PCR MP Method for Differentiation of *Clavibacter michiganensis* Subsp. *sepedonicus* Isolates

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Abstract

The published data indicate that the bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) shows a notably low degree of intraspecific variation, and most of the molecular diagnostic methods could differentiate among subspecies groups but lacked the resolution to detect genetic diversity within subspecies. The present study describes effective differentiation of 50 isolates of *C. michiganensis* subsp. *sepedonicus* by the PCR MP method using five restriction enzymes (*Apa*I, *Pst*I, *Bam*HI, *Xma*I, *Hind*III). By analysing the electrophoretic patterns obtained by the PCR MP method performed with the applied restriction enzymes, each of the 50 tested isolates could be identified and distinguished from the remaining isolates. The PCR MP method of bacterial strain identification can be used in epidemiological studies, particularly for excluding the isolates of *C. michiganensis* subsp. *sepedonicus* as a source of primary infection.

Resumen

Los datos publicados indican que la bacteria Clavibacter michiganensis subsp. sepedonicus (Cms) muestra un grado notablemente bajo de variación intraespecífica y la mayoría de los métodos de diagnóstico molecular podrían diferenciar entre los grupos de subespecies pero carecían de la resolución para detectar la diversidad genética dentro de las subespecies. El presente estudio describe la diferenciación de 50 aislamientos de C. michiganensis subsp. sepedonicus por el método PCR MP usando cinco enzimas de restricción (ApaI, PstI, BamHI, XmaI, HindIII). Al analizar los patrones electroforéticos obtenidos por el método de PCR MP realizado con las enzimas de restricción aplicadas, cada uno de los 50 aislamientos probados se pudo identificar y distinguir de los demás aislamientos. El método PCR MP de identificación de cepas bacterianas se puede utilizar en estudios epidemiológicos, particularmente para excluir los aislamientos de C. michiganensis subsp. sepedonicus como fuente de infección primaria.

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Introduction

Bacterial potato ring rot caused by *C. michiganensis* subsp. *sepedonicus (Cms)* (Spieckermann & Kotthoff) is a highly important quarantine disease in the potato industry, primarily in economic terms (Van der Wolf et al. 2005). This disease causes large economic losses, both directly and indirectly. The direct loses occur during potato cultivation and storage, the indirect losses are caused by the denied access to the current export markets and restricted or denied access to new markets. Zero tolerance for ring rot is obligatory in all certified EPPO potato seed production areas, both in the European Union and in North America (Smith et al. 1997; Commission Directive 2006/56/EC of 12 June 2006). The routine detection techniques, such as immunofluorescence microscopy (IF) (De

Boer and McCann 1990), molecular methods based on PCR reaction (Pastrik 2000; Gudmestad et al. 2009), hybridization (Drennan et al. 1993), and pathogenicity tests on eggplants (Black Beauty) (Olsson 1976) do not have sufficient discriminatory power to efficiently differentiate *Cms* isolates. The close intraspecific affinity of *Cms* isolates was observed in morphology, biochemistry, protein, plasmid profiles and in DNA hybridization (Carlson and Vidaver 1982; Davis et al. 1984; Mogen et al. 1987; Mogen et al. 1985; Snieszko and Bonde 1943; Vantomme et al. 1987). RFLP PCR of three housekeeping gene fragments (*rec*A, *rpo*B, and *rpo*D) differentiated between *C. michiganensis* subspecies however, no differences were demonstrated within *C. michiganensis* subsp. *sepedonicus* (Waleron et al. 2011). The cluster analysis of the combined sets of BOX-, ERIC- and Rep-PCR experiments used by a group of researchers from the USA showed that subspecies *C. michiganensis* can be divided into five different groups (Louws et al. 1998). Genetic similarities between the obtained amplification profiles of Rep-PCR between the five subspecies of *Clavibacter* were lower than 40%. Therefore, the rep-PCR technique uniquely distinguished the subspecies. However, the electrophoretic patterns obtained for the isolates of each *C. michiganensis* subspecies were similar and the isolates of the subspecies *Clavibacter michiganensis* subsp. *sepedonicus* showed over 94.7% similarity (Louws et al. 1998; Smith et al. 2001). It has been indicated that each subspecies of *C. michiganensis* had distinct Rep-PCR pattern and that isolates within individual subspecies had almost identical Rep-PCR patterns. Therefore, Rep-PCR is effective for the differentiation between the subspecies, not within the subspecies (Carlson and Vidaver 1982; Louws 1998). Most of the molecular methods did not differentiate the *Cms* isolates, even those coming from different geographical regions (Lee et al. 1997; Palomo et al. 2000; Smith et al. 2001; Waleron et al. 2011). The analysis of 16S and 23S rRNA coding sequences showed a close phylogenetic relationship between the five subspecies of *C. michiganensis* (Fessehaie et al. 2003; Li and De Boer 1995a, b; Mirza et al. 1993). However, based on the whole-genome and multi-locus sequence analyses it was suggested that the subspecies of *Clavibacter michiganensis* should be raised to species status (Li et al. 2018). Using molecular methods, the *Cms* isolates were classified into virulent and avirulent groups (Lee et al. 1997).

CHEF analysis, detected differences between virulent and non-virulent strains, which were correlated with alterations in the genomic regions responsible for hypersensitivity response and cellulase synthesis (Brown et al. 2002). However, there was no correlation between the morphology of bacterial colonies and pathogenicity (Brown et al. 2002). The effect of mucoids on the degree of differentiation of *Cms* isolates has not been observed either (Fousek and Mraz 2003; Fousek et al. 2002).

Thus, there was a consensus concerning low genetic variation among *Cms* isolates which has been confirmed by numerous scientific teams using various genotyping techniques (Carlson and Vidaver 1982; Davis et al. 1984; Lee et al. 1997; Li and De Boer 1995a, b; Pastrik and Rainey 1999; Louws et al. 1998; Smith et al. 2001).

The PCR melting profiles (PCR MP) method, has been confirmed efficient in the differentiation of the plant pathogen *Pseudomonas syringae pv. lachrymans* (Olczak-Woltman et al. 2007) and other *Pseudomonas* species pathogenic to plants (Bultreys and Kałużna 2010; Kałużna et al. 2016 a; Kałużna et al. 2010; Kałużna et al. 2016,b). Previously, the PCR MP method was used to differentiate clinical bacterial strains in epidemiological studies (Krawczyk et al. 2007; Stojowska et al. 2009) and demonstrated discriminatory power at least equivalent to the discriminatory power of pulsed field gel electrophoresis (PFGE), which was considered to be the gold standard (Krawczyk et al. 2007; Krawczyk et al. 2009). The ability to analyse genomes with unknown nucleotide sequence and the ease of analysing results on agarose gels are the main advantages of the PCR MP method (Masny and Płuciennczak 2003). There were also reports on applicability of PCR MP to *Cms* isolate differentiation (Żaczek et al. 2018).

There is little published data regarding the genetic diversity among *Cms* isolates recovered from potato tubers in Poland. Gathering information regarding the genetic diversity within the populations of *Cms* in our country and worldwide would help to study the epidemiology of these bacteria and to define its economic threat to potato production.

The objective of this study was to determine the discriminatory power of the PCR MP method in genotyping subspecies of *Cms* isolates.

Materials and Methods

Origin and Growth Conditions of *Cms* Strains

This study presents the results of genotyping 50 isolates of *Cms* bacteria. Year of isolation, place of origin and morphology of the bacterial colonies are shown in Table 1. Isolates were organized into three groups, depending on the degree of pathogenicity to eggplant. Degree of pathogenicity of *Cms* isolate was assessed in a biological assay based on the five-stage symptom scale and appendage proposed by Stead and Janse (2000). In this test, the rate of their growth in plants and the production of virulence factors inducing wilt in controlled conditions were used as the basis for isolation of isolates. The first group of isolates were characterized by the highest degree of pathogenicity (group A), the second group were medium pathogenic isolates (group B) and the third group were isolates exhibiting the lowest degree of pathogenicity (group C). The third group contained the reference strain most frequently

Table 1 Origin and identification year of *Cms* isolates and morphology of *Cms* cells

The name of the isolate	Year of identification	Geographical region of Poland (province)	Morphology of the bacterial cell	Pathogenic Group
5289	2010	mazowieckie	fluidal	A
20	2005	śląskie	mucoidal	A
K05	2007	zachodnio-pomorskie	mucoidal	A
Wa-wa 4	2009	mazowieckie	mucoidal	A
Błonie 1	2007	mazowieckie	fluidal	A
Vineta 5.1	2006	świętokrzyskie	fluidal	A
2389	2008	podlaskie	fluidal	A
P2	2006	wielkopolskie	dry	A
2427	2007	dolnośląskie	fluidal	A
P1	2006	wielkopolskie	fluidal	A
CL 555	2009	małopolskie	fluidal	A
KO1	2007	zach.-pomorskie	fluidal	A
KO1(150)	2007	zach.-pomorskie	fluidal	A
Bydgoszcz 15	2009	kujawsko-pomorskie	mucoidal	A
2897	2005	dolnośląskie	dry	A
46	2006	pomorskie	fluidal	A
CL 511	2010	łódzkie	fluidal	A
CL 611	2009	lubelskie	mucoidal	B
CL 612	2009	lubelskie	fluidal	B
7/Rz	2007	podkarpackie	fluidal	B
CL 552	2009	małopolskie	fluidal	B
1656	2010	świętokrzyskie	fluidal	B
7453	2006	pomorskie	fluidal	B
P4	2006	wielkopolskie	mucoidal	B
1850	2005	świętokrzyskie	fluidal	B
1455	2013	małopolskie	fluidal	B
CL 622	2010	wielkopolskie	fluidal	B
6269	2010	pomorskie	fluidal	B
Bydgoszcz12047	2011	kujawsko-pomorskie	fluidal	B
NCPPB 3917	1968	Canada	fluidal	B
3091	2005	dolnośląskie	fluidal	C
88	2006	śląskie	fluidal	C
25	2005	śląskie	fluidal	C
11,198	2006	pomorskie	fuidal	C
Wa-wa 5	2009	mazowieckie	mucoidal	C
147	2007	łódzkie	mukidal	C
8706	2006	kujawsko-pomorskie	mucoidal	C
5006	2009	pomorskie	dry	C
4 K	2007	śląskie	mucoidal	C
6490	2006	kujawsko-pomorskie	fluidal	C
1.1	2006	warmińsko-mazurskie	fluidal	C
CL 584	2009	warmińsko-mazurskie	fluidal	C
CL 588	2009	świętokrzyskie	fluidal	C
8711	2006	kujawsko-pomorskie	fluidal	C
1625	2010	małopolskie	fluidal	C
CL 616	2009	lubelskie	fluidal	C
CL 607	2009	świętokrzyskie	mucoidal	C
1365 Kielce	2011	świętokrzyskie	fluidal	C
NCPPB 3324	1985	Belgium	fluidal	C
Ref (BPRIOR 527)	1994	Poland	fluidal	C

used for research in Polish laboratories (Ref BPRIOR 527). All tested *Cms* isolates were derived from sixteen Voivodship Inspectorates of Plant Health and Seed Inspection (WIORiN). These isolates were derived from the Polish bacterial collection of IHAR-PIB in Radzików collected in the years 2005–2010. Two foreign isolates were also included in the study (NCPPB 3324 - an isolate from Belgium and NCPPB 3917 – an isolate from Canada).

These isolates were grown on yeast - peptone glucose agar (YPGA) and yeast extract mineral salt medium (YGM) at

22 °C for 5 days. All bacteria were stored in glycerol (70% final concertation) at −80 °C.

Identification Tests

PCR with Species-Specific Primers

To identify the *Cms* bacterial isolates, PCR reactions were performed according to the protocol by (Pastrik and Rainey 1999), with specific primers PSA-1 (5'-CTCCTTGT

GGGGTGGGAAAA-3′) and PSA-R (5'-TACTGAGA TGTTTCACTTCCCC-3′) targeting a portion of the ITS region with the expected amplicon size of 502 bp. The PCR reaction was performed in total volume of 15 μL: 1x reaction buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl) 1.5 mM MgCl$_2$, 100 μM of each dNTP; 0.2 μM of each primer (PSA-1 and PSA-R), 1 U Taq DNA polymerase (Fermentas) and 1 ng of DNA. The following PCR conditions were used: initial denaturation at 94 °C for 2 min 30 s, followed by 10 reaction cycles of 95 °C for 1 min, 65 °C for 1 min and 72 °C for 1 min. The initial 10 cycles were followed by 25 more cycles of amplification: 95 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min and incubation in 72 °C for 5 min after the last cycle.

DNA Extraction

The extraction of bacterial DNA was performed with the Easy-DNA TM Kit (Invitrogen, Netherlands) following the protocol provided by the manufacturer. Concentration and the purity of DNA was determined spectrophotometrically by measuring the absorbance at 260 nm and 280 nm using NanoDrop ND-1000 (Thermo Scientific).

PCR MP Procedure

Five restriction endonucleases (ApaI, PstI, BamHI, XmaI, HindIII) were used to obtain a variable level of discrimination of the isolates in PCR MP. For all restriction enzymes applied the number of the created genomic DNA restriction fragments could be predicted by in silico restriction digestion of the C. michiganensis subsp. sepedonicus reference genomes (NCBI Reference Sequence: NC_010407.1 and C. michiganensis subsp. sepedonicus strain CFIA-Cs3N NCBI Reference Sequence: NZ_MZMM01000001.1) using free software (tools.neb.com /REBsites/).

Prior to the PCR amplification, the genetic material was digested with five restriction enzymes in separate reactions in their recommended buffers: ApaI (Buffer B, Fermentas), PstI (Buffer O, Fermentas), XmaI (Buffer Tango, Thermo Scientific), HindIII (Buffer R, Thermo Scientific) and BamHI (Buffer BamHI Lsp 1109I, Thermo Scientific). The digestion of 200 ng of bacterial DNA was performed in total volume of 10 μL by adding 1 μL of restriction buffer to the reaction and 0.2 μL (10 U/μL) of endonuclease. The digestion was performed from 4 to 16 h at 16–37 °C depending on the restriction enzyme used. After incubation, a ligation mix was prepared in a total volume of 15 μL as follows: 1 μl of 20 μM adapters matching the sticky ends of the DNA fragments produced by the restriction enzyme used 3 μL of 5x Ligase Buffer (Invitrogen), and 0.2 μL of DNA ligase (5 U /μL, Invitrogen). The samples were incubated 3 min. at 37 °C and 3 h at 16 °C. The PCR was performed in a volume of 15 μL. The reaction

mixture contained 1 μL of ligation solution, 1.5 μl 10x Dream Taq Green Buffer (Fermentas), 0.3 μL dNTP (10 mM), 0.08 μL Dream Taq DNA Polymerase 5 U/μL (Fermentas) and 1.5 μL (10 μM) primer (Table 2). Denaturation temperature ranges were optimized for each enzyme separately in the thermocycler with gradient functions (Mastercycler ep Gradient S; Eppendorf). It has to be emphasized that a number of gradients has been tested and only those with the highest level of electrophoretic pattern reproducibility and an analysable number of bands produced in PCR MP were chosen for further analyses. From each gradient single denaturation temperature was selected for each restriction endonuclease: ApaI - 93.3 °C, PstI - 92.7 °C, BamHI - 91.8 °C and 92.8 °C, XmaI -94 °C, HindIII - 95 °C, in which the number of the obtained bands was analysable and the electrophoretic band pattern was reproducible in at least two independent experiments performed on DNA extracted from two independent cultures of a Cms isolate.

The ranges of denaturation temperature gradients applied for PCR MP were: 90 °C - 95 °C for ApaI, 90 °C - 94 °C for PstI, 92.6 °C - 94.1 °C for XmaI 92.6- 94.1. The PCR MP reactions for the enzyme BamHI were performed for two denaturation temperature ranges: 90 °C - 92 °C and 91 °C - 93 °C. PCR reactions were carried out as follows for the ApaI enzyme: initial elongation of 72 °C – 5 min, 30 cycles (denaturation in the gradient 90 °C - 95 °C – 40 s, annealing 55 °C – 40 s, elongation 68 °C – 1 min 30 s) and a final elongation of 72 °C – 30 min; for the PstI enzyme initial elongation of 72 °C – 5 min, 30 cycles (denaturation in the gradient 90 °C - 94 °C – 40 s, annealing 55 °C – 40 s, elongation 68 °C – 1 min 30 s) and a final elongation of 72 °C – 30 min; for the XmaI enzyme initial elongation of 72 °C – 5 min, 27 cycles (denaturation in the gradient of 92.6 °C - 94.1 °C – 40 s, annealing 55 °C – 40 s, elongation 68 °C – 1 min30 s) and a final elongation of 72 °C – 30 s; for the BamHI enzyme initial elongation of 72 °C – 5 min, 30 cycles (denaturation in the gradient of 90 °C - 92 °C or 91 °C - 93 °C – 40 s, annealing 55 °C – 40 s, elongation 68 °C – 1 min 30 s), and a final elongation of 72° - 30 min.

For HindIII enzyme PCR reactions were conducted as follows: initial elongation 72 °C - 5 min, denaturation at 95 °C - 2 min, 23 cycles (denaturation and annealing: 95 °C - 1 min, elongation: 72 °C – 2 min).

For the PCR MP products, 6 μL or 15 μL were electrophoresed on a 2% agarose gel (24 × 20 cm) with TBE buffer and stained in ethidium bromide (10 mg/mL aqueous solution) for 5 min. The electrophoresis was performed at 120 V for 4 h and 45 min. The images of the gels were analysed using a Kodak Gel Logic 200 Imagine System (Eastman Kodak Company, Rochester, NY 14650, USA).

The images of the agarose gels with PCR MP profiles were analysed using BioNumerics v6.6 software (Applied Maths, Belgium). The similarity of the profiles obtained for the

Table 2 The Oligonucleotides and PCR primers used in PCR MP

RestrictionEnzyme	Oligonucleotide1 5′-3'	Oligonucleotide2 5′-3'	primer 5′-3'
*Apa*I	TCGTAGACTGCGTACAGGCC	TGTACGCAGTCTAC	GACTGCGTACAGGCCC
*Pst*I	TGTACGCAGTCTAC	TCGTAGACTGCGTACATGCA	TCGTAGACTGCGTACATGCAG
*Xma*I	CCTTCATCCACCAACGTCGAC	CCGGGTCGACGTT	CCTTCATCCACCAACGTCGACCCGGG
*Hind*III	CTCACTCTCACCAACGTCGAC	AGCTGTCGACGTTGG	CTCACTCTCACCAACGTCGACAGCTT
*Bam*HI	GATCGTCGACGTTGG	CTCACTCTCACCAACAACGTCGAC	CTCACTCTCTCACCAACGTCGACGATCC

reactions with individual restriction enzymes were determined using the Dice correlation coefficient (using optimization and tolerance parameters equal to 1% and 1%, respectively). The dendrograms were prepared using the UPGMA clustering algorithm. Additionally a summary analysis was performed for all of the tests based on a similarity analysis for averaged data values from individual experiments. The dendrogram was prepared using the UPGMA clustering algorithm, as well.

PCR Product Cloning and Sequencing

The PCR products obtained from DNA of *Cms* isolate CL 511 using previously described PCR MP for *Hind*III were separated in a 6% polyacrylamide gel. Two fragments of DNA (approximately 600 bp and approximately 1000 bp) were excised from the gel and eluted (Dybczynski and Pluciennczak 1988). The extracted DNA fragments were phosphorylated with T4 polynucleotide kinase (BioLabs), subsequently the enzyme was heat inactivated, the reactions were carried out according to the manufacturer's recommendations. Phosphorylated DNA fragments were ligated to the blunt ended linear vector pUC19, previously digested with *Sma*I (BioLabs) and dephosphorylated with calf intestinal alkaline phosphatase CIAP (Promega). The ligation was carried out with the T4 DNA ligase (Biolabs) according to the manufacturer's recommendations. Ligation products were transformed into *E.coli* NEB Turbo Competent (BioLabs) following the recommendations from the user's manual, and bacteria were plated on LB/ampicillin plates. The positive clones were identified by colony PCR with universal M13 forward and reverse primers. Overnight bacterial cell cultures of positive clones were grown in the liquid LB medium with ampicillin. Plasmids were isolated using Plasmid Mini kit (A&A Biotechnology) and subjected to Sanger sequencing with M13 forward and reverse primers using 3130 Genetic Analyzer (Applied Biosystems).
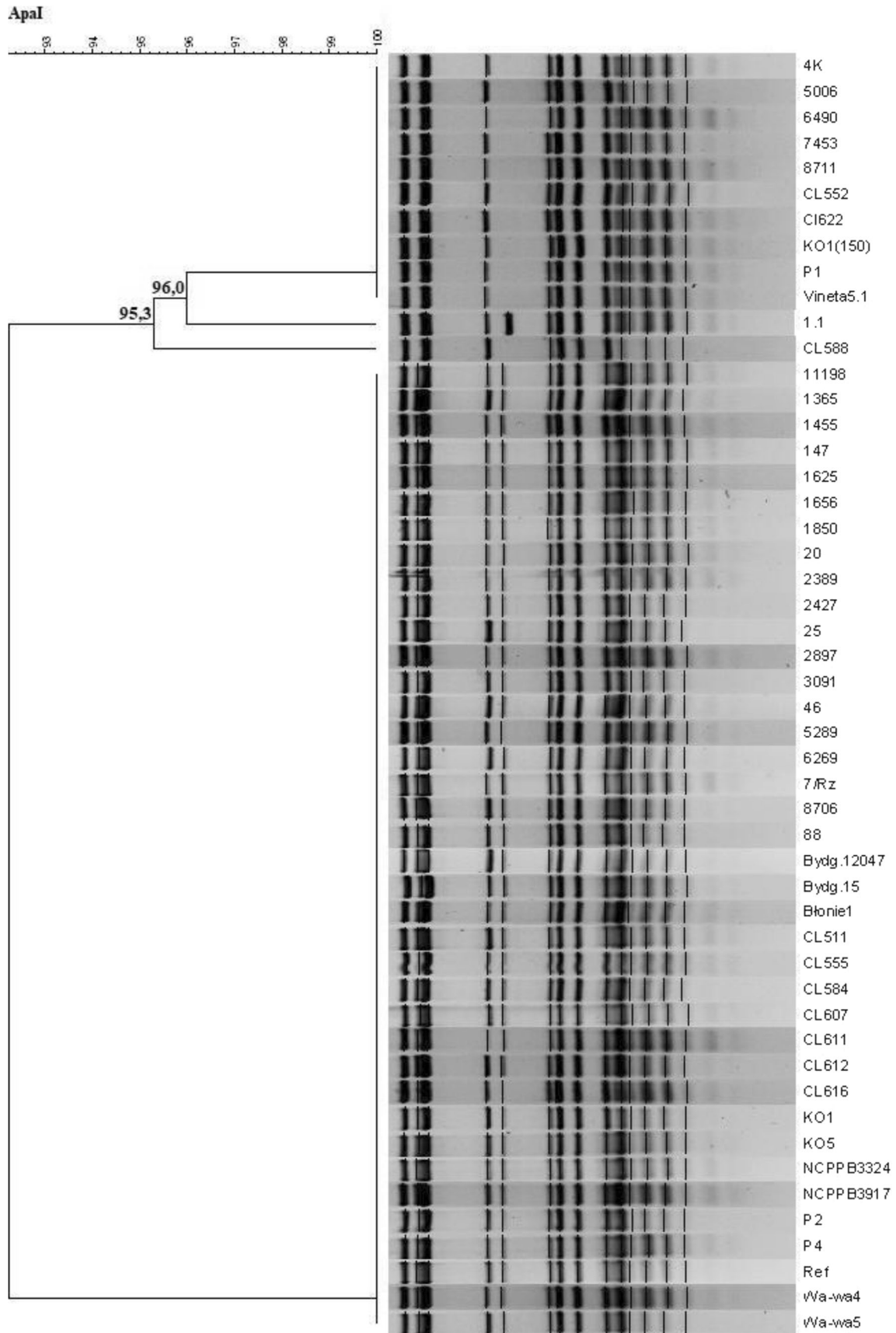
Results

Specific PCR for *Cms* Bacteria

Specific primers PSA-1 and PSA-R were used for all the tested isolates to obtain the 502 bp amplification product - a characteristic fragment for *C. michiganensis* subsp. *sepedonicus* used for *Cms* identification according to Commission Directive 2006/56 / EC of 12 June 2006.

Genetic Polymorphism Obtained by PCR MP

The results of all the PCR analyses performed for all the restriction enzymes and the conditions of the denaturation temperature are shown as electrophoretic patterns in Figs. 1, 2, 3, 4, 5 and 6.

The analysis of the genetic diversity based on the results obtained for the single enzyme versions of the PCR MP showed a relatively low discrimination power using the *Apa*I, *Hind*III and *Pst*I enzymes. Each of those enzymes allowed to obtain four distinct electrophoretic patterns for the Polish *C. michiganensis* subsp. *sepedonicus* isolates and the reference strains *Apa*I, *Hind*III and *Pst*I enzymes (Figs. 1, 2 and 3). *Apa*I PCR MP enzyme enabled us to distinguish two primary clusters grouping the majority of the studied isolates (internally indistinguishable by the adopted research methodology) and showing a 92% similarity level of the profiles. Analysis using the *Hind*III enzyme did not allow differentiation of the majority of the tested isolates (identical profiles were obtained for 46 isolates) however, four distinct clusters were identified. Analysis using the *Pst*I enzyme enabled distinguishing two primary clusters grouping the majority of the studied isolates (internally indistinguishable by the adopted research methodology) showing a 94% similarity level of the profiles. Higher discriminating power was demonstrated by analysis using the *Bam*HI enzyme; in denaturation temperature gradient 90 °C - 92 °C in single temperature of 91.8°C at least 9 unique electrophoretic patterns were obtained (Fig. 4). The increase of the denaturation temperature to the level of 91 °C - 93 °C additionally increased the discriminating power of this method and in single denaturation temperature of 92.8 °C 12 unique electrophoretic patterns or clusters were obtained (Fig. 5). The highest discriminatory power of the PCR MP differentiation of the isolates was achieved using the *Xma*I enzyme - 26 distinct electrophoretic patterns were obtained (Fig. 6). The dendrogram showing a summary analysis of the average PCR MP results obtained for all five enzymes is shown in Fig. 7. Only the dendrogram based on the results of analyses with all five restriction endonucleases

◀ **Fig. 1** Dendrogram showing the genetic similarity among 50 isolates of *Clavibacter michiganensis* subsp. *sepedonicus* generated on the basis of PCR MP genotyping results obtained for *Apa*I enzyme and denaturation temperature 93.3 °C (denaturation temperature range: 90- 95 °C)

based PCR MP allowed discrimination to the level of single, unique isolates Fig. 7.

Bioinformatic Restriction Fragment Analysis

The results of the restriction fragment analysis of the *Cms* genomes are presented in Table 3. Theoretically predicted restriction fragments, of size below 4000 bp, obtained from the reference genomes digested with *Hind*III were as follows: NCBI Reference Sequence NC_010407.1 - two fragments of the same size 3428 bp, 1728 bp, 945 bp and 608 bp, *C. michiganensis* subsp. *sepedonicus* strain CFIA-Cs3N (NZ_MZMM01000001.1) – 3428 bp (two fragments of the same size), 1728 bp and 608 bp.

Discussion

The bacterial species differentiation methods used so far for *Clavibacter michiganensis* subsp. *sepedonicus* isolates: rep-PCR (Louws et al. 1998; Fousek et al. 2002), RFLP (Mogen et al. 1990), AFLP (De León et al. 2009), RAPD PCR (Pastrik and Rainey 1999), PFGE (Milijašević-Marčić et al. 2012) and genetic marker based methods (Fessehaie et al. 2003) did not assure satisfactory discrimination between the isolates of the pathogen. Therefore, we decided to apply a method of bacterial species differentiation based on a different principle than rep-PCR, PFGE and RAPD PCR for discriminating between *C. michiganensis* subsp. *sepedonicus* isolates. The principle of the PCR MP is worth explaining since it differs from the principle of standard PCR techniques. In the PCR MP procedure used entire genome is converted into restriction fragments and to all those fragments artificial oligonucleotides are ligated. Thus, entire genome is converted into fragments which have primer binding sites on both ends and each of this fragments could be potentially amplified by PCR using single primer – such amplifiable fragments are called amplicons. It is not the presence of the primer binding site which determines amplification of the amplicon in PCR MP. In every PCR technique the essential step is denaturation of dsDNA in order to obtain ssDNA to which the primers can bind. The melting temperature of the DNA fragments depends on: the GC composition of those fragments and the sequence of those fragments. Two fragments of exactly the same length can have distinct melting temperature such as in the case of two PCR primers of the same length and different nucleotide sequence. In PCR MP the denaturation temperature is decreased below 95 °C and only less thermally stable DNA fragments become

ssDNA and only those fragments can be amplified by PCR because primers can bind only to ssDNA (Masny and Płucienniczak 2003). In PCR MP gradients of denaturation temperature are used in PCR to establish the temperature in which the number of obtained PCR products is small enough to be analyzable by electrophoresis (Masny and Płucienniczak 2003) in contrast to most PCR methods in which the denaturation temperature is 94- 95 °C and the primer annealing and primers sequences determine which target sequences are amplified. Each DNA fragment amplified in PCR MP is characterized by two features: the size (position on the gel after electrophoresis) and the lowest denaturation temperature in which the fragment is amplified. Thus, DNA fragments of the same length and distinct sequence can be differentiated by PCR MP by the difference in the lowest denaturation temperature in which those fragments are amplified (Krystynowicz et al. 2005). *C. michiganensis* subsp. *sepedonicus* has a very high percentage of GC in its genome, and the frequency of restriction sites in a genome of a bacterial species depends on the percentage of GC pairs (Masny and Płucienniczak 2003). Identifying a restriction enzyme enabling the differentiation of *C. michiganensis* subsp. *sepedonicus* by the MP PCR method posed a problem caused by a very high (over 72%) GC content of the *Cms* genome (Gartemann et al. 2008). Therefore, a series of restriction enzymes and oligonucleotide adapters were tested. In each denaturation temperature gradient single denaturation temperature was used for PCR MP in our study. Selection of such single denaturation temperature in which the obtained electrophoretic pattern is reproducible in at least two independent repeats of the PCR MP procedure, performed starting from the DNA extraction from the *Cms* bacterial culture, has to be done experimentally in series of experiments.

The electrophoretic patterns obtained in PCR MP for the *Bam*HI enzyme are good example of the impact of the denaturation temperature on the selective amplification of the discriminatory bands. Approximately 5.900 restriction fragments are created by *Bam*HI digestion of *Cms* genome Table 3. Applying gradient 90- 92 °C allowed us to amplify limited number of DNA particles out of 5.900 restriction fragments and obtain at least nine distinct clusters or unique electrophoretic patterns (Fig. 4) and in the gradient 91- 93 °C twelve distinct clusters or unique electrophoretic patterns were obtained (Fig. 5). In denaturation temperature 91.8 °C (Fig. 4) less bands were present in the electrophoretic patterns and in Tm 92.8 °C (Fig. 5) more bands were present. We have not performed direct evaluation of PCR MP against another genotyping or DNA fingerprinting method. However, the restriction endonuclease *Bam*HI was used in PCR MP by another research group investigating *C. michiganensis* subsp. *sepedonicus*, also (Żaczek et al. 2018). The level of discrimination obtained by PCR MP in the latter study was higher (11 clusters in a set of 93 isolates) than the discrimination level
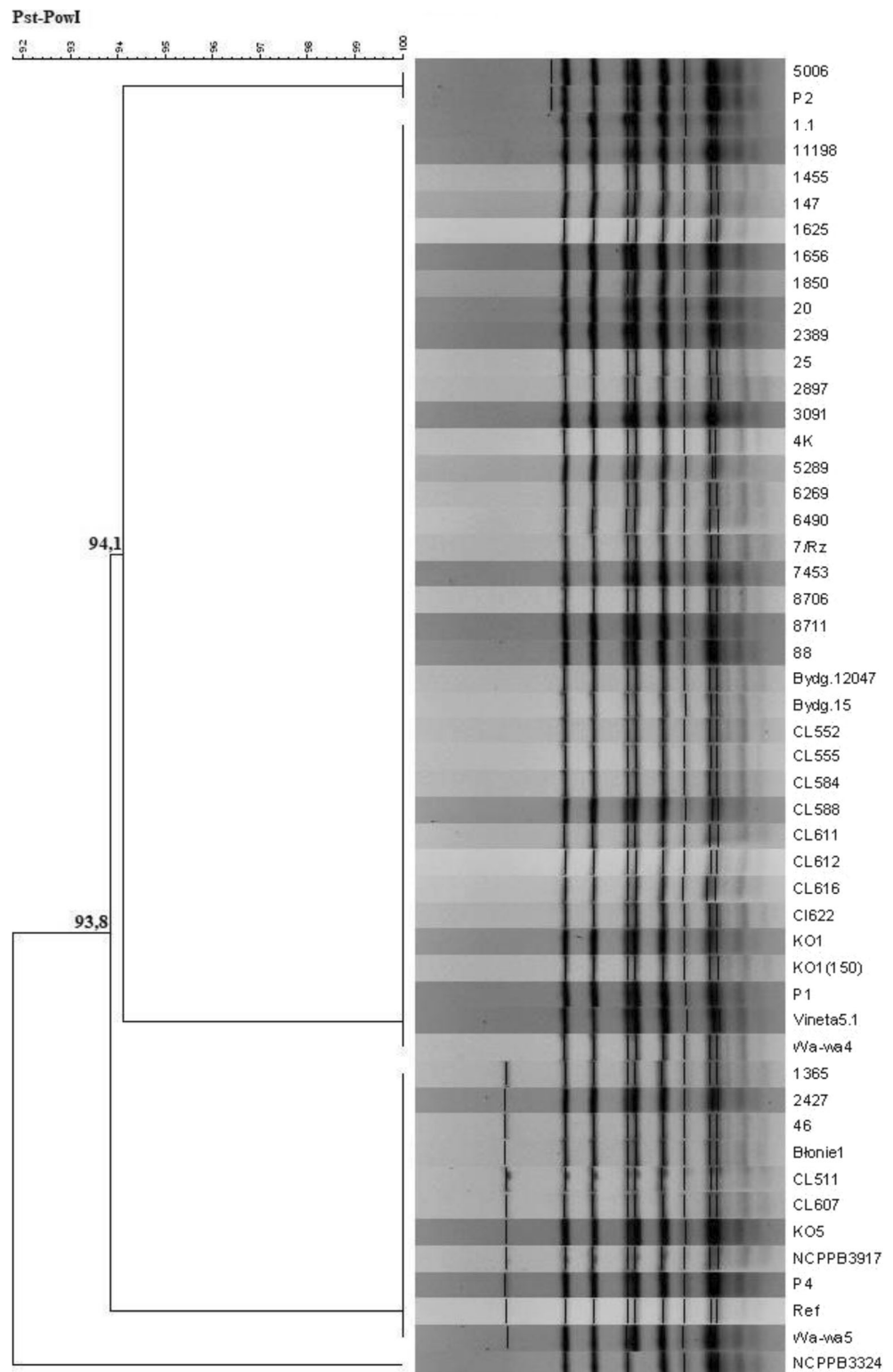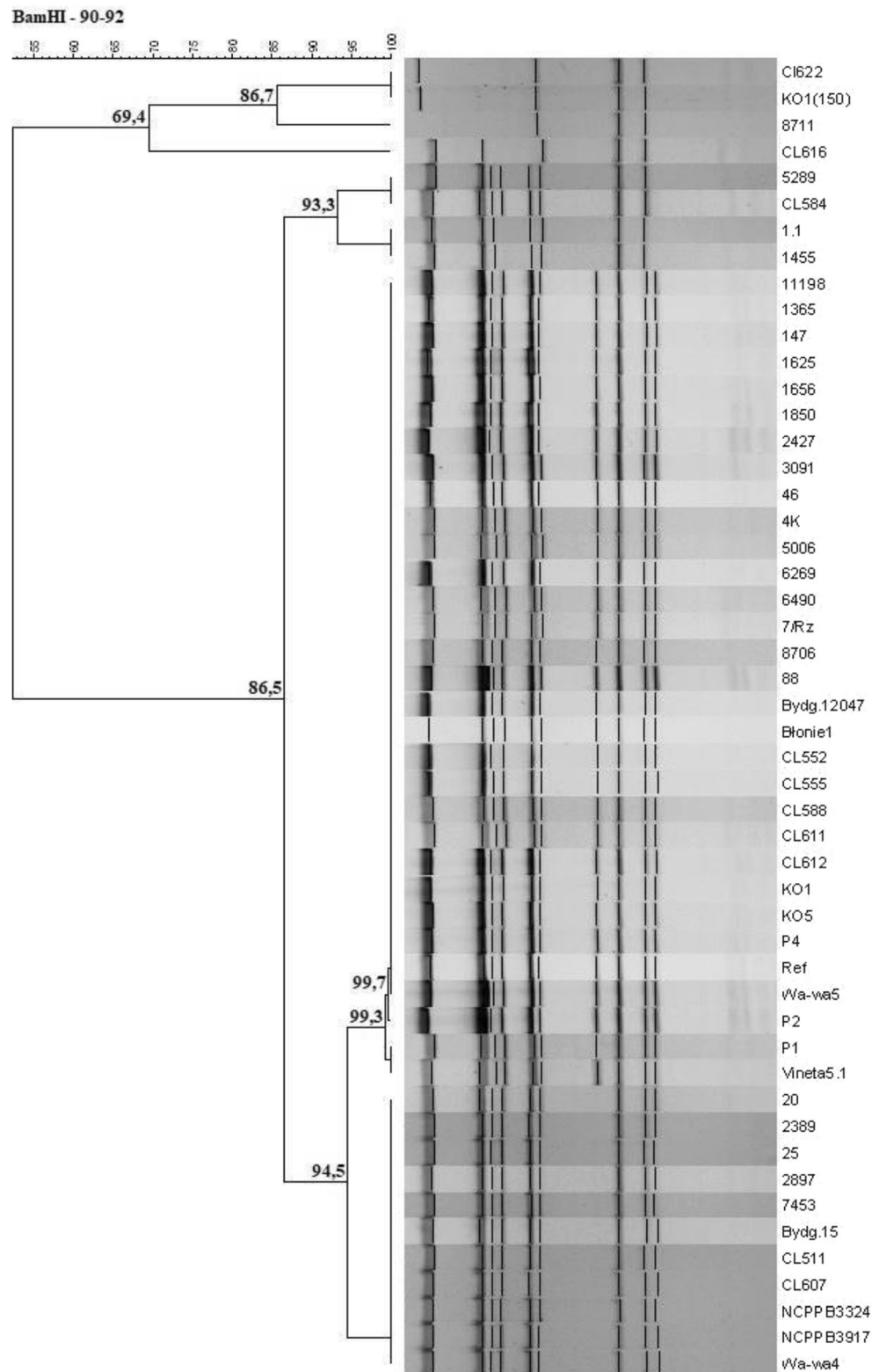
Fig. 2 Dendrogram showing the genetic similarity among 50 isolates of *Clavibacter michiganensis* subsp. *sepedonicus* generated on the basis of PCR MP genotyping results obtained for *Hind*III enzyme and denaturation temperature 95 °C
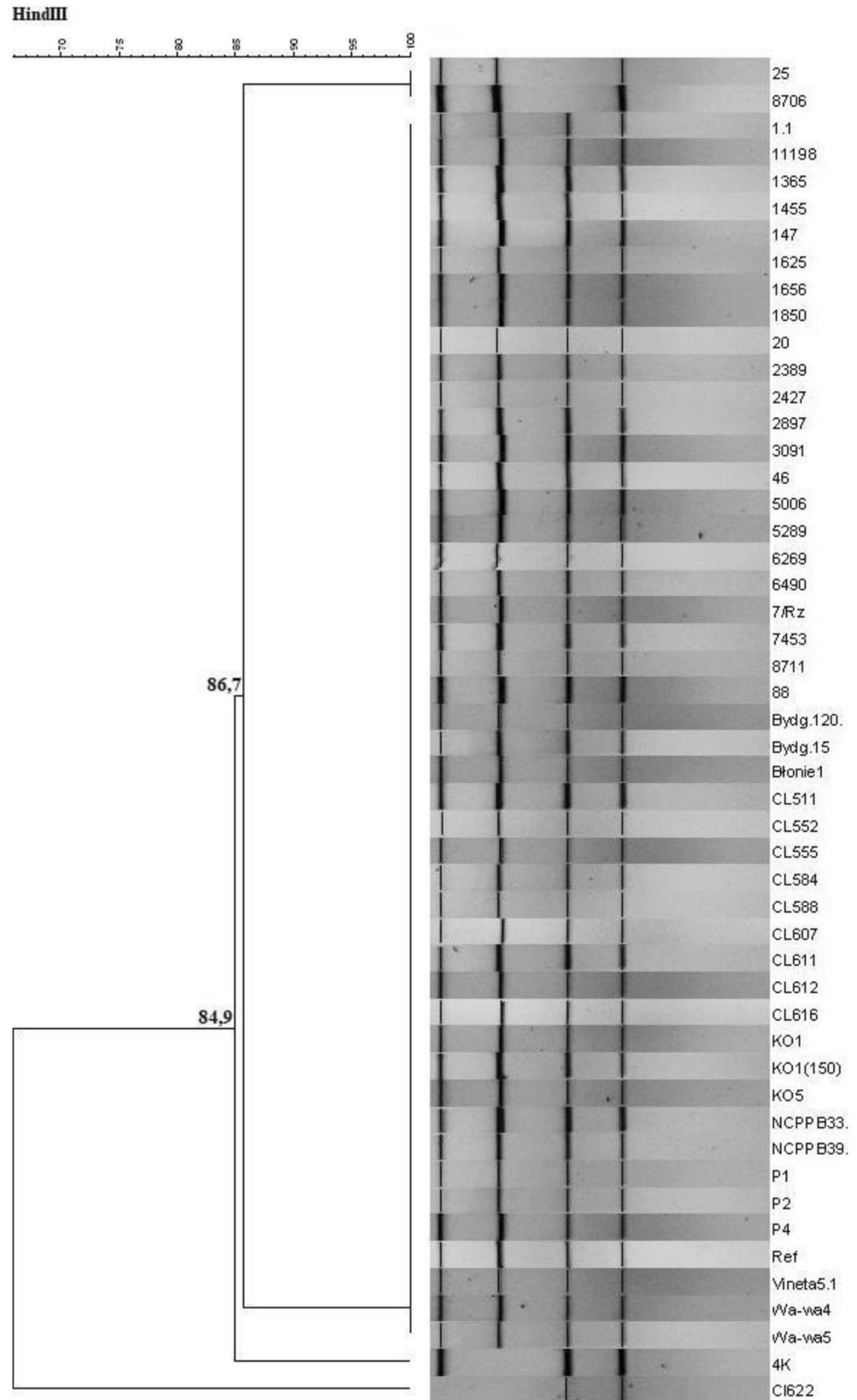
achieved by application of VNTR analysis (7 clusters in the set of 93 isolates) which was considered, by the authors, efficient discrimination (Żaczek et al. 2018). We obtained at least 9 unique electrophoretic patterns in *Bam*HI PCR MP in

Fig. 3 Dendrogram showing the genetic similarity among 50 isolates of *Clavibacter michiganensis* subsp. *sepedonicus* generated on the basis of PCR MP genotyping results obtained for *Pst*I enzyme and denaturation temperature 92.7 °C (denaturation temperature range: 90- 94 °C)

single denaturation temperature of 91.8 °C in the gradient 90 °C – 92 °C. Using in another denaturation temperature gradient 91 °C – 93 °C, in temperature 92.8 °C, 12 unique electrophoretic patterns or clusters were obtained. Thus, both sets of experiments performed by us and those performed by

Żaczek et al. 2018 proved that *Bam*HI PCR MP allowed discrimination of *C. michiganensis* subsp. *sepedonicus* into clusters. We and Żaczek et al. 2018 used distinct oligonucleotides sets and temperature gradients yet we obtained similar results regarding the achieved discriminatory power of the *Bam*HI
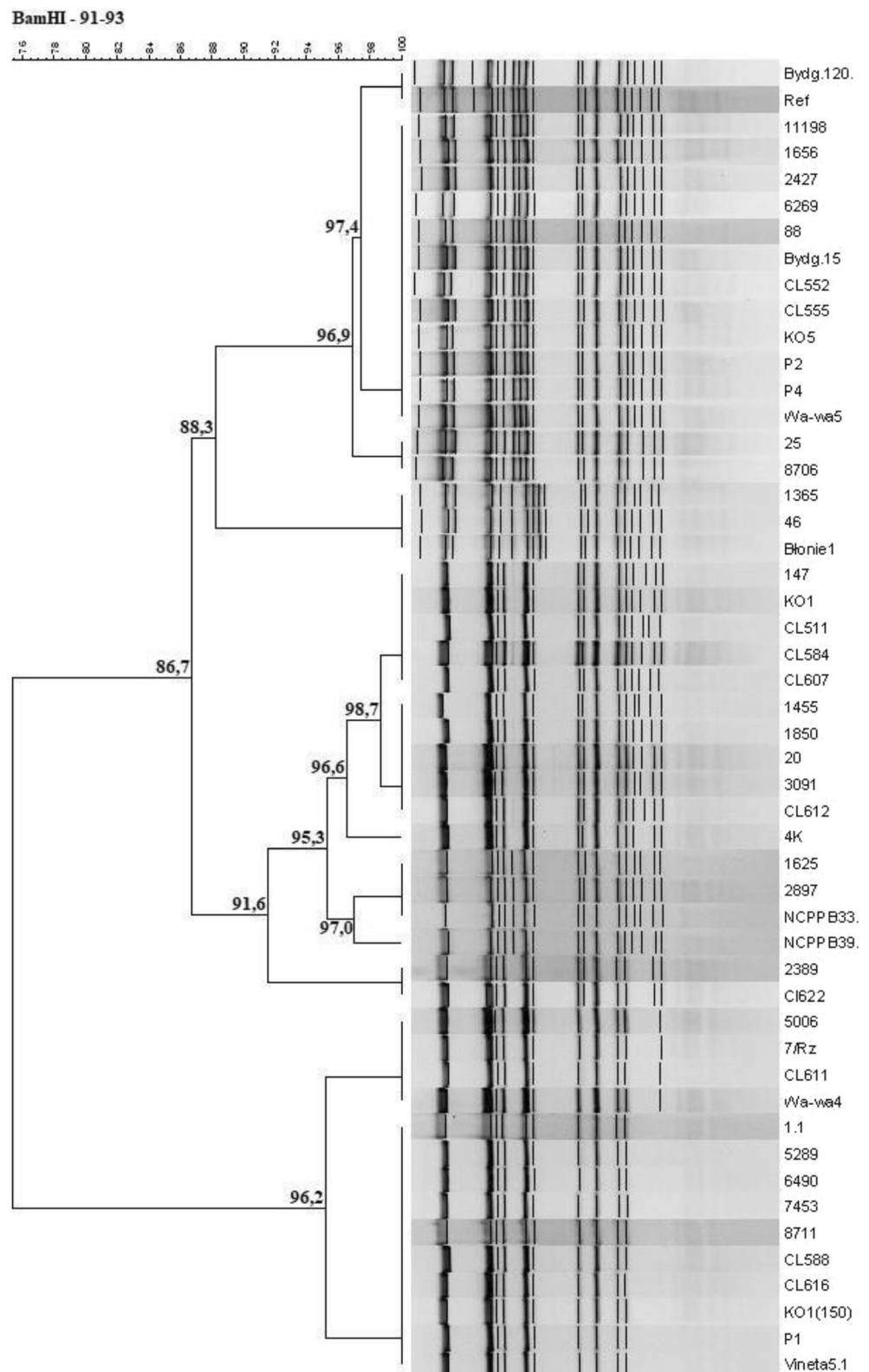
Fig. 4 Dendrogram showing the genetic similarity among 50 isolates of *Clavibacter michiganensis* subsp. *sepedonicus* generated on the basis of analysis of PCR MP genotyping results obtained for *BamH*I enzyme and denaturation temperature 91.8 °C (denaturation temperature range: 90- 92 °C)

PCR MP assay. Other enzymes used in the present study enabled obtaining electrophoretic patterns that differentiated the isolates and/or groups of isolates, as well. For *Bam*H1 PCR MP in denaturation temperature 92.8 °C 12 distinct electrophoretic patterns were obtained for 50 isolates analyzed and for the same group of isolates 26 distinct electrophoretic patterns were obtained in *Xma*I PCR MP which corresponds to approximately 2 fold higher discriminatory power of *Xma*I
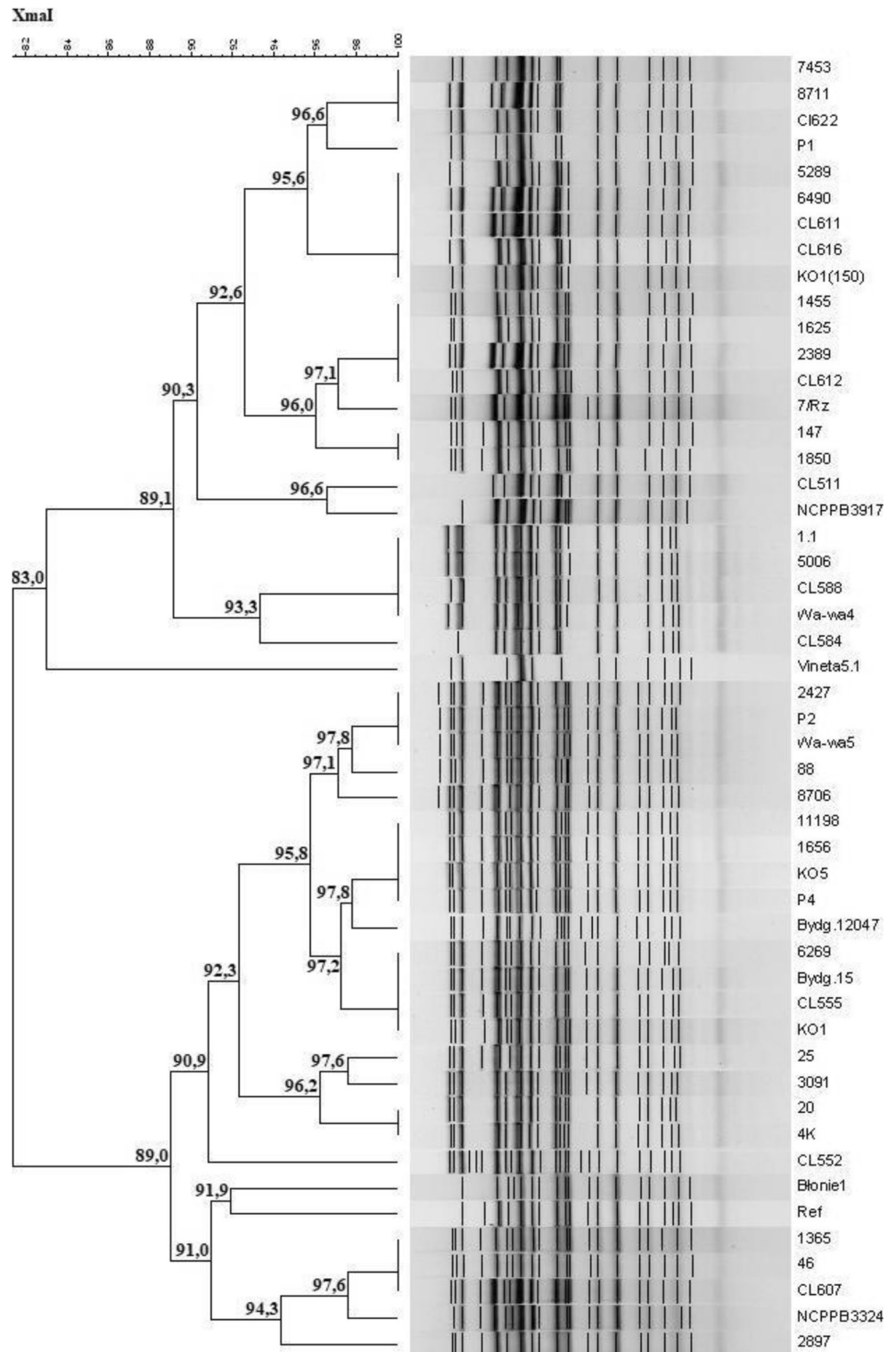
Fig. 5 Dendrogram showing the genetic similarity among 50 isolates of *Clavibacter michiganensis* subsp. *sepedonicus* generated on the basis of analysis of PCR MP genotyping results obtained for *BamH*I enzyme and denaturation temperature 92.8 °C (denaturation temperature range: 91- 93 °C)

PCR MP compared to *Bam*HI PCR MP. All electrophoretic patterns obtained in PCR MP performed for all five restriction enzymes applied were used to construct a dendrogram (Fig. 7). It should be noted that the dendrograms for the PCR MP

results served as a tool to visualize the degree of genetic differentiation of the isolates and as a tool for epidemiological studies, exclusively. Differences in the electrophoretic patterns obtained for all variants of PCR MP produced a unique

Fig. 6 Dendrogram showing the genetic similarity among 50 isolates of *Clavibacter michiganensis* subsp. *sepedonicus* generated on the basis of analysis of PCR MP genotyping results obtained for *Xma*I enzyme and denaturation temperature 94 °C (denaturation temperature range: 92.6- 94.1 °C)

combination of electrophoretic patterns for each of the 50 *Cms* isolates. This finding means that the ability to differentiate a single isolate within a group of isolates has been achieved which, to the best of our knowledge, has been reported for *C. michiganensis* subsp. *sepedonicus* isolates by the first time. It should also be noted that the degree of differentiation was

Fig. 7 Dendrogram showing the genetic similarity among isolates of *Clavibacter michiganensis* subsp. *sepedonicus* generated on the basis of a summary analysis of the average of PCR MP genotyping results obtained for five enzymes using the Dice similarity coefficient and the UPMGA clustering method
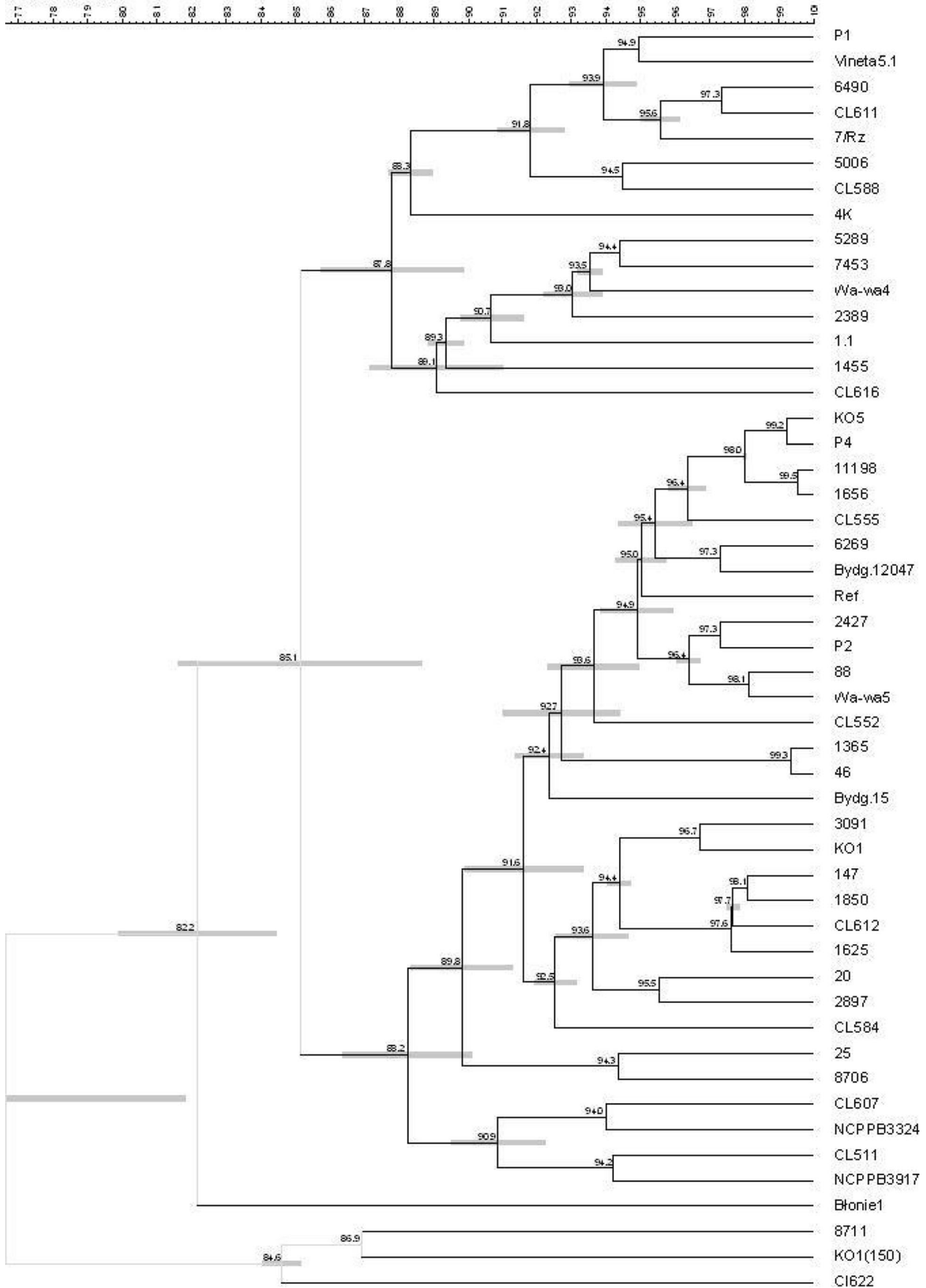
Table 3 Results of restriction analysis and PCR MP for *Clavibacter michiganensis* subsp. *sepedonicus*

Genomic DNA Restriction Enzyme		50 isolates of *Clavibacter michiganensis* subsp. *sepedonicus*	*Clavibacter michiganensis* subsp. *sepedonicus* NCBI Ref. Seq.:NC_010407.13,258,645 bp	*Clavibacter michiganensis* subsp. *sepedonicus* strain CFIA-Cs3N NCBI Ref. Seq.:NZ_MZMM01000001.13,205,544 bp
Digestion with *Apa*I	Predicted number of all restriction fragments	-	1577	1569
	Predicted number of restriction fragments from 1 bp to 4000 bp	-	1349	1346
	Number of amplicons obtained by PCR MP method	11–16	-	-
Digestion with *Hind*III	Predicted number of all restriction fragments	-	28	26
	Predicted number of restriction fragments from 1 bp to 4000 bp	-	5*	4*
	Number of amplicons obtained by PCR MP method	2–4	-	-
Digestion with *Pst*I	Predicted number of all restriction fragments	-	1541	1518
	Predicted number of restriction fragments from 1 bp to 4000 bp	-	1301	1282
	Number of amplicons obtained by PCR MP method	9–12	-	-
Digestion with *Xma*I	Predicted number of all restriction fragments	-	2279	2238
	Predicted number of restriction fragments from 1 bp to 4000 bp	-	2116	2077
	Number of amplicons obtained by PCR MP method	7–20	-	-
Digestion with *Bam*HI	Predicted number of all restriction fragments	-	6012	5912
	Predicted number of restriction fragments from 1 bp to 4000 bp	-	5994	5896
	Number of amplicons obtained by PCR MP method (90–92 °C)	3–12	-	-
	Number of amplicons obtained by PCR MP method (91–93 °C)	9–16	-	-

*two DNA fragments of the same size

low, and for most of the isolates, the degree of similarity was 95% or higher. However, groups (clusters) of *C. michiganensis* subsp. *sepedonicus* isolates could be distinguished with every restriction nuclease applied to PCR MP just the discriminatory power varied depending on the restriction endonuclease used.

Of course, the summary analysis was based on the combination of results of series individual electrophoreses, which increases the noise that can sometimes significantly affect the quality of the results. However, this problem applies to many bacterial genotyping techniques, such as the gold standard in typing many pathogens - PFGE, which was also used in the *C. michiganensis* subsp. *sepedonicus* genotyping apart from BOX-, ERIC- or Rep-PCR techniques.

We have determined that the oligonucleotides used for *Hind*III PCR MP of *Enetrobacteriaceae* (Masny and Płucienniczak 2003) allowed reproducible, theoretically predictable differentiation of *C. michiganensis* subsp. *sepedonicus* isolates (Fig. 2). Other authors described *Hind*III enzyme as unsuitable for PCR MP analysis of *Cms* strains (Żaczek et al. 2018). The PCR MP results obtained with *Hind*III enzyme were exceptional because in the size range between 100 bp and 4000 bp maximum of four PCR products were amplified and observed as discrete bands after electrophoresis (Fig. 3). According to our analysis, except for *Hind*III, all restriction enzymes used in PCR MP produced hundreds or thousands (from 1282 to 5994) of fragments in the size range between 6 and 4000 bp (Table 3) and *Hind*III produced just 5 DNA fragments in this size range including two same size fragments (Table 3). Therefore, in case of *Hind*III PCR MP reduction of denaturation temperature was not necessary to obtain limited number of PCR products analysable by gel electrophoresis. The results of the bioinformatic analysis, in-silico digestion of the genomes of the reference strains of *C. michiganensis* subsp. *sepedonicus*, matched the results we obtained; most strains we investigated produced electrophoretic patterns with four bands in *Hind*III PCR MP (Fig. 2) whose size matched the theoretically predicted restriction fragments obtained from the reference genome (NCBI Reference Sequence: NC_010407.1): 3428 bp (two fragments of the same size), 1728 bp, 945 bp and 608 bp. In-silico restriction digestion with *Hind*III revealed that only 28 fragments were created by the enzyme from entire NCBI Reference Sequence: NC_010407.1. Thus, the theoretical predictions matched our results. We analysed another full genome sequence of *C. michiganensis* subsp. *sepedonicus* strain CFIA-Cs3N (NZ_MZMM01000001.1) and in this genome 26 fragments in total were created and the following DNA fragments shorter than 4000 bp were obtained: 3428 (two fragments of the same size), 1728 bp and 608 bp. Thus, three PCR products would be visible on the electrophoretic pattern matching the sizes of PCR products obtained for isolates 25 and 8076 (Fig. 2). Concordant results of the in silico analyses

and the results of the experiments confirm that the differences in the electrophoretic patterns correspond to differences in the genomic sequences. We cloned and sequenced two (approximately 600 bp an approximately 1000 bp) out of four PCR products obtained by PCR MP after digestion of isolate CL 511 with *Hind*III and the obtained sequences were identical to the 608 bp sequence region 1,791,286–1,791,893 and 945 bp region 3,116,062–3,117,006 of the *Clavibacter michiganensis* subsp. *sepedonicus* complete genome (NCBI Reference Sequence: NC_010407.1). Thus both the sizes of PCR MP products and the sequences were in agreement with the predictions based on the available genomic data. The most unique electrophoretic patterns were obtained for isolates CL 622 and 4 K in *Hind*III PCR MP (Fig. 2). Thus, the genomes of those isolates differ from the reference genomes described above and could be interesting objects for further analyses. Despite the low number of electrophoretic bands produced *Hind*III PCR MP proved to be useful in detection of polymorphisms in the *Cms* reference genomes and the genomes of the investigated *Cms* isolates.

In the presented analysis, the lowest degree of similarity between the groups of isolates was 82.2%, which is still a high level of similarity. In our study, PCR MP allowed the differentiation of the isolates of *C. michiganensis* subsp. *sepedonicus* both from the same and distant geographical regions. Two of the least similar isolates, P1 and CL 622, were isolated in the same region of Poland (Wielkopolska) in 2006 and 2010, respectively. The ability to differentiate isolates from the same region or different regions has important practical consequences because it allows us to conclude that a developed set of PCR MP tests might be applied in epidemiological studies. Other authors also considered PCR MP a tool suitable for epidemiological analyses of *C. michiganensis* subsp. *sepedonicus* (Żaczek et al. 2018). Using the developed PCR MP tests with a set of five distinct restriction enzymes, we could determine whether any two isolates were genetically distinct or not in contrast to differentiation of *C. michiganensis* subsp. *sepedonicus* isolates by single restriction enzyme PCR MP analysis combined with VNTR analysis allowing discrimination of isolate clusters, only (Żaczek et al. 2018). The lack of differences does not determine the identity of the compared isolates, but the occurrence of the difference confirms the distinctness, which is important when a proof is necessary that the source of infection in a given region was not an isolate from another region. Reproducibility is always an important issue in bacterial isolate differentiation methods. Larger number of obtained bands in an electrophoretic profile increases the noise of the analysis and contributes to the possibility of ambiguous interpretation of the patterns of bands, which is known phenomenon common for the DNA fingerprinting methods (Koopman 2005). Reproducibility of PCR MP in *C. michiganensis* subsp. *sepedonicus* isolate differentiation was confirmed by other authors (Żaczek et al. 2018).

Based on the obtained results, we may claim that the designed PCR MP tests are currently the most efficient PCR based method for genetic differentiation isolates of *C. michiganensis* subsp. *sepedonicus*. In addition, despite the very low diversity among *Cms* individual isolates, these can be unambiguously identified within this subspecies. It is probable that PCR MP can allow the identification of genetic markers, enabling the differentiation of *Cms* isolates by cloning and sequencing of the isolate differentiating DNA bands. The differentiation of *Cms* isolates and the detection of unique DNA polymorphisms in their genome can be highly important in epidemiological studies, in which it would be possible to track the spread of a particular strain. Integrated diagnostics based on the combination of biochemical and genetic features would help in achieving better diagnosis of quarantine plant organisms.

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