The development of a specific Real-Time TaqMan for the detection of *Clavibacter michiganensis* subsp. *michiganensis*

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Introduction

Clavibacter michiganensis subsp. *michiganensis* (Cmm), a seed borne bacterial pathogen, is the causal agent of bacterial canker of tomato. The most commonly used methods for screening plant material and seeds include a plating step on semi selective medium as first screening (1, 2). The confirmation of Cmm is done by Immunofluorescence (3), PCR or a pathogenicity test on tomato plants.

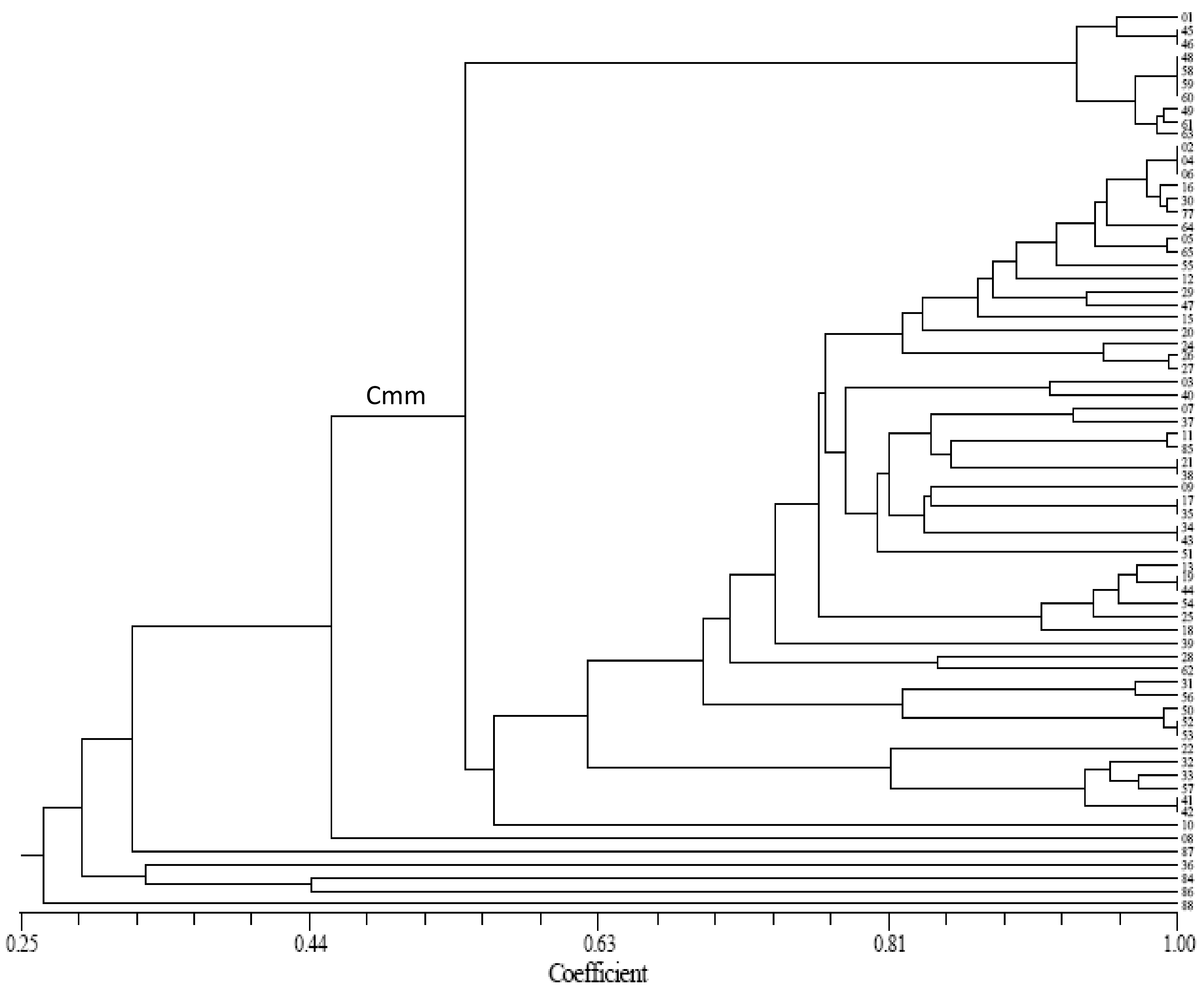
All confirmation methods currently used have drawbacks. Monoclonal antibodies (4) and known PCR's are not specific enough, and a pathogenicity test is time consuming. So the development of better confirmation methods is still needed. Within the framework of ISHI/ISF Cmm marker development using AFLP® technology was reported previously (5). Primers developed in this project were recently found to cross-react with Clavibacter-like bacteria recovered from tomato seed. Data from this AFLP® project were re-evaluated aiming for development of a new, more specific marker.

Methods and Results

63 Cmm strains of diverse geographic origin and 6 *Clavibacter michiganensis* strains representing other subspecies were selected from culture collections. Total DNA was isolated and sent to KeyGene to generate AFLP® patterns using six different primer combinations.

A dendrogram based on 307 markers, using the "Jaccard" similarity coefficient, was constructed (Fig. 1).

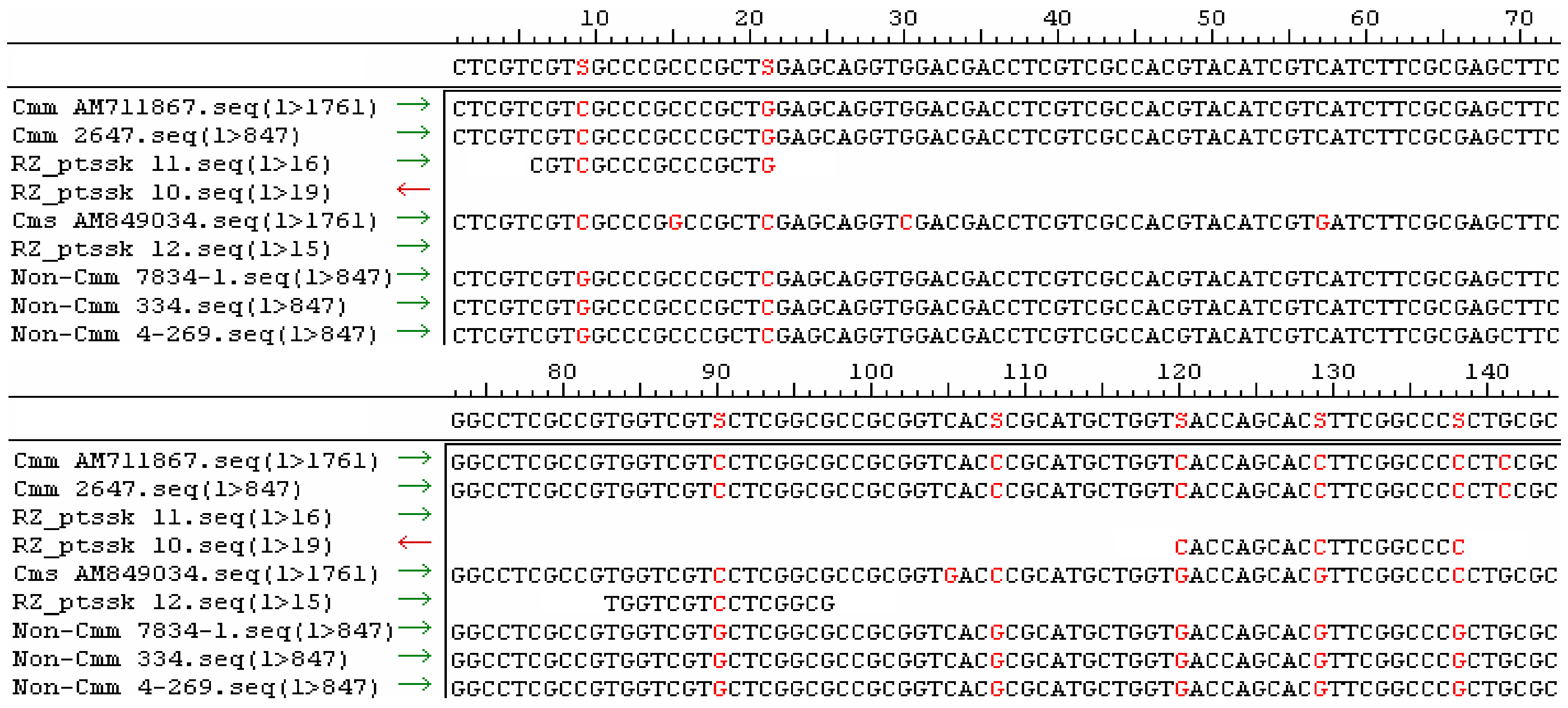
Fig 1. Dendrogram of Clavibacter sp. strains based on AFLP® patterns (5).



Only 1 AFLP® fragment, not previously investigated, was found to be present in all 63 Cmm strains and absent in the 6 non-Cmm strains. This fragment was sequenced and a BLAST search was performed. A 100% match was found with Cmm strain NCPPB 382 (AM711867) on position 2792386 – 2792516, partially coding for the gene producing a protein two-component system sensor kinase (ptssk).

Several primer combinations were designed, based on the 1761 bp ptssk gene of NCPPB 382. A PCR was performed on a collection of 24 strains, consisting of 7 Cmm and 21 non-Cmm. All fragments amplified by PCR were sequenced. The sequences were aligned and analyzed for Cmm specific SNP's. A combination of SNP's, specific for Cmm strains, was found and used for development of a MGB based TaqMan® (Fig. 2).

Fig 2. The alignment of a small selection of Cmm and non-Cmm strains and the position of created TaqMan®.



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                                    10        20        30        40        50        60        70
                            CTCGTCGTSGCCCGCCCGCTSGAGCAGGTGGACGACCTCGTCGCCACGTACATCGTCATCTTCGCGAGCTTC
Cmm AM711867.seq(1>1761)  → CTCGTCGTCGCCCGCCCGCTGGAGCAGGTGGACGACCTCGTCGCCACGTACATCGTCATCTTCGCGAGCTTC
Cmm 2647.seq(1>847)       → CTCGTCGTCGCCCGCCCGCTGGAGCAGGTGGACGACCTCGTCGCCACGTACATCGTCATCTTCGCGAGCTTC
RZ_ptssk 11.seq(1>16)     →      CGTCGCCCGCCCGCTG
RZ_ptssk 10.seq(1>19)     ←
Cms AM849034.seq(1>1761)  → CTCGTCGTCGCCCGGCCGCTCGAGCAGGTCGACGACCTCGTCGCCACGTACATCGTGATCTTCGCGAGCTTC
RZ_ptssk 12.seq(1>15)     →
Non-Cmm 7834-1.seq(1>847) → CTCGTCGTGGCCCGCCCGCTCGAGCAGGTGGACGACCTCGTCGCCACGTACATCGTCATCTTCGCGAGCTTC
Non-Cmm 334.seq(1>847)    → CTCGTCGTGGCCCGCCCGCTCGAGCAGGTGGACGACCTCGTCGCCACGTACATCGTCATCTTCGCGAGCTTC
Non-Cmm 4-269.seq(1>847)  → CTCGTCGTGGCCCGCCCGCTCGAGCAGGTGGACGACCTCGTCGCCACGTACATCGTCATCTTCGCGAGCTTC
                                  80        90       100       110       120       130       140
                            GGCCTCGCCGTGGTCGTSCTCGGCGCCGCGGTCACSCGCATGCTGGTSACCAGCACSTTCGGCCCSCTGCGC
Cmm AM711867.seq(1>1761)  → GGCCTCGCCGTGGTCGTCCTCGGCGCCGCGGTCACCCGCATGCTGGTCACCAGCACCTTCGGCCCCCTCCGC
Cmm 2647.seq(1>847)       → GGCCTCGCCGTGGTCGTCCTCGGCGCCGCGGTCACCCGCATGCTGGTCACCAGCACCTTCGGCCCCCTCCGC
RZ_ptssk 11.seq(1>16)     →
RZ_ptssk 10.seq(1>19)     ←                                                CACCAGCACCTTCGGCCCC
Cms AM849034.seq(1>1761)  → GGCCTCGCCGTGGTCGTCCTCGGCGCCGCGGTGACCCGCATGCTGGTGACCAGCACGTTCGGCCCCCTGCGC
RZ_ptssk 12.seq(1>15)     →           TGGTCGTCCTCGGCG
Non-Cmm 7834-1.seq(1>847) → GGCCTCGCCGTGGTCGTGCTCGGCGCCGCGGTCACGCGCATGCTGGTGACCAGCACGTTCGGCCCGCTGCGC
Non-Cmm 334.seq(1>847)    → GGCCTCGCCGTGGTCGTGCTCGGCGCCGCGGTCACGCGCATGCTGGTGACCAGCACGTTCGGCCCGCTGCGC
Non-Cmm 4-269.seq(1>847)  → GGCCTCGCCGTGGTCGTGCTCGGCGCCGCGGTCACGCGCATGCTGGTGACCAGCACGTTCGGCCCGCTGCGC
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Sequence of the TaqMan® design:

RZ_Ptssk 10:	5'-GGG GCC GAA GGT GCT GGT G-3'
RZ_Ptssk 11:	5'-CGT CGC CCG CCC GCT G-3'
RZ_Ptssk 12:	5'-Fam-TGG TCG TCC TCG GCG-NFQ-MGB-3'

The developed RZ_Ptssk TaqMan® was validated on a collection of 67 strains, 27 Cmm and 40 non-Cmm strains, of which several had been found to cross-react with Cmm primers published by Pastrik and Rainey (8) and/or Rijlaarsdam et al. (5). Additionally, 3 other Cmm-specific PCR's were tested on this collection. The validation results showed that the RZ_Ptssk TaqMan® was the only PCR method that correlated 100% with the pathogenicity test on tomato plants conducted by Naktuinbouw (Table 1).

The RZ_Ptssk primer and probe sequences were distributed in January 2010 within ISHI-Veg. SNES- GEVES has validated the RZ_Ptssk TaqMan® on an collection of 139 Cmm and 66 non-Cmm strains and found a 100% correlation.

	Cmm (27 strains)		Non Cmm (40 strains)	
	Pos.	False Neg.	False Pos.	Neg.
ZTO55/56 (5)	27	0	20	20
Bach (6)	27	0	27	13
Luo (7)	17	10	0	40
PSA-8/R*	27	0	1	39
RZ_Ptssk	27	0	0	40

Table 1. Comparison of several Real-Time PCR methods to detect Cmm using 67 strains.

* PSA-8 (5'-TTGGTCAATTCTGTCTCCCTTC-3') is a modification of PSA-4 from Pastrik and Rainey (8) (Woudt, B. unpublished).

Discussion and Conclusion

This study shows that false positive and false negative results (Table 1) can be obtained with several widely used PCR's. AFLP® provided a new single marker that was present in all selected Cmm strains. Partial sequencing of the ptssk gene showed that this gene is also present in related Clavibacter-like bacteria. However, we did find sequences within the gene that are specific for Cmm. It was possible to design a TaqMan® and we chose for a MGB. Validation of the RZ_Ptssk Real-Time PCR is the first, to our knowledge, that showed 100% correlation with the pathogenicity test on tomato plants.

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