

SHORT COMMUNICATION

CHARACTERIZATION AND GENETIC DIVERSITY OF *PSEUDOMONAS SYRINGAE* FROM STONE FRUITS AND HAZELNUT USING REPETITIVE-PCR AND MLSTM. Kaluzna¹, P. Ferrante², P. Sobiczewski¹ and M. Scortichini²¹Research Institute of Pomology and Floriculture, Department of Plant Pathology, Pomologiczna 18 str., Skierniewice, Poland²CRA, Istituto Sperimentale per la Frutticoltura, Via di Fioranello 52, 00040 Ciampino Aeroporto, Roma, Italy

SUMMARY

A total of 33 bacterial isolates from sweet cherry (*Prunus avium* L.), sour cherry (*P. cerasus* L.), plum (*P. domestica* L.), and hazelnut (*Corylus avellana* L.) showing disease symptoms in Poland and Italy were first identified phenotypically using conventional techniques. Sixteen isolates were classified as *Pseudomonas syringae* pv. *syringae* (*Pss*), 12 as *P. syringae* pv. *morsprunorum* (*Psm*) race 1, and five as *Psm* race 2. Detection of toxin production, showed that out of 16 *Pss* strains, 11 possessed the *syrB* gene, whereas three strains of *Psm* race 1 had the *cfl* gene and three strains of *Psm* race 2 had the gene encoding yersiniabactin (*irp1*). Repetitive-sequence PCR (rep-PCR) using BOX and ERIC primer sets revealed three separate clusters that were consistent with the phenotypically identified pathovars and races. The only exception were two strains of *Psm* race 2 that did not possess *irp1*. In this case, both BOX and ERIC-PCR fingerprints showed that their patterns were more similar to those obtained from *Pss* strains. Twenty representative strains were also used for multilocus sequence typing (MLST). In particular, MLST of *gyrB*, *gapA*, *gltA* and *rpoD* genes allowed a clear allocation of the strains into three separate clusters corresponding to *Pss* and *Psm* race 1 and 2.

Key words: phytotoxins, biochemical tests, *Pseudomonas syringae* pv. *syringae*, *Pseudomonas s. pv. morsprunorum*, races, BOX-PCR, ERIC-PCR, MLST.

Pseudomonas syringae has a wide host range as it causes diseases to over 180 plant species. In some stone fruits such as sweet cherry (*Prunus avium* L.), sour cherry (*P. cerasus* L.) and plum (*P. domestica* L.) and in hazelnut (*Corylus avellana* L.), it attacks all the above-ground organs causing cankers and necrosis of woody tissue, blight of buds and blossoms, spots on the leaves and wilting of twigs. Based on pathogenicity and host

range, *P. syringae* has been divided into over 50 pathovars (Bradbury, 1986; Young *et al.*, 1996; Young, 2010) which, based on DNA:DNA homology, are grouped into nine genomospecies (Gardan *et al.*, 1999). On sweet and sour cherry and plum, two pathovars, pv. *syringae* (*Pss*) and pv. *morsprunorum* (*Psm*) occur (Young, 1991; Gardan *et al.*, 1999), whereas pvs. *syringae* and *corylina* are found on hazelnut (Scortichini *et al.*, 2002, 2005). Within pv. *morsprunorum*, races 1 and 2 have been determined (Freigoun and Crosse, 1975; Vicente *et al.*, 2004).

Identification and differentiation of *P. syringae* pathovars were reported by Garrett *et al.* (1966), Little *et al.* (1998), Scortichini *et al.* (2002, 2003), Stead *et al.* (2004), Vicente *et al.* (2004), Cirvilleri *et al.* (2006), Kaluzna *et al.* (2010). Strains of *Psm* races 1 and 2 form separate clusters, but those of *Pss* are more heterogeneous (Weingart and Völksch, 1997; Sawada *et al.*, 1999; Sarkar and Guttman, 2004). Variability of aggressiveness was also found between *Pss* strains (Gilbert *et al.*, 2010).

Dendrograms based on REP and ERIC-PCR as well as BOX-PCR showed clear separation of *Psm* race 1 and *Psm* race 2 from non-pathogenic *P. syringae* strains (Vicente *et al.*, 2004). Determination of genetic diversity of strains from stone fruits using melting profile PCR (PCR MP) showed that the patterns obtained strongly correlated with phenotypically distinct pathovars and races (Kaluzna *et al.*, 2010). Multilocus sequence typing (MLST) is a rather new technique used for the characterization of bacteria (Maiden *et al.*, 1998; Enright and Spratt, 1999; Hwang *et al.*, 2005; Wang *et al.*, 2007). It is a recommended method for the determination of genomic relatedness among bacterial strains, where sequences of some housekeeping genes of the bacterial core genome encoding proteins necessary for the microorganism's survival are compared (Sarkar and Guttman, 1999; Stackebrandt *et al.*, 2002).

The purpose of this study was the identification and differentiation of 33 *Pseudomonas syringae* strains from diseased sweet and sour cherry, plum and hazelnut trees from Italy and Poland, using repetitive-sequence PCRs and MLST.

Twenty five isolates from sweet and sour cherry and

Table 1. Origin and phenotypic characteristics of *Pseudomonas syringae* isolates used in this study.

Isolate	Host	Year of isolation	Place of isolation	Grouping based on LOPAT, GATTa and L-lactate	Grouping based on rep-PCR	Yersiniabactin (PCR)	Syringomycin production (PCR)	Coronatine presence (PCR)
RIPF 25	Sweet cherry	2007	Poland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	+
RIPF 38	Sweet cherry	2007	Poland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-
RIPF 202	Plum	2007	Poland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-
RIPF 213	Plum	2007	Poland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-
RIPF 214	Sweet cherry	2007	Poland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-
RIPF 217	Sweet cherry	2007	Poland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-
RIPF 671	Sweet cherry	2008	Poland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	+
RIPF 702	Plum	1994	Poland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-
RIPF 710	Sweet cherry	1996	Poland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	+
RIPF 782	Sweet cherry	2001	Poland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-
I-433	<i>Prunus avium</i> (twig)	1992	Italy	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-
I-434	<i>Prunus avium</i> (leaf)	1992	Italy	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-
RIPF 77	Sour cherry	2007	Poland	<i>Psm</i> race 2	<i>Psm</i> race 2	+	-	-
RIPF 732	Sour cherry	1997	Poland	<i>Psm</i> race 2	<i>Psm</i> race 2	+	-	-
RIPF 764	Sour cherry	1999	Poland	<i>Psm</i> race 2	<i>Psm</i> race 2	+	-	-
RIPF 165	Sweet cherry	2007	Poland	<i>Psm</i> race 2	<i>Pss</i>	-	+	-
RIPF 96	Sour cherry	2007	Poland	<i>Psm</i> race 2	<i>Pss</i>	-	-	-
RIPF 59	Sour cherry	2007	Poland	<i>Pss</i>	<i>Pss</i>	-	-	-
RIPF 68	Sour cherry	2007	Poland	<i>Pss</i>	<i>Pss</i>	-	+	-
RIPF 81	Sour cherry	2007	Poland	<i>Pss</i>	<i>Pss</i>	-	-	-
RIPF 90	Sour cherry	2007	Poland	<i>Pss</i>	<i>Pss</i>	-	-	-
RIPF 110	Plum	2007	Poland	<i>Pss</i>	<i>Pss</i>	-	+	-
RIPF 240	Plum	2007	Poland	<i>Pss</i>	<i>Pss</i>	-	+	-
RIPF 244	Plum	2007	Poland	<i>Pss</i>	<i>Pss</i>	-	+	-
RIPF 663	Sour cherry	2008	Poland	<i>Pss</i>	<i>Pss</i>	-	+	-
RIPF 760	Sour cherry	1999	Poland	<i>Pss</i>	<i>Pss</i>	-	+	-
RIPF 791	Sour cherry	2001	Poland	<i>Pss</i>	<i>Pss</i>	-	-	-
Pss I-1431	Hazelnut		Italy	<i>Pss</i>	<i>Pss</i>	-	-	-
Pss I-1365	Hazelnut		Italy	<i>Pss</i>	<i>Pss</i>	-	+	-
Pss I-1386	Hazelnut		Italy	<i>Pss</i>	<i>Pss</i>	-	+	-
Pss I-1367	Hazelnut		Italy	<i>Pss</i>	<i>Pss</i>	-	+	-
Pss I-1394	Hazelnut		Italy	<i>Pss</i>	<i>Pss</i>	-	+	-
Pss PA4a	Hazelnut		Italy	<i>Pss</i>	<i>Pss</i>	-	+	-
NCPPB 2427	<i>P.armeniaca</i>	1965	Switzerland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-
NCPPB 2787	<i>Prunus avium</i>	1961	Greece	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-
NCPPB 2427 str.+	<i>P.armeniaca</i>	1965	Switzerland	<i>Psm</i> race 1	<i>Psm</i> race 1	-	-	-

GATTa (gelatin hydrolysis - G, aesculin hydrolysis - A, tyrosinase activity - T and utilization of tartrate - Ta)//L-lactate results for each group - *Pss*-G⁺ A⁺T⁻ Ta⁻//+, *Psm* race 1-G⁻ A⁻T⁺ Ta⁺//-, *Psm* race 2-G⁺ A⁻T⁻ Ta⁻//-, +positive; - negative.

plum growing in various regions in Poland (RIPF) and eight from Italy (two from *Prunus avium* and six from hazelnut) (Table 1) were studied. The reference strains of *Psm*: NCPPB 2427, NCPPB 2787 and NCPPB 2427 were also included (Table 1). All isolates were identified using LOPAT (Lelliott and Stead, 1987), GATTa and L-lactate utilization tests (Latorre and Jones, 1979). The possible presence of genes encoding yersiniabactin (*irp1*) (Bultreys *et al.*, 2006), syringomycin (*syrB*) (Sorensen, 1998), and coronatine (*cfl*) (Bereswill, 1994) was also checked. All PCR reactions were conducted according to Kaluzna *et al.* (2010), with slight modifications of annealing temperatures.

The genetic diversity of strains was studied by means of repetitive-sequence PCR using ERIC and BOX primer sets (Versalovic *et al.*, 1991, 1994; Louws *et al.*, 1994). The 25 μ l reaction mix contained 1x PCR buffer (GoTaq Flexi Buffer, Promega, USA), 1.25 mM deoxyribonucleoside triphosphate, 6.7 mM MgCl₂, 60 pmol of each primer, 2 U *Taq* DNA polymerase and 1 μ l DNA. PCR was carried out in a thermal cycler (MJ Mini, Biorad, USA). The ERIC and BOX programs comprised: initial denaturation at 95°C for 7 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 65°C for 8 min, and a single final extension cycle at 65°C for 8 min. PCR amplifications were done twice with all strains. PCR products were separated by gel electrophoresis on 1.5% agarose in 0.5x TAE buffer. Patterns (after staining for 5 min in 0.5 mg l⁻¹ of ethidium bromide) were visualized under UV camera (Kodak Gel apparatus, Biorad, USA) and analyzed. Each band was scored as present (1) or absent (0) for all strains. A dendrogram with distance matrix was constructed using the Jaccard's coefficient and UPGMA algorithm with the MVSP software.

For MLST analyses, primer sequences for these loci as well as the protocol described by Sarkar and Guttman (2004) were used. Four housekeeping genes, namely *rpoD* encoding sigma factor 70, *gyrB* encoding DNA gyrase B, *gltA* (also known as *cts*) encoding citrate synthase, and *gapA* encoding glyceraldehyde-3-phosphate dehydrogenase were used. Small modifications of the PCR protocol were made, e.g. increasing the annealing temperature by 1°C for the *rpoD* gene.

Amplifications were performed in a total volume of 25 μ l. Reaction mixture consisted of 1 μ l of DNA, 1.5 U of GoTaq DNA polymerase (Promega, USA), GoTaqFlexi Buffer at the final 1x concentration, 0.2 mM concentrations of each dNTP and 0.5 μ M of primers for each gene. Amplifications were carried out in the Biorad thermal cycler under the following conditions: initial step at 95°C for 5 min, 35 cycles of denaturation at 62°C for 30 sec, annealing at 62°C, 52°C, 73°C and 76°C for 40 sec for *gyrB*, *gapA*, *gltA* and *rpoD*, respectively, and extension at 72°C for 1.5 min. The final step was at 72°C for 5 min. DNA sequencing was by PRIMM (Mi-

lano, Italy). Chromatograms were reviewed and edited using the Bioinformatics software for sequence alignment, Geneious Basic 4.6.5. A neighbor-joining tree was constructed using the same software.

All 33 isolates were identified as *Pseudomonas syringae* – LOPAT group Ia. GATTa and L-lactate utilization tests allowed further discrimination of pathovars and races: 16 isolates were classified as *Pss*, 12 as *Psm* race 1 and five as *Psm* race 2 (Table 1).

Out of 33 strains tested for the presence of the yersiniabactin gene, only two of *Psm* race 2 (RIPF 96 and RIPF 165) did not possess it (Table 1). Although the phenotypic features defined these isolates as *Psm* race 2, strains with lack of this gene could be not regarded as race 2 of *pv. morsprunorum*. Moreover, RIPF 165 showed the presence of syringomycin gene (*syrB*) which is characteristic only for strains of pathovar *syringae*. The gene encoding for the coronatine (*cfl*) was

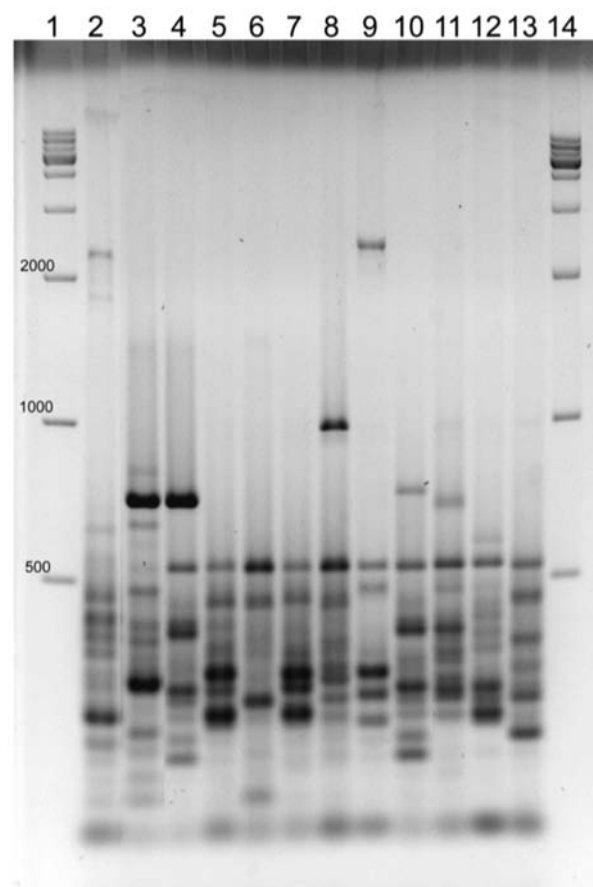


Fig. 1. PCR fingerprinting patterns of representative *Pseudomonas syringae* isolates obtained using ERIC primers set: lane 1, Marker (1kb Sharp ladder); lane 2, *Psm* race 1 RIPF 25-*P. avium*; lane 3, *Psm* race 2 RIPF 732-*P. cerasus*; lane 4, *Pss* RIPF 59-*P. cerasus*; lane 5, *Pss* RIPF 68-*P. cerasus*; lane 6, *Pss* RIPF 110-*P. domestica*; lane 7, *Pss* RIPF 240-*P. domestica*; lane 8, *Pss* RIPF 663-*P. cerasus*; lane 9, *Pss* RIPF 760-*P. cerasus*; lane 10, *Pss* RIPF 791-*P. cerasus*; lane 11, *Pss* Pss I-1431-*C. avellana*; lane 12, *Pss* Pss I-1365-*C. avellana*; lane 13, *Pss* Pss I-1386-*C. avellana*; lane 14, Marker (1kb Sharp ladder).

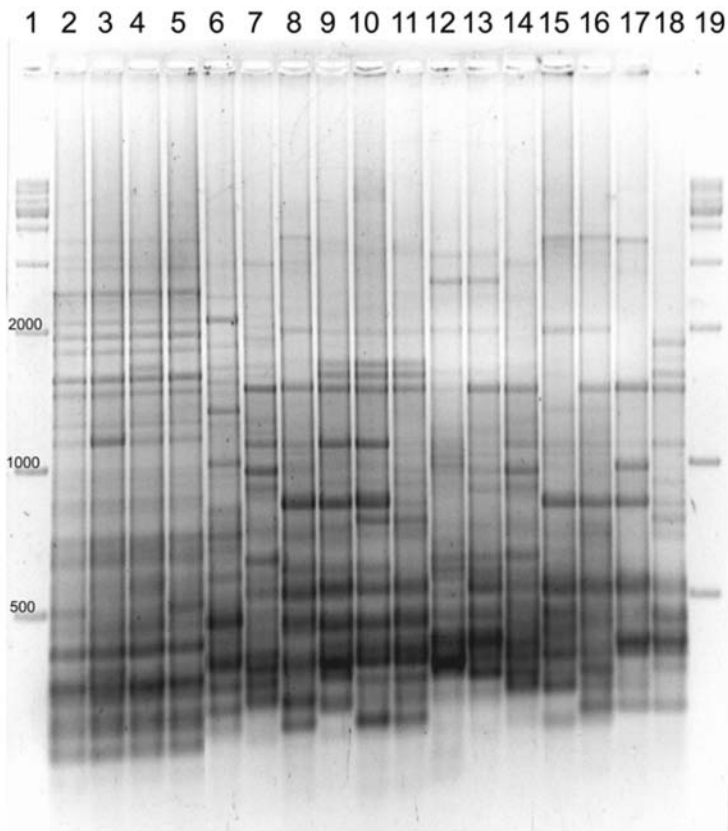


Fig. 2. PCR fingerprinting patterns of representative *Pseudomonas syringae* isolates obtained using BOX primer: lane 1, Marker (1kb Sharp ladder); lane 2, *Psm* race 1 RIPF 25-*P. avium*; lane 3, *Psm* race 1 RIPF 202-*P. domestica*; lane 4, *Psm* race 1 RIPF 213-*P. domestica*; lane 5, *Psm* race 1 NCPPB 2727-*P. armeniaca*; lane 6, *Psm* race 2 RIPF 732-*P. cerasus*; lane 7, *Pss* RIPF 96-*P. cerasus*; lane 8, *Pss* RIPF 165-*P. avium*; lane 9, *Pss* RIPF 110-*P. domestica*; lane 10, *Pss* Pss I-1386-*C. avellana*; lane 11, *Pss* RIPF 663-*P. cerasus*; lane 12, *Pss* RIPF 240-*P. domestica*; lane 13, *Pss* RIPF 760-*P. cerasus* L.; lane 14 – *Pss* RIPF 59-*P. cerasus* L.; lane 15 – *Pss* Pss I-1365-*C. avellana* L., lane 16 – *Pss* Pss I-1367-*C. avellana*; lane 17, *Pss* Pss I-1431-*C. avellana*; lane 18, *Pss* PssP14a-*C. avellana*; lane 19, Marker (1kb Sharp ladder).

found only in *Psm*1 isolates RIPF 25, RIPF 671 and RIPF 710 (Table 1).

Rep-PCR protocols allowed differentiation of the strains. BOX-PCR proved more suitable for determining the genetic diversity of the strains in comparison with ERIC-PCR. In fact, BOX-PCR yielded 17 patterns compared to 12 obtained with ERIC (Fig. 1 and 2). The higher resolution of BOX-PCR had been reported in previous studies on *Pss* and *Psm* from sweet and sour cherry (Vicente and Roberts, 2007) and *Pseudomonas syringae* pv. *coryli* from hazelnut (Scortichini *et al.*, 2005). By contrast, Gilbert *et al.* (2009) showed that for others *Pseudomonas* BOX-PCR was the less effective in assessing the genetic variability of *Pseudomonas syringae* and *P. viridiflava* strains from pear, plum and cherry.

The results of ERIC and BOX PCR showed that all strains were consistent with the phenotypic identification of pathovars and races, with the exceptions of two (RIPF 96, RIPF 165) which were classified phenotypically as *Psm* race 2 but did not possess the yersiniabactin gene. Noteworthy, their patterns were more similar [identical to *Pss* strains RIPF 240 and RIPF 244 according to ERIC PCR (data not shown)] to those obtained for pathovar *syringae* strains (Fig. 1 and 2).

Three strains possessing the yersiniabactin gene and identified as *Psm* race 2 (RIPF 77, RIPF 732, RIPF 764) had the same pattern in both ERIC and BOX-PCR. Although the year (ten and eight years between strains RIPF 77 as compared to RIPF 732 and RIPF 764, respectively) and place of isolation were different, no differences in their fingerprints were found.

All strains of *Psm* race 1 gave the same fingerprint by ERIC-PCR (Fig. 1), by contrast BOX-PCR (Fig. 2) showed slightly different patterns. Although based on BOX-PCR *Psm* race 1 isolates could be grouped according to the host of origin, no relation was found with the year and place of isolation.

Pss strains were the most heterogeneous and intermingled in relation to host plant, year and place of isolation as shown by Weingart and Völksch (1997), Little *et al.* (1998), Scortichini *et al.* (2003). In fact, their fingerprints were diverse in both ERIC and BOX-PCR with 10 and 12 patterns, respectively (Fig. 1 and 2).

All strains representing *Pss*, *Psm* race 1 and *Psm* race 2 formed separate clusters (Fig. 3 and 4). It should be pointed out that the very high overall similarity of *Psm* strains, especially those of race 2 found in our studies, was also reported by Vicente and Roberts (2007) and Kaluzna *et al.* (2010). MLST afforded the highest discrimination among the strains studied, especially in the case of *Psm* race 2, as compared to both rep-PCRs. Strains of *Psm* race 1 slightly differed from each other but the high heterogeneity of the *Pss* group was confirmed also by MLST analysis. No correlation between *Pss* strains from hazelnut and stone fruits was obtained, and differences deriving from the place and year of isolation were not observed.

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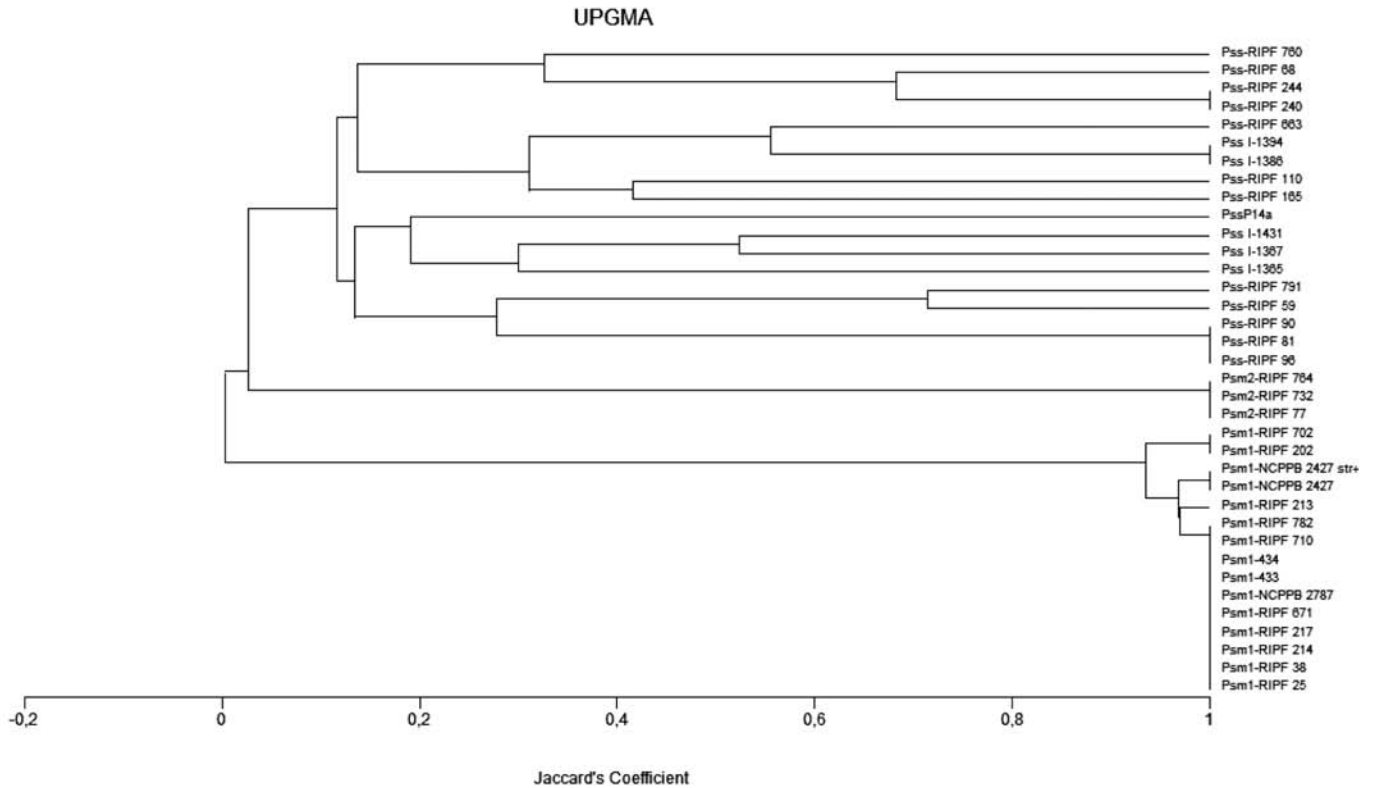


Fig. 3. Dendrogram of genetic similarity of 36 *Pseudomonas syringae* isolates obtained from stone fruits and hazelnut. The combined data sets from ERIC and BOX-PCR using UPGMA analysis and Jaccard's coefficient are shown.

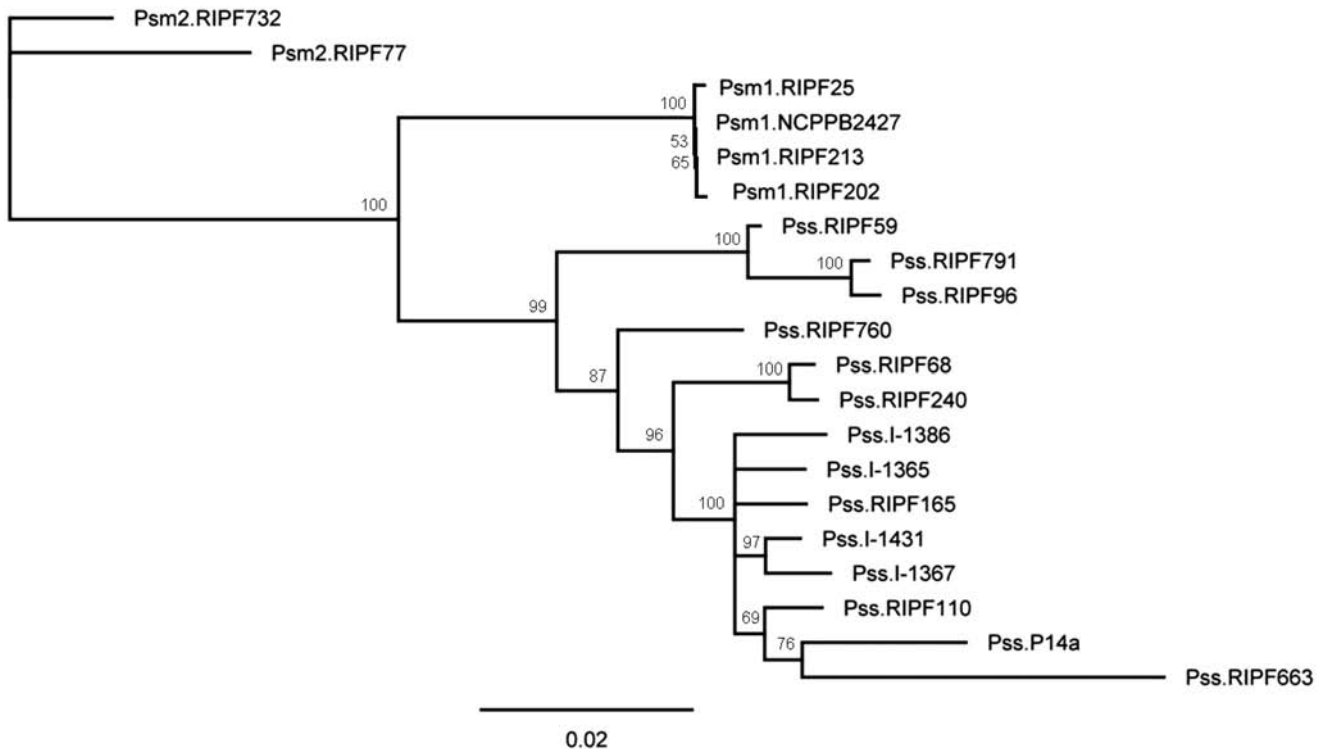


Fig. 4. Neighbour-joining tree of *Pseudomonas syringae* isolates obtained from stone fruit and hazelnut based on the concatenated data set of MLST analysis of the *gyrB*, *gapA*, *gltA* and *rpoD* genes. Bootstrap values are indicated at each node. *Pss*: *Pseudomonas syringae* pv. *syringae*; *Psm1*: *P. s.* pv. *morsprunorum* race 1; *Psm2*: *P. s.* pv. *morsprunorum* race 2.

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