown).

of 98 primers assessed) and BOX-PCR were able to differentiate between the strains, whereas no differences were observed with REP, ERIC and RFLP. Using RAPD primers Pastrik & Rainey (1999) reported that primer 180–4 (included in this study) provided differentiation among *Clavibacter michiganensis* subspecies. Furthermore, these authors also identified and discriminated strains within the subspecies *C. michiganensis* subsp. *sepedonicus*, *C. michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *insidiosus* with this primer.

Another genomic fingerprinting technique, rep-PCR, has proven useful in characterizing the genetic diversity of Cmm (Louws *et al.*, 1998; Smith *et al.*, 2001; Nazari *et al.*, 2007). In addition, Louws *et al.* (1998) identified four distinct groups of Cmm strains from different zones of the USA by BOX-PCR, which were not observed by using either ERIC-PCR or REP-PCR. McDonald & Wong (2000) found that strains of *C. michiganensis* subspecies subject to rep-PCR analysis (ERIC, REP and BOX) showed high similarity levels within each

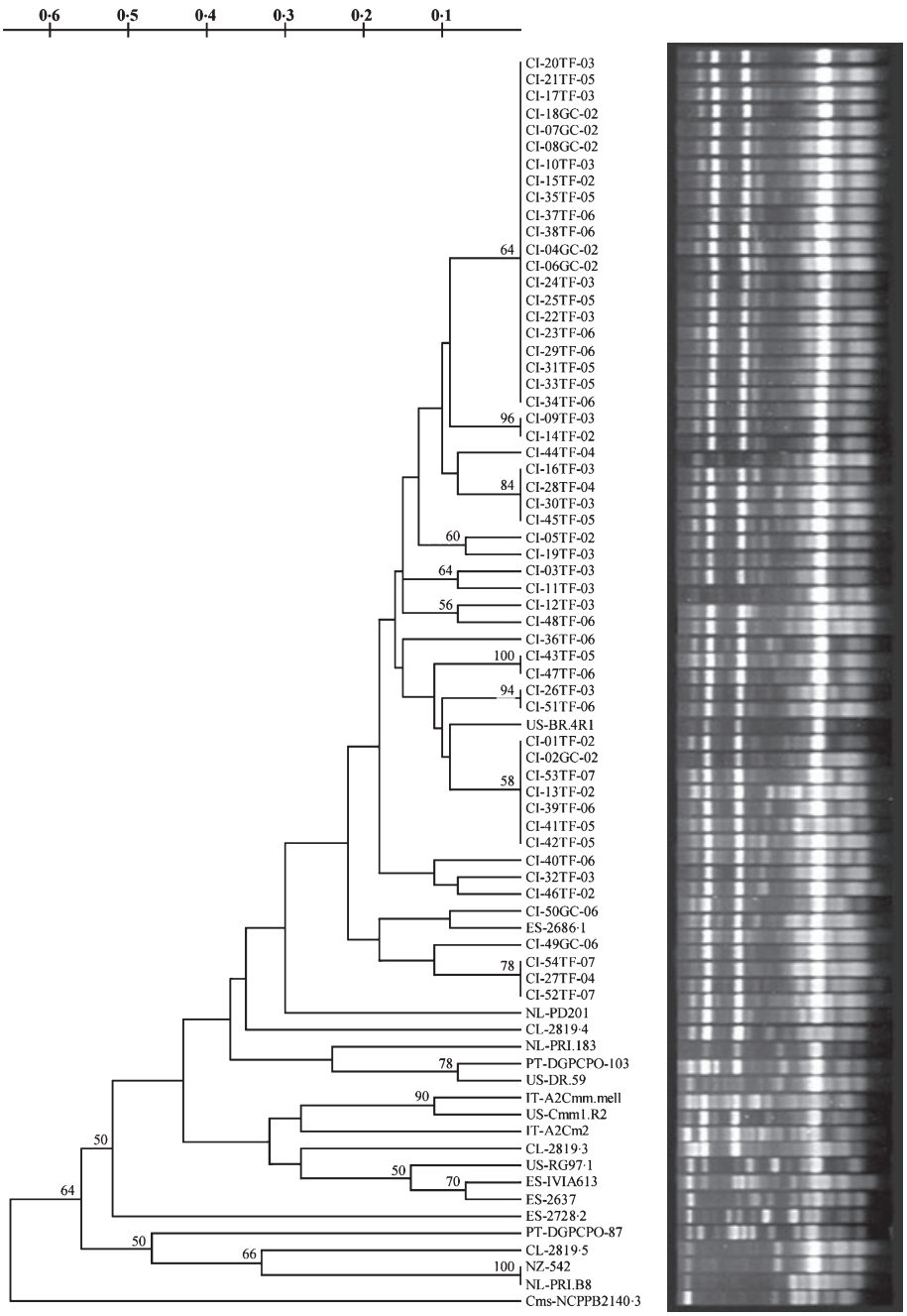


Figure 3 Dendrogram based on UPGMA clustering with Dice association coefficient for BOX fingerprint patterns generated by 73 strains of *Clavibacter michiganensis* subsp. *michiganensis*. A strain of *C. michiganensis* subsp. *sepedonicus* was included as out-group. The scale at the top indicates the degree of genetic relatedness between strains. Numbers at the nodes of clusters represent bootstrap values generated from 1000 replications (only values greater than 50% are shown).

subspecies, but their study was performed with only three Cmm strains. Smith *et al.* (2001) observed that Cmm showed the highest polymorphism within *C. michiganensis* subspecies using BOX-PCR. Likewise, similar analysis of 10 Cmm strains from Azerbaijan and Golestan (Iran) gave six different fingerprint patterns (Nazari *et al.*, 2007) suggesting that this technique can be used to evaluate diversity within this subspecies as confirmed by the present results.

In the study conducted here, PCR-RFLP amplification of the intergenic spacer between 16S and 23S rRNA provided low diversity among Cmm strains. These data are in agreement with those reported by Lee *et al.* (1997), who used different restriction enzymes but obtained little

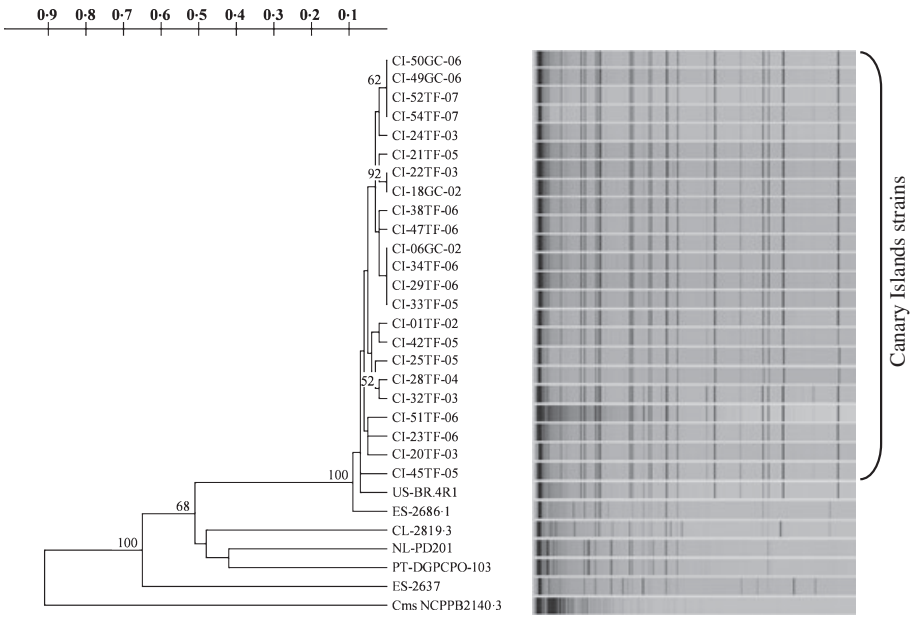


Figure 4 Dendrogram based on UPGMA clustering with Dice association coefficient of AFLP data from 29 *Clavibacter michiganensis* subsp. *michiganensis* strains. A strain of *C. michiganensis* subsp. *sepedonicus* was included as out-group. The scale at the top indicates the degree of genetic relatedness between strains. Numbers at the nodes of clusters represent bootstrap values generated from 1000 replications (only values greater than 50% are shown).

genetic divergence among the five subspecies of *C. michiganensis*. Similarly, analysis of nearly complete sequences of 16S rRNA sequences have also shown high homogeneity between *C. michiganensis* subspecies (Li & de Boer, 1995). The conservative nature of the 16S rRNA genes and the intergenic spacer limit the discriminatory interest of this region for distinguishing closely related strains (Pastrik & Rainey, 1999).

In the present work, 73 Cmm strains were characterized by RAPD-PCR and BOX-PCR, and cluster analysis of the data provided comparable results. The Canary Island strains showed high homogeneity with similarity levels of 80% and 75% in RAPD-PCR and BOX-PCR, respectively. According to Tailliez *et al.* (1996) strains with RAPD profiles of over 80% similarity are considered extremely close genetically, perhaps even identical. These Cmm selected strains, highly representative of the Canary Island population, could not be distinguished based on tomato cultivar, location or year of isolation. By contrast, RAPD-PCR and BOX-PCR fingerprints of 19 Cmm strains obtained from different geographic origins revealed high variability. Cluster analysis of these strains provided 18 and 12 types by RAPD-PCR and BOX-PCR, respectively, with most types being composed of a single strain. The results indicate the ability of these two techniques to discriminate among strains of this subspecies, with the selected primers and amplification protocols.

A selection of Cmm strains were also subjected to AFLP analysis. This method provides many polymorphic bands and consequently isolate grouping is highly reliable. As occurred with RAPD-PCR and BOX-PCR analysis, AFLP gave a high level of similarity (>90%) among the strains obtained from the Canary Islands. This would confirm that probably only one bacterial clone was present in the contaminated seed batches and was responsible for the severe outbreaks of the disease in 2002. Subsequently it was disseminated throughout these islands where it remained for several years. In addition, US-BR-4R1 (USA) and ES-2686·1 (Granada, Spain) strains were clustered with the Canary Island strains by BOX-PCR and AFLP, and related by RAPD-PCR, suggesting that they could have a common origin. Interestingly, strain ES-2686·1 was isolated in 2002, coinciding with the first reported event of bacterial canker in the Canary Islands.

Other Spanish strains isolated in different areas of the Iberian Peninsula showed marked differential genotypes by RAPD-PCR (ES-IVIA613, ES-2637, ES-2728·2 and ES-2686·1) and by AFLP (ES-2637 and ES-2686·1). This finding, although obtained from a small number of strains, may suggest that each strain originated from different sources and was introduced into the country separately. However, many other strains from different Spanish locations must be assessed to support this hypothesis.

Contaminated seeds, the main long-distance vector of the pathogen, are reported to be the crucial factor preceding new outbreaks of bacterial canker in many parts of the world (Tsiantos, 1987; Gleason *et al.*, 1993; Hadas *et al.*, 2005). Each year, at the beginning of the tomato growing season, commercial seed lots are imported into the Canary Islands from different countries. From 2002

to 2007 many lots came from Bolivia, Chile, Denmark, India, Israel, Latvia, Mexico, Peru, Thailand or the Netherlands. The bacterium was probably transmitted from one or several of those seed lots to seedlings and multiplied in nurseries, which provided latently infected tomato transplants to commercial greenhouses. As a result, many tomato crops on the islands were severely affected by bacterial canker and significant yield losses occurred. The rapid action taken by the local authorities in adopting methods to prevent the disease from spreading (analysis of tomato seeds, careful plant management and disinfection of greenhouse materials and facilities), led to a drastic reduction in the incidence of this disease in the following years. However, it has occasionally appeared in different locations since then.

*Clavibacter michiganensis* subsp. *michiganensis* can survive on tomato plant debris for several months (Tsiantos, 1987; Gleason *et al.*, 1991; Fatmi & Schaad, 2002) which could serve as a major inoculum in each growing season (McKeen, 1973; Kleitman *et al.*, 2008). Thus, bacterial canker of tomato is a severe and constant threat to tomato-producing areas and epidemics are unpredictable (Nazari *et al.*, 2007). The high homogeneity observed among the Canary Island strains suggests that a single introduction of the pathogen occurred in the islands and tomato plant debris infected with the pathogen was most likely the inoculum source for the disease in the years following the first outbreak. Residual infected tomato plants have been proposed as the major inoculum source in each growing season in the Besor region of Israel, where two haplotypes remained invariable for at least 10 years (Kleitman *et al.*, 2008).

The results presented here demonstrate that the RAPD, BOX and AFLP protocols evaluated are suitable for the characterization of Cmm diversity in epidemiological studies. The advantage of RAPD-PCR and BOX-PCR techniques resides in their efficiency in terms of simplicity, speed and economy. However, AFLP generated more bands per strain and resulted in more reproducible and robust discriminatory clustering of the strains. Results from this work provide the foundations for a better understanding of the genetic structure and diversity among strains of Cmm, in particular the population of this pathogen in the Canary Islands. These data may serve as a base-line for monitoring populations of this quarantine organism and, in the hypothetical scenario of new Cmm outbreaks in the Canary Islands, the detection of strains with different fingerprint patterns could indicate the introduction of exotic seedborne inoculum.

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