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METHODS AND PROTOCOLS

Development of SCAR markers for rapid and specific detection of *Pseudomonas syringae* pv. *morsprunorum* races 1 and 2, using conventional and real-time PCR

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Abstract Specific primers were developed to detect the causal agent of stone fruit bacterial canker using conventional and real-time polymerase chain reaction (PCR) methods. PCR melting profile (PCR MP) used for analysis of diversity of *Pseudomonas syringae* strains, allowed to pinpoint the amplified fragments specific for *P. syringae* pv. *morsprunorum* race 1 (*Psm*1) and race 2 (*Psm*2), which were sequenced. Using obtained data, specific sequence characterised amplified region (SCAR) primers were designed. Conventional and real-time PCRs, using genomic DNA isolated from different bacterial strains belonging to the *Pseudomonas* genus, confirmed the specificity of selected primers. Additionally, the specificity of the selected DNA regions for *Psm*1 and *Psm*2 was confirmed by dot blot hybridisation. Conventional and real-time PCR assays enabled accurate detection of *Psm*1 and *Psm*2 in pure cultures and in plant material. For conventional PCR, the detection limits were the order of magnitude ~10^0 cfu/reaction for *Psm*1 and 10^1 cfu/reaction for *Psm*2 in pure cultures, while in plant material were 10^0–10^1 cfu/reaction using primers for *Psm*1 and 3×10^2 cfu/reaction using primers for *Psm*2. Real-time PCR assays with SYBR Green I showed a higher limit of detection (LOD) − 10^0 cfu/reaction in both pure culture and in plant material for each primer pairs designed, which corresponds to 30–100 and 10–50 fg of DNA of *Psm*1 and *Psm*2, respectively. To our knowledge, this is the first PCR-based method for detection of the causal agents of bacterial canker of stone fruit trees.

Keywords Dot blot hybridisation · Stone fruit tree pathogens · PCR MP · SCAR primers · Real-time PCR

Introduction

Bacterial canker of fruit trees occurs in stone fruit growing areas all over the world (Agrios 2005). In Poland, the disease incidence on stone fruit trees orchards is observed every year with different intensity and is becoming more economically significant. Moreover, in the last vegetative seasons, bacterial canker was dangerous not only to stone fruit trees, but also to apple and pear trees. The causal agents of the disease belong to the polyphagous *Pseudomonas syringae* species, able to infect more than 180 plant species, both annual and perennial, including fruit trees, ornamental plants and vegetables. *P. syringae* affects all organs of the aboveground parts of trees (i.e. the branches and main trunk as well as buds, blossoms, leaves and fruits), which causes reduction of yield and sometimes leads to death of the trees.

P. syringae is composed of plant pathogens divided into 60 pathovars (Young 2010) belonging to nine genomospecies, as determined by DNA:DNA hybridisation (Gardan et al. 1999). On King's B medium, the majority of these bacteria produce a fluorescent pigment visible under UV light (King et al. 1954). Bacteria that cause bacterial canker on stone fruit trees belong to three genomospecies (gs): gs 1—*P. syringae* pv. *syringae* (*Pss*); gs 2—*P. syringae* pv. *morsprunorum* race 1 (*Psm*1); and gs 3—*P. syringae* pv. *morsprunorum* race 2 (*Psm*2),

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P. syringae pv. *avii* (*Psa*) and *P. syringae* pv. *persicae* (*Psp*) (reviewed in Bultreys and Kałużna 2010). In Poland, three taxa were already described as present: *Pss*, *Psm*1 and *Psm*2. Recently, the new atypical taxon including bacteria that infect only cherries (mainly sour cherry) was also found (Kałużna data not published).

The diagnostics of bacterial canker are commonly based on isolation and phenotypic characterisation of the causal agent, including pathogenicity (Bultreys and Gheysen 1999; Vicente et al. 2004). The phenotypic tests LOPAT (Lelliott et al. 1966), GATTa and L-lactate utilisation (Lattore and Jones 1979) enable the determination of morphological, physiological and biochemical features of the bacteria. These features are used for identification of species and their discrimination into pathovars and races. However, this methodology requires the implementation of a high number of often laborious and time-consuming tests. Moreover, the obtained results can sometimes be ambiguous or difficult to interpret, and they are often not sufficient for proper strain classification (Vicente et al. 2004).

Concerning serological methods, the slide agglutination test, immunofluorescence and indirect-enzyme-linked immunosorbent assay (ELISA), with the antisera produced from live whole-cell antigens, were widely adopted for routine bacterial identification. However, nowadays these methods are less frequently used for the identification of bacteria that cause bacterial canker because of frequent cross-reactions with non-pathogenic bacteria. Furthermore, serological tests do not always provide a response in distinguishing isolates of *P. syringae* (Vicente et al. 2004).

Molecular methods are currently the most widely adapted and are considered very useful for the identification of bacterial canker causal agents and for studying their genetic diversity. For many years, the identification of the pathogen has been based on detection of genes encoding the toxins coronatine, syringomycin and the siderophore yersiniabactin (Bereswill et al. 1994; Sorensen et al. 1998; Bultreys and Gheysen 1999). However, it should be noted that the determination of presence of genes encoding for toxin production is not reliable for identification in itself and thus cannot be the only criterion for the classification of strains. In fact, strains of *Psm*1 and *Pss*, which do not have the ability to produce coronatine or syringomycin, respectively, are quite common (Ullrich et al. 1993; Renick et al. 2008; Kałużna et al. 2010a). On the other hand, although production of the siderophore yersiniabactin is now considered a stable feature of all *Psm*2 strains and could be a criterion for their identification, is should be mentioned that it is not an exclusive feature of strains of *Psm*2, since positive amplification with primers for the *irp1* gene (encoding this siderophore) was also confirmed in other pathovars of *P. syringae*, including the following: *antirrhini*, *apii*, *berberidis*, *delphinii*, *lachrymans*, *passiflorae*, *persicae*, *tomato*, *viburni*, *helianthi*, *tagetis* and *theae* (Bultreys et al. 2006).

In recent years, fingerprinting methods have been widely applied for the identification and genotyping of *P. syringae* through the analysis of repetitive regions (i.e. Enterobacterial Repetitive Intergenic Consensus (ERIC), BOX, Repetitive Extragenic Palindromic Elements (REP) and Insertion Sequence (IS50) sequences) (Ullrich et al. 1993; Weingart and Völksch 1997) and through PCR MP (Kałużna et al. 2010b). However, it should be taken into account that all fingerprinting methods require inclusion of the reference strains for comparison of obtained amplification patterns (Vicente and Roberts 2007; Gilbert et al. 2009), and, in the case of heterogeneous strains of *Pss* (Vicente et al. 2004; Renick et al. 2008; Kałużna et al. 2010a, b), it is difficult to determine affiliation of analysed strains to this taxon.

Despite the availability of different approaches for characterisation and genotyping of *P. syringae*, they require time-consuming and labour-intensive classical microbiological methods or complex analyses including comparison of amplification patterns and housekeeping gene sequencing. Therefore, there is still the need to develop a rapid and specific method of diagnosis that would allow the detection and identification of the causal agent of stone fruit bacterial canker (López et al. 2010). This specific, fast diagnostic system would be invaluable in the study on etiology of cankers on trunks and branches, which are similar to those caused by fungi of the genus *Leucostoma* (*Valsa*) and *Monilinia*, and also necrotic spots on leaves, which may be mistaken with those caused by *Prunus necrotic ring spot virus* or *Clasterosporium carpophilum*, especially late in the growing season. Moreover, the occurrence of gummosis on woody tissue often associated with bacterial infection may be related to the physiological response of the trees to damage caused by abiotic factors, such as frost, sunburn, periodic water flooding or mechanical damage, and is not due to biotic factors only (Saniewski et al. 2006).

Ideally, a novel diagnostic system would apply specific primers and the PCR technique, both conventional and real-time, making them more useful for a wide group of researchers according to available lab equipment, which allows for the detection and identification of the pathogen within a short amount of time. Additionally, such a system would undoubtedly be very useful in enforcing appropriate programmes to prevent and control disease occurrence in nurseries and orchards of stone fruit trees, especially sweet and sour cherry, where the damage is the most severe.

The aim of this study was to design and validate novel specific primers and to develop conventional and real-time PCR-based methodologies for rapid and specific detection of *Psm*1 and *Psm*2, with the aim of enhancing bacterial canker diagnostic procedures.

Materials and methods

Bacterial strains

Species and pathovar identification of previously uncharacterised *Pseudomonas* strains from our collection, obtained from stone fruit trees in Poland, was determined on the basis of phenotypic tests (i.e. Gram reaction with 3 % KOH (Suslow et al. 1982), LOPAT (Lelliott et al. 1966), GATTa and L-lactate utilisation (Lattore and Jones 1979). A total of 168 isolates were analysed. The reference strains *P. syringae* pv. *syringae*—LMG 1247, *P. syringae* pv. *morsprunorum* race 1—LMG 2222 and *P. syringae* pv. *morsprunorum* race 2—CFBP 3800 were included in all tests (Table 1). Additionally, type and not-type strains of other *P. syringae* pathovars (79) and related species (three) were included in the analysis (Table 2). The strains were kept at −75 °C in a mixture of glycerol (200 μl/ml) and phosphate-buffered saline (PBS) and streaked on King's B medium (3.8 % Pseudomonas Agar F Difco, 1 % glycerol) (King et al. 1954) for routine culturing.

DNA isolation

Bacterial DNA was isolated using the method described by Aljanabi and Martinez (1997), with slight modifications described by Kałużna et al. (2012). DNA was diluted to a final concentration of 10 ng/μl and kept at −20 °C for further analysis.

PCR melting profile

A slightly modified method of PCR MP described by Masny and Płucienniczak (2003) was used. An amount of 100 ng of DNA from 23 *Pseudomonas* strains (Figs. 1 and 2) was digested with *Pst*I endonuclease (10 U/μl; Promega Corporation, Madison, WI, USA) or *Taq*I (10 U/μl; Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's instructions. Digested DNA was ligated with two oligonucleotides forming an adaptor: DNA digested by *Pst*I endonuclease with a PstI adaptor—5′-TGTACGCAGTCTAC-3′/5′-CTCGTAGACTGCGTACATGCA-3′ (Waugh et al. 1997) and DNA digested by *Taq*I endonuclease with a TaqI adaptor—5′-GACGATGAGTCCTGAC-3′/5′-CGGTCAGGACTCAT-3′ (Ajmone-Marsan et al. 1997). PCR amplification was performed separately for *Pst*I- or *Taq*I-digested DNA in a 25-μl reaction mixture containing the following: 1 μl of ligation mixture; 0.4 U of GoTaq DNA polymerase (Promega, Madison, WI, USA) for *Pst*I and 0.4 U of Dream Taq Green DNA Polymerase (Thermo Scientific, Vilnius, Lithuania) for *Taq*I; and 1× of appropriate Taq polymerase buffer, 0.2 mM of dNTPs and 1 μM of each primer (PstI-0—5′-GACTGCGTACATGCAG-3′ for *Pst*I-digested DNA (Waugh et al. 1997) or TaqI-0—5′-GACGATGAGTCCTGACCGA-3′ for *Taq*I-digested DNA (Ajmone-Marsan et al. 1997)). The amplification reactions were conducted in a Biometra T3000 thermocycler (Biometra, Göttingen, Germany) with the following conditions: initial step of 72 °C for 5 min; 30 cycles at 86.5 °C for *Pst*I and 83 °C for *Taq*I for 40 s, 55 °C for 40 s and extension at 72 °C for 90 s; and final extension at 72 °C for 10 min. PCR products from each reaction and the O'GeneRuler 100-bp DNA Ladder Plus (Thermo Scientific, Vilnius, Lithuania) were separated on a 1.5 % agarose gel in 0.5× TBE buffer (0.045 M tris-boric acid, 0.001 M EDTA, pH 8.0) and electrophoresis was ran at 5–7 V/cm of gel. After staining with an ethidium bromide solution (0.5 μg/ml), the obtained amplification profiles were visualised under UV light. The same conditions were used in all subsequent electrophoresis.

Selection of specific fragments

Based on the results of genetic analyses using PCR MP, DNA fragments characteristic of *Psm*1 and *Psm*2 strains were selected. The fragments were excised from the gel, purified with the DNA AxyPrep Gel Extraction Kit (Axygen Scientific, Inc. Union City, CA, USA) and cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. The resulting ligation mixture was used to transform *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA). The cloned fragments were sequenced with universal primers M13Rev 5′-CAGGAAACAGCTATGAC-3′ and M13 (−40) 5′-GTTTTCCCAGTCACGAC-3′ at Genomed S.A. (Warsaw, Poland). The sequences obtained were assembled using the SeqMan software package LASERGENE (DNASTAR, Madison, USA).

Design of SCAR primers

The sequences of specific fragments for *Psm*1 and *Psm*2 were used to design the SCAR primers, for both conventional and real-time PCR, with the PrimerSelect programme of the LASERGENE package (DNASTAR). Different primer pairs were designed for conventional PCR (five for *Psm*1 and 7 for *Psm*2) and real time PCR (four for each taxa). All primer sequences and their potential amplification reaction products were checked for homology (June 2015) to other sequences deposited in the GenBank database using the 'blastn' algorithm (Altschul et al. 1997). Selected primers were synthesised at Genomed S.A.

Table 1 Strains of *P. syringae* used in this study

Lp.	Strain number	Place (voivodeship/country) and year of isolation		Host-plant	Taxon based on LOPAT, GATTa/L
1.	58	Łódzkie, PL	2007	Sour cherry	Atypical taxon
2.	59	Łódzkie, PL	2007	Sour cherry	Atypical taxon
3.	61	Łódzkie, PL	2007	Sour cherry	Atypical taxon
4.	64	Łódzkie, PL	2007	Sour cherry	Atypical taxon
5.	65	Łódzkie, PL	2007	Sour cherry	Atypical taxon
6.	66	Łódzkie, PL	2007	Sour cherry	Atypical taxon
7.	69	Łódzkie, PL	2007	Sour cherry	Atypical taxon
8.	71	Łódzkie, PL	2007	Sour cherry	Atypical taxon
9.	72	Łódzkie, PL	2007	Sour cherry	Atypical taxon
10.	73	Łódzkie, PL	2007	Sour cherry	Atypical taxon
11.	74	Łódzkie, PL	2007	Sour cherry	Atypical taxon
12.	75	Łódzkie, PL	2007	Sour cherry	Atypical taxon
13.	76	Łódzkie, PL	2007	Sour cherry	Atypical taxon
14.	78	Łódzkie, PL	2007	Sour cherry	Atypical taxon
15.	80	Łódzkie, PL	2007	Sour cherry	Atypical taxon
16.	81	Łódzkie, PL	2007	Sour cherry	Atypical taxon
17.	82	Łódzkie, PL	2007	Sour cherry	Atypical taxon
18.	83	Łódzkie, PL	2007	Sour cherry	Atypical taxon
19.	86	Łódzkie, PL	2007	Sour cherry	Atypical taxon
20.	87	Łódzkie, PL	2007	Sour cherry	Atypical taxon
21.	88	Łódzkie, PL	2007	Sour cherry	Atypical taxon
22.	89	Łódzkie, PL	2007	Sour cherry	Atypical taxon
23.	90	Łódzkie, PL	2007	Sour cherry	Atypical taxon
24.	91	Łódzkie, PL	2007	Sour cherry	Atypical taxon
25.	93	Łódzkie, PL	2007	Sour cherry	Atypical taxon
26.	94	Łódzkie, PL	2007	Sour cherry	Atypical taxon
27.	95	Łódzkie, PL	2007	Sour cherry	Atypical taxon
28.	96	Łódzkie, PL	2007	Sour cherry	Atypical taxon
29.	118	Mazowieckie, PL	2007	Sour cherry	Atypical taxon
30.	119	Mazowieckie, PL	2007	Sour cherry	Atypical taxon
31.	120	Łódzkie, PL	2007	Sour cherry	Atypical taxon
32.	122	Łódzkie, PL	2007	Sour cherry	Atypical taxon
33.	211	Łódzkie, PL	2007	Sour cherry	Atypical taxon
34.	271	Silesian, PL	2007	Sour cherry	Atypical taxon
35.	374	Łódzkie, PL	2008	Sour cherry	Atypical taxon
36.	439	Łódzkie, PL	2008	Sour cherry	Atypical taxon
37.	909	Łódzkie, PL	2009	Sour cherry	Atypical taxon
38.	910	Łódzkie, PL	2009	Sour cherry	Atypical taxon
39.	949	Łódzkie, PL	2009	Sour cherry	Atypical taxon
40.	963	Lubelskie, PL	2009	Sweet cherry	Atypical taxon
41.	966	Lubelskie, PL	2009	Sour cherry	Atypical taxon
42.	967	Lubelskie, PL	2009	Sour cherry	Atypical taxon
43.	968	Lubelskie, PL	2009	Sour cherry	Atypical taxon
44.	969a	Lubelskie, PL	2009	Sour cherry	Atypical taxon
45.	969b	Lubelskie, PL	2009	Sour cherry	Atypical taxon
46.	970a	Lubelskie, PL	2009	Sour cherry	Atypical taxon
47.	970b	Lubelskie, PL	2009	Sour cherry	Atypical taxon
48.	971a	Lubelskie, PL	2009	Sour cherry	Atypical taxon

Table 1 (continued)

Lp.	Strain number	Place (voivodeship/country) and year of isolation		Host-plant	Taxon based on LOPAT, GATTa/L
49.	971b	Lubelskie, PL	2009	Sour cherry	Atypical taxon
50.	972	Lubelskie, PL	2009	Sour cherry	Atypical taxon
51.	973	Lubelskie, PL	2009	Sour cherry	Atypical taxon
52.	981	Lubelskie, PL	2009	Sour cherry	Atypical taxon
53.	982	Lubelskie, PL	2009	Sour cherry	Atypical taxon
54.	1017	Łódzkie, PL	2009	Sour cherry	Atypical taxon
55.	1021	Łódzkie, PL	2009	Sour cherry	Atypical taxon
56.	791	No data	2001	Sour cherry	Atypical taxon
57.	441	Łódzkie, PL	2008	Plum	*Psm*1
58.	LMG 2222	No data, UK	1958	*Prunus avium*	*Psm*1
59.	25b	Łódzkie, PL	2007	Sweet cherry	*Psm*1
60.	28a	Łódzkie, PL	2007	Sweet cherry	*Psm*1
61.	29a	Łódzkie, PL	2007	Sweet cherry	*Psm*1
62.	38a	Łódzkie, PL	2007	Plum	*Psm*1
63.	98	Łódzkie, PL	2007	Sweet cherry	*Psm*1
64.	100	Łódzkie, PL	2007	Plum	*Psm*1
65.	107	Łódzkie, PL	2007	Plum	*Psm*1
66.	158	West Pomerania, PL	2007	Sweet cherry	*Psm*1
67.	174	West Pomerania, PL	2007	Sweet cherry	*Psm*1
68.	175	West Pomerania, PL	2007	Sweet cherry	*Psm*1
69.	177	West Pomerania, PL	2007	Peach	*Psm*1
70.	199	West Pomerania, PL	2007	Plum	*Psm*1
71.	201	West Pomerania, PL	2007	Plum	*Psm*1
72.	202	West Pomerania, PL	2007	Plum	*Psm*1
73.	203	West Pomerania, PL	2007	Plum	*Psm*1
74.	204	West Pomerania, PL	2007	Plum	*Psm*1
75.	205	West Pomerania, PL	2007	Plum	*Psm*1
76.	206	West Pomerania, PL	2007	plum	*Psm*1
77.	209	West Pomerania, PL	2007	Plum	*Psm*1
78.	213	Świętokrzyskie, PL	2007	Plum	*Psm*1
79.	214	Kuyavian-Pomeranian, PL	2007	Sweet cherry	*Psm*1
80.	215	Kuyavian-Pomeranian, PL	2007	Sweet cherry	*Psm*1
81.	216	Kuyavian-Pomeranian, PL	2007	Sweet cherry	*Psm*1
82.	217	Kuyavian-Pomeranian, PL	2007	Sweet cherry	*Psm*1
83.	218	Kuyavian-Pomeranian, PL	2007	Sweet cherry	*Psm*1
84.	219	Kuyavian-Pomeranian, PL	2007	Sweet cherry	*Psm*1
85.	220	Kuyavian-Pomeranian, PL	2007	Plum	*Psm*1
86.	221	Kuyavian-Pomeranian, PL	2007	Plum	*Psm*1
87.	250	Kuyavian-Pomeranian, PL	2007	Plum	*Psm*1
88.	274	Silesian, PL	2007	Plum	*Psm*1
89.	276	Silesian, PL	2007	Plum	*Psm*1
90.	280	Silesian, PL	2007	Plum	*Psm*1
91.	283	Silesian, PL	2007	Sweet cherry	*Psm*1
92.	291	Łódzkie, PL	2007	Sweet cherry	*Psm*1
93.	527	Mazowieckie, PL	2008	Sweet cherry	*Psm*1
94.	528	Mazowieckie, PL	2008	Sweet cherry	*Psm*1
95.	671	Lubelskie, PL	2008	Sweet cherry	*Psm*1
96.	1061	Łódzkie, PL	2009	Plum	*Psm*1

Table 1 (continued)

Lp.	Strain number	Place (voivodeship/country) and year of isolation		Host-plant	Taxon based on LOPAT, GATTa/L
97.	701A	No data, PL	2005	Sweet cherry	*Psm*1
98.	702	No data, PL	1994	Plum	*Psm*1
99.	704	No data, PL	1994	Sweet cherry	*Psm*1
100.	710	Lower Silesian, PL	1996	Sweet cherry	*Psm*1
101.	755	No data, PL	1999	Plum	*Psm*1
102.	771	Łódzkie, PL	1999	Plum	*Psm*1
103.	782	No data, PL	2001	Sweet cherry	*Psm*1
104.	787	Mazowieckie, PL	2001	Plum	*Psm*1
105.	788	Łódzkie, PL	2001	Plum	*Psm*1
106.	793	Łódzkie, PL	2001	Plum	*Psm*1
107.	CFBP 3800	No data, UK	ND	*Prunus cerasus*	*Psm*2
108.	77	Łódzkie, PL	2007	Sour cherry	*Psm*2
109.	117	Mazowieckie, PL	2007	Sour cherry	*Psm*2
110.	266	Silesian, PL	2007	Sour cherry	*Psm*2
111.	417	Mazowieckie, PL	2008	Sour cherry	*Psm*2
112.	701	No data, PL	1994	Sour cherry	*Psm*2
113.	719	Łódzkie, PL	1997	Sour cherry	*Psm*2
114.	732	Łódzkie, PL	1997	Sour cherry	*Psm*2
115.	733	Łódzkie, PL	1997	Sour cherry	*Psm*2
116.	745	Łódzkie, PL	1999	Sour cherry	*Psm*2
117.	764	Mazowieckie, PL	1999	Sour cherry	*Psm*2
118.	LMG 1247	No data, UK	ND	*Syringa vulgaris*	*Pss*
119.	2905	No data/PL	1978	Sour cherry	*Pss*
120.	68	Łódzkie, PL	2007	Sour cherry	*Pss*
121.	103	Łódzkie, PL	2007	Sour cherry	*Pss*
122.	106	Łódzkie, PL	2007	Plum	*Pss*
123.	109	Łódzkie, PL	2007	Plum	*Pss*
124.	110	Łódzkie, PL	2007	Plum	*Pss*
125.	112	Łódzkie, PL	2007	Plum	*Pss*
126.	115	Łódzkie, PL	2007	Plum	*Pss*
127.	141	West Pomerania, PL	2007	Peach	*Pss*
128.	147	West Pomerania, PL	2007	Peach	*Pss*
129.	165	West Pomerania, PL	2007	Sweet cherry	*Pss*
130.	184	West Pomerania, PL	2007	Peach	*Pss*
131.	192	West Pomerania, PL	2007	Plum	*Pss*
132.	210	Łódzkie, PL	2007	Sour cherry	*Pss*
133.	222	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
134.	226	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
135.	227	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
136.	229	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
137.	233	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
138.	234	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
139.	235	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
140.	236	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
141.	237	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
142.	239	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
143.	240	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
144.	242	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*

Table 1 (continued)

Lp.	Strain number	Place (voivodeship/country) and year of isolation		Host-plant	Taxon based on LOPAT, GATTa/L
145.	244	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
146.	245	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
147.	247	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
148.	248	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
149.	256	Kuyavian-Pomeranian, PL	2007	Plum	*Pss*
150.	257	Kuyavian-Pomeranian, PL	2007	Sour cherry	*Pss*
151.	258	Kuyavian-Pomeranian, PL	2007	Sour cherry	*Pss*
152.	259	Łódzkie, PL	2007	Sweet cherry	*Pss*
153.	264	Łódzkie, PL	2007	Peach	*Pss*
154.	286	Silesian, PL	2007	Sweet cherry	*Pss*
155.	373	Łódzkie, PL	2008	Sour cherry	*Pss*
156.	376	Łódzkie, PL	2008	Sour cherry	*Pss*
157.	415	Świętokrzyskie, PL	2008	Plum	*Pss*
158.	420a	Mazowieckie, PL	2008	Sour cherry	*Pss*
159.	435	Mazowieckie, PL	2008	Sour cherry	*Pss*
160.	437	Łódzkie, PL	2008	Sour cherry	*Pss*
161.	442	Łódzkie, PL	2008	Plum	*Pss*
162.	460	Podkarpackie, PL	2008	Sour cherry	*Pss*
163.	663	Lubelskie, PL	2008	Sour cherry	*Pss*
164.	914	Kuyavian-Pomeranian, PL	2009	Sour cherry	*Pss*
165.	959	Lubelskie, PL	2009	Sour cherry	*Pss*
166.	702A	Łódzkie, PL	2005	Plum	*Pss*
167.	753	Łódzkie, PL	1999	Apricot	*Pss*
168.	757	Mazowieckie, PL	1999	Plum	*Pss*
169.	760	Mazowieckie, PL	1999	Sour cherry	*Pss*
170.	762	No data, PL	1999	Apricot	*Pss*
171.	763	No data, PL	1999	Sour cherry	*Pss*

LOPAT—levan production from sucrose (*L*), presence of oxidase (*O*), ability to cause rot on potato tubers (*P*, pectolytic activity), presence of arginine dihydrolase (*A*), hypersensitive reaction (HR) on tobacco plants; *GATTA*—gelatine hydrolysis (*G*), aesculin hydrolysis (*A*, activity of the β-glucosidase), tyrosinase activity (*T*), utilisation of tartrate (*Ta*); test of L-lactate utilisation (*L*); *PL* Poland, *UK* United Kingdom

Dot blot hybridisation

High-throughput specificity assays were carried out using a dot blot platform, essentially as previously described (Albuquerque et al. 2011). PCR amplicons obtained using primers Psm1-6F/6R, with template DNA from strain *Psm* 28a (race 1), and primers Psm2-8F/8R, with *Psm* 77 (race 2), were purified using the GFX PCR and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and labelled with digoxigenin, using the DIG-High Prime DNA labelling kit (Roche, Basel, Switzerland) in order to obtain the two tested hybridisation probes Psm1 and Psm2, respectively.

Amounts of 100 ng of heat-denatured DNA from each bacterial strain were transferred to a nylon membrane using a Bio-Dot apparatus (Bio-Rad, Hercules, USA). Hybridisation was carried out overnight at 68 °C with a final probe concentration of 100 ng/mL, and the washing and detection steps were carried out according to the DIG application manual (Roche). The chemiluminescent signal indicative of probe–target hybrids was detected using a Molecular Imager ChemiDoc XRS+ System (Bio-Rad), with all pixels below saturation point.

Conventional and real-time PCR amplifications

Amplification reactions with the two selected primer pairs, one specific for the strains of *Psm*1 and the second specific for *Psm*2, were performed in a Biometra T3000 thermocycler (Biometra, Göttingen, Germany). The reaction mixture in 15 μl of total reaction volume contained 10 ng of DNA, 0.4 U of Dream DNA Polymerase (Promega, Madison, WI, USA), 1× reaction Dream Taq Green buffer (Thermo Scientific, Vilnius, Lithuania), 0.15 mM dNTPs and 0.7 mM of each primer. The following

Table 2 Results of specificity of designed primers in reactions with DNA of different pathovars of *Pseudomonas syringe* and other *Pseudomonas* species tested

Pathovar of *P. syringae*	Strain number*	Host	Origin-place/year of isolation	PCR result with primers Psm1-6F/6R and Psm1-1F/R-RT and Psm2-8F/8R and and Psm2-1F/1R-RT	Reference/ source
aceris	CFBP 2339^{PT}	*Acer* sp.	1961	–**	CFBP***
actinidiae	CFBP 4909^{PT}	*Actinidia deliciosa*	Japan/1984	–	CFBP
	MAFF 302135	*Actinidia argute*	Japan/1987	–	MAFF
	MAFF 302145	*Actinidia deliciosa*	Japan/1988	–	MAFF
	MAFF 613005	*Actinidia deliciosa*	Japan/1986	–	MAFF
aesculi	CFBP 2894^{PT}	*Aesculus indica*	India/1980	–	CFBP
	6617	*Aesculus hippocastanum*	UK/2006	–	R. W. Jackson (UK)
	2250	*Aesculus hippocastanum*	UK/2008	–	
	H3	*Aesculus hippocastanum*	Germany/2007	–	Schmidt et al., 2008
	H4	*Aesculus hippocastanum*	Germany/2007	–	
	2190	*Aesculus hippocastanum*	UK	–	
antirrhini	CFBP 1620^{PT}	*Antirrhinum majus*	UK/1956	–	CFBP
apii	CFBP 2103^{PT}	*Apium graveolens*	USA/1942	–	CFBP
	BS 426	*Petroselinum crispum*	USA/2003	–	Bull et al. 2011
	BS 463	*Flat-leaf parsley*	USA/2002	–	
aptata	CFBP 1617^{PT}	*Beta vulgaris*	USA/1959	–	CFBP
atrofaciens	CFBP 2213^{PT}	*Triticum aestivum*	New Zealand/1968	–	CFBP
atropurpurea	CFBP 2340^{PT}	*Lolium multiflorum*	ND/1967	–	CFBP
	1304			–	K.Geider (Germany)
avii	CFBP 3846^{PT}	*Prunus avium*	France/1991	–	CFBP
berberidis	CFBP 1727^{PT}	*Berberis* sp.	New Zealand/1972	–	CFBP
broussonetiae	CFBP 5140^{PT}	*Broussonetia kazinoki Sieb.X Broussonetia papyrifa Vent.*	Japan/1980	–	CFBP
	MAFF 810038	*Broussonetia kazinoki Sieb.*	Japan/ 1996	–	MAFF
	MAFF 810044	*Broussonetia kazinoki Sieb.*	Japan/ 1996	–	MAFF
castaneae	CFBP 4217^{PT}	*Castanea crenata*	Japan/1977	–	CFBP
cerasicola	CFBP 6109^{PT}	*Prunus X yedoensis*	Japan/1995	–	CFBP
ciccaronei	CFBP 2342^{PT}	*Ceratonia siligua*	Italy/1942	–	CFBP
coriandricola	CFBP 5010^{PT}	*Coriandrum sativum*	Germany/1990	–	CFBP
	BS 456	*Curled-leaf parsley*	USA/2003	–	Carolee T. Bull (USA)
	BS 462	*Flat-leaf parsley*	USA/2002	–	

Table 2 (continued)

coronafaciens	CFBP 2216^{PT}	*Avena sativa*	UK/1958	–	CFBP
cunninghamiae	CFBP 4218^{PT}	*Cunninghamia lanceolata*	China/1995	–	CFBP
daphniphylli	CFBP 4219^{PT}	*Daphniphyllum teigsmanni*	Japan/1981	–	CFBP
delphinii	CFBP 2215^{PT}	*Delphinium sp.*	New Zealand/1957	–	CFBP
dendropanacis	CFBP 3226^{PT}	*Dendropanax trifidus*	Japan/1979	–	CFBP
dysoxyli	CFBP 2356^{PT}	*Dysoxylum spectabile*	New Zealand/1949	–	CFBP
eriobotryae	CFBP 2343^{PT}	*Eriobotrya japonica*	USA/1970	–	CFBP
garcae	CFBP 1634^{PT}	*Coffea arabica*	Brasil/1958	–	CFBP
helianthi	CFBP 2067^{PT}	*Helianthus annuus*	Mexico/ND	–	CFBP
hibisci	CFBP 2895^{PT}	*Hibiscus rosa-sinensis*	USA/1984	–	CFBP
japonica	MAFF 301159	*Triticum aestivum (L.) Thell.*	Japan/ND	–	MAFF
	MAFF 301166	*Hordeum vulgare L.*	Japan/ND	–	MAFF
lachrymans	CFBP 6463^{PT}	*Cucumis sativus*	Hungary/1958	–	CFBP
	B 01557	*Cucumis* L.	ND	–	M. Hevesi (Hungary)
lapsa	CFBP 1731^{PT}	*Zea* sp.	ND/1968	–	CFBP
maculicola	LMG 5071^{PT}	*Brassica oleracea*	New Zealand/1965	–	LMG
	LMG 2208	*Brassica oleracea*	UK/1965	–	LMG
mellea	CFBP 2344^{PT}	*Nicotiana tabacum*	Japan/1968	–	CFBP
mori	CFBP 1642^{PT}	*Morus alba*	Hungary/1958	–	CFBP
	MAFF 302756	*Morus bombycis Koidz.*	Japan/ND	–	MAFF
	MAFF 810010			–	MAFF
morsprunorum	CFBP 2351^{PT}	*Prunus domestica*	USA/1931	– (Psm1) + (Psm2)	CFBP M. Hevesi
	B 01835	*Prunus* sp.	Hungary/1995	+ (Psm1) – (Psm2)	(Hungary)
	PD5329	*ND*	ND	+ (Psm1) – (Psm2)	J.D.Janse (Netherlan
	LMG 2222	*Prunus avium*	ND/1958	+ (Psm1) – (Psm2)	d) LMG
	CFBP 3800	*Prunus cerasus*	UK/ND	– (Psm1) + (Psm2)	CFBP
myricae	CFBP 2897^{PT}	*Myrica rubra*	Japan/1978	–	CFBP
	MAFF 302457	*Myrica rubra Sieb. et Zucc.*	Japan/ND	–	MAFF MAFF
	MAFF 302944	*Myrica rubra Sieb. et Zucc.*	Japan/ND	–	
oryzae	CFBP 3228^{PT}	*Oryza sativa*	Japan/1983	–	CFBP
papulans	CFBP 1754^{PT}	*Malus sylvestris*	Canada/1973	–	CFBP
passiflorae	CFBP 2346^{PT}	*Passiflora edulis*	New Zealand/1962	–	CFBP
persicae	LMG 5184^{PT}	*Prunus persica*	France/1974	–	LMG
philadelphi	CFBP 2898^{PT}	*Philadelphus coronarius*	UK/1985	–	CFBP
photiniae	CFBP 2899^{PT}	*Photinia glabra*	Japan/1976	–	CFBP
pisi	CFBP 2105^{PT}	*Pisum sativum*	New Zealand/1969	–	CFBP M. Hevesi

Table 2 (continued)

	B 01685	*Pisum* L.	USA/1957	–	(Hungary)
porri	CFBP 1908^{PT}	*Allium porrum*	France/1978	–	CFBP
primulae	CFBP 1660^{PT}	*Primula sp.*	USA/ND	–	CFBP
rhaphiolepidis	CFBP 4220^{PT}	*Rhaphiolepis umbellata*	Japan/1980	–	CFBP
ribicola	CFBP 2348^{PT}	*Ribes aureum*	ND/1946	–	CFBP
sesami	CFBP 1671^{PT}	*ND*	Yugoslavia/1961	–	CFBP
spinaceae	CFBP 5524^{PT}	*ND*	Japan/ND	–	CFBP
striafaciens	CFBP 1674^{PT}	*Avena sativa*	ND	–	CFBP
syringae	LMG 1247^{PT}	*Syringa vulgaris*	UK/1950	–	LMG
	B 01461	*ND*	ND	–	M. Hevesi (Hungary)
	B 01558	*Prunus* sp.	Hungary/1978	–	
	B 1893	*Prunus armeniaca L.*	Hungary/1999	–	
tabaci	CFBP 2106^{PT}	*Nicotiana tabacum*	Hungary/1959	–	CFBP
	B 01606	*Nicotiana tabacum*	Hungary/1995	–	M.Hevesi (Hungary)
tagetis	CFBP 1694^{PT}	*Tagetes erecta*	Zimbabwe/1972	–	CFBP
theae	CFBP 2353^{PT}	*Thea sinensis*	Japan/1970	–	CFBP
	MAFF 302853	*ND*	Japan/1975	–	MAFF
tomato	CFBP 2212^{PT}	*Lycopersicon esculentum*	UK/1960	–	CFBP
	KFB 145	*ND*	ND	–	A. Prokić, A Obradovic (Serbia)
ulmi	CFBP 1407^{PT}	*Ulmus* sp.	Yugoslavia/1958	–	CFBP
viburni	CFBP 1702^{PT}	*Viburnum* sp.	USA/ND	–	CFBP
zizaniae	CFBP 4117^{PT}	*Zizania aquatica*	USA/1983	–	CFBP
Pseudomonas savastanoi pv. *savastanoi*.	CFBP 1670 ^{T}	*Olea europaea*	Yugoslavia/ND	–	CFBP
Pseudomonas cannabina pv. *alisalensis*	CFBP 6866 ^{T}	*Brassica rapa subsp. Rapa*	USA/1995	–	CFBP
	CFBP 6869	*Eruca vesicaria subsp. sativa*	USA/1995	–	CFBP Bull et al. 2010
Pseudomonas corrugata	B 01638	*Lycopersicon esculentum*	Hungary/1996	–	M. Hevesi (Hungary)

**ND* no data, *PT* pathotype strain, *T* type strain; ** − negative result of amplification with tested primer pairs; + positive result of amplification with tested primer pairs; ***CFBP* Collection Francaise des Bacteries Phytopathogenes, Institut National de la Recherche Agronomique, Beaucouzé Cedex, France; *MAFF* Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan, *LMG* Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium

experimentally determined amplification conditions were used: initial denaturation at 94 °C for 4 min; 30 cycles at 94 °C for 45 s, 55–62 °C for 45 s for primers Psm1-6F and Psm1-6R (for detection of *Psm*1 strains) and 50–58 °C for 45 s for primers Psm2-8F and Psm2-8R (for detection of *Psm*2 strains) and 72 °C for 1 min; and final extension at 72 °C for 10 min. The resulting PCR products were separated by electrophoresis on 1.5 % agarose gels as described above.

Real-time PCR with SYBR Green I was conducted in the Bio-Rad CFX96 with SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, USA). The reaction mixture in 20 μl of total volume contained 1× reaction SYBR

Green Supermix and 0.5 mM of each of the following primers: Psm1-1F-RT/Psm1-1R-RT for *Psm*1 and Psm2-1F-RT/Psm2-1R-RT for *Psm*2. Bacterial DNA was used as a template (10 ng per PCR reaction). No-template reactions were used as negative controls. The PCR programme was started from one cycle of denaturation at 98 °C for 130 s, followed by 35 cycles at 95 °C for 10 s and then 60 °C for 15 s, finished by a melting curve analysis for verification of the specificity of amplification in real-time PCR products. Progressive denaturation of products was carried out at a rising temperature, starting from 65 °C and continuing to 95 °C, with 0.5 °C of increment for 5 s each.

Specificity of designed primers and their usefulness in detection in plant material

In the first stage of this part of the study, the specificity of the two designed primer pairs was determined with PCR using DNA from all strains of *Psm*1, *Psm*2 and *Pss* as well as strains of atypical taxa (Table 1). In the second stage, the primers were tested with DNA from other *P. syringae* pathovars and related species (Table 2).

In order to assess the suitability of the designed primers for the detection of *Psm*1 and *Psm*2 strains in plant material, several leaves, shoots and fruits of sweet cherry, sour cherry and plum were collected. Amounts of 100 mg of crushed/cut plant tissue of each organ were placed in 1.9 ml of PBS buffer. For each type of tissue (organ) and host plant, two tubes were prepared (18 tubes in total). One hundred microlitres of bacterial suspension (10^5 cfu/ml) of the *Psm*1 reference strain (LMG 2222) or the *Psm*2 reference strain (CFBP 3800) were added to nine of the samples (one of each organ and of each plant). One hundred microlitres of sterile water were added to the remaining nine samples, which were tested to verify the purity of the plant material. After 1 h of shaking incubation at 26 °C, 1 ml of washing liquid separate from each of all 18 samples was centrifuged; the resulting pellet was suspended in 100 μl of TE buffer, and the DNA was isolated using a Genomic Mini DNA Extraction Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions.

PCR limit of detection

The limit of detection of PCR using the SCAR primers was evaluated using DNA extracted from pure bacterial cultures, DNA extracted from plant material that was mixed with suspensions of bacteria and bacterial genomic DNA (gDNA). A PCR assay was carried out with decimal dilutions of bacterial suspensions of strain LMG 2222 or CFBP 3800 (from ~10^8 to 10^0 cfu/ml). DNA was isolated from 1 mL of each dilution using a Genomic Mini DNA Extraction Kit (A&A Biotechnology) according to the protocol supplied by the manufacturer. To determine the limit of detection of bacteria in the plant material, 100-mg portions of stems and leaves of sweet cherry (for *Psm*1 primers) or sour cherry (for *Psm*2 primers) and 100 μl of the previously prepared 10-fold serial dilutions of bacterial suspensions (from ~10^8 to 10^0 cfu/ml) or 100 μl of sterile water, used as a control of material purity, were added to 1.9 mL of PBS buffer and shaken for 30 min at 26 °C. After incubation, the washings were centrifuged (8,000 rpm, 5 min); the resulting pellet was suspended in 100 μl of TE buffer, and DNA was isolated using the Genomic Mini DNA Extraction Kit (A&A Biotechnology) according to the manufacturer's instructions. The sensitivity of gDNA detection was checked using 2-fold serial dilutions of gDNA isolated (11 ng to ~11 fg per PCR reaction for *Psm*1 and 14 ng to ~14 fg per PCR reaction for *Psm*2) using the method described by Aljanabi and Martinez (1997), with slight modifications described by Kałużna et al. (2012). The PCR efficiency was calculated from the slope of the standard curve generated for each run in the following equation $E = 10^{(-1/\text{slope})}$ where $E = 2$ and corresponds to 100 % efficiency (Ramakers et al. 2003).

Results

Phenotypic characterisation

All 168 isolates have been classified into species *P. syringae* LOPAT group Ia. GATTa and L-lactate utilisation tests allowed further discrimination of pathovars and races: 49 isolates were identified as *P. syringae* pv. *morsprunorum* race 1 (*Psm*1), 10 as race 2 of this pathovar, 53 as pathovar *syringae* (*Pss*) and 56 as belonging to atypical taxa, having most of the features of *Pss* without, however, the ability of esculine hydrolysis (lack of β-glucosidase activity) (Table 1).

PCR MP

To select specific fragments of the taxon, the PCR MP method was applied using DNA from different strains of *P. syringae* (Figs. 1 and 2; Table 1). The obtained PCR MP patterns corresponded to phenotypically determined pathovars and races. Similar electrophoretic patterns were obtained for races within pathovar *morsprunorum*, confirming their homogeneity; however, different patterns were observed for strains belonging to pathovar *syringae*. For *Psm*1 and *Psm*2, the products that were specific and were shared between all strains of each taxa were selected, cloned and sequenced. Two products specific for *Psm*1 (after digestion by *Pst*I) had sizes of 1,208 and 1,128 bp, while the unique amplification product (after digestion by *Taq*I) for strains of *Psm*2 was 781 bp long. No specific and unique band was found for strains of *Pss*.

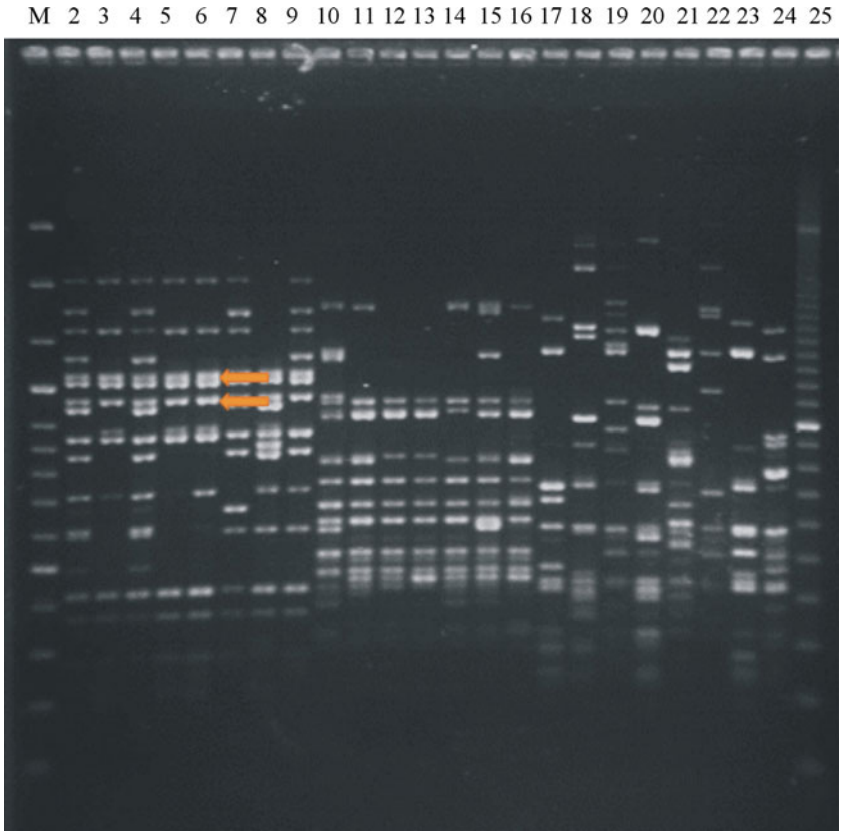

Fig. 1 Electrophoretic patterns obtained after polymerase chain reaction melting profile (PCR MP) of fluorescent Pseudomonads with primer Pst1: Lane 1—*M*—marker 100-bp ladder (Genoplast, Rokocin, Poland); pathovar *morsprunorum* race 1 isolates: *2*—LMG 2222, *3*—702, *4*—710, *5*—755, *6*—787, *7*—782, *8*—793, *9*—701A; pv. *morsprunorum* race 2 isolates: *10*—CFBP 3800, *11*—719, *12*—733, *13*—732, *14*—745, *15*—764, *16*—701; pv. *syringae* isolates: *17*—LMG 1247, *18*—2905, *19*—760; *20*—762, *21*—702A, *22*—757, *23*—753, *24*—763, *25*—M—marker 100-bp PCR Molecular Ruler (Bio-Rad, Hercules, USA)

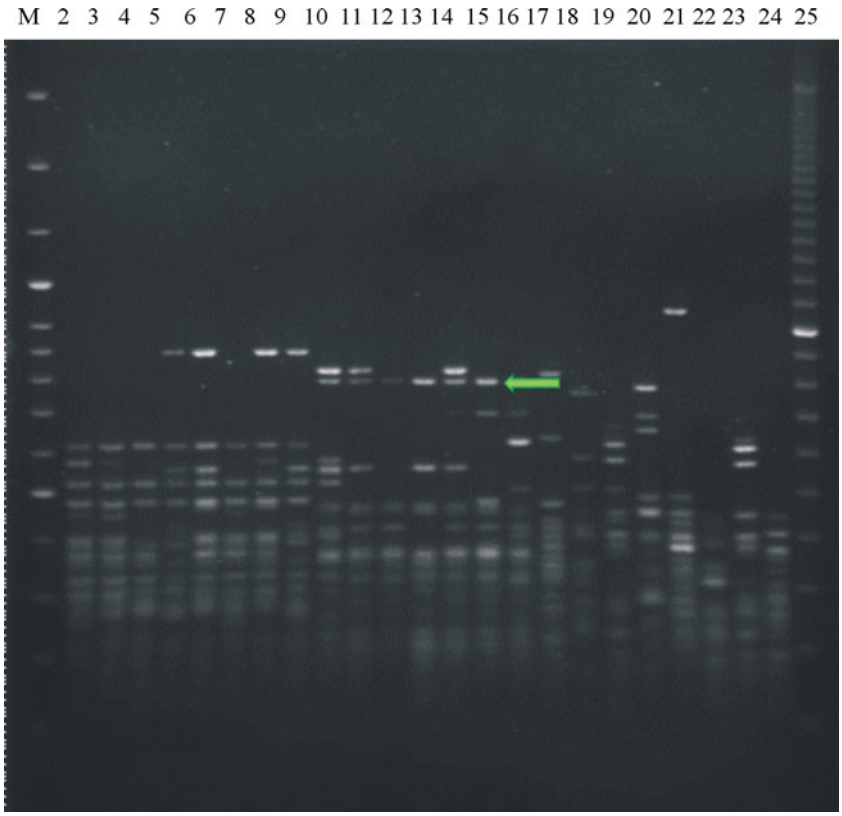

Fig. 2 Electrophoretic patterns obtained after polymerase chain reaction melting profile (PCR MP) of fluorescent Pseudomonads with primer Taq1: Lane 1—*M*—marker 100-bp ladder (Genoplast, Rokocin, Poland); pathovar *morsprunorum* race 1 isolates: *2*—LMG 2222, *3*—25b, *4*—28a , *5*—107, *6*—201, *7*—701A, *8*—755, *9*—771; pv. *morsprunorum* race 2 isolates: *10*—CFBP 3800, *11*—77, *12*—701, *13*—732, *14*—733, *15*—745, *16*—764; pv. *syringae* isolates: *17*—LMG 1247, *18*—2905, *19*—68; *20*—110, *21*—141, *22*—286, *23*—415, *24*—763, *25*—M—marker 100-bp PCR Molecular Ruler (Bio-Rad, Hercules, USA)

Design of SCAR primers

The nucleotide sequences obtained for the *Psm*1 and *Psm*2 fragments were used to design different SCAR primers. After validation, the most specific primers for conventional and real-time PCR were selected (Table 3). A BLAST analysis of selected primer sequences showed no similarity to any bacterial sequences in GenBank.

Dot blot hybridisation

The dot blot results confirmed the high specificity of the selected markers towards the target pathogens. Using probe Psm1, positive hybridisation results (dark dots) were observed with all tested Psm1 strains, and no unspecific hybridisation was observed with DNA from any non-*Psm*1 pseudomonads. Similarly, probe *Psm*2 was exclusively specific for the tested *Psm*2 strains. Additionally, the hybridisation results showed that the selected DNA regions were present in all their respective target strains, confirming their stability (Fig. 3).

Specificity of designed primers and usefulness in detection in plant material

The PCR assays using DNA from all tested *P. syringae* strains including reference strains (Table 1), as well as DNA from strains of other species within the *Pseudomonas* genus (Table 2), showed that all the designed primers were specific for their respective taxa. PCR assays using primers Psm1-6F/6R and Psm1-1F-RT/1R-RT, specific for *Psm*1, successfully amplified the expected PCR products 793 bp (Fig. 4) and 101 bp (Fig. 5), respectively, using DNA from all strains of *Psm*1. No amplification was observed when DNA from strains identified as *Psm*2 or *Pss* and strains of atypical taxa were used. Amplification using primers Psm2-8F/8R and Psm2-1F-RT/1R-RT, designed for detection of *Psm*2, was achieved with DNA from all strains of *Psm*2, resulting in PCR products of expected lengths of 410 bp (Fig. 6) and 104 bp, respectively. No increase in fluorescence was observed with DNA from *Psm*1 or *Pss* and strains of atypical taxa. The melting curves of the reaction products obtained from real-time PCR revealed a single peak with a melting temperature of 80 °C or 77 °C for *Psm*1 and *Psm*2, respectively. Also, neither unexpected nor additional peaks in the product melting curves were observed, which clearly excluded possibilities or tendency of the primers to form dimers. Moreover, none of the four tested primer pairs amplified the DNA of 79 strains of other pathovars of *P. syringae* and other species (Table 2).

The usefulness of the designed primers for detection of *Psm*1 and *Psm*2 strains in plant material was assessed with PCR assays using DNA extracted from a mixture of plant tissues and a suspension of target bacteria. The results confirmed the specificity of selected primer-pairs since positive amplification was achieved in mingled samples, while no nonspecific amplification was observed in samples without bacteria addition. Additionally, these assays showed that the proposed PCR detection methodology was not affected by potential inhibitors present in plant samples.

Limit of detection of *P. syringae* pv. *morsprunorum* for conventional and real-time PCR

Both tested primer pairs designed for conventional PCR allowed for the detection of 10^0 cfu/reaction of *Psm*1 and 10^1 *Psm*2 in pure culture. Regarding the presence of bacteria in different organs of sweet and sour cherries, it was possible to detect 10^0 and 10^1 cfu/reaction for sweet cherry leaves and shoots, respectively, using the *Psm*1-specific primers and 10^2 cfu/reaction for sour cherry leaves and shoots using the *Psm*2-

Table 3 Primers specific for strains of *Psm*1 and *Psm*2

	Primer name	Primer sequence	T_m	Product length
Conventional PCR	Psm1-6F	5′-TGTTCCCGGCCATCCAATA-3′	51.1 °C	793 bp
	Psm1-6R	5′-ATCCGCATCAGTCAAAATAGTCAT-3′	52.3 °C	
	Psm2-8F	5′-CTTTTTAGATGGTGAGGTTTTGTA-3′	50.6 °C	410 bp
	Psm2-8R	5′-ACTTTCGGATCATCGTTTTCTA-3′	49.2 °C	
Real-time PCR	Psm1-1F-RT	5′-TCCCGGCCATCCAATACTTTACG-3′	57.1 °C	101 bp
	Psm1-1R-RT	5′-ACGCTTCATGGTGTCTTGTTTA-3′	51.1 °C	
	Psm2-1F-RT	5′-GGTTTGCCTTTTCCTCAG-3′	48 °C	104 bp
	Psm2-1R-RT	5′-ATTGCATTACTTCTTTGTTGC-3′	46.5 °C	

F forward primer, *R* reverse primer, *RT* real-time, *Tm* melting temperature

	1	2	3	4	5	6	7	8	9	10	11	12
A	25a	28a	29a	58	59	61	66	68	69	71	72	83
B	86	87	88	89	90	91	93	94	95	96	98	100
C	103	106	107	109	110	112	115	117	118	119	120	122
D	141	147	158	165	174	177	184	192	199	201	202	203
E	204	205	206	209	210	211	213	214	215	216	217	218
F	219	220	221	222	226	227	229	233	234	235	236	237
G	239	240	242	244	245	247	248	250	256	257	258	259
H	264	266	271	274	276	280	283	291	373	374	376	415
I	417	420a	435	437	439	441	442	460	663	671	701	701A
J	702	702A	710	719	732	733	745	753	755	757	760	762
K	763	764	771	782	787	788	791	793	909	910	914	949
L	959	963	966	967	968	969a	970a	970b	971a	971b	972	973
M	982	1017	1021	1061	1247	2222	2905	3800	CFBP1407	CFBP1617	CFBP1694	CFBP1731
N	CFBP1754	CFBP2067	CFBP2105	CFBP2213	CFBP2215	CFBP2339	CFBP2346	CFBP3226	CFBP5010-2	LMG2208	MAFF301159	C-

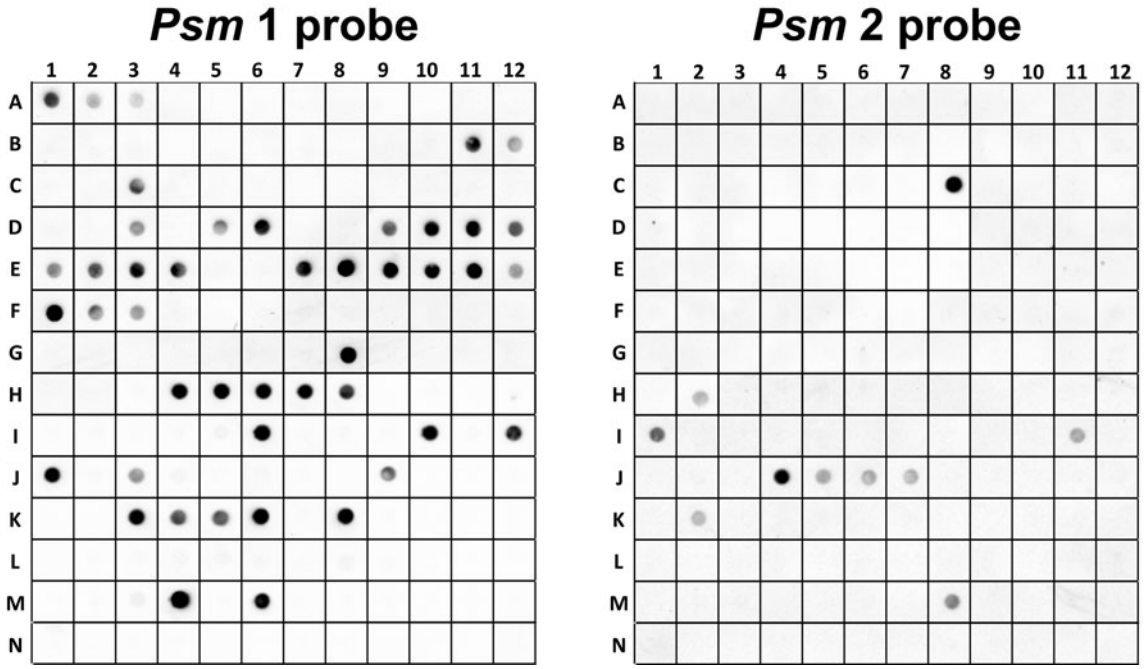

Fig. 3 Dot blot validation of probes Psm1 and Psm2. The probes were evaluated with total DNA from 167 strains; including *P. syringae* strains isolated from stone fruit trees, reference strains and others pathovars of *P. syringae* from the CFBP culture collection. The *table grid* above represents the coordinates of each strain tested in the dot blot, which are identified by their abbreviations further detailed in Tables 1 and 2. Positive hybridization signals are visualised as *dark dots*

specific primers. The sensitivity (LOD, limit of detection) of the detection in the conventional PCR assay was ~4 pg for *Psm*1 strain 199 and ~5 pg for *Psm*2 strain 745 when aliquots of serial 2-fold dilutions of purified DNA were used which corresponds to the order of magnitude ~10^1–10^2 cfu/reaction.

Both tested primer pairs designed for *Psm*1 and *Psm*2 strains using real-time PCR allowed the detection of 10^0 cfu/reaction of *Psm*1 or *Psm*2 in pure culture and in plant material. Only the expected products and a single peak with melting temperature were obtained. Standard curves using template DNA from bacterial suspensions, DNA from plant material with additions of bacterial suspensions and bacterial gDNA showed high amplification efficiency and linearity of the data (Table 4). An exception occurred for the products obtained from shoots of sweet cherry with additions of bacterial suspensions of *Psm*1. Although linearity was quite good, the noted efficiency of 83 % was not in the range considered acceptable (90–110 %). Moreover, the efficiency obtained for the mixture of shoots of sour cherry and *Psm*2 suspension when testing with primers for *Psm*2 was also lower compared to DNA template from sour cherry leaves and bacterial suspension alone. The sensitivity (LOD) of the detection in the real-time PCR assay when using gDNA ranged from ~30 to 100 fg for *Psm*1 strain 199 and ~10 to 50 fg for *Psm*2 strain 745 when 1.0-μl aliquots of serial 2-fold dilutions of

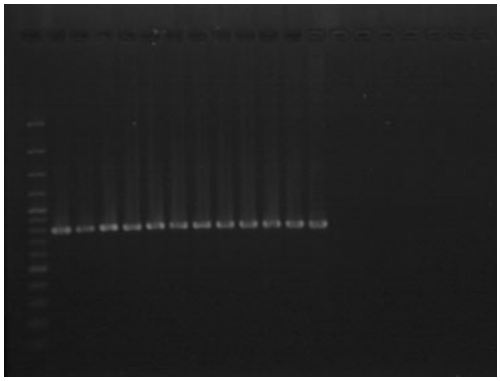

Fig. 4 Evaluation of primers Psm1-6F and Psm1-6R for identification of *P. syringae* pv. *morsprunorum* race 1: M—O'GeneRuler 100–3000 bp (Thermo Scientific, Vilnius, Lithuania), strains *Psm*1: strains *Psm*1: 2—LMG 2222, 3—28a, 4—29a, 5—38a, 6—175, 7—199, 8—201, 9—203, 10—205, 11—274, 12—755, strains *Psm*2:13—CFBP 3800, 14—732, strains *Pss*: 15—LMG 1247, 16—760, strains of atypical taxon: 17—58, 18—970a, 19—K-

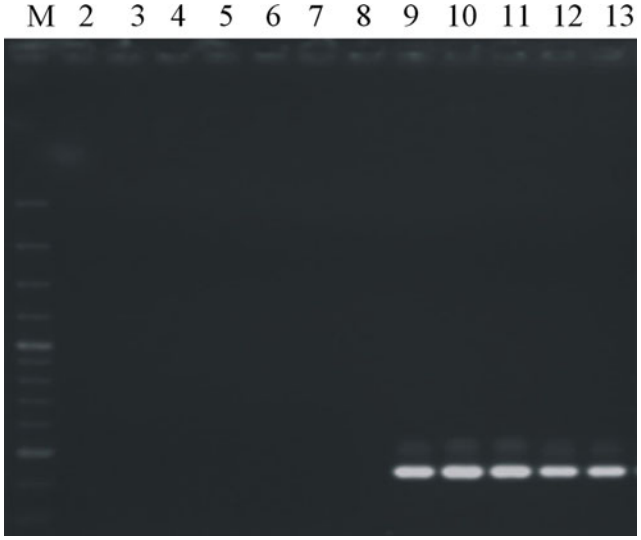

Fig. 6 Evaluation of primers Psm2-8F and Psm2-8R for identification of *P. syringae* pv. *morsprunorum* race 2: *M*—O'GeneRuler 100–3000 bp (Thermo Scientific, Vilnius, Lithuania), strains *Psm*1: *2*—25b, *3*—250, *4*—788; strains *Pss*: *5*—68, *6*—110; strains of atypical taxon: *7*—61, *8*—970a; strains *Psm*2: *9*—77, *10*—CFBP3800, *11*—77, *12*—745, *13*—764

purified DNA were used which corresponds to the order of magnitude ~10^0 cfu/reaction (Table 4).

Discussion

In this study, the methods and tools enabling the rapid and highly specific identification and detection of bacterial canker causal agent *P. syringae* pv. *morsprunorum* races 1 and 2 were developed. The methods based on the use of specific primers designed for conventional and real-time PCR allow in routine testing for omitting the application of often time-consuming methods of classical microbiology, fingerprinting methods or housekeeping gene sequence analysis used until now by other authors (Vicente and Roberts 2007; Gilbert et al. 2009). Of course in critical cases (i.e. first reports, claims, etc.) these other methods are still indispensable. Our newly developed

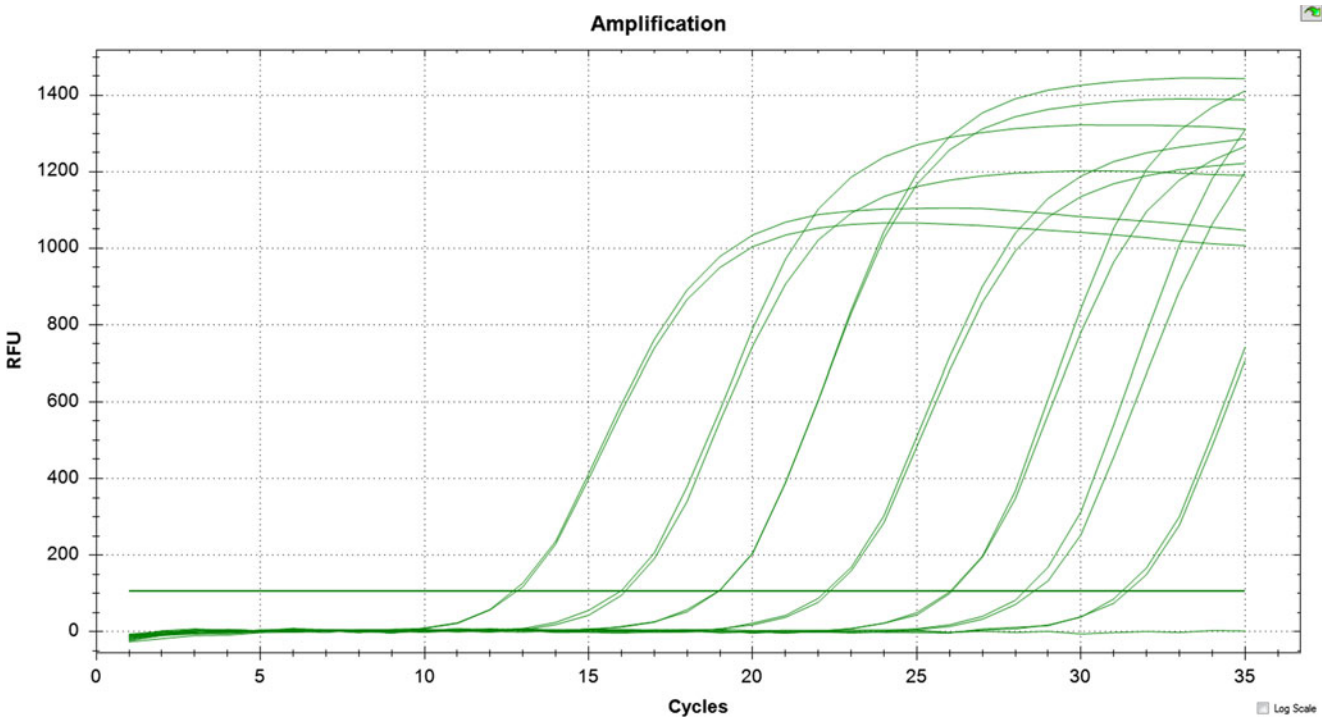

Fig 5 Real-time PCR with SYBR Green I (Bio-Rad, Hercules, USA) for specific detection of DNA from suspension of *Psm*1 strain LMG2222 (example). Fluorescence signal is related to the amount of template. Samples from 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 cfu/reaction

Table 4 Important parameters of real-time polymerase chain reaction (PCR) runs evaluated through the analysis of standard curves generated with different DNA templates of *P. syringae* pv. *morsprunorum* races 1 and 2

Template	E (%)[a]	R2 [b]	Slope [c]	*Y* = int [d]
*Psm*1 (DNA from bacterial suspension)	103	0.998	−3.252	35.445
*Psm*1+sweet cherry leaves	99.7	0.965	−3.328	36.551
*Psm*1+sweet cherry shoots	83.0	0.989	−3.810	43.932
*Psm*1 gDNA	99.2	0.997	−3.342	18.425
*Psm*2 (DNA from bacterial suspension)	99.8	0.995	−3.326	33.093
*Psm*2+sour cherry leaves	99.3	0.999	−3.338	32.451
*Psm*2+sour cherry shoots	91.4	0.994	−3.548	35.130
*Psm*2 g DNA	99.2	0.991	−3.342	17.805

[a] E = PCR efficiency; ideally the efficiency should be 100 %, meaning that for each cycle the amount of product doubles; high/acceptable amplification efficiency (90–110 %). Efficiency = $10^{(-1/\text{slope})} - 1$

[b] R2 is a measure of data linearity amongst technical replicates of serial dilutions; indicates how good one value is in predicting another; R2 = 1 is perfect

[c] The slope of the log-linear phase of the amplification reaction is a measure of reaction efficiency. To obtain accurate and reproducible results, reactions should have an efficiency as close to 100 % as possible, equivalent to a slope of −3.32

[d] Y = int represents the value of Ct where the curve crosses the y-axis

methods and tools are very useful and invaluable in both epidemiological studies and in development of protection programmes for stone fruits against bacterial canker.

Using the genetic fingerprinting PCR MP method, we demonstrated the diversity of *P. syringae* strains, which was very important in the selection of specific DNA fragments for two races of *P. syringae* pv. *morsprunorum*. Based on the obtained nucleotide sequences of these fragments, *Psm*1- and *Psm*2-specific SCAR primers were designed. The specificity of the designed primers for *Psm* and amplified regions was confirmed by BLAST, since the fragments did not show (at present) any significant similarity hits within the NCBI database. Due to the high electrophoretic profile heterogeneity obtained for *Pss* strains arising from their high genetic diversity confirmed already by other authors (Vicente and Roberts 2007; Gilbert et al. 2009; Kałużna et al. 2010a, b), it was not possible to find a common DNA fragment for all strains belonging to this taxon.

Commonly used methods for designing SCAR primers include rep-PCR (repetitive PCR) (Sangdee et al. 2013), randomly amplified polymorphic DNA (RAPD) (Liu et al. 2012; Cheng et al. 2015), amplified fragment length polymorphism (AFLP) (Zhang et al. 2012), PCR with universal rice primers (URP-PCR) (Lim et al. 2009) and inter-simple sequence repeat (ISSR) (Giaj Merlera et al. 2015). Although the PCR MP method was described so far as helpful in the study of genetic diversity of bacteria and yeast (Leibner-Ciszak et al. 2010; Kałużna et al. 2010b, 2014; Zasada et al. 2014), it has not been previously reported to be used for the selection of SCAR markers. In this work, the PCR MP is for the first time used for the design of SCAR primers specific for detection of plant pathogenic bacteria.

The results obtained in this study showed that the designed SCAR primers can be applied for specific, direct detection of strains belonging to *Psm*1 or *Psm*2, both in pure culture and infected plant material. Their specificity was confirmed by PCR, using DNA from several *Pseudomonas* spp. strains, which showed that positive amplification occurred only with DNA of the targeted taxa strains. This is especially significant in the case of strains of atypical taxa and pathovars of *P. syringae* (i.e. pv. *syringae* and pv. *avii*, which also infect cherry (Ménard et al. 2003; Renick et al. 2008)) to exclude that symptoms are connected to another taxa/pathogen or to abiotic factors. Importantly, when testing the developed primers in conventional PCR, using DNA isolated from a mixture of plant material and bacteria of *Psm*1 or *Psm*2, the suppression of amplification by potential plant inhibitors like polyphenols and pesticide residues, as reported by Puławska et al. (1997), was not found. Additionally, for DNA from the asymptomatic plant material without addition of bacterial DNA, no positive amplification was observed. This means that the designed primers did not react with DNA of potential bacteria naturally inhabiting the plant material, which is essential to prevent false-positive diagnostic results. However, in the case of real-time PCR, which is the more sensitive method, some effects of plant material were noted. Although standard curves using different template DNA showed the high amplification efficiency and linearity of the data for the majority of DNA tested, for shoots of sweet cherry with additions of bacterial suspensions the efficiency was below the range considered acceptable, indicating higher dilution of those templates than expected. Also, a decrease of efficiency (Table 4) in the case of sour cherry shoots was observed. The results therefore may indicate the influence of shoots for more sensitive real-time PCR reactions.

The designing of primers for both systems, conventional and real-time PCR, makes the developed diagnosis system more accessible to a wider group of researchers, as many laboratories do not have access to special equipment or specialised personnel to perform the real-time PCR or have less funds. However, as described, the real-time PCR procedure is much faster (whole reaction with melting curve analysis is about 1 h from the beginning with SsoAdvanced SYBR Green Supermix); it allows the use of DNA quickly extracted from pure culture by the boiling method, without loss of detection resolution, and also excludes additional time-consuming post-PCR processes (i.e. agarose gel electrophoresis). Therefore, using this technique, it is possible to obtain a very fast response about the causal agent of the disease. However, it should be noted that this system is highly sensitive and that false-positive results can occur. The risk of false-positive results due to cross-contamination during preparation of the PCR can be minimised by using negative controls and high discipline during work (e.g., application of tips with filters during the DNA isolation step). Additionally, positive results obtained during those of the final PCR cycles should be treated as suspect only, for which additional, more detailed investigations should be conducted. Moreover, during all the assays the melting curve analysis is recommended to exclude nonspecific amplicons (as a consequence of which are visible in each run as the rest of the analysed specific ones). Dot blot hybridisation confirmed that the two selected DNA regions were highly specific for their target genomospecies and stable amongst all tested isolates of either *Psm*1 or *Psm*2, which is essential for preventing false-positive and false-negative results, respectively as much as possible.

In summary, when compared with so-far available methods for identification and differentiation of causal agents of stone fruit bacterial canker based on phenotypic characters, fingerprinting methods or MLST, the use of pathovar-specific primers allowed for greatly shortening the time required for diagnosis, while highly increasing assay accuracy and lowering detection limit. Moreover, this PCR-based method is relatively simple and inexpensive, and it does not require the time-consuming step of pre-incubation on microbiological media (Schaad et al. 1995). Even in the presence of potential inhibitors present in plant material, which can affect the limit of detection, we could detect 1 and 3×10^2 cfu/reaction using primers specific for *Psm*1 and *Psm*2 in conventional PCR. A similar detection sensitivity in conventional PCR was obtained by other authors in their identification systems for other phytopathogens (Catara et al. 2000; Kerkoud et al. 2002; Biondi et al. 2013). The sensitivity of real-time PCR was higher than in the case of conventional ones, as 1 cfu/reaction was detected when different templates were used. This is especially important in the case of naturally infected material in the presence of a small amount of pathogen DNA, which be detected in a very short time. The limit of detection when using gDNA was in the range from ~4–5 pg in conventional and ~10–100 fg in real-time PCR for both taxa, which are similar to results obtained for *P. syringae* pv. *actinidiae* (Gallelli et al. 2014) and *Clavibacter michiganensis* subsp. *sepedonicus* (Cho et al. 2015). The high sensitivity of the developed assay (obtained in our hands) will be invaluable for detecting the target bacteria in the early latent period of the disease, allowing growers to undertake appropriate prevention or protection programmes.

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Compliance with ethical standards

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

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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