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Polymorphism analysis of housekeeping genes for identification and differentiation of *Clavibacter michiganensis* subspecies

Malgorzata Waleron · Krzysztof Waleron · Joanna Kamasa · Wlodzimierz Przewodowski · Ewa Lojkowska

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Abstract The utility of polymorphism analysis was determined for differentiation of the following subspecies of the Gram-positive plant pathogenic bacterium, *Clavibacter michiganensis*: *C. m.* subsp. *michiganensis*, *C. m.* subsp. *sepedonicus*, *C. m.* subsp. *insidiosus C. m.* subsp. *nebraskensis*, and *C. m.* subsp. *tessellarius*. Specific primers designed for amplification of the housekeeping genes *recA*, *rpoB*, and *rpoD* generated 827-, 1037-, and 862-bp DNA fragments, respectively. PCR products obtained from 40 *C. michiganensis* strains were analysed using RFLP with four restriction endonucleases, and those PCR products with specific RFLP patterns were sequenced. The genotypes discriminated after PCR–RFLP were specific for each subspecies and also allowed for differentiation of *C. m.* subsp. *michiganensis* strains. Sequence analysis of the *recA*, *rpoB*, and *rpoD* gene fragments also distinguished *C. michiganensis* subspecies and was useful for phylogenetic analysis of all subspecies. For rapid, inexpensive, and effective differentiation of the five subspecies in this research, we recommend the amplification of *recA* and/or *rpoD* gene fragments and digestion of the PCR products with the restriction endonuclease *Fnu*DII.

Keywords Plant pathogenic bacteria · *recA* · *rpoB* · *rpoD* · PCR-RFLP

M. Waleron (✉) · K. Waleron · E. Lojkowska
Department of Biotechnology, Intercollegiate Faculty of Biotechnology of the University of Gdansk and Medical University of Gdansk,
Kladki 24,
80–822 Gdansk, Poland
e-mail: goniawu@biotech.ug.edu.pl

J. Kamasa
Department of Virology and Bacteriology,
Institute of Plant Protection, National Research Institute,
Wladyslawa Wegorka 20,
60–318 Poznan, Poland

W. Przewodowski
Department of Potato Protection and Seed Science at Bonin, Plant Breeding and Acclimatization Institute at Radzikow,
76–009 Bonin 3, Poland

Introduction

The genus *Clavibacter* contains Gram-positive plant pathogenic bacteria that cause serious diseases and heavy economic losses (Davis et al. 1984). The genus consists of only one species, *Clavibacter michiganensis* (Cm), which is subdivided into five subspecies according to host specificity: *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) causes bacterial wilt and canker of tomato; *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) causes potato ring rot; *Clavibacter michiganensis* subsp. *insidious* (Cmi) causes wilting and stunting in alfalfa; *Clavibacter michiganensis* subsp. *nebraskensis* (Cmn) causes wilt and blight of maize; and *Clavibacter michiganensis*

subsp. *tessellarius* (Cmt) causes leaf freckles and pots in wheat. These subspecies also differ in phenotypic traits such as pigmentation, growth on different media, total protein profile, serological reactions (Carlson and Vidaver 1982; Vidaver and Davis 1988; Gitaitis 1990; Louws et al. 1998), as well as in genetic markers (Metzler et al. 1997). Although bacteria from one subspecies are highly host specific, they may cause disease symptoms on other plant species after artificial inoculation. Latent infection with no or mild symptoms can also occur on host plants. The subspecies Cmm, Cms, and Cmi are listed as quarantine organisms worldwide on tomato, potato, and alfalfa, respectively (Van der Wolf et al. 2005).

Serological tests: enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody staining test (IFAS) have been used to screen plant tissue for Cms, Cmm, and Cmi (De Boer 1982; De Boer and Wieczorek 1984; De Boer et al. 1988; De Boer and McCann 1990; Slack et al. 1978). Diagnostic kits are available for some subspecies, but false positive results and limited sensitivity remain problems for serological detection of Cm (Crowley and De Boer 1982; De Boer and Wieczorek 1984; Mills et al. 1997; Pastrik 2000). Identification of Cm has also been based on fatty acid methyl ester analysis (FAME). Unfortunately identification of Cm subspecies using this approach is difficult because fatty acid profiles within the species overlap (Henningson and Gudmestad 1993; Steed 1992). Application of protein profiles analysis is useful but labor intensive (Stead et al. 1998).

Several DNA-based detection protocols including Southern blot hybridization and the analysis of polymerase chain reaction (PCR)-amplified DNA products have also been used for the identification of Cm. For example, various primer sets have been developed to detect and identify Cmm and Cms (Thompson et al. 1989; Rademaker and Janes 1994; Firrao and Locci 1994; Dreier et al. 1997; Rademaker et al. 1997; Rivas et al. 2002; Arahal et al. 2004; Hu et al. 1995; Palacio-Bielsa et al. 2009); unfortunately, the primers were insufficiently specific for screening environmental samples. *In silico* analysis of the primers recommended for identification of Cms revealed that they might not be specific enough or that they might react with only a narrow group of strains (Arahal et al. 2004). In addition, they may amplify the DNA of closely related species and even the DNA of other nonpathogenic soil bacteria (Van der Wolf et al. 2005). Other techniques developed for *C. michiganensis* identification include fluorescent *in situ* hybridization (FISH) (Van Beuningen et al. 1995), real-time PCR (Schaad et al. 1999; Bach et al. 2003), and nucleic acid sequence based amplification (NASBA) (Van Beckhoven et al. 2002). The methods that are applied to differentiate *C. michiganensis* species are restriction fragment length polymorphisms (RFLP) analysis of the amplified internal transcribed spacer (ITS) between 16S and 23S rRNA genes region of the rrn operon (Lee et al. 1997a; b; Borowicz 2001) and rep-PCR (Louws et al. 1998).

Cm subspecies diversity is relatively low with regard to many phenotypic and genetic characteristics. The exception is subspecies Cmm: strains from different countries displayed significant diversity and could be divided into several clusters. Strains were successfully distinguished from other subspecies by analysis of repetitive sequences like rep, BOX, or ERIC (Louws et al. 1998; Nazari et al. 2007; Kleitman et al. 2008; De León et al. 2009; Kawaguchi et al. 2010); by random DNA amplification techniques such as RAPD (Pastrik and Rainey 1999; De León et al. 2009); by pulsed field gel electrophoresis (PFGE) (Kleitman et al. 2008), by PCR-RFLP (De León et al. 2009), and by amplified fragment length polymorphisms (AFLP), (De León et al. 2009).

Although strains of Cms vary in phenotypic features like disease-causing ability, colony morphology on nutrient-rich media (fluidal, mucoid, dry), production of extracellular polysaccharides and extracellular enzymes, and ability to elicit a hypersensitive response (HR) in tobacco (De Boer and McCann 1990; Westra and Slack 1992; Henningson and Gudmestad 1993; Baer and Gudmestad 1995; Nissinen et al. 2001), molecular studies indicate that the taxon is genetically uniform. A low level of genetic variability among Cms strains was confirmed by PCR-RFLP of a repetitive sequence (IS1121), which is present on the circular Cms plasmid pCS1 (Mogen et al. 1990), as well as by a rep-PCR genomic fingerprinting approach (Louws et al. 1998). The rRNA gene sequences (Lee et al., 1997a; b) and low-molecular weight RNA profiles of Cms (Palomo et al. 2000) are also very uniform. The strong homogeneity was also confirmed by application of clamped homogenous electric fields (CHEF) gel electrophoresis of restriction digested high-molecular weight DNA (Brown et al. 2002).

Apart from 16S and 23S rDNA, other molecular markers that have been recommended for the identification and differentiation of bacterial pathogens include *groEL*, *hsp*60, *recA*, *gyrA*, and *rpoS* (Ludwig and Schleifer 1999; Zeigler 2003). The use of housekeeping genes like *gyrB*, *rpoB*, *ppk*, 16S, and *recA* was proposed for studying the phylogeny of the family *Microbiaceae* (Stackebrandt et al. 2007) but housekeeping genes have not been previously used for genetic characterization of Cm strains.

As noted earlier, three of the five Cm subspecies are quarantined, and their spread is controlled in the European Union and North America. A rapid and reliable method for identification of Cm to the subspecies level is not currently available but is needed.

This study investigates whether molecular markers based on PCR-RFLP analysis and sequencing of *rec*A, *rpoB*, and *rpo*D genes are useful for the differentiation of Cm subspecies. The specific objective was to develop a rapid and inexpensive method for identification and differentiation of *C. michiganensis* subspecies.

Methods

Bacterial strains, DNA preparation

This study used a total of 40 strains of five *C. michiganensis* subspecies, including 16 strains of Cmm, 15 of Cms, 5 of Cmi, and 2 each of Cmn and Cmt (Table 1). Strains of Cmm and Cms subspecies were selected from a collection of 188 isolates (123 Cmm and 78 Cms), which were characterized genetically by rep-PCR with BOX1R primer (Kamasa 2004) and RAPD (Burokiene et al. 2005b). Strains used in this study belong to different fingerprinting groups (data not shown).

The morphology of the colonies growing on YGM was recorded. According to the colony morphology strains were divided into three categories: fluidal, mucoid and dry (Table 1).

The identity of Cmm strains was confirmed by biochemical characterization (Kamasa and Pospieszny 2002; Burokiene et al. 2005a) according to Davis and Vidaver (2001) and by the ELISA reaction following the protocol provided by manufacture (Agida, Inc., Elkhart, IN). All strains of Cms were biochemically characterized and tested by the IFAS reaction with monoclonal antibody 9A1 supplied by Agida, Inc., Elkhart, IN and in the eggplant bioassay. Cmi strains were identified by biochemical and the alfalfa bioassay (Davis and Vidaver 2001). All other bacteria used in this study were well-characterized strains from international collections of plant pathogens. The hypersensitive response and pathogenicity of Cmm, Cms and Cmi strains were determined earlier by Kamasa and Pospieszny (2002), and Burokiene et al. (2005a).

For DNA preparation, bacterial strains were grown overnight at 26°C in M6 and M39 (http://bccm.belspo.be/db). Cells were harvested by centrifugation and were suspended in hot TE buffer (50 mM Tris–HCl, 40 mM EDTA pH 8.0). The total genomic DNA was extracted using the CTAB method (Ausubel 1992).

Primer design and PCR amplification

Oligonucleotide primers were designed on the basis of the published sequences for the *recA*, *rpoB*, and *rpoD* genes of Cmm (AM711867) and Cms (AM849034). The sequences of the following primers were checked for homology to other sequences, which might also be amplified, in the GenBank and EMBL databases using the BLAST-n program: recAF1 5′- TCGGCAAGGGCTCGGTCATGC -3′, recAR2 5′- GGTCGCCRTCGTASGTGTACCA -3′, rpoBF1 5′- CATCATCAACGGCACCGAGC -3′, rpoBR2 5′- AAGCCGAAGGGGTTGATGCG -3′, rpoDF1 5′- ATGGTGCTGTCGAACAAGGA -3′ and rpoDR2 5′- CGATCTGGTCGAGSGTCTT -3′. DNA amplification was performed in 50-µl reaction volumes containing 5 µl of 10× reaction buffer (Fermentas), 2.0 mM $MgCl_2$, 250 µM each of dNTPs, 20 pmol of each primer, bovine serum albumin (BSA) (1.0 mg ml^{-1}); 6% (v/v) glycerol, 50–100 ng of DNA, and 1 U of recombinant Taq DNA polymerase (Fermentas). Amplification was performed using a UNOII Biometra thermocycler, with initial denaturation (95°C, 3 min); followed by 32 cycles of denaturation (94°C, 1 min), annealing (72°C for 1 min for *rec*A, 66°C for 1 min for *rpoD*, and 62°C for 1 min for *rpoD*), and extension (72°C, 2 min); and with a final extension (72°C, 5 min). The amplified products were electrophoretically separated in a 1.5% (w/v) agarose gel at 75 V for 2 h in 1X Tris-borate EDTA (TBE) buffer (pH 8.3) and were visualized with UV light after staining in ethidium bromide (0.5 µg ml^{-1}).

Table 1 Host plant, geographical origin, year of isolation, RFLP genotype, and RFLP group based on three house keeping genes for 40 *Clavibacter michiganensis* strains used in this study

Bacterial strain	Host plant	Geographical origin	Year of isolation	Virulence[a]	HR[b]	Colony morphology	RFLP genotype	RFLP groups based on:		
								recA	*rpoB*	*rpoD*
Clavibacter michiganensis subsp. *michiganensi*										
1L	Eggplant	Lithuania	2000	HV	+	Mucoid	1	1	1	1
5	Tomato	Gorsk, Poland	1984	HV	+	Mucoid	1	1	1	1
3L	Tomato	Lithuania	2000	HV	+	Mucoid	1	1	1	1
10L	Tomato	Lithuania	2000	HV	+	Mucoid	1	1	1	1
61	Tomato	Kalisz, Poland	2000	HV	+/−	Mucoid	1	1	1	1
LMG2891	Tomato	Hungary	1968	nt	nt	Mucoid	1	1	1	1
7	Tomato	Lodz, Poland	1984	IV	+	Mucoid	2	1	1	2
80	Tomato	Skaryszew, Poland	2001	HV	+	Mucoid	2	1	1	2
114	Tomato	Przyborowo, Poland	2002	IV	+	Mucoid	2	1	1	2
136	Tomato	Leszno, Poland	2002	HV	+	Mucoid	2	1	1	2
137	Tomato	Skaryszew, Poland	2003	HV	+	Mucoid	2	1	1	2
153	Tomato	Lodz, Poland	2003	HV	+	Mucoid	3	1	2	2
78	Tomato	Bijewo, Poland	2001	HV	+	Mucoid	4	1	2	3
63	Tomato	Lodz, Poland	2000	LV	+	Mucoid	5	2	2	1
2L	Eggplant	Lithuania	2000	LV	+	Mucoid	5	2	2	1
5L	Tomato	Lithuania	2000	IV	+	Mucoid	6	3	1	2
Clavibacter michiganensis subsp. *sepedonicus*										
LMG2889	Potato	Canada,	1968	V	+	Mucoid	7	4	3	4
527	Potato	Poland,	1994	V	+/−	Dry	7	4	3	4
529	Potato	Leszno, Poland	1993	LV	+	Dry	7	4	3	4
759	Potato	Finland	1983	V	+	Dry	7	4	3	4
LMG6385	Potato	Norway	1982	nt	nt	Dry	7	4	3	4
15814	Potato	Poland	2005	V	+	Fluidal	7	4	3	4
165	Potato	Poland	2006	V	+	Fluidal	7	4	3	4
18795	Potato	Poland	2005	V	+	Fluidal	7	4	3	4
18849	Potato	Poland	2005	V	+	Fluidal	7	4	3	4
NCPPB2140	Potato	Czech Republic	1942	nt	nt	Dry	7	4	3	4
NCPPB4216	Potato	Czech Republic	1997	nt	nt	Mucoid	7	4	3	4
152	Potato	Poland	2006	V	+	Fluidal	7	4	3	4
NCPPB3917	Potato	Canada	1977	nt	nt	Fluidal	7	4	3	4
NCPPB3324	Potato	Belgium	1985	V	+	Fluidal	7	4	3	4
NCPPB4053	Potato	Poland	2007	nt	nt	Fluidal	7	4	3	4
Clavibacter michiganensis subsp. *insidiosus*										
G	Alfalfa	Poland	1964	V	nt	Fluidal	8	5	4	5
18a1	Alfalfa	Poland	2008	V	nt	Fluidal	8	5	4	5
18a2	Alfalfa	Poland	2008	V	nt	Fluidal	8	5	4	5
18b1	Alfalfa	Poland	2008	V	nt	Fluidal	8	5	4	5
18b2	Alfalfa	Poland	2008	V	nt	Fluidal	8	5	4	5
Clavibacter michiganensis subsp. *nebraskensis*										
LMG3697	Corn	United States	1971	nt	nt	Mucoid	9	6	5	6
LMG3700	Corn	United States	1971	nt	nt	Fluidal	9	6	5	6
Clavibacter michiganensis subsp. *tessellarius*										
LMG7294	Wheat	United States	1978	nt	nt	Fluidal	10	7	6	7
LMG7295	Wheat	United States	1978	nt	nt	Fluidal	10	7	6	7

[a] *HV* highly virulent strain; *IV* intermediate virulent, *LV* low virulent, *AV* avirulent

[b] *HR* hyper sensitive reaction; + = positive, - = negative; +/− inconsistent between trials; *nt* not tested

Restriction fragment length analysis

The amplified DNA fragments of *recA*, *rpoB*, and *rpoD* were digested with restriction endonucleases selected on the basis of the nucleotide sequences of the genes using Vector NTI software. The amplified fragments of all three genes were separately digested with *Bsu*RI, *Fnu*DII, *Hpa*II, and *Hin*6I. All applied restriction endonucleases were purchased from Fermentas. Restriction analysis was performed overnight with 2.5 U of each endonuclease using the buffer and temperature recommended by the manufacturer (Fermentas). Restriction fragments were separated in a 12% (w/v) polyacrylamide gel at 120 V for 10 h in 1X TBE buffer and were visualized with UV light after staining in ethidium bromide (0.5 μg ml^{-1}).

Sequencing of recA, rpoB, and rpoD genes

Nucleotide sequences of *recA*, *rpoB*, and *rpoD* genes were determined directly from PCR fragments amplified by PCR primers described in Table 2. Sequencing was carried out using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI3730XL DNA Sequencer (Perkin-Elmer) according to the manufacturer's instructions.

RFLP analysis

Following electrophoresis of polyacrylamide gels, RFLP images were digitized and band profiles were analyzed using the software Bionumerics v 6.0 (Applied Maths, Kortrijk, Belgium). The Pearson product-moment correlation coefficient was used to estimate levels of similarity between RFLP patterns for each strain. The unweighted pair-group method of averages (UPGMA) algorithm was used to construct dendrograms from similarity matrices.

Phylogenetic analysis

The partial *recA*, *rpoD*, and *rpoB* gene sequences obtained for the 40 Cm strains were assembled, aligned and deposited in GenBank under accession numbers HQ585634 to HQ585670, HQ585701 to HQ585725, HQ585741 to HQ585755, HQ585772 to HQ585795, HQ585816 to HQ585830, HQ585846 to HQ585855. For comparison, sequences were searched in GenBank using software BLASTn. The sequences of the Cmm NCPPB382 (AM711867) and Cms ATCC33113 (AM849034) and the nearest neighbour, *Leifsonia xyli* subsp. *xyli* CTCB07 (AE016822), obtained from the GenBank according to BLAST analysis, were aligned using the MUSCLE algorithm with the default settings in Geneious Pro 5.0.4 (Drummond et al. 2009; available at www.geneious.com/). The phylogenetic analysis was performed with the same software. Genetic distances were calculated and corrected for multiple base exchanges and excluding insertions and deletions by the two parameters model of Kimura (Kimura 1980) with transition/

Table 2 RFLP groups of five *Clavibacter michiganensis* subspecies based on restriction analysis using four restriction endonucleases on amplified fragments of *recA, rpoB*, and *rpoD* genes

RFLP* groups	PCR-RFLP patterns for specific restriction Restriction endonucleases:				*Clavibacter* subspecies	No of strains
	*Bsu*RI	*Fnu*DII	*HpaII*	*Hin*6I		
recA-PCR-RFLP						
1	1	1	1	1	Cmm	13
2	1	2	1	1	Cmm	2
3	1	3	1	1	Cmm	1
4	2	4	2	2	Cms	15
5	3	5	3	3	Cmi	5
6	3	6	4	4	Cmn	2
7	4	7	5	5	Cmt	2
rpoB-PCR-RFLP						
1	1	1	1	1	Cmm	12
2	1	2	1	1	Cmm	4
4	2	2	1	2	Cms	1
5	3	2	2	2	Cmi	5
6	4	2	3	2	Cmn	2
7	5	2	1	3	Cmt	2
rpoD PCR-RFLP						
1	1	1	1	1	Cmm	7
2	1	1	1	2	Cmm	7
3	2	1	1	1	Cmm	2
4	3	2	2	3	Cms	1
5	4	3	3	4	Cmi	5
6	5	4	4	5	Cmn	2
7	6	5	5	6	Cmt	2

Numbers of RFLP groups based on the combined PCR-RFLP patterns.

transversion ratio estimated from the analysed sequences. A distance tree was constructed by the Neighbour-joining method of Saitou and Nei (1987), including bootstrap analysis (Felsenstein 1985).

For the parsimony analysis, all characters in the alignment were included at equal weight, gaps were scored as missing data, and a heuristic search was performed with starting trees obtained by random stepwise addition and TBR branch swapping. Bootstrapping was performed with 1000 replications. Trees were rooted with an outgroup composed of the *recA*, *rpoD*, or *rpoB* gene sequences of *Leifsonia xyli* subsp. *xyli* CTCB07 (AE016822).

Results

Phenotypic characterization of *C. michiganensis* strains

Cmm strains were very homogeneous in their morphological, physiological and biochemical characteristic excluding strains Cmm 80 and Cmm 114, which did not exhibit endocellulase activity. All of them were tested for virulence on tomato and HR on four o'clock plants and indicated high, intermediate and low virulence (Table 1). All of Cmm isolates were virulent and induced HR activity. Only two mucoid Cmm strains indicated low virulence (Table 1).

All tested strains of Cms were virulent (Table 1) and caused disease symptoms on eggplant (data not shown). In case of Cmi strains, the biochemical proprieties of analyzed strains were typical with three exceptions. Strain Cmi 18b1, unlike the others, was able to reduce nitrates, strain Cmi 18b2 did not exhibit pyrazinamidase activity, while Cmi 18a1 was not able to produce pyrrolidonyl arylamidase (data not shown). All Cmi strains were virulent (Table 1) and induced symptoms on alfalfa (data not shown). Both strains of Cmn and Cmt were not tested in our laboratories for pathogenicity.

Comparison of *recA* gene fragment amplified from *C. michiganensis*

DNA isolated from the cells of Cm strains (Table 1) was used as a target in the PCR reactions. The primers for the amplification of the *recA* gene fragment generated an amplification product estimated to be 830 bp for all 40 Cm strains.

PCR products were digested by four restriction endonucleases: *Bsu*RI, *Fnu*DII, *Hpa*II, and *Hin*6I. The application of all endonucleases enabled us to distinguish the five subspecies (Fig. 1a and Table 2). Restriction analysis of the PCR product of *recA* indicated seven RFLP groups (Tables 1 and 2, and Fig. 1a). RFLP groups 1, 2, and 3 were described only for Cmm strains (Tables 1 and 2, and Fig. 1a). Restriction endonucleases *Bsu*RI, *Hpa*II, and *Hin*6I generated a single, characteristic RFLP pattern for each subspecies (Fig. 1a and Table 2). Only application of endonuclease *Fnu*DII allowed the differentiation of the Cmm strains into three RFLP groups (Table 2 and Fig. 1a).

RFLP group 4 was unique for Cms strains. The fifth RFLP group was characteristic only for Cmi strains. Strains of Cmn and Cmt belonged to RFLP group 6 and 7, respectively (Table 2 and Fig. 1a).

The obtained RFLP groups indicated high homogeneity within *C. michiganensis* subspecies. The comparison of nucleotide sequences of the *recA* gene fragment amplified from 40 Cm strains and type strains Cmm NCPPB382 (AM711867) and Cms ATCC33113 (AM849034), for which genomes are known, showed that sequences obtained for strains belonging to the same subspecies were identical with the exception of sequences from Cmm strains. Based on *recA*, the genetic distance between these five subspecies ranged from 2 to 5% (Table 3).

Of the *recA* sequences of the 16 Cmm strains, the sequence for Cmm 63 had two polymorphic sites and that for Cmm 5 L had one polymorphic site. For each of these strains, one polymorphic position enabled them to be differentiated from the other strains based on restriction analysis with *Fnu*DII (Fig. 1a). Among the 15 Cms strains, only one polymorphic position occurred in sequences from two strains. In spite of these differences in nucleotide sequences, the translation of the obtained *recA* gene sequences shows that the amino acid sequences of the RecA protein are identical. This illustrates an extreme homogeneity among *Cms* strains.

Comparison of *rpoB* gene fragment amplified from *C. michiganensis*

The primers for the *rpoB* gene amplified a 1037-bp fragment for all 40 Cm strains (Table 1). When PCR products were digested by the four restriction endo-

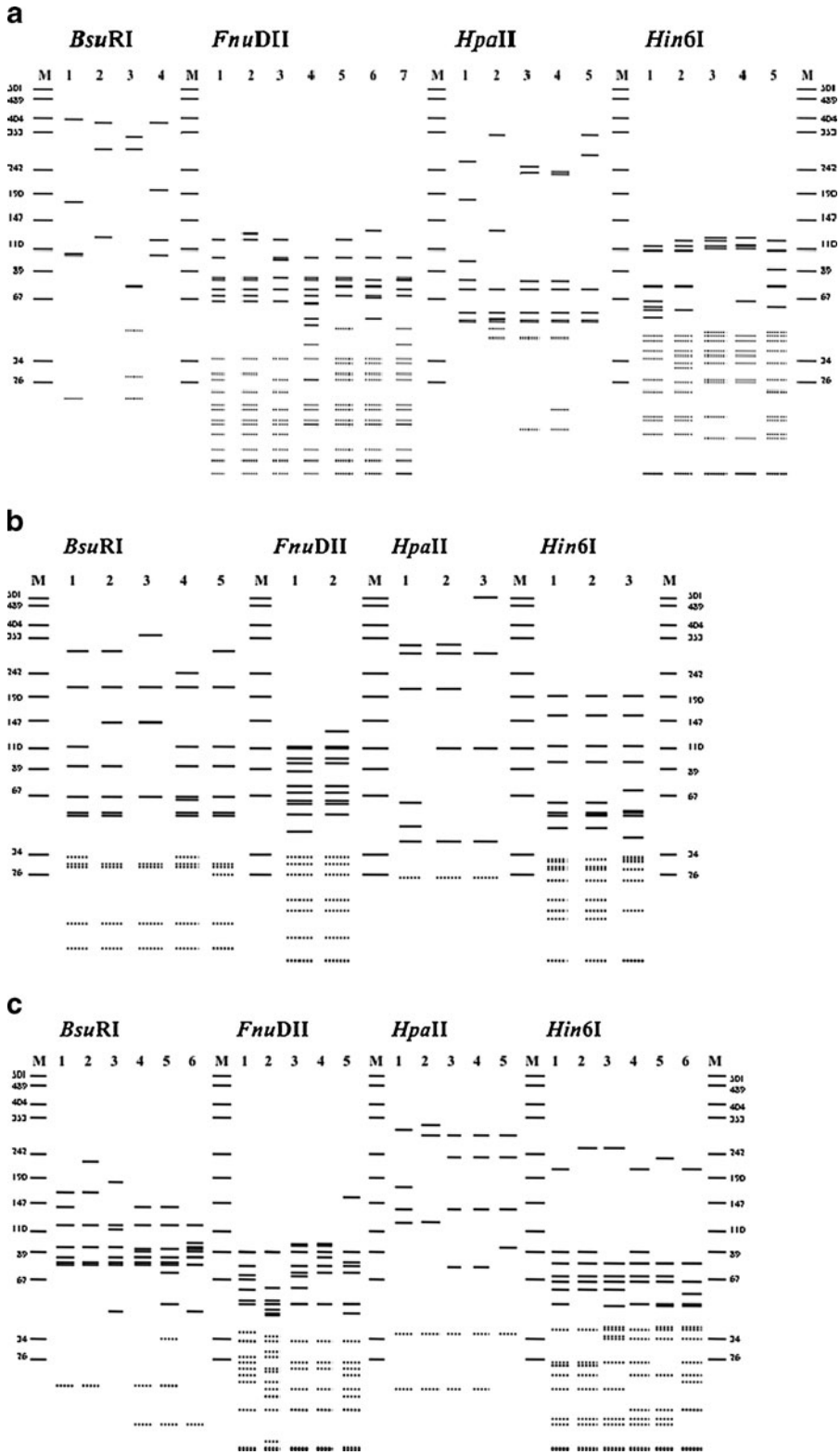

Fig. 1 The database of RFLP patterns obtained after RFLP analysis of PCR products from three housekeeping genes: *recA* (**a**), *rpoB* (**b**), and *rpoD* (**c**). Restriction analysis of RFLP patterns obtained after digestion of PCR products with the following endonucleases: *Bsu*RI; *Fnu*DII; *HpaII*; *Hin*6I. Lanes: M - molecular size markers (pUC18/*Msp*I, Fermentas); Line numbers correspond to RFLP patterns obtained for each endonuclease listed in Table 2. The figures were constructed from normalized and background-subtracted computer-digitalized images of gel strips processed in Dendron 3.0 for Windows software (Solltech Inc.). The RFLP patterns were verified by sequence analysis. Bands, which might not be visible on the electrophoresis gel, are marked as *dotted lines*

Table 3 Identity between the nucleotide sequences of *recA, rpoB*, and *rpoD* genes fragment from *C. michiganensis* subspecies

Gene	Size of the analyzed gene fragment	Subspecies	Identity (%) between gene fragments from *C. michiganensis* subspecies				
			Cmm	Cms	Cmi	Cmn	Cmt
recA	708 bp	Cmm	99–100	96	95	95	96
		Cms		99–100	96	96	96
		Cmi			100	98	96
		Cmn				100	96
		Cmt					100
rpoB	1009 bp	Cmm	99–100	98	98	98	98
		Cms		100	98	98	98
		Cmi			100	98	98
		Cmn				100	98
		Cmt					100
rpoD	795 bp	Cmm	99–100	95	95	95	95
		Cms		100	96	96	96
		Cmi			100	97	97
		Cmn					96
		Cmt					

nucleases, only *Bsu*RI produced a single, characteristic RFLP pattern for each subspecies. *Hpa*II and *Hin*6I produced three RFLP patterns, and *Fnu*DII produced only two patterns for the 40 strains and five subspecies (Table 2). For *Fnu*DII, the first RFLP pattern was unique only for Cmm strains (Fig. 1b and Table 2), and the second pattern was common for three Cmm strains (63, 78, and 153) and the other Cm subspecies. Overall, restriction analysis of the PCR fragments of the *rpoB* gene allowed the description of six RFLP groups (Tables 1 and 2, Fig. 1b).

The nucleotide sequences of the *rpoB* gene obtained for the 40 Cm strains and the type strains of Cmm NCPPB382 (AM711867) and Cms ATCC33113 (AM849034) indicated 98% identity. Based on *rpoB*, the genetic distance between the Cm subspecies ranged from 1 to 2% (Table 3).

In general, strains from the same subspecies had identical sequences but sequences for Cmm strains had four polymorphic positions. Three of the 16 Cmm strains could be distinguished based on a change in only one position: strains 63, 78, and 153 could be distinguished from the other strains of this subspecies based on the restriction analysis using *Fnu*DII (Fig. 1b and Table 1).

Comparison of *rpoD* gene fragment amplified from *C. michiganensis*

The primers designed from the *rpoD* gene generated an amplification product of about 862 bp. After digestion by *Bsu*RI, *Fnu*DII, *Hpa*II, and *Hin*6I in separate reactions, RFLP analysis revealed seven restriction groups (Tables 1 and 2, and Fig. 1c). The first three RFLP groups (1–3) were unique for Cmm strains. Strains from each of the other four subspecies belonged to a separate, single RFLP group (Tables 1 and 2, Fig. 1c). *Fnu*DII and *Hpa*II produced a different RFLP pattern for each subspecies (Fig. 1c and Table 2). Consideration of restriction endonucleases *Bsu*RI and *Hin*6I allowed the differentiation of the Cmm strains into three RFLP groups (Table 2 and Fig. 1c).

Forty PCR products for the *rpoD* gene of the 40 Cm strains were sequenced and compared with the *rpoD* sequences of Cmm NCPPB382 (AM711867) and Cms ATCC33113 (AM849034). Based on the *rpoD* gene, the genetic distance between subspecies ranged from 3 to 5% (Table 3). Strains belonging to the same subspecies had identical sequences, except for strains of Cmm, whose sequences had four

polymorphic positions. Two of these polymorphisms enabled the Cmm strains to be differentiated into three RFLP groups. Application of *Hin*6I revealed the first polymorphic position, which was characteristic for 9 of 15 Cmm strains (strains 5, 14, 21, 56, 61, 63, 80, LMG2891 and 78), which belong in RFLP groups 1 and 3 (Table 1). *Bsu*RI revealed the second polymorphic position, which was unique for RFLP group 3, a group containing only one strain (Cmm 78) (Table 1).

Compilation of *recA*, *rpoB*, and *rpoD* RFLP analysis

When *recA*, *rpoB*, and *rpoD* genes were subjected to PCR and the products were subsequently subjected to restriction analysis, a characteristic RFLP pattern was obtained for each gene product of each subspecies. The compilation of the RFLP analysis for all three genes indicated 10 RFLP genotypes for the five Cm subspecies (Tables 1 and 2). Strains of Cms, Cmi, Cmn, and Cmt generated a subspecies-specific RFLP genotype for each of the three genes. In contrast, Cmm strains generated six RFLP genotypes after compilation of three RFLP groups, which were found for *rpoD* and *recA* and two RFLP groups, which were found for *rpoB* (Tables 1 and 2). Strains belonging into the same RFLP genotype have an identical sequences of the tested genes.

A consensus dendrogram was constructed based on the RFLP patterns of *recA*, *rpoD*, and *rpoD* genes of all 40 strains. The dendrogram revealed that strains belonging to the same subspecies formed separate clusters (Fig. 2).

The same results were obtained when phylogenetic analysis of the concatenated sequences of the three genes was performed. The analyzed sequences formed five main phylogroups, one for each subspecies. The identity of the sequences between phylogroups was 96% (Fig. 3).

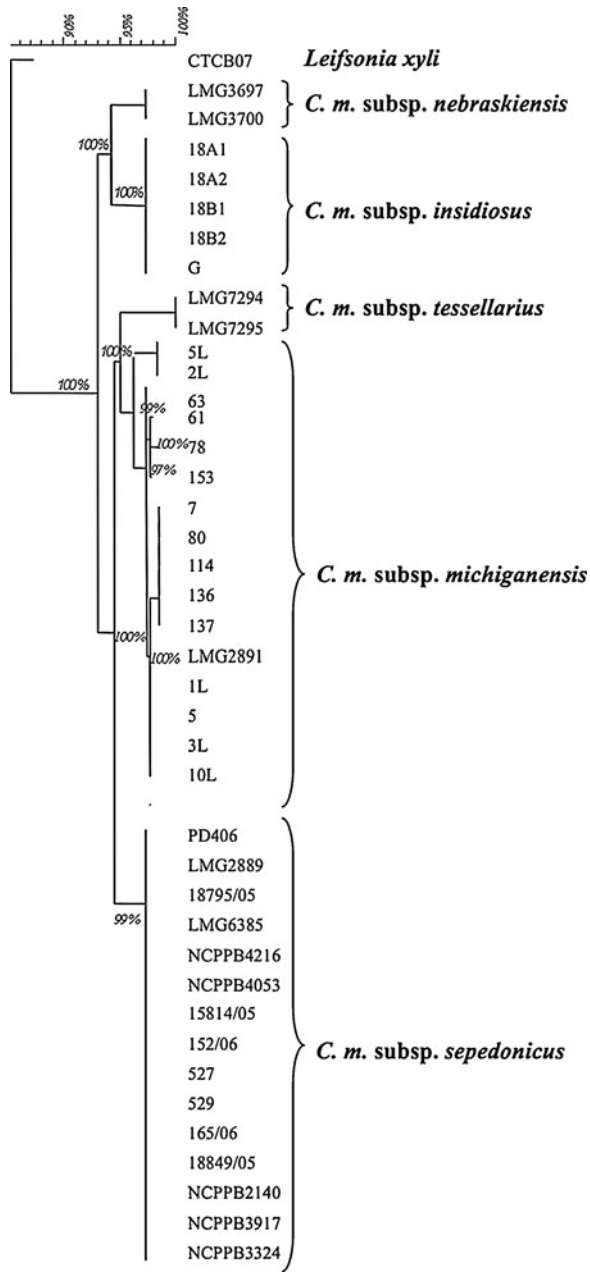

Fig. 2 A consensus dendrogram derived from the UPGMA linkage of correlation coefficients for *recA*, *rpoB*, and *rpoD* RFLP profiles

Discussion

The amplified fragments of the three housekeeping genes, *recA*, *rpoB*, and *rpoD*, from five subspecies of *C. michiganensis* were compared using PCR-RFLP and sequence analysis. Our earlier work indicated the usefulness PCR-RFLP of housekeeping genes such as *recA*, *gyrA*, and *rpoS* for identification of subspecies of the former genus *Erwinia* (Waleron et al. 2002a; b; Waleron et al. 2008). The current results demonstrate that polymorphism analysis of housekeeping genes, either by PCR-RFLP or by sequencing, is useful for identifying subspecies of Cm and also for evaluating their diversity. For each of the three genes, each of the five subspecies had a unique RFLP pattern after PCR

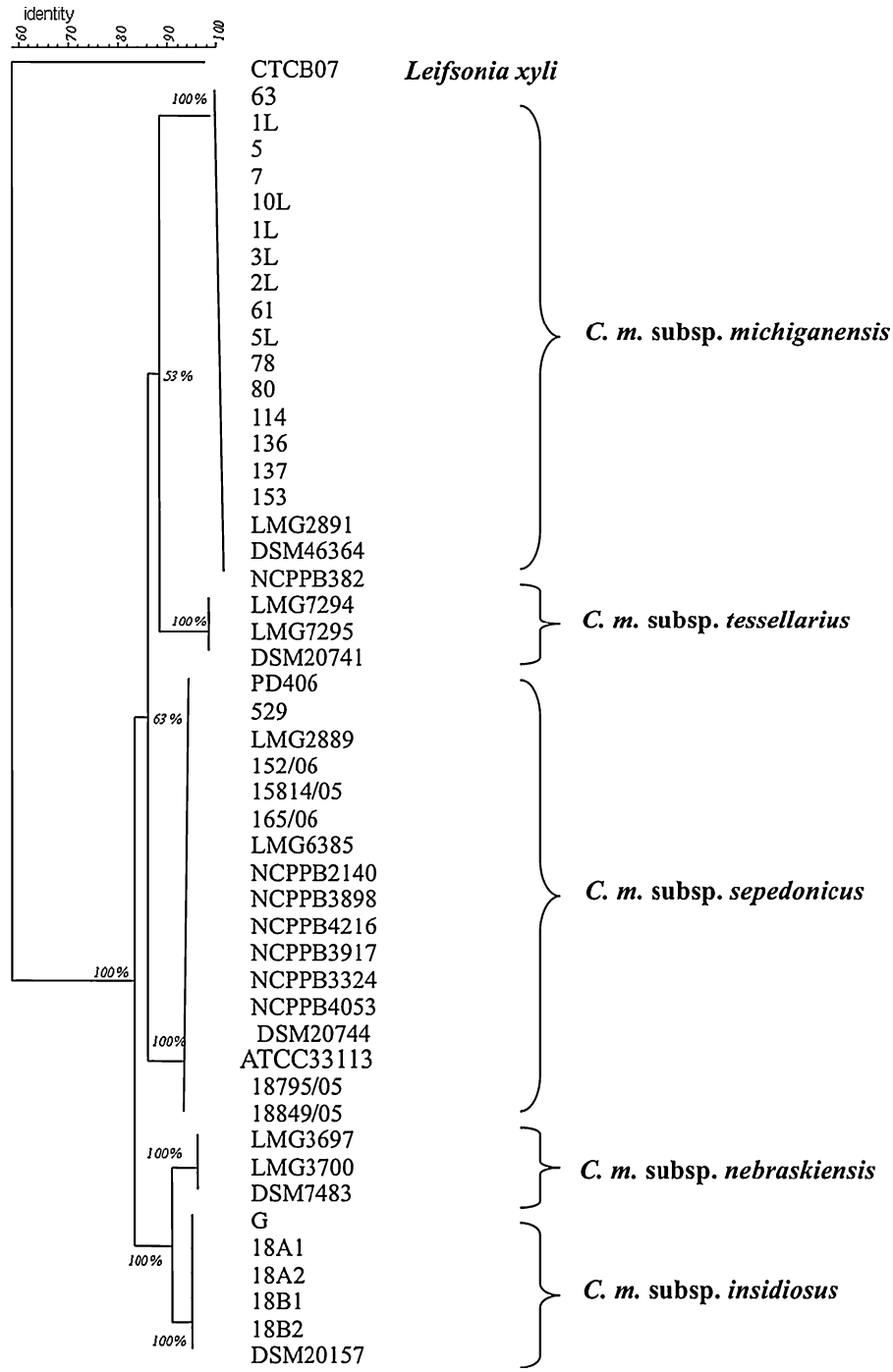

Fig. 3 The distance genetic tree based on the concatenated nucleotide sequences of the *recA*, *rpoB*, and *rpoD* genes from 40 *C. michiganensis* strains and 7 sequences obtained from the GenBank. Bootstrap values after 1000 replicates are expressed as percentages. *Leifsonia xyli* was included as an outgroup

amplification and restriction analysis. Isolates of each subspecies Cms, Cmi, Cmn, and Cmt generated one specific RFLP group in the case of all three genes.

Cms strains, which differed in mucus production, the level of virulence and geographical origin, generated identical RFLP pattern for all tested genes. Also all of five analyzed Cmi strains generated the same RFLP pattern although three of them differed in single biochemical properties. This low genetic variability among Cms, Cmi, Cmn, and Cmt strains was previously reported (Li and De Boer 1995; Lee et al., 1997a; b; Pastrik and Rainey 1999).

In the case of Cms strains, some heterogeneity was previously observed by CHEF analysis (Brown et al. 2002), which detected differences between virulent and avirulent strains. The *rrn* operon gene sequences (Lee et al., 1997b) and low-molecular weight RNA profiles (Palomo et al. 2000) of Cms were also uniform. BOX PCR demonstrated the absence of fingerprint variation among Cms strains from different geographical locations or from strains isolated from potato and asymptomatic sugar beets (Smith et al. 2001); in the same study, fingerprint variation for strains of Cmi, Cmn, and Cmt was similar to or slightly higher than that of Cms strains. No conclusions based on polymorphism analysis of analyzed housekeeping genes regarding Cmn, Cmt can be stated because only two strains from these subspecies were used in this study.

For Cmm strains, the compilation of the RFLP analyses of all three genes (*recA*, *rpoB*, and *rpoD*) enabled the description of six RFLP genotypes. There was no obvious relationship between RFLP group and geographic origin, biochemical features or virulence level of the Cmm strains. The colonies of all tested Cmm strains were mucoid. Strains, which differed in virulence, biochemical properties and geographical origin, were belonged to the same genotypes.

This is in contrast to previous reports, which indicated that variation in Cmm strains was related to geographic distribution. BOX PCR revealed four distinct groups of Cmm strains from four regions in the USA (Louws et al. 1998) and from four regions in Japan (Kawaguchi et al. 2010). A similar analysis of Cmm strains from Iran produced six fingerprint patterns (Nazari et al. 2007). When Cmm strains from the Canary Islands were characterized and compared with strains from seven other countries using BOX-PCR, 12 genotypes were detected, while the use of RAPD techniques increased the number of different profiles to 18, with five AFLP types (De León et al. 2009). The relatively high genetic diversity among Cmm strains was also confirmed by PFGE analysis, which distinguished 11 haplotypes among 58 Cmm strains from Israel and 18 haplotypes from four other countries (Kleitman et al. 2008).

It is difficult to compare results of these studies with the data obtained in other laboratories because of the lack of a common pool of reference strains. Although DNA fingerprinting techniques can be useful for characterizing the genomic diversity in bacterial populations, they can produce variable patterns (Busch and Nitschko 1999). In addition, the results of the fingerprinting methods are often difficult to compare between laboratories (Busch and Nitschko 1999).

The results reported here were based on PCR of isolated DNA, but we have obtained similar results when bacterial lysate rather than isolated DNA was used for the PCR reaction (unpublished data). This modified PCR assay significantly reduced the time and cost of the identification procedures. For rapid, inexpensive, and effective differentiation of the five subspecies in this research, we also recommend the amplification of the *recA* and/or *rpoD* gene and the digestion of the PCR products with the restriction endonuclease *Fnu*DII.

The current study demonstrated that polymorphisms of three housekeeping genes could be used to identify subspecies of the plant-pathogenic bacterium *C. michiganensis*. The study also demonstrated that polymorphisms in these genes can be used to measure genomic diversity among the subspecies. The results indicated that both PCR-RFLP analysis and sequencing of the conservative housekeeping genes *recA*, *rpoB*, and *rpoD* are simple and accurate methods for identification and differentiation of *C. michiganensis* subspecies. In addition, the sequencing results can be used to differentiate Cmm strains and to investigate phylogenetic relationships among *C. michiganensis* subspecies. The genetic distance observed between subspecies was quite small but was sufficient for precise identification of subspecies.

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