CrossMark

# Phenotypic and genotypic characterization of *Clavibacter michiganensis* subsp. *michiganensis* causing tomato bacterial canker and wilt disease in Turkey

**Hüseyin Basım · Esin Basım**

**Abstract** Tomato bacterial canker and wilt disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is among one of the major bacterial diseases associated with tomato (*Solanum lycopersicum* L.) in the western Mediterranean region of Turkey. A total of 118 *Cmm* isolates were obtained from the petiole and the main vein of leaves of different cultivars of diseased tomato plants, and these isolates were cultured in semiselective medium (mSCM). The identity of *Cmm* isolates was confirmed through gas chromatography-fatty acid methyl-esters (GC-FAME) analysis and polymerase chain reaction (PCR) using the primers, CMM5 and CMM6. The fatty acid analysis of all the Turkish isolates yielded major components that included anteisoheptadeconic acid (a15:0), palmitic acid (i16:0) and anteisoheptadeconic acid (a17:0); the analysis detected and categorized all the isolates into 10 different FAME groups. Among repetitive element sequence PCR (rep-PCR) analysis, Box primer yielded the most reproducible genomic profiles with band sizes that ranged from ~200 bp to 2 kb. The isolates were also separated into 12 groups by pulsed-field gel electrophoresis (PFGE) after digesting the total genomic DNA with *Spe*I, a rare cutting enzyme. The genome sizes of the different strains of *Cmm* were also determined after running unrestricted total genomic DNA, which yielded average values between 3.0 and 3.5 MB. All the *Cmm* isolates had pCM1 and pCM2 plasmids. This is the first report on the detailed characterization of the *Cmm* population in Turkey.

H. Basım (✉)
Faculty of Agriculture, Department of Plant Protection, Akdeniz University, Antalya, Turkey
e-mail: hbasim@akdeniz.edu.tr

E. Basım
Korkuteli Vocational School, Horticulture Department, Akdeniz University, Korkuteli, Antalya, Turkey

## Introduction

Tomato (*Solanum lycopersicum* L.) bacterial canker and wilt, caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), a gram-positive and xylem-invading bacterium, is one of the most economically important disease of the tomato worldwide (Jahr et al. 1999; Kawaguchi et al. 2010). The disease can become very destructive, and economic loss, due to this pathogen, in commercial tomato production in both fields and greenhouses has been reported worldwide (Gitaitis et al. 1991; Chang et al. 1992; de Leon et al. 2011). As a result of severe infection by this pathogen, yield losses up to 84% have been reported (Poysa 1993; Hausbeck et al. 2000). The world's total tomato production is 164,492,970 thousand tons over 4,762,457 ha. The major tomato-producing countries are China, USA, Italy, Turkey, India, and

Springer

Egypt. Turkey is one of the world's leading producers of tomato and ranked fourth globally with the production of 11,820 thousand tons over 311,000 ha (FAO 2013). More than 100 tomato varieties are cultivated in every growing season in Turkey. The total tomato seedling production of Turkey is 1.6 billion, and ~0.6 billion tomato seedlings are produced in Antalya, which makes it the largest tomato production province of Turkey. Approximately 70 commercial vegetable seedling companies of the 114 companies in the Mediterranean region of Turkey are located in Antalya; thus, Antalya plays a major role in tomato production in Turkey. The most economically important bacterial disease in tomato occurring during its production in Turkey is tomato bacterial wilting and canker. *Cmm* is among the most well-known quarantine plant pathogens in Asia, Africa, the Caribbean, and Europe (de Leon et al. 2011). The pathogen can rapidly spread through infected seeds over long distances (de Leon et al. 2011; Tancos et al. 2013). *Cmm* invades the xylem and spreads systematically throughout a host plant (Chalupowicz et al. 2012; Tancos et al. 2013). Secondary infections and epiphytic spread of the pathogen occurs through stomata, hydathodes, roots, injured trichomes, and cultural practices including pruning, clipping, mechanical contact of healthy and infected plants, rain, splashing, irrigation, and fertigation practices (Carlton et al. 1998; Ricker and Riedel 1993; Van der Wolf et al. 2012). None of the present control measures of the disease, including the use of pathogen-free seeds and seedlings (Jahr et al. 1999) and applying antibiotics and other chemical compounds, such as copper, on the infected plants in the fields and the greenhouses (Werner et al. 2002) have been found to be completely effective against this pathogen (Gleason et al. 1993). So far, none of the commercial tomato varieties are known to be effectively resistant to the *Cmm* pathogen (Xu et al. 2010). Several studies of population heterogeneity and genetic differences among *Cmm* pathogens from other parts of the world have been documented and reported (Nazari et al. 2007; Kleitman et al. 2008; de Leon et al. 2009; Kawaguchi et al. 2010; Bella et al. 2012; Milijasevic-Marcic et al. 2012; Quesada-Ocampo et al. 2012; Tancos et al. 2015; Croce et al. 2016; Ialacci et al. 2016; Wassermann et al. 2017). In Turkey however, only a few studies have been carried out on the diversity of *Cmm* species (Baysal et al.

2011). DNA fingerprinting methods such as randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), BOX-PCR, amplified fragment length polymorphism (AFLP), multilocus sequence analysis (MLSA), and pulsed-field gel electrophoresis (PFGE) have shown to be effective in characterizing the genetic differences among the populations of *Cmm* pathogen (Pastrik and Rainey 1999; Louws et al. 1998; Smith et al. 2001; de Leon et al. 2009; Kleitman et al. 2008; Milijasevic-Marcic et al. 2012). Analysis of fatty acid methyl-esters (FAME) of bacterial cell walls is also helpful for the identification and phenotypic characterization of *Cmm* populations (Gitaitis and Beaver 1990).

The objectives of this study were to reveal the phenotypic and genotypic features of the *Cmm* populations in Turkey by means of gas chromatography (GC) - FAME, repetitive element sequence PCR (rep-PCR) and PFGE, and to compare their phenotypic and genotypic differences with those of several strains of *Cmm* isolated from different countries.

## Material and methods

### Bacterial strains

A total of 128 *Cmm* strains was examined in this study (Online Resource 1). Samples of diseased tomato plants were collected from greenhouses and fields in the Mediterranean region of Turkey between 2003 and 2015. A total of 118 isolates was recovered from the samples and cultured on semiselective medium (mSCM) (Waters and Bolkan 1992) and nutrient broth yeast extract (NBY) agar (Schaad 1988) at 27 °C for 3 days. Additionally, 114 of these strains were collected from 88 greenhouses located in the various districts of the Antalya province, whereas four of the strains were obtained from open tomato fields within the province between 2003 and 2015 (Online Resource 1; Fig. 1). The remainder of the 10 strains were obtained from other countries. The strains were preserved in glycerol (30%) at -80 °C for further analysis.

The *Cmm* strains were identified and confirmed using traditional or classical characterization techniques (Davis and Vidaver 2001), gas chromatographic (GC)-FAME analysis (Gitaitis and Beaver 1990), and PCR using CMM5 and CMM6 primers (Dreier et al. 1995).
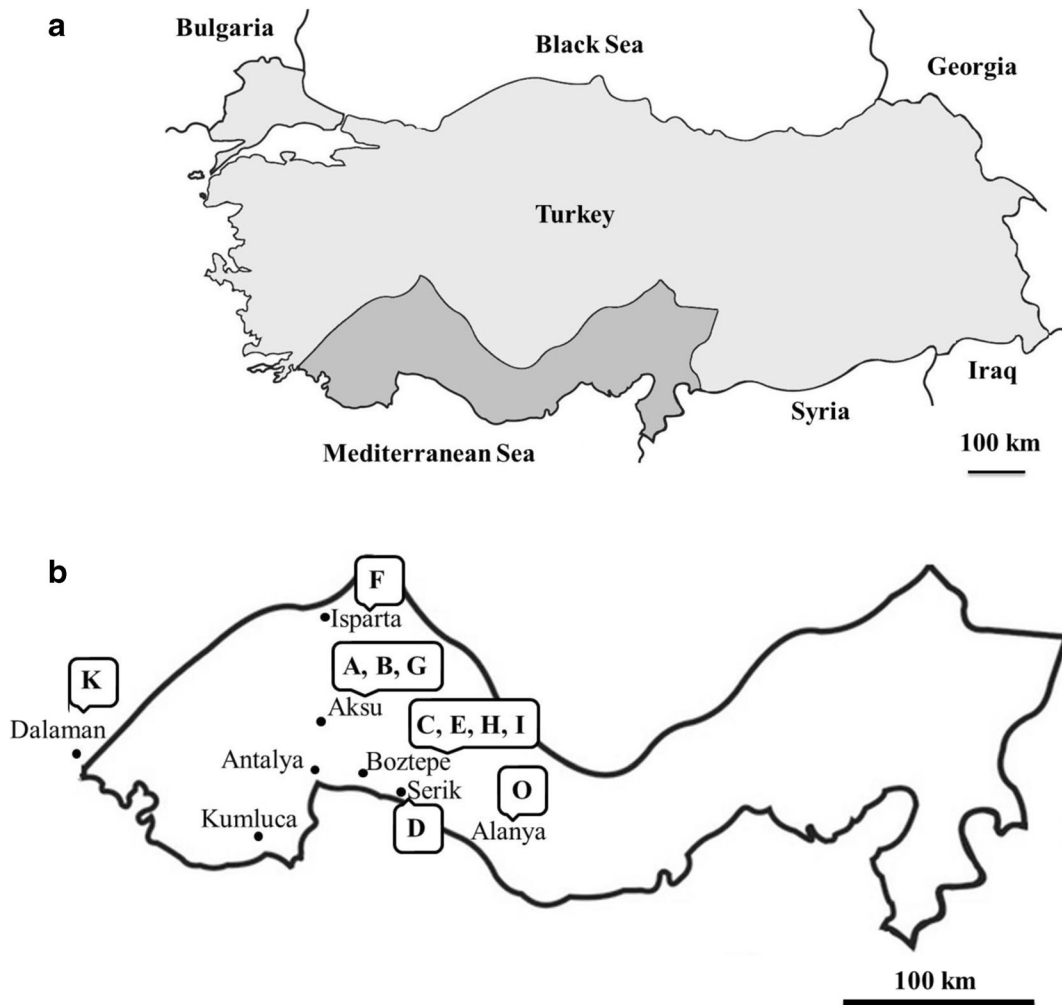
**Fig. 1** Distribution of *Cmm* in the West Mediterranean region of Turkey as determined by PFGE analysis. Schematic map of the region showing the 7 districts studied. Letters next to the district names indicate the haplotype groups (haplotypes A, B, and G strains from Aksu, haplotypes C, E, H, and I strains from Boztepe, haplotype D strains from Serik, haplotype J strains from KumLuca, haplotype K strains from Dalaman, haplotype F strains from Isparta, and haplotype O strains from Alanya based on the PFGE analysis

Pathogenicity and hypersensitive response (HR) assay

*Cmm* strains were cultured in liquid NBY medium at 27 °C for 24 h with shaking. The bacterial suspensions were prepared as described by Gitaitis (1990). Pathogenicity of each *Cmm* strain was confirmed by inoculating 6-week-old tomato seedlings (*Solanum lycopersicum* cv. Selin) according to Thyr (1968) in a greenhouse and maintained at 26-29 °C with relative humidity in the range of 65-85%. Ten tomato seedlings were inoculated with each strain. Symptom development was monitored for 3-4 weeks. For the HR test, four o'clocks (*Mirabilis jalapa* L.) plants were grown in a growth chamber maintained at 24-25 °C with relative humidity ranging from 60 to 75%.

The HR was determined according to Gitaitis (1990). The type strain of *Cmm*, NCPPB 2979, and sterile deionized H$_2$O were used as both positive and negative controls for pathogenicity and HR assays, respectively. All tests were performed in duplicate. The virulence of each *Cmm* strain tested on tomato plants was assessed by following the standard infection procedure described by Van Steekelenburg (1985) and Meletzus et al. (1993).

FAME profiling

Extraction and GC-FAME analysis were conducted on each strain according to manufacturer's specifications. The *Cmm* strains were cultured on tryptic soy broth agar

(Difco Laboratories, Detroit, MI, USA) at 27 °C for 24 h. A loopful of bacteria was mixed with 1.2 N NaOH in methanol: $H_2O$ in a screw cap tube, briefly vortexed and heated in a boiling water bath for 5 min, and the tubes vigorously vortexed for 10 s and returned to the water bath for a total of 30 min heating at 100 °C for saponification. The samples were cooled to room temperature, uncapped, and 2 mL methylation solution (325 mL 6.0 N hydrochloric acid and 275 mL methyl alcohol) was added. The tubes were capped, briefly vortexed and heated at 80 °C for 10 min. The tubes were cooled rapidly inside an icebox. Then, 1.25 mL FAME extraction solution (N-hexane/ methyl tert-butyl ether (1:1; *v/v*) was added to the tubes, and the solution was gently mixed on a clinical rotator for 10 min. The lower aqueous phase was removed using a pipette, and discarded, then 3 mL of sample clean-up solution (10.8 g sodium hydroxide dissolved 900 mL $dH_2O$) was added, and the tubes were tumbled for 5 min. Approximately 2/3 of the organic phase was pipetted into a dark GC vial, which was then capped. The extracts were analyzed using GC System (HP 6980, Microbial Identification System (MIDI) equipped with a 25 m × 0.2 mm phenly methyl silicone fused silica capillary column. The analyses of the samples started after the GC system was calibrated with a commercial reference standard mix of FAMEs (Supelco, Bellefone, PA, USA). Based on FAME composition, the relationships among the 128 strains of *Cmm* were determined by the cluster analysis using the Microbial Identification System software version 6.0 (Microbial ID, Inc., Newark, DE, USA).

Plasmid profile

Bacterial strains were grown at 27 °C with shaking at 140 rpm for 36 h in 4 mL of nutrient broth. Cells were harvested by centrifugation at 14,000 rpm for 2 min, the cell density was adjusted to 0.3 $A_{600,}$ and the cells were sterilized by the method reported by Kado and Liu (1981) with the modifications described by Minsavage et al. (1990). Plasmids were electrophoresed using 0.5% agarose gels (Sea Kem, DNA grade; FMC Corporation, Rockland, ME, USA) in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0) with an electrical current of 3 V $cm^{-1}$. Electrophoresis of the plasmid DNA was run until the bromophenol blue dye moved 10 cm away from the gel wells. The gel was stained with ethidium bromide (0.5 mg/mL) for 30 min, and photographed using transmitted UV light. A plasmid preparation of *Erwinia stewartii* strain SW2 was used as a size marker. The 13

plasmids of *E. stewartii,* which range in size from 4.1 kb to 318 kb (Coplin et al. 1981) were isolated with a PureLink, HiPure Plasmid DNA Purification kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The *Cel*A gene localized in pCM1plasmid was amplified by PCR using the PFC3/ PFG5 primer pair according to Kleitman et al. (2008).

Pulsed Field Gel Electrophoresis (PFGE)

Bacterial strains were grown on YDCA (Yeast Extract-Dextrose-CaCO3 Agar) medium at 27 °C for 72 h, and 4 mL NBY were inoculated with cells from a single colony of each culture. After incubation at 27 °C for 12 h, cell suspensions were adjusted to an optical density of 0.3 (approximately $3 × 10^8$ CFU/mL) at 600 nm using a Biophotometer (Eppendorf, AG 22331 Hamburg, Germany). Aliquots of 1.5 mL of each suspension were pelleted by centrifugation at 14,000 rpm for 2 min, washed by resuspending in 1 mL sterile distilled $H_2O$, and pelleted again. The washing was repeated twice. The pellet was resuspended in 1000 μL Cell Suspension (CS) buffer (1 M NaCl, 10 mM Tris HCl, pH 7.6). Subsequently, 100 μL of Pulsed-field certified, ultra pure DNA-grade agarose (Bio-Rad Laboratories, Fullerton, CA, USA) (10 mM Tris HCl, pH 8.0; 10 mM $MgCl_2$; 25 mM EDTA, pH 8.0, and 2% [wt/vol]) was mixed with the cell suspensions and pipetted into disposable plastic plug-molds (Bio-Rad Laboratories). Agarose plugs were solidified at 4 °C for 20 min and transferred to microfuge tube (1.5 mL) containing 1 mL of lysing solution (1 mg lyzosyme, 1 mL RNase A, 1 M NaCl, 100 mM EDTA, 6 mM Tris HCl, pH 7.6). The embedded cells were lysed by incubation at 37 °C for 12 h. The plugs were transferred to new microfuge tubes containing 1 mL ES solution (0.1 mg proteinase K, 1% *N*-lauryl sarcosine, 0.5 M EDTA), and incubated at 50 °C for 12 h. Following this, they were transferred to new microfuge tubes containing 1.5 mL sterile 250 mM EDTA (pH 8.0) and stored 4 °C.

For DNA digestion, DNA plugs were cut into 5x5x1-mm pieces and rinsed 4 times with 1 mL of 1× TE (1 M Tris HCl, pH 8.0; 0.5 M EDTA, pH 8.0) (30 min rinses). The 1× TE was replaced with 200 μL of 1× restriction enzyme buffer solution [Buffer B (Roche Applied Science, Mannheim, Germany)] and BSA (Albumin Fraction V, Merck, Kenilworth, NJ, USA 1 mg/mL) and the plugs were incubated at 37 °C for 15 min. The above step was repeated twice, after which 200 μL fresh restriction buffer each containing 25 units *Spe*I (Roche Diagnostics), *Ase*I,

XbaI (New England Biolabs, Ipswich, MA, USA), and DraI and VspI (Fermentase, Vilnius, Lithuania) were added separately, and the agarose plugs were incubated at 37 °C for 20 h. The restriction buffer was then replaced with 500 μL of washing solution (1.25% Sarcosyl, 12.5 mM EDTA, pH 9.5) without proteinase K, and the agarose plugs were incubated at 50 °C for 2 h. The washing solution was replaced with fresh washing solution containing proteinase K, and the agarose plugs were incubated at room temperature for 3 h.

The restriction enzyme step was omitted for undigested samples. The plugs were then loaded into the wells of a 1% (for digested plugs) and 0.8% (for undigested plugs) (wt/vol) pulsed field certified agarose (Bio-Rad Laboratories) gel, and sealed with molten low-melting agarose. Yeast Chromosomal PFGE markers (*Saccharomyces cerevisiae*, *Saccharomyces pombe*) and Low–Range marker (New England Biolabs) were used as molecular weight markers. Electrophoresis was conducted on a contour-clamped homogenous electric field gel electrophoresis unit (CHEF-DR III; Bio-Rad Laboratories) (Chu et al. 1986) for 8 h at 5 V/cm in 0.5× TBE buffer, which was cooled to 14 °C throughout the run. The electrophoresis conditions for the genome size analysis included an initial switch time of 5 s and a final switch time of 45 s (with a gradient of 6 V/cm and an angle of 120° at 14 °C) for 22 h. The gel was then stained with ethidium bromide (0.5 μg) for 30 min, and the images captured under ultraviolet transillumination using a Lourma Monochrome ½" IT CCD camera (Vilber, Collegien, France).

The DNA fingerprint patterns and sizes of the fragments were analyzed using the Lourma Bio-gene (version 11.04) gel imaging software (Vilber). DNA fingerprint matching and dendrogram generation using UPGMA (Sneath and Sokal 1973) analysis were carried out using Dice coefficient (2a/(2a + b + c) (Dice 1945), with a tolerance window of between 1.0 and 1.5% for comparison of the generated profiles.

Rep-PCR analysis

Rep-PCR protocols using REP, BOX, and ERIC primers were conducted according to the Versalovic et al. (1991). Single colonies of the *Cmm* strains cultured in NBY agar media were used for rep-PCR reactions. The amplified fragments were fractionated by horizontal gel electrophoresis at 4 °C in 2% agarose gels in 0.5× TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) buffer with 80 V for 4 h. The gels were stained in 0.5×

TAE buffer containing 0.5 μg of ethidium bromide per mL for 30 min and destained in 0.5× TAE buffer for 30 min. After electrophoresis, the gel images were photographed under ultraviolet transillumination using a Vilber Lourma Monochrome ½" IT CCD camera.

## Results

### Identification and pathogenicity characteristics of the *Cmm* strains

A total of 118 *Cmm* isolates was isolated from tomato plant samples which showed bacterial canker and wilting symptoms. The *Cmm* produced typical mucoid and gray colonies with distinguishable internal flecks on mSCM (Waters and Bolkan 1992). All 118 strains were gram-positive, and produced convex, mucoid and yellow-pigmented colonies on yeast dextrose carbonate agar (YDCA) (Table 1). The strains also showed full virulence on tomato seedlings (causing cankers and wilting) and induced strong HR on *Mirabilis jalapa*. Among all the strains, *Cmm* 2 was found to be the most virulent based on the wilting index as described by Van Steekelenburg (1985) and Meletzus et al. (1993), because this pathogen caused early wilting of 50% of the 10 tomato plants 2 weeks post-inoculation of the tomato seedlings, as compared to the rest of the strains which started symptom production after 3 weeks. All the strains also produced the expected fragments of 614 bp with CMM5 and CMM6 primers (Table 1) (Dreier et al. 1995).

### FAME analysis

The fatty acid compositions of the 118 *Cmm* strains isolated from infected tomato plants in the western Mediterranean region of Turkey varied; groups of 10 different FAMEs were identified in Turkish and foreign *Cmm* strains, respectively, by GC-FAME analysis (Table 1). The 118 *Cmm* strains were identified as *Cmm/Cms* by MIDI (Table 1). All strains contained high proportions of anteisoheptadeconic acid (a15:0) (41.8-55.89%), palmitic acid (i16:0) (7.12-15.41%) and anteisoheptadeconic acid (a17:0) (24.83-30.99%) (Online Resource 2). The MIDI dendrogram tree of unweighted pair grouped all 128 *Cmm* strains by fatty acid composition into a cluster showing different profiles: A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, and P profiles at a Euclidian distance of 13 U (Fig. 2). Out of

**Table 1** *Cmm* strains used in this study

| Group of strains | Origin | Source | Color on YDC | Pathogenicity | HR | PCR CMM5/CMM6 | Plasmid pCM1 | Plasmid pCM2 | MIDI | FAME profile | BOX-PCR[b] haplotypes | PFGE[a] haplotypes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 (16)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | K | A | A |
| 2 (12)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | G | B | G |
| 3 (27)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | G | F | B |
| 4 (4)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | B | I | F |
| 5 (8)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | E | E | E |
| 6 (5)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | C | D | H |
| 7 (5)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | A | E | C |
| 8 (7)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | M | B | I |
| 9 (23)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | H | E | J |
| 10 (3)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | K | E | K |
| 11 (4)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | F | B | D |
| 12 (4)* | Turkey | H. Basim | Yellow | + | + | + | + | + | *Cmm/Cms* | D | C | O |
| 13 (1)* | Netherlands | J. D. Janse | Yellow | + | + | + | + | + | *Cmm/Cms* | I | G | P |
| 14 (1)* | Netherlands | J.D. Janse | Yellow | + | + | + | + | + | *Cmm/Cms* | J | J | L |
| 15 (1)* | Spain | Valencia Inst. | Yellow | + | + | + | + | + | *Cmm/Cms* | F | H | N |
| 16 (1)$^{T,*}$ | Hungary | J. D. Janse | Yellow | + | + | + | + | + | *Cmm/Cms* | L | N | M |
| 17 (1)* | USA | N. Cohen | Yellow | + | + | + | + | + | *Cmm/Cms* | O | K | U |
| 18 (1)* | USA | N. Cohen | Yellow | + | + | + | + | + | *Cmm/Cms* | P | L | R |
| 19 (1)* | USA | N. Cohen | Yellow | + | + | + | + | + | *Cmm/Cms* | N | K | V |
| 20 (1)* | USA | N. Cohen | Yellow | + | + | + | + | + | *Cmm/Cms* | M | N | Y |
| 21 (1)* | New Zealand | ICMP | Yellow | + | + | + | + | + | *Cmm/Cms* | K | M | S |
| 22 (1)* | New Zealand | ICMP | Yellow | + | + | + | + | + | *Cmm/Cms* | D | M | T |

[a] based upon BOX primer
[b] based upon *Spe*I
[c] Type culture (NCPPB 2979)
*Number of *Cmm* strains

the 16 profiles, 10 profiles from profiles A (= 5, group 7), profiles B (= 4, group 4), profiles C (= 5, group 6), profiles D (= 4, group 12), profiles E (= 8, group 5), profiles F (= 4, group 11), profiles G (= 39, group 2, 3), profiles H (= 23, group 9), profiles K (= 19, group 1, 10) and profiles M (= 7, group 8) constituted Turkey's strain groups and profiles, whereas the remaining six profiles, belonging to strain groups profiles I (= strain PD807, group 13), profiles J (= strain PD3925, group 14), profiles L (= strain NCPPB 2979, group 16), profiles O (= *Cmm*-2-5, group 17), profiles P (= *Cmm*-9-6, group 18), profiles N (= *Cmm*-4a 39, group 19), represented strains obtained from other countries (Table 1, Fig. 2). According to the FAME analysis, Turkish strains from the Serik district belong to group D with New Zealand strain 1899. Strains from Isparta, Keçiborlu district belongs to group F, with the strain from Spain. Strains from Serik, Isparta, Dalaman and Boztepe districts shared the same FAME groups with strains from New Zealand, Spain, New Zealand and USA, respectively.
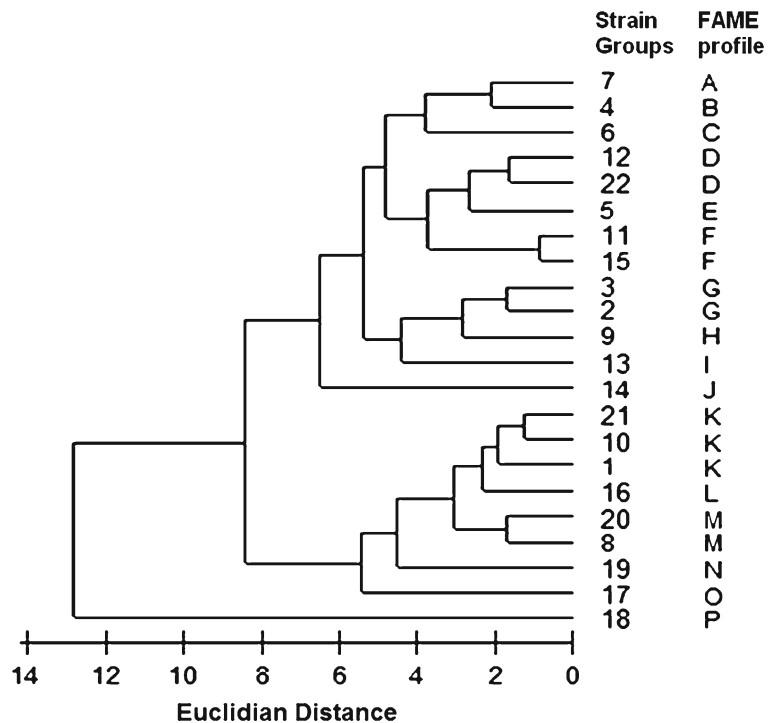
Plasmid profile

The plasmid content of the 128 *Cmm* strains was analyzed in this study. All plasmid profiles of the *Cmm* strains were same as type strain NCPPB 2979, and they all had the 70-kb plasmid named pCM2. The *E. stewartii* plasmid, which contains 13 plasmids, was used as a size marker (Fig. 3). The *Cmm* strains are known to contain two plasmids, pCM1 and pCM2, but after the plasmid isolation, we found only pCM2. The plasmid isolation method followed in this study did not allow the detection of both plasmids, so we amplified the subgenic fragment of *CelA* gene in the pCM1 plasmid as described by Kleitman et al. (2008) using PCR, which produced amplification products of approximately 562 bp (Fig. 3).

PFGE analysis

PFGE of genomic DNA macrorestricted with *Spe*I, *Xba*I and *Ase*I produced various fragments for comparison of fingerprinting and a visual score of genomic DNA size distribution of *Cmm* strains in this study. Digestion of the DNA of *Cmm* strains from 7 different geographical locations in the western Mediterranean region of Turkey with the *Spe*I restriction enzyme produced 12-18 fragments, with an average cumulative genome size of ~2.55 to 4.10 Mbp (Table 2, Fig. 5a, b). All 128 *Cmm* strains listed in Online Resource 1 were separated into 22 haplotypes using *Spe*I (Fig. 5c). The PFGE cluster analysis grouped the



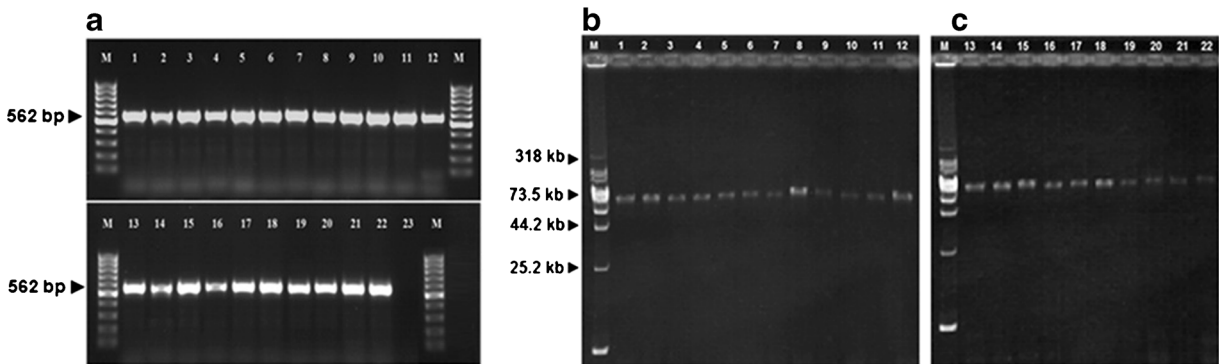**Fig. 2** Dendrogram generated by cluster analysis of *Cmm* strains based on fatty acid composition

Fig. 3 **a** PCR amplification products of *Cmm* using *Cel*A gene localized in pCM1 plasmid, M; Molecular marker (100 bp) **b** Plasmid profiles of *Cmm* (pCM2); M; Molecular marker (*Erwinia* *stewartii* SW2 plasmid used as a marker); Lanes 1-12; *Cmm* strain groups from Turkey; 13-22; *Cmm* foreign strains

**Table 2** Number of DNA fragments restricted by *Ase*I, *Spe*I and *Xba*I and predicted genome sizes of *Cmm* strains by PFGE

| Strain groups | Number of *Ase*I fragments | Genome size in Mbp[a] | Number of *Spe*I fragments | Genome size in Mbp[b] | Number of *Xba*I fragments | Genome size in Mbp[c] | Genome size in Mbp[d] |
|---|---|---|---|---|---|---|---|
| 1 | 9 | 2.85 | 15 | 3.47 | 12 | 3.52 | 3.0 |
| 2 | 10 | 2.83 | 16 | 3.87 | 15 | 4.07 | 3.0 |
| 3 | 9 | 2.62 | 15 | 3.91 | 13 | 3.40 | 3.0 |
| 4 | 8 | 2.51 | 15 | 3.78 | 12 | 3.52 | 3.0 |
| 5 | 8 | 2.78 | 16 | 4.05 | 15 | 3.97 | 3.0 |
| 6 | 11 | 2.79 | 13 | 3.55 | 13 | 3.86 | 3.0 |
| 7 | 7 | 2.33 | 14 | 3.63 | 15 | 3.61 | 3.0 |
| 8 | 7 | 2.76 | 15 | 3.99 | 15 | 4.05 | 3.0 |
| 9 | 7 | 2.86 | 14 | 3.15 | 11 | 3.10 | 3.0 |
| 10 | 10 | 2.82 | 13 | 3.56 | 14 | 3.70 | 3.0 |
| 11 | 8 | 2.86 | 12 | 3.62 | 12 | 3.46 | 3.0 |
| 12 | 12 | 2.68 | 14 | 3.31 | 14 | 3.50 | 3.0 |
| 13 | 8 | 3.02 | 14 | 2.81 | 12 | 2.60 | 3.0 |
| 14 | 6 | 2.31 | 14 | 3.02 | 13 | 2.92 | 3.0 |
| 15 | 10 | 2.84 | 15 | 3.87 | 15 | 4.10 | 3.0 |
| 16 | 11 | 2.74 | 17 | 3.55 | 12 | 2.65 | 3.0 |
| 17 | 7 | 2.25 | 17 | 3.56 | 14 | 2.55 | 3.0 |
| 18 | 9 | 2.66 | 18 | 2.72 | 12 | 2.96 | 3.0 |
| 19 | 7 | 2.25 | 17 | 3.56 | 14 | 2.55 | 3.0 |
| 20 | 7 | 2.45 | 13 | 3.97 | 14 | 4.04 | 3.0 |
| 21 | 9 | 2.93 | 14 | 3.12 | 13 | 2.55 | 3.0 |
| 22 | 8 | 2.55 | 16 | 3.44 | 14 | 2.82 | 3.0 |
|  |  | *2.67 |  | *3.52 |  | *3.34 |  |

[a] Measured by addition of the sizes of *Ase*I restriction fragments

[b] Measured by addition of the sizes of *Spe*I restriction fragments

[c] Measured by addition of the sizes of *Xba*I restriction fragments

[d] Measured by migration of undigested DNA

*Average total genome size in Mb of 22 *Cmm* strain groups

Fig. 4 **a** BOX-PCR analysis of *Cmm* strains **b** Unweighted average linkage dendrogram of the cluster analysis of *Cmm* strains based on BOX-PCR analysis
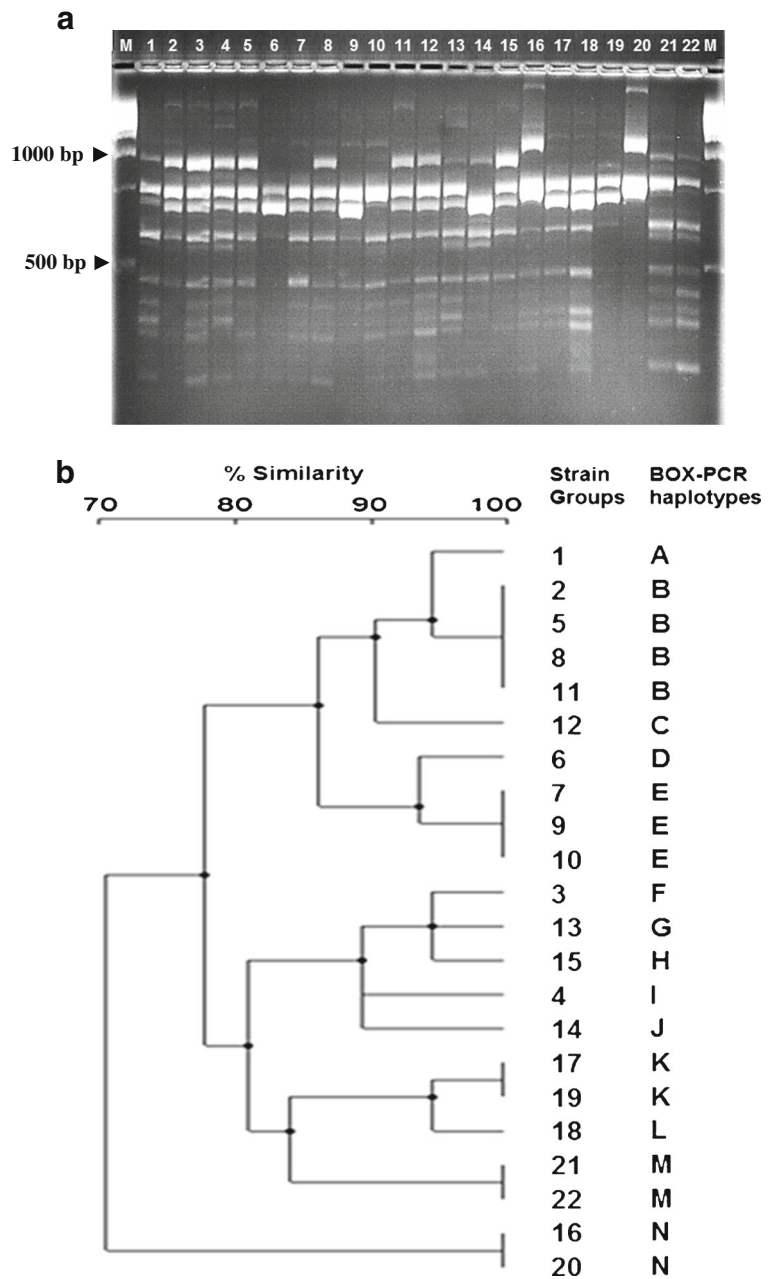


Turkish strains into 12 haplotypes; haplotype A (= 16; group 1), haplotype B (= 27; group 3), haplotype C (= 5; group 7), haplotype D (= 4; group 11), haplotype E (= 8; group 5), haplotype F (= 4; group 4), haplotype G (= 12; group 2), haplotype H (= 5; group 6), haplotype I (= 7; group 8), haplotype J (= 23; group 9), haplotype K (= 3; group 10), haplotype O (= 4; group 12). All isolates were isolated between the years 2003 and 2015 from the various districts of Antalya province. The foreign strains each were grouped into haplotype P (= strain PD807, group 13), haplotype L (= strain PD3925, group 14), haplotype N (= strain 1803-6, group 15), haplotype M (= strain NCPPB 2979, group 16), haplotype U (= *Cmm*-2-5, group 17), haplotype R (= *Cmm*-9-6, group 18), haplotype V (= *Cmm*-4a39, group 19), haplotype Y (= *Cmm*-4-27, group 20), haplotype S (= strain 762, group 21) and haplotype T (= strain 1899, group 22). Each strain group shown in the cluster analysis represents a number of strains, which

generated the same profile. The strain groups, strain designation and the geographical locations, from which the strains were collected and analyzed by the PFGE, are shown in Online Resource 1.

The close relationship between the strains within each group was 66-89%. The similarity among the major groups of Turkish strains, namely, A, B, G and J was 58-64%. (Table 1, Fig. 5c). None of the Turkish strains showed close similarity to the strains from other countries (Fig. 5c). Apart from *Spe*I all the other restriction enzymes, namely *Ase*I, *Xba*I, *Dra*I and *Vsp*I resulted in fewer profiles, which failed to type all the strains into a large number of groups or profiles. For this reason, the *Spe*I restriction enzyme produced the most promising results in this study.
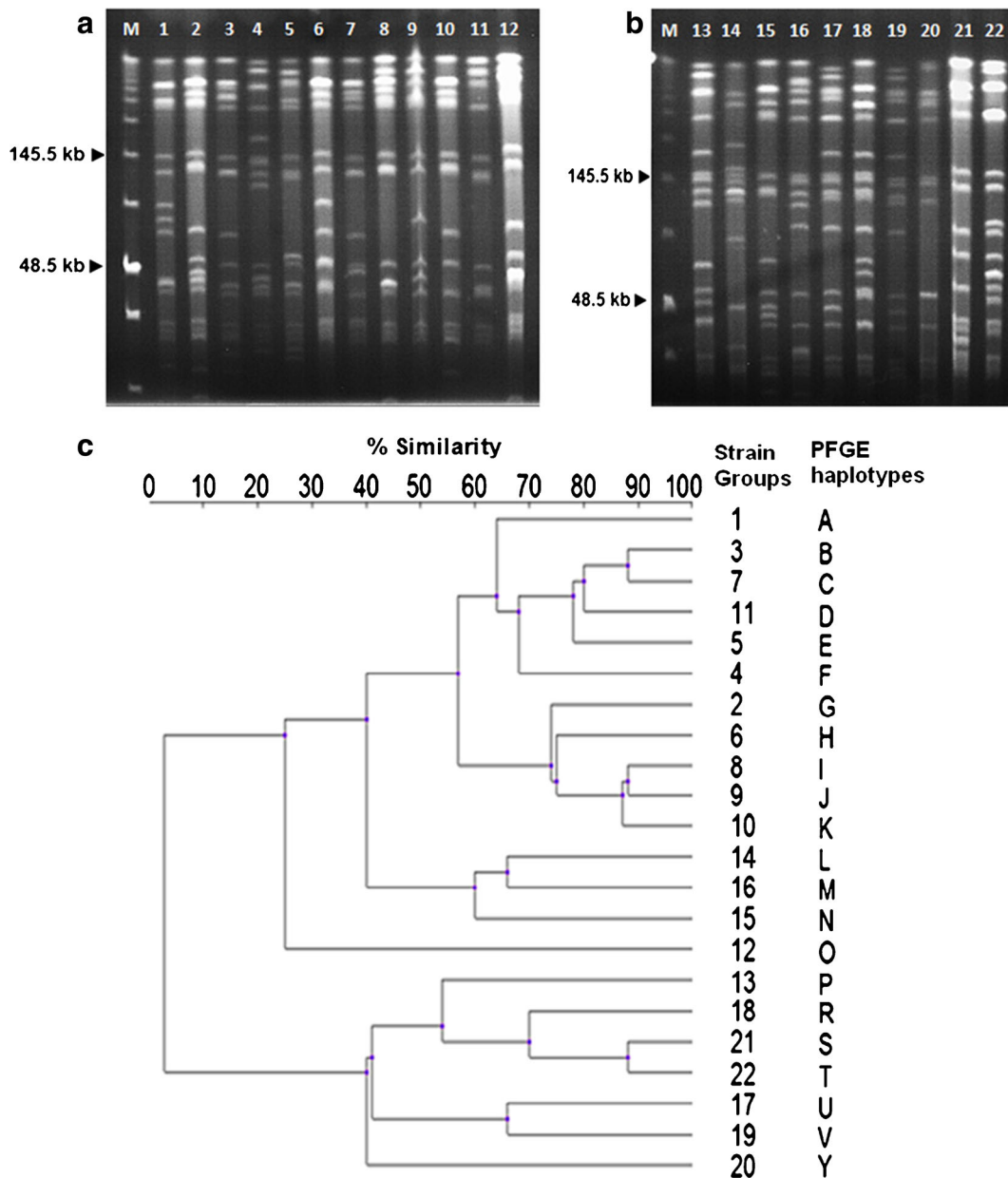


**Fig. 5** **a**, **b** PFGE of *Spe*I digests of genomic DNA of *Cmm* strains. Lanes: M; Low-range PFGE marker, 1-12; *Cmm* strains from Turkey, 13-22; *Cmm* strains from other countries **c** Unweighted average linkage dendrogram of the cluster analysis of *Cmm* strains based on PFGE patterns with *Spe*I. Number of horizontal axes indicate percentage similarity as determined by Dice coefficient (Each strain group represents a number of strains, see Online Resource 1)

A total of 118 strains were recovered from both greenhouses and open fields in the various districts of Antalya province. The PFGE results based on the distribution of the strains across the seven geographical locations showed that the districts of Aksu (A, B, G) and Boztepe (C, E, H, I) together constituted seven haplotypes of the 12 haplotypes; the rest of the five haplotypes were found in Kumluca, Dalaman, Isparta, Alanya, and Serik districts (Fig. 1).

The analysis of the whole and restricted genomes of the 22 strain groups (Online Resource 1, Table 1) containing 128 *Cmm* strains both from Turkey and foreign countries were determined in this study. The size of each restricted fragment was estimated using imaging software based on the value of the distance migrated by each DNA fragment of each strain from gel images captured using the Lourma Bio-gene (version 11.04) gel imaging software (Vilber). The genome sizes of *Cmm* strains were automatically calculated using Bioprofil version 10.03 (Bio1D++, Bio1D and Biogene) computer software system by inputting the lengths of the various fragments into the software obtained by *Ase*I, *Spe*I, and *Xba*I digestion, which yielded mean average values of 2.67 Mb, 3.52 Mb and 3.34 Mb, respectively, as shown in Table 2. Additionally, the whole genome sizes of the *Cmm* strains were verified by PFGE of the undigested genome of the strains. A *S. pombe* CHEF DNA was used a size marker. The migration of the whole genome within the gel, which yielded 3.0 Mb products, was accurately measured using the Vilber Lourma Bio-gene (version 11.04) gel imaging software analyzer (Online Resource 3; Table 2).

Rep-PCR analysis

Rep-PCR analysis of the genomes of the 128 *Cmm* strains produced different patterns. BOX-PCR yielded reproducible genomic fingerprints that consisted of bands that ranged in size ~200 bp to 2 kb. The distinct DNA polymorphisms for each group of *Cmm* strains were in the region of ~500 bp to 1000 bp. Based on the Box-PCR cluster analysis, the 118 *Cmm* strains from Turkey were typed into seven different haplotypes; haplotype A with designated strain group 1 consisting of 16 strains, haplotype B (31 strains with strain group numbers 2, 5, 8, and 11 from Aksu, Boztepe, and Serik districts), haplotype C (four strains with strain group number 12 from Alanya district), haplotype D (five strains with strain group number 6 from Boztepe district), haplotype E (31 strains with strain group numbers 7, 9, and 10 from Boztepe, Kumluca and Dalaman districts), haplotype F (27 strains

with strain group number 3 from Aksu district), haplotype I (four strains with strain group number 4 from Isparta district). All isolates were obtained between the years 2003 and 2015 from the various districts of Antalya province. The foreign strains each were grouped into G (strain PD807), H (strain 1803-6), J (strain PD3925), K (*Cmm*-2-5; *Cmm*-4a39), L (*Cmm*-9-6), M (strain 762; strain 1899), and N (strain NCPPB 2979; *Cmm*-4-27) haplotypes as shown in the dendrogram (Table 1, Fig. 4). The cluster analysis grouped the strains based on the banding patterns generated on the gel. BOX-PCR proved to be more effective than rep-PCR and ERIC PCR; thus, it was used in addition to PFGE for the analysis of all 128 strains of *Cmm* in this study (Table 1). However, BOX-PCR was less efficient in typing all the strains into a large number of haplotypes compared to PFGE.

The number of strains in each group and their geographical origins are presented in Online Resource 1. Strain 3 from Turkey had 95% similarity with strains, 13 and 15 from the Netherlands and Spain, respectively. Strain 4 from Turkey had 90% similarity with strain 14 from the Netherlands (Fig. 4).The *Cmm* isolates from Aksu district shared similar DNA fingerprinting patterns with the strains from the Netherlands and Spain, whereas isolates from Boztepe district shared similar patterns with the strains from the Netherlands only. The Turkish strains did not share the same DNA fingerprinting patterns with the strains from Hungary, USA, and New Zealand.

Discussion

A total of 118 isolates from Turkey collected from diseased tomato plants over a 13-year period were highly diverse and were divided into 12 haplotypes by the PFGE. Similarly, the PFGE dendrogram grouped the foreign strains into 10 haplotypes. The *Cmm* isolates were pathogenic to all the inoculated tomato plants. There was no absolute correlation between the virulence and phenotypic features, genomic diversity, or plasmids of the isolate. Several isolates with the same phenotypic and genotypic group, and same plasmid profile produced symptoms non-identical to each other. One of the strains, *Cmm*2, isolated from the Aksu district of Antalya, was much more virulent than all the other strains. No variations were observed among the rest of the isolates with respect to their virulence in tomato plants, in this study.

Understanding the population structure of *Cmm* may help to develop an efficient pathogen control management system for *Cmm* in the future. Recent studies on *Cmm* strains from various geographical locations, have attempted to explain the population structure of *Cmm* (Nazari et al. 2007; Kleitman et al. 2008; de Leon et al. 2009; Kawaguchi et al. 2010; Baysal et al. 2011; Quesada-Ocampo et al. 2012; Milijasevic-Marcic et al. 2012; Tancos et al. 2015). There is little information about the epidemiological and *Cmm* population structure in the areas with common outbreaks of this pathogen. The *Cmm* isolates collected from diseased tomato plants growing in different areas of the world were highly diverse. In the last decade, various *Cmm* strain populations collected from different parts of the world, including one strain each from the Netherlands, France, and United Kingdom, 15 strains from USA, and 58 strains from Israel, were investigated using macrorestriction analysis and PFGE (Kleitman et al. 2008). The majority of the strains collected from Israel were classified into four different groups, and 2 of the major *Cmm* groups were collected from one region, implying that the seasonal occurrence of the *Cmm* epidemic in this zone arises from bacteria subsisting on the plant residue, instead of infected seeds. A study carried out involving large numbers of *Cmm* strains collected from various locations on the Canary Islands indicated that the pathogen was imported into the country only through infected seeds, and it subsequently continued to persist in those islands (de Leon et al. 2009). Similar findings have also been reported in greenhouses in Japan (Kawaguchi et al. 2010). The above findings showed that seeds serve as the main sources of inoculum for the dissemination of *Cmm* over a long distance. The high diversity among *Cmm* strains as resolved by PFGE, together with the same strains or related pathogens, such as *C. michiganensis* subsp. *sepedonicus,* has been reported (Brown et al. 2002). Similarly, plasmid profiles that were unique and diverse from both Serbian and Israel *Cmm* populations have also been documented (Kleitman et al. 2008; Milijasevic-Marcic et al. 2012).

*Cmm* usually spreads into tomato-producing areas primarily via contaminated seeds or by latent diseased transplants of tomato (Gleason et al. 1993). The origin of *Cmm* inocula in the Antalya province of Turkey might have mainly originated from tomato plant debris in addition to contaminated tomato seeds or seedlings. In the districts of Kumluca, Isparta, Serik, Alanya and Dalaman, only one haplotype remained unchanged for

at least 13 years (Fig. 1). These districts are close to each other; the west district of Dalaman is ~370 km far away from and to the east district of Alanya (Fig. 1). The infected tomato plant tissues or materials have been suggested to be the primary inoculum sources originating in each planting season in the districts of the Besor region of Israel, where two haplotypes remained unchanged for ~10 years (Kleitman et al. 2008). The high uniformity seen between the *Cmm* strains of the Canary Island shows that the single introduction of this pathogen was through infected plant seeds, which have served as an inoculum sources for many years following the first disease outbreak in this region. The highest heterogeneity has been determined in the districts of Aksu and Boztepe, where 7 haplotypes have been characterized for at least 13 years (Fig. 1). This might be the result of the presence of large numbers of commercial seedling companies in these two districts and infected seed or latently infected tomato transplants produced by these companies.

The diversity among different plant pathogenic bacteria are determined using different techniques. Unfortunately, FAME, rep-PCR, inter simple sequence repeat (ISSR) markers, RAPD, AFLP and MLSA as methods of determining the genetic diversity of these pathogen populations, either *in situ* or *in vitro*, are constrained by their low discriminatory capabilities and interlaboratory reproducibility (Louws et al. 1998). In our study, GC-FAME analysis produced similar findings to those reported by Gitaitis and Beaver (1990), was useful for the identification and phenotypic characterization of *Cmm* isolates, and can be a helpful technique for strain differentiation among the *Cmm* populations. Rep-PCR, specifically using BOX primer partially helped to characterize the isolates of the *Cmm* population. Unfortunately, the technique could adequately type all the *Cmm* strains into various haplotypes. Our results suggest that both the GC-FAME and Rep-PCR techniques did not have a powerful discriminatory feature as compared to PFGE. Therefore, in this work, PFGE using rare-cutting endonucleases provided the most comprehensive results.

PFGE is a powerful genomic analysis method with high reproducibility, and allowed for excellent DNA typing, which requires accurate interpretation of the heterogeneity and potential migration of *Cmm* isolates. Rare-cutters, *Ase*I, *Dra*I, *Spe*I, *Xba*I, and *Vsp*I used in PFGE analysis in this study were very useful for obtaining genomic diversity between *Cmm* populations. The *Cmm* genomic analysis by PFGE using *Spe*I

resulted in more restricted DNA fragments compared to PFGE using any other restriction enzyme used in this study, and therefore, it is suitable for epidemiological studies of *Cmm*. Although the other enzymes used produced some profile patterns, they were not adequate and fell short of our expectations. The various groups obtained in the present study by PFGE could not be correlated with plasmid profiles. All the *Cmm* isolates had only the 70 kb pCM2 plasmid (Fig. 3). *Cmm* is known to have two plasmids, pCM1 (27 kb) and pCM2 (70 kb) (Gartemann et al. 2008). After plasmid isolation, we found only the pCM2 plasmid, so we amplified the subgenic fragment of *Cel*A gene localized in the pCM1 plasmid as described by Kleitman et al. (2008), by PCR using the primers PFC3/PFC5. This resulted in the expected amplification of the fragment of approximately 562 bp, signifying that the pCM1 plasmid might have been integrated into the *Cmm* genome. Two virulence factors, *Cel*A on pCM1 (Gartemann et al. 2008) and *Pat*-1 on pCM2 (Dreier et al. 1995), have been identified using deletion mutation and complementation analysis. Horizontal gene transfer of plasmids and chromosomal genes, as seen in pathovars of plant pathogenic bacterial genus of *Xanthomonas* and *Pseudomonas* (Canteros et al. 1995; Basım et al. 1999; Sundin 2007) may explain the high heterogeneity among *Cmm* populations from different origins. Pathogenicity of *Cmm* appeared to have been gained through horizontal gene transfer (Şen et al. 2015). From all the results analyzed by the various methods, it could be seen that the cluster analysis by FAME recorded 16 profiles, BOX-PCR recorded 14 profiles, whereas PFGE recorded 22 profiles for the same group of strains. The simple explanation for this variation was that the FAME and BOX-PCR techniques were not powerful enough to separate these strains into a large number of profiles as compared to PFGE, which typed all the strains into a large number of profiles.

In this study, genome sizes of *Cmm* haplotypes are clearly shown to be approximately 3.0-3.5 Mbp by PFGE using rare-cutters, *Ase*I, *Spe*I, and *Xba*I, which have restriction sites rich in A + T, and G + C content in *Cmm*, which was 72.7% (Eichellaub and Gartemann 2011). The *Spe*I restriction enzyme produced the most efficient results than all the other enzymes and grouped all the Turkish strains into 12 haplotypes. The usage of two different running parameters for two running gels in PFGE analysis, developed in this study, was very useful for the observation of all restricted fragments of the *Cmm* genome. This helped to clearly determine truly different haplotypes and

whole genome sizes of *Cmm* strains (Online Resource 3). The genome sizes of *Cmm* strains were verified by PFGE using the unrestricted genomic DNA of *Cmm* strains, and were compared with the total unrestricted genomes of *Xanthomonas axonopodis* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* (Online Resource 3). Our results were supported by the nucleotide sequence of the *Cmm* type strain, which has been fully sequenced and partially annotated with the determined genomic size of 3.3 Mb (Eichellaub and Gartemann 2011).

In conclusion, the results from this study provide the ground for better understanding of phenotypic, genotypic structure, and differences between strains of the *Cmm* population in the western Mediterranean region of Turkey. These data may be useful for tracing possible future strain shiftings among the *Cmm* populations in Turkey, and for tomato breeding programs aimed at the development of a resistant tomato variety against this pathogen.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Human and animal rights** The authors declare that the research does not involve any Human Participants and/or Animals.

**Grants** Our institution (Akdeniz University) is fully aware and granted permission before this research was conducted.

## References

Basım, H., Stall, R. E., Minsavage, G. V., & Jones, J. B. (1999). Chromosomal gene transfer by conjugation in the plant pathogen *Xanthomonas axonopodis* pv. *vesicatoria*. *Phytopathology, 89*, 1044–1049.

Baysal, Ö., Mercati, F., İkten, H., Yıldız, R. Ç., Carimi, F., Aysan, Y., & Silva, J. A. T. (2011). *Clavibacter michiganensis* subsp. *michiganensis*: Tracking strains using their genetic differentiations by ISSR markers in Southern Turkey. *Physiological and Molecular Plant Pathology, 75*, 113–119.

Bella, P., Ialacci, G., Licciardello, G., La Rosa, R., & Catara, V. (2012). Characterization of atypical *Clavibacter michiganensis* subsp. *michiganensis* populations in greenhouse tomatoes in Italy. *Journal of Plant Pathology, 94*, 635–642.

Brown, S. E., Knudson, D. L., & Ishimaru, C. A. (2002). Linear plasmid in the genome of *Clavibacter michiganensis* subsp. *sepedonicus*. *Journal of Bacteriology, 184*, 908–911.

Canteros, B. I., Minsavage, G. V., Jones, J. B., & Stall, R. E. (1995). Diversity of plasmids in *Xanthomonas campestris* pv. *vesicatoria*. *Molecular Plant Pathology, 85*, 1482–1486.

Carlton, W. M., Braun, E. J., & Gleason, M. L. (1998). Ingress of *Clavibacter michiganensis* subsp. *michiganensis* into tomato leaves through hydathodes. *Phytopathology, 88*, 525–529.

Chang, R. J., Ries, S. M., & Pataky, J. K. (1992). Local sources of *Clavibacter michiganensis* subsp. *michiganensis* in the development of bacterial canker on tomatoes. *Phytopathology, 82*, 553–560.

Chu, G., Volrath, D., & Davis, R. W. (1986). Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science, 234*, 1582–1685.

Chalupowicz, L., Zellermann, E. M., Fluegel, M., Dror, O., Eichenlaub, R., Gartemann, K. H., Savidor, A., Sessa, G., Iraki, N., Barash, I., & Manulis-Sasson, S. (2012). Colonization and movement of GFP-labeled *Clavibacter michiganensis* subsp. *michiganensis* during tomato infection. *Phytopathology, 102*, 23–31.

Coplin, D. L., Rowan, R. G., Chisholm, D. A., & Whitmoyer, R. E. (1981). Characterization of plasmids in *Erwinia stewartii*. *Applied and Environmental Microbiology, 42*, 599–604.

Croce, V., Pianzzola, M. J., Durand, K., González-Arocs, M., Jacques, M. A., & Inés Siri, M. (2016). Multilocus sequence typing reveals high variability among *Clavibacter michiganensis* subsp. *michiganensis* strains affecting tomato crops in Uruguay. *European Journal of Plant Pathology, 144*, 1–13.

Davis, M. J., & Vidaver, A. K. (2001). Gram positive bacteria coryneform plant pathogens. In N. W. Schaad, J. B. Jones, & W. Chun (Eds.), *Laboratory Guide for Identification of Plant Pathogenic Bacteria* (3rd ed., pp. 218–235). St. Paul: APS Press.

de Leon, L., Rodriguez, A., Llop, P., Lopez, M. M., & Siveri, F. (2009). Comparative study of genetic diversity of *Clavibacter michiganensis* subsp. *michiganensis* isolates from the Canary islands by RAPD-PCR, Box-PCR and AFLP. *Plant Pathology, 58*, 862–871.

de Leon, L., Siverio, F., Lopez, M. M., & Rodriguez, A. (2011). *Clavibacter michiganensis* subsp. *michiganensis*, a seed-borne tomato pathogen: Healthy seeds are still the goal. *Plant Disease, 95*, 1328–1338.

Dice, L. R. (1945). Measures of the amount of ecologic association between species. *Ecology, 26*, 297–302.

Dreier, J., Bermpohl, A., & Eichenlaub, R. (1995). Southern hybridization and PCR for spesific detection of phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. *Phytopathology, 85*, 462–468.

Eichellaub, R., & Gartemann, K. H. (2011). The *Clavibacter michiganensis* subspecies: molecular investigation of gram-postive bacterial plant pathogens. *Annual Review of Phytopathology, 49*, 445–464.

FAO. (2013). FAO statistics division. http://faostat3.fao.org/download/Q/QC/E, Accessed 24 October 2016.

Gartemann, K. H., Abt, B., Bekel, T., Burger, A., Engemann, J., Flügel, M., Gaigalat, L., Goesmann, A., Grafen, I., Kalinowski, J., Kaup, O., Kirchner, O., Krause, L., Linke, B., McHardy, A., Meyer, F., Pohle, S., Rückert, C., Schneiker, S., Zellermann, E. M., Pühler, A., Eichenlaub, R., Kaiser, O., & Bartels, D. (2008). The genome sequence of the tomato-pathogenic actinomycete *C. michiganensis* subsp. *michiganensis* NCPPB382 reveals a large island involved in pathogenicity. *Journal of Bacteriology, 190*, 2138–2149.

Gitaitis, R. D. (1990). Induction of a hypersensitive-like reaction in four-o'clock by *Clavibacter michiganensis* subsp. *michiganensis*. *Plant Disease, 74*, 834–838.

Gitaitis, R. D., & Beaver, R. W. (1990). Characterization of fatty acid methyl ester content of *Clavibacter michiganensis* subsp. *michiganensis*. *Phytopathology, 80*, 318–321.

Gitaitis, R. D., Beaver, R. W., & Voloudakis, A. E. (1991). Detection of *Clavibacter michiganensis* subsp. *michiganensis* in symptomLess tomato transplants. *Plant Disease, 75*, 834–838.

Gleason, M. I., Gitaitia, R. D., & Ricker, M. K. (1993). Recent progress in understanding and controlling bacterial canker of tomato in eastern North America. *Plant Disease, 77*, 1069–1076.

Hausbeck, M. K., Bell, J., Medina-Mora, C., Podolsky, R., & Fulbright, D. W. (2000). Effect of bactericides on population sizes and spread of *Clavibacter michiganensis* subsp. *michiganensis* on tomatoes in the greenhouse and on disease development and crop yield in the field. *Phytopathology, 90*, 38–44.

Ialacci, G. M., Bella, P., Licciardello, G., Strano, C. P., Eichenlaub, R., Gartemann, K. H., La Rosa, R., & Catara, V. (2016). Clonal populations of Clavibacter michiganensis subsp. michiganensis are responsible for the outbreaks of bacterial canker in greenhouse tomatoes in Italy. *Plant Pathology, 65*, 484–495.

Jahr, H., Bahro,R., Burger, A., Ahlemeyer, J., & Eichenlaub, R. (1999). Interactions between *Clavibacter michiganensis* and its host plants. Environmental Microbiology, 1, 113-118.

Kado, C. I., & Liu, S. T. (1981). Rapid procedure for detection and isolation of large and small plasmids. *Journal of Bacteriology, 145*, 1365–1373.

Kawaguchi, A., Tanina, K., & Inoue, K. (2010). Molecular typing and spread of *Clavibacter michiganensis* subsp. *michiganensis* in greenhouses in Japan. *Plant Pathology, 59*, 76–83.

Kleitman, F., Barash, I., Burger, A., Iraki, N., Falah, Y., Sessa, G., Weinthal, D., Chalupowica, L., Gartemann, K. H., Eichenlaub, R., & Manulis-Sasson, S. (2008). Characterization of a *Clavibacter michiganensis* subsp. *michiganensis* population in Israel. *European Journal of Plant Pathology, 121*, 463–475.

Louws, F. J., Bell, J., Medina-Mora, C. M., Smart, C. D., Opgenorth, D., Ishimaru, C. A., Hausbeck, M. K., de Bruijn, F. J., & Fulbright, D. W. (1998). Rep-PCR-mediated genomic fingerprinting: A rapid and effective method to identify *Clavibacter michiganensis*. *Phytopathology, 88*, 862–868.

Meletzus, D., Bermphol, A., Dreier, J., & Eichenlaub, R. (1993). Evidence for plasmid-encoded virulence factors in the phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382. *Journal of Bacteriology, 175*, 2131–2136.

Milijasevic-Marcic, S., Gartemann, K. H., Frohwitter, J., Eichenlaub, R., Todorovic, B., Rekanovic, E., & Potocnik, I. (2012). Characterization of *Clavibacter michiganensis* subsp. *michiganensis* strains from recent out-breaks of

bacterial wilt and canker in Serbia. *European Journal Plant Pathology, 134*, 697–711.

Minsavage, G. V., Canteros, B. I., & Stall, R. E. (1990). Plasmid-mediated resistance to streptomycin in *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology, 80*, 719–723.

Nazari, F., Niknam, G. R., Ghasemi, A., Taghavi, S. M., Momeni, H., & Torabi, S. (2007). An investigation on strains of *Clavibacter michiganensis* subsp. *michiganensis* in North and North West of Iran. *Journal of Phytopathology, 147*, 687–693.

Pastrik, K. H., & Rainey, F. A. (1999). Identification and differentiation of *Clavibacter michiganensis* subspecies by Polymerase Chain Reaction-based techniques. *Journal of Phytopathology, 147*, 687–693.

Poysa, V. (1993). Evaluation of tomato breeding lines resistant to bacterial canker. *Canadian Journal of Plant Pathology, 15*, 301–304.

Quesada-Ocampo, L. M., Landers, N. A., Lebeis, A. C., Fulbright, D. W., & Hausbeck, M. K. (2012). Genetic structure of *Clavibacter michiganensis* subsp. *michiganensis* populations in Michigan commercial tomato fields. *Plant Disease, 96*, 788–796.

Ricker, M. D., & Riedel, R. M. (1993). Effect of secondary spread of *Clavibacter michiganensis* subsp. *michiganensis* on yield of northern processing tomatoes. *Plant Disease, 77*, 364–366.

Schaad, N. W. (1988). *Laboratory Guide for Identification of Plant Pathogenic Bacteria* (2nd ed.). St. Paul: American Phytopathological Society.

Smith, N. C., Hennessy, J., & Stead, D. E. (2001). Repetitive sequence-derived PCR profiling using the BOX-AIR primer for rapid identification of the pathogen *Clavibacter michiganensis* subspecies *sepedonicus*. *European Journal of Plant Pathology, 107*, 739–748.

Sneath, P. H. A., & Sokal, R. R. (1973). *Numerical taxonomy: the principles and practice of numerical classification* (p. 573). San Francisco: W. H. Freeman.

Sundin, G. W. (2007). Genomic insights into the contribution of phytopathogenic bacterial plasmids to the evolutionary history of their hosts. *Annual Review of Phytpathology, 45*, 129–151.

Şen, Y., van der Wolf, J., Visser, R. G. F., & van Heusden, S. (2015). Bacterial canker of tomato current knowledge of detection, management, resistance, and interactions. *Plant Disease, 99*, 4–13.

Tancos, M. A., Chalupowicz, L., Barash, I., Manulis-Sasson, S., & Smart, C. D. (2013). Tomato fruit and seed colonization by *Clavibacter michiganensis* subsp. *michiganensis* through external and internal routes. *Applied Environmental Microbiology, 79*, 6948–6957.

Tancos, M. A., Lange, H. W., & Smart, C. D. (2015). Characterizing the genetic diversity of the *Clavibacter michiganensis* subsp. *michiganensis* population in NewYork. *Phytopathology, 105*, 169–179.

Thyr, B. D. (1968). Bacterial canker of tomato: Inoculum level needed for infection. *Plant Disease Report, 52*, 741–743.

Van der Wolf, J. M., van der Zouwen, P. S., van der Ludeking, D., Hamelink, M. R., Schenk, M. (2012). Onderzoeksverslag 'Distributie van *Clavibacter michiganensis* subsp. *michiganensis* in tomatenplanten'. *Plant Research International*, onderdeel van Wageningen UR, Business Unit Bio-interacties en Plantgezondheid, Rapport 448. Wageningen, Netherlands, pp. 1-31.

Van Steekelenburg, N. A. M. (1985). Resistance to *Corynebacterium michiganense* in tomato genotypes. *Euphytica, 34*, 245–250.

Versalovic, J., Koeuth, T., & Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research, 19*, 6823–6831.

Wassermann, E., Montecchia, M. S., Correa, O. S., Damián, V., & Romero, A. M. (2017). *Clavibacter michiganensis* subsp. *michiganensis* strains virulence and genetic diversity. a first study in Argentina. *European Journal of Plant Pathology*. https://doi.org/10.1007/s10658-017-1159-z.

Waters, C. M., & Bolkan, H. A. (1992). An improved selective medium and method of extraction for detecting *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds. *Phytopathology, 82*, 1072.

Werner, N. A., Fulbright, D. W., Podolsky, R., Bell, J., & Hausbeck, M. K. (2002). Limiting populations and spread of *Clavibacter michiganensis* subsp. *michiganensis* on seedling tomatoes in the greenhouse. *Plant Disease, 86*, 535–542.

Xu, X. L., Miller, S. A., Baysal-Gürel, F., Gartemann, K. H., Eichenlaub, R., & Rajashekara, G. (2010). Bioluminescence imaging of *Clavibacter michiganensis* subsp. *michiganensis* infection of tomato seeds and plants. *Applied Environmental Microbiology, 76*, 3978–3988.