MOLECULAR AND PHENOTYPIC VARIABILITY AMONG *PSEUDOMONAS AVELLANAE, P. SYRINGAE* pv. *ACTINIDIAE* AND *P. SYRINGAE* pv. *THEAE*: THE GENOMOSPECIES 8 *SENSU* GARDAN *et al.* (1999)

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SUMMARY

Genomospecie 8, *sensu* Gardan *et al.* (1999), includes *Pseudomonas avellanae*, *P. syringae* pv. *theae* and *P. s*. pv. *actinidiae*. To further characterize this genomospecies, 14 *P. avellanae*, three *P. s*. pv. *theae* and 18 *P. s.* pv. *actinidiae* strains were analysed by multilocus sequence typing (MLST) using *gapA, gltA, gyrB* and *rpoD* gene fragments. These strains were also checked for the presence/absence of 38 effector protein genes based on the corresponding sequences of *P. syringae*. pv. *tomato* DC3000 and *P. syringae* pv. *phaseolicola* 1448A. Nutritional tests and a comparison of the 16S rDNA gene sequences deposited at NCBI database were also done to detect possible differences. MLST analysis, based on 2.5 kb sequences, revealed that *P. s.* pv. *theae* and *P. s.* pv. *actinidiae* are more closely-related to one another than to *P. avellanae*. This technique clearly revealed that the *P. s*. pv. *actinidiae* strains causing the current severe epidemics in Italy are different from those of past outbreaks in Japan and central Italy. Nine effector protein genes were displayed by all strains of genomospecies 8. However, each pathogen of this genomospecies displays some distinctive effector protein genes. *HopA1* and *hopH1* are unique to *P. s.* pv. *actinidiae* strains of the recent epidemics of bacterial canker on *Actinidia chinensis* and *A. deliciosa* in Italy. A triplet, in position 461-463 of the 16S rDNA gene, is different in *P. s.* pv. *actinidiae*, namely GAT, and in *P. avellanae* and *P. s.* pv. *theae,* namely ATC. Contrarily to *P. s.* pv. *theae* and *P. s.* pv. *actinidiae, P. avellanae* did not utilize sorbitol.

Key words: Multilocus sequence typing, effector proteins, bacterial canker and decline of hazelnut, bacterial canker of kiwifruit, bacterial shoot blight of tea.

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INTRODUCTION

Based on DNA-DNA hybridization analysis and ribotyping Gardan *et al.* (1999) circumscribed nine genomospecies among 48 pathovars of *Pseudomonas syringae* and eight related species of *Pseudomonas*. However, these nine genetically distinct groups showed no clear distinguishing phenotypic traits that could enable their differentiation. Consequently, and according to the recommendations of a taxonomic committee (Wayne *et al*., 1987), they could not be formally elevated to the species level. Genomospecies 8 included *Pseudomonas avellanae* and *Pseudomonas syringae* pv. *theae*. *P. avellanae* is the causal agent of bacterial canker and decline of hazelnut (*Corylus avellana*), an economically important disease so far found solely in northern Greece and central Italy (Scortichini, 2002). *P. s*. pv. *theae* causes bacterial shoot blight of tea (*Camellia sinensis*) and it has been reported only from Japan (Takikawa *et al*., 1989; Tomihama *et al*., 2009).

Subsequently, it was shown that also *Pseudomonas syringae* pv. *actinidiae* belongs to genomospecies 8 (Scortichini *et al*., 2002; Manceau and Brin, 2003). This bacterium is the causal agent of bacterial canker of kiwifruit (*Actinidia deliciosa*) and yellow kiwifruit (*A. chinensis*) reported as a destructive pathogen from Japan, South Korea and Italy (Takikawa *et al*., 1989; Koh *et al*., 2003; Ferrante and Scortichini, 2010). Studies on the genetic structure of these pathogens revealed two distinct lineages of *P. avellanae* in Greece and Italy (Scortichini *et al*., 1998, 2006), which may represent a case of pathogenic convergence to the same host plant (Wang *et al*., 2007). Moreover, different *P. s*. pv. *actinidiae* populations causing mild and severe losses to *A. deliciosa* in Italy have been found (Ferrante and Scortichini, 2009, 2010).

The three bacteria belonging to genomospecies 8 are distinguishable either by repetitive-sequence PCR with BOX and ERIC primer sets or by means of nutritional and biochemical tests (Scortichini *et al.,* 2002). However, a rapid detection method enabling their effective discrimination is still lacking. In fact, primers targeting the 16S rDNA gene of *P. avellanae* detect also *P. s*. pv. *actinidiae* either with conventional PCR or TaqMan real-time PCR (Scortichini and Marchesi, 2001; Gervasi and

Scortichini, 2009), whereas primers directed to gene fragments of 16S-23S internal transcribed spacer region (ITS), *gyrB*, *acnB*, *rpoD*, *pgi, cts* or to a putative lipoprotein, are unable to distinguish *P. s*. pv. *actinidiae* from *P. s*. pv. *theae* (Rees-George *et al*., 2010).

To further characterize this genomospecies and to determine differences among these bacteria linked to their pathogenic life style, we performed multilocus sequence typing (MLST) with four housekeeping genes and a thorough screening of 39 effector proteins presence on 35 strains of the genomospecies 8. In addition, we compared the sequences of gene fragments (i.e. 16S rDNA) of the genomospecies 8 bacteria deposited at NCBI for identitifying significant differences for designing specific primers to be utilized in the detection of the three phytopathogens. Finally, further nutritional tests were performed to provide phenotypic traits that can help with the identification procedures.

MATERIALS AND METHODS

Bacterial strains. The *P. avellanae*, *P. s*. pv. *actinidiae* and *P. s*. pv. *theae* strains used in this study are listed in Table 1. They were previously identified or received from international culture collections or individuals scientists. *P. s*. pv. *tomato* DC3000 was used as positive control for the effector detection. All strains were maintained on nutrient agar with 3% sucrose (NSA) at 25-26°C.

Table 1. Strains belonging to genomospecies 8 *sensu* Gardan *et al.* (1999) (*Pseudomonas avellanae*, *P. syringae* pv. *theae*, *P. s.* pv. *actinidiae*) used in this study.

Species/Pathovar	Strain name	Origin	Year of isolation	Host
P. avellanae	CRA-PAV 2058	Italy-Viterbo	1994	*Corylus avellana*
P. avellanae	CRA-PAV 037	Italy-Viterbo	1992	*Corylus avellana*
P. avellanae	CRA-PAV 040	Italy-Viterbo	1993	*Corylus avellana*
P. avellanae	CRA-PAV 1113	Italy-Viterbo	1998	*Corylus avellana*
P. avellanae	CRA-PAV 1267	Italy-Viterbo	2003	*Corylus avellana*
P. avellanae	CRA-PAV 011	Italy-Rome	1991	*Corylus avellana*
P. avellanae	CRA-FRU VIVTGR1	Italy-Rome	2007	*Corylus avellana*
P. avellanae	BPIC 631 ^{T}	Greece	1976	*Corylus avellana*
P. avellanae	BPIC 710	Greece	1987	*Corylus avellana*
P. avellanae	BPIC 665	Greece	1976	*Corylus avellana*
P. avellanae	BPIC 641	Greece	1976	*Corylus avellana*
P. avellanae	BPIC 715	Greece	1987	*Corylus avellana*
P. avellanae	BPIC 1422	Greece	1987	*Corylus avellana*
P. avellanae	BPIC 1435	Greece	1990	*Corylus avellana*
P. s. pv. *theae*	SUPP 2709PT1 ^{T}	Japan	1983	*Camellia sinensis*
P. s. pv. *theae*	SUPP68	Japan	1983	*Camellia sinensis*
P. s. pv. *theae*	CFBP 4092	Japan	1983	*Camellia sinensis*
P. s. pv. *actinidiae*	CRA-FRU 8.52	Italy-Latina	2008	*Actinidia chinensis*-CK3
P. s .pv. *actinidiae*	CRA-FRU 8.43	Italy-Latina	2008	*Actinidia chinensis*-Hort16A
P. s. pv. *actinidiae*	CRA-FRU 8.57	Italy-Latina	2009	*Actinidia chinensis*-Hort16A
P. s. pv. *actinidiae*	CRA-FRU 1.2	Italy-Latina	2009	*Actinidia chinensis*-CK3
P. s. pv. *actinidiae*	CRA-FRU 3.1	Italy-Latina	2009	*Actinidia chinensis*-Hort16A
P. s. pv. *actinidiae*	CRA-FRU 10.14	Italy-Latina	2009	*Actinidia chinensis*-Jin Tao
P. s .pv. *actinidiae*	CRA-FRU 10.15	Italy-Latina	2009	*Actinidia chinensis*-Jin Tao
P. s. pv. *actinidiae*	CRA-FRU 10.16	Italy-Latina	2009	*Actinidia chinensis*-Jin Tao
P. s. pv. *actinidiae*	CRA-FRU 5.1	Italy-Latina	2009	*Actinidia deliciosa*-Hayward
P. s. pv. *actinidiae*	CRA-FRU 8.69	Italy-Latina	2009	*Actinidia deliciosa*-Hayward
P. s. pv. *actinidiae*	CRA-FRU 8.75	Italy-Latina	2009	*Actinidia deliciosa*-Hayward
P. s. pv. *actinidiae*	4252 A.1	Italy-Ravenna	2009	*Actinidia chinensis*-Hort16A
P. s. pv. *actinidiae*	4649.1	Italy-Ravenna	2009	*Actinidia chinensis*-Hort16A
P. s. pv. *actinidiae*	NCPPB 3871	Italy-Rome	1992	*Actinidia deliciosa*-Hayward
P. s. pv. *actinidiae*	NCPPB 3873	Italy-Rome	1992	*Actinidia deliciosa*-Hayward
P. s. pv. *actinidiae*	KW11 ^{T}=NCPPB 3739	Japan	1984	*Actinidia deliciosa*-Hayward
P. s. pv. *actinidiae*	KW31	Japan	1984	*Actinidia deliciosa*-Hayward
P. s. pv. *actinidiae*	KW30=NCPPB 3740	Japan	1984	*Actinidia deliciosa*-Hayward

^{T}: type-strain

Multilocus sequence typing. For multilocus sequence typing (MLST), fragments of *gapA, gltA, gyrB*, and *rpoD* genes coding for glyceraldehyde-3-phosphate dehydrogenase, citrate synthase, DNA gyrase B and sigma factor 70, respectively, were amplified from genomic DNA of *P. avellanae*, *P. s.* pv. *actinidiae* and *P. s*. pv. *theae* strains listed in Table 1. DNA was extracted as already described (Ferrante and Scortichini, 2010). Gene fragments were amplified and sequenced with primers described by Sarkar and Guttman (2004). All PCR reactions were performed in a Bio-Rad MJ Mini thermal cycler. The annealing temperature used for *gapA*, *gltA*, *gyrB* and *rpoD* was 54°C, 72°C, 62°C, and 76°C, respectively and the PCR-amplified products were custom sequenced (Primm, Italy). A neighbor-joining dendrogram was generated with the concatenated data of the four housekeeping genes using the SplitsTree4 software (Huson and Bryant, 2006) to infer the genetic relationships among the genomospecies 8 strains. *P. s.* pv. *tomato* DC3000 (genomospecies 3), indicated as the closest genomospecies by Gardan *et al.* (1999), was used as outgroup. Bootstrap values were obtained using the PhyML method by means of the TOPALi program version 2.5 (Milne *et al.*, 2008), available at http://www. topali.org/.

Effector protein genes detection. The presence of 38 effector genes in *P. avellanae*, *P. s.* pv. *actinidiae* and *P. s*. pv. *theae* strains was evaluated by PCR using the primers listed in Table 2. Two *P. s*. pv. *syringae* strains, Pss P14a and CRA-FRU 10.31, obtained from *C. avellana* and *A. chinensis*, respectively, were also analysed for comparison. Effector primers were designed using the Primer3 program based on the sequences of *P. s.* pv. *tomato* DC3000 and *P. s*. pv. *phaseolicola* 1448A effector genes, and available at *Pseudomonas*-PlantInteraction website (www.pseudomonas-syringae.org). For *hrpK1*, *hopAF1* and *hopAN1* effectors, the primers described by Ferrante *et al.* (2009) were used. PCR was carried out in a total volume of 25 µl containing 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% TritonX-100, pH 9), 50 pmol of each primer, 1.25 U GoTaq DNA Polymerase (Promega, USA), 0.2 mM each dNTPs (Promega, USA), 2 mM $MgCl_2$ and 1 µl of 50 ng DNA extracted by alkaline lysis. All PCR reactions were performed in the above mentioned thermal cycler at the following condition: denaturation at 95°C for 5 min, 30 sec of annealing at 58°C, extension at 72°C for 1 min for 35 cycles, followed by 5 min final extension at 72°C. For *hrpK1* and *hopAF1* and for *hopAN1* amplification annealing temperatures of 64°C and 68°C, respectively, was used. Amplification products were separated on 1% agarose gels, in Tris-acetate-EDTA (TAE) buffer

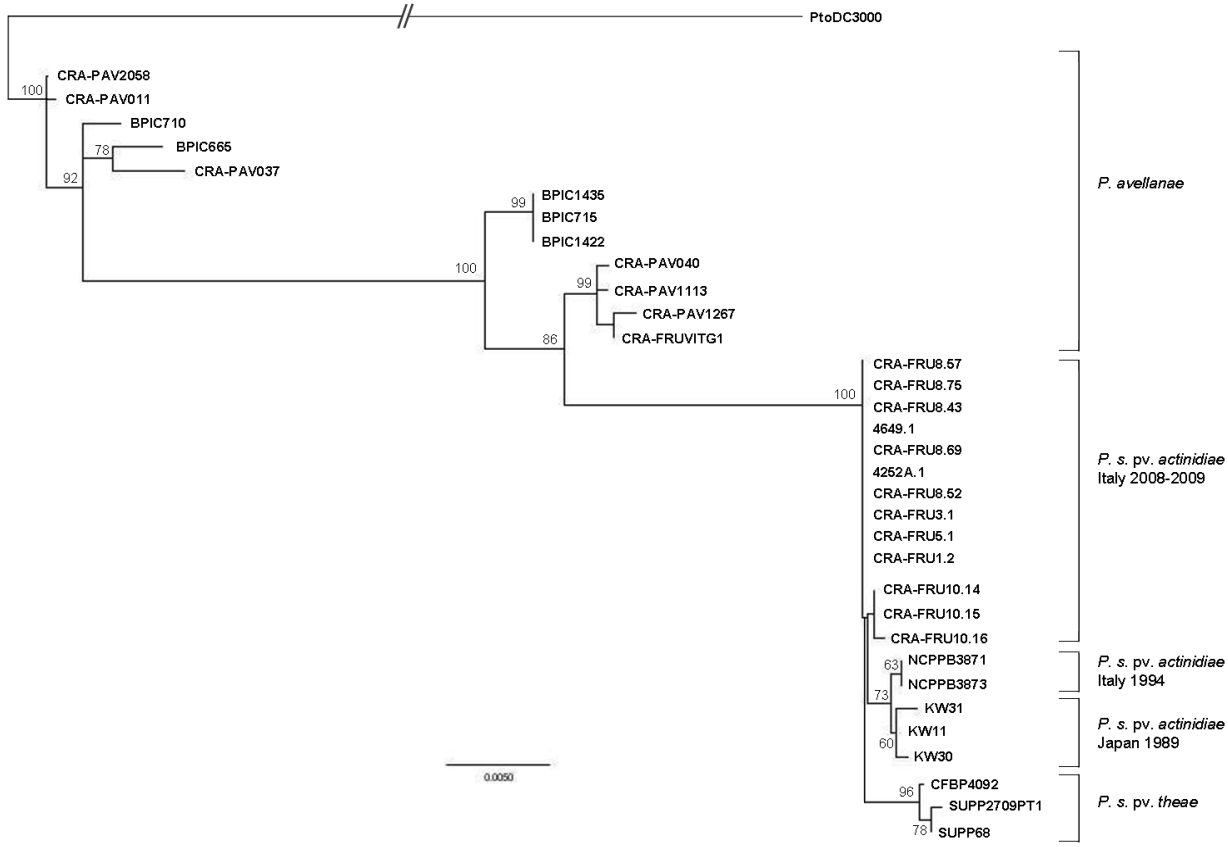

Fig. 1. Dendrogram of relationships using concatenated data and the neighbour-joining algorithm obtained with *gapA, gltA, gyrB*, and *rpoD* nucleotidic gene sequences among strains of genomospecies 8. Bootstrap values are reported at the main nodes.

Table 2. Primers for detecting the presence of effector proteins in genomospecies 8 used in this study.

Primer name	Primer sequence	Reference
avrPto-F	5'-GAACTCCCCAGACCGAGTTA-3'	Ferrante and Scortichini, 2010
avrPto-R	5'-CCAGTGTTCTTTGCGTATGTG-3'	
hopA1-F	5'-CGGCAAGAGGTACGAGATTC-3'	
hopA1-R	5'-TTCAATGCCTTTAGCGTGTG-3'	
hopB1-F	5'-AGGCTATTATCCGCCAACCT-3'	
hopB1-R	5'-TCTTGCAACAGGATGCTCAC-3'	
hopC1-F	5'-TCTGGACACATCGGAAAACA-3'	
hopC1-R	5'-GGAAGGGTAGGCCTGAGTTC-3'	
hopD1-F	5'-GTTACTGAGCTCGCCAGACC-3'	
hopD1-R	5'-TGGTGGCTACATGCAACAAT-3'	
hopF2-F	5'-GGTAATATTTGCGGCACCTC-3'	
hopF2-R	5'-AATCTCCCAGTCGCATTTTG-3'	
hopG1-F	5'-TAATGCCTCGCCTAAGATGG-3'	
hopG1-R	5'-AGCTCACCTTTCAGGCACAT-3'	
hopAN1-F	5'-GCGCACCGTGCAGAGCAT-3'	Ferrante *et al.*, 2009
hopAN1-R	5'-GTCAGCGGGTGGTTCTGC-3'	
hopAF1-F	5'-CAAGCAGAAAGACGGCATC-3'	
hopAF1-R	5'-GCACACGCGACAGCAATG-3'	
hrpK1-F	5'-GACARTGCCGACAAGGACK-3'	
hrpK1-R	5'-ATCKGCGGTTTGCAGAGACT-3'	
avrE1-F	5'-TGGTCAAGGAACTGGAAACC-3'	This study
avrE1-R	5'-CAGAGCCCTGTAGCCTTGTC-3'	
hopE1-F	5'-GAGTTTCCGGTAGCTCGTCA-3'	
hopE1-R	5'-GCCGTATCTCTCGATCCACT-3'	
hopH1-F	5'-CGTCTCGATATCCAGGCATC-3'	
hopH1-R	5'-TTCAGCTCGGATGGAGTTCT-3'	
hopK1-F	5'-ACAACATCTATCGCCCAAGC-3'	
hopK1-R	5'-AGCCATGGTATCCTCTGGTG-3'	
hopM1-F	5'-ATCCGGTAGGTGAGCTGATG-3'	
hopM1-R	5'-ATACCGCTCAACGTGCTCTT-3'	
hopN1-F	5'-CGCTAAGACGCAACACGATA-3'	
hopN1-R	5'-AATAGGCCTGCTGCTCGATA-3'	
hopO1-1-F	5'-TCTGGGACGATGCTTTCTCT-3'	
hopO1-1-R	5'-TGTGCAGACCGGTTACTTTG-3'	
hopO1-2-F	5'-TCAGCTCAATCCACAGCATC-3'	
hopO1-2-R	5'-TACCCGGTTTGAAACGACTC-3'	
hopQ1-1-F	5'-TCACACAAGCCCCTTTTACC-3'	
hopQ1-1-R	5'-ACGTCCGAAAGAACCATGAG-3'	
hopR1-F	5'-CATACGGCTTGAGAACAGCA-3'	
hopR1-R	5'-GTGGTATCGGGATGGTTGAC-3'	
hopS1'-F	5'-GCGCAGAAGTCCTCTTCAAA-3'	
hopS1'-R	5'-ACCTTCCCAAGCTCTGGATT-3'	
hopS2-F	5'-TTTTGCACCCAACAAGACTG-3'	
hopS2-R	5'-CTCGCCACAGAATGAAGTGA-3'	
hopT1-1-F	5'-GAAATCGTCCAGGCAGTCTC-3'	
hopT1-1-R	5'-TCACTTCAAGCATCGAATGG-3'	
hopT1-2-F	5'-AGTGCGTTGGTAGAGGCATT-3'	
hopT1-2-R	5'-ATAGCGGACCAGCTCTTTGA-3'	
hopQ1-2-F	5'-TTCAAGCTGGATTGCTTCCT-3'	
hopQ1-2-R	5'-GACTGGACAAGCGCAGATTT-3'	
hopT2-F	5'-CACGGGTTGGAAGTCCTAAA-3'	
hopT2-R	5'-TGACGCTTTATTGGCTGTTG-3'	
hopV1-F	5'-GACGTCACACACGATGTTCC-3'	
hopV1-R	5'-TTTTTCCAGTGCATCAGCAG-3'	
hopX1-F	5'-CTTTGCCCGGCTACTTACTG-3'	
hopX1-R	5'-CGGACAAATGCATCATCAAG-3'	
hopY1-F	5'-ATGATCAATGCCGCTTCAAT-3'	
hopY1-R	5'-AGTGGCGCTGATCTTCTTGT-3'	
hopAA1-1-F	5'-GATAAATGCGATTGCCGATT-3'	
hopAA1-1-R	5'-GAGCCTTGGGCTCTTTATCC-3'	
hopAA1-2-F	5'-TGCATTACCTGAGCACTTCG-3'	

hopAA1-2-R	5'-TGACTGTTTTCGCCAGTTTG-3'
hopAB2-F	5'-GAGCAGGGTATGCCTACAGC-3'
hopAB2-R	5'-CAAATTCAGCAGGGGATGTT-3'
hopAD1-F	5'-GAGCTTAATGGTCGCTTTGC-3'
hopAD1-R	5'-TCCGCATCTAGCACCTTCTT-3'
hopAH1-F	5'-AGTCCGACTTCAGGCTCAAA-3'
hopAH1-R	5'-CGTCACGCTGTTCGAAGTTA-3'
hopAI1-F	5'-AGAAAAATGCGGAAGCTGAA-3'
hopAI1-R	5'-ATCAGCGAACGGCTTGAC-3'
hopAM1-F	5'-CCAAGTTAAATCGGCAGGAA-3'
hopAM1-R	5'-TCAGGTCAACGCTATTTTCG-3'
hopAO1-F	5'-GCAACCTATTCAGCACAGCA-3'
hopAO1-R	5'-AGGTGATAGGCAAACCGTTG-3'
hopAQ1-F	5'-TCGAATTTCAACCAGCTCAG-3'
hopAQ1-R	5'-CGTCCGACCGAGATTAAATG-3'

0.5X, and visualized by a Bio-Rad Gel Logic 100 UV transilluminator. Presence/absence of a band of the expected size was taken as indication of the presence or absence of the gene in the tested strain. *P. s.* pv. *tomato* DC3000 and *P. s.* pv. *phaseolicola* 1448A were used as positive controls. The analysis was performed twice.

Comparison of 16S rDNA gene. Comparisons were performed with sequences of 16S rDNA (1.434 bp) gene of genomospecies 8 strains deposited at NCBI databank. The type-strains of species and pathovars belonging to genomospecies 8 were included. The following accessions were compared. 16S rDNA: X95745, AJ889838, and AJ889839 for *P. avellanae*; AB001450 for *P. s.* pv. *theae*; AJ889840, EU906856, GQ914994, and D86357 for *P. s.* pv. *actinidiae*. All ambiguous and terminal sequences were edited before data analysis with Geneious 4.7.4. (http://www.geneious.com) and aligned using ClustalW 1.83 (http://www.ebi.ac.uk/tools/ clustalw2/).

Nutritional tests. For determining possible discriminative nutritional tests within genomospecies 8, the utilization of the following compounds was assessed, as described by Lelliott and Stead (1987): L-alanine, dulcitol, erythritol, D-fructose, glycerol, D-glucose, mannitol, D-mannose, maltose, melibiose, myoinositol, L-rhamnose, D-ribose, sorbitol, D-sucrose, D-threalose.

RESULTS

Multilocus sequence typing (MLST). MLST of *gapA*, *gltA*, *gyrB*, and *rpoD* gene fragments provided 2.5 kb of sequences. MLST analysis based on the concatenated data and using the NJ algorithm is shown in Fig. 1. *P. avellanae* strains clustered separately from *P. s.* pv. *actinidiae* and *P. s.* pv. *theae* strains. Within this species, two subgroups were found. One subgroup includes only strains from Greece, whereas another only strains from Italy. In addition, other five strains showed single sequence types. *P. s.* pv. *actinidiae* and *P. s.* pv. *theae* formed a tighter cluster. The *P. s.* pv. *actinidiae* strains from Japan and from past bacterial canker infections in Italy grouped in subclusters separate from the other strains causing recent outbreaks in Italy. The three *P. s.* pv. *theae* strains clustered separately. High bootstrap values supported dendrogram branching. *P. s.* pv. *tomato* DC3000, used as outgroup, was located away from the genomospecies 8.

Detection of effector protein genes. The detection of 38 effector proteins revealed remarkable difference among strains of genomospecies 8 (Fig. 2). Nine effectors, namely *hopO1-1, hopAN1, hopS2, hopY1, hopAA1-1, hopR1, hopB1* and *hrpK1,* are common to all 35 strains tested. In contrast, five effectors, namely *hopC1, hopG1, hopK1, hopT2* and *hopAB2* are unique to all *P. avellanae* strains. Two effector protein genes, *avrE1* and *hopY1*, appear unique to *P. s.* pv. *theae*, whereas other two (*hopA1* and *hopH1*) are unique to the *P. s.* pv. *actinidiae* strains of recent epidemics by *A. chinensis* and *A. deliciosa* in Italy. By contrast, all the *P. s.* pv. *actinidiae* strains previously isolated in Japan, and NCPPB 3871 and 3873, isolated in Italy in 1992, had two effectors (*hopAH1* and *hopAM1*) that are not found in the strains of recent epidemics in Italy. *P. s.* pv. *syringae* strains isolated from *C. avellana* and *A. chinensis* showed a limited number of the effector genes herein assessed.

Table 3. Differences among *Pseudomonas avellanae*, *P. syringae* pv. *actinidiae* and *P. s.* pv. *theae* based on the comparison of 16S rDNA gene sequences obtained from accessions deposited at the National Center for Biotechnology Information (NCBI) and ClustalW algorithm.

Gene position	*P. avellanae*	*P. s.* pv. *actinidiae*	*P. s.* pv. *theae*
16S rDNA			
461-463	ATC	GAT	ATC
1025	A	G	G
1125	T	A	A
1128	A	T	T

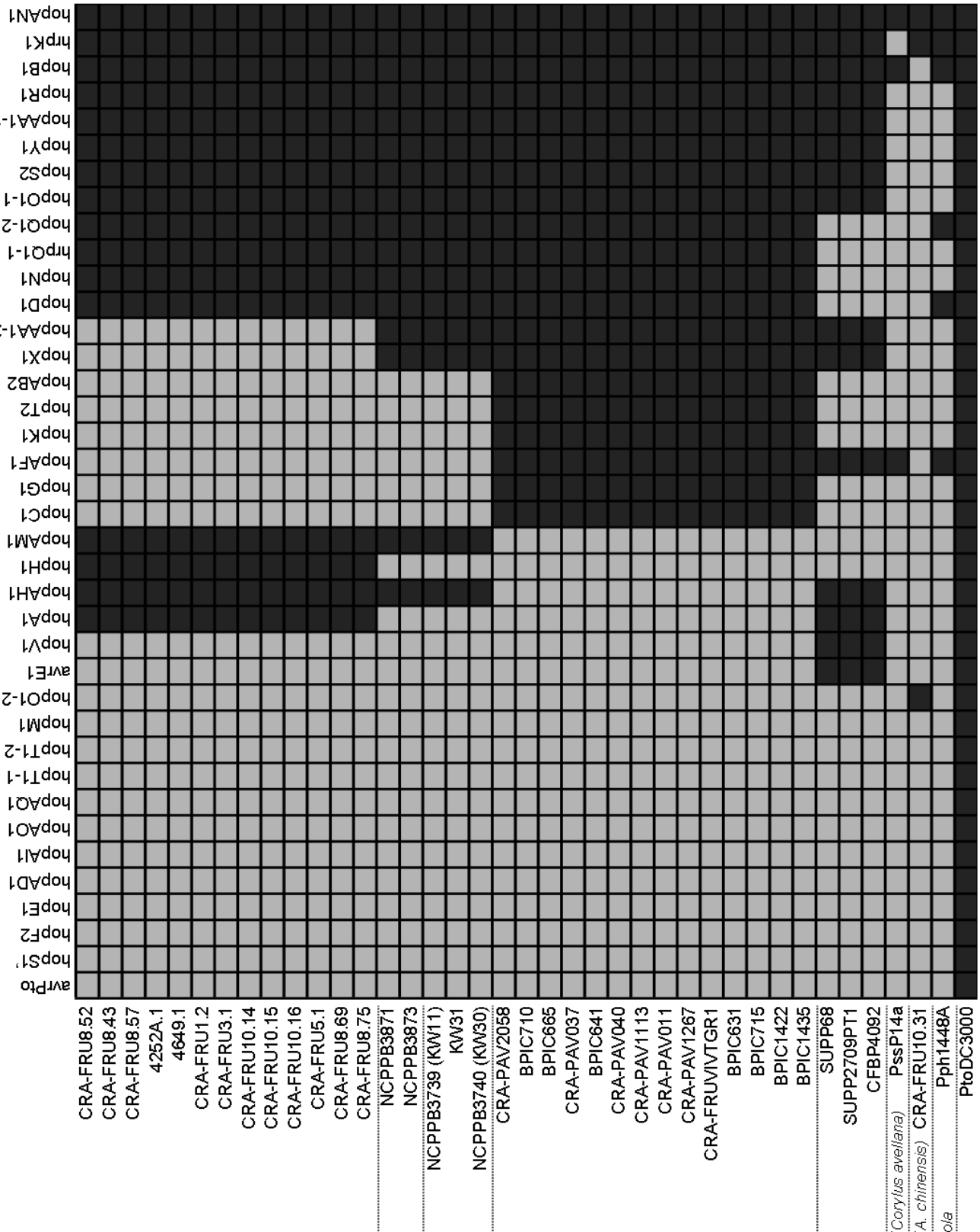

Fig. 2. Presence/absence of effector proteins in strains of genomospecies 8 (*Pseudomonas avellanae*, *P. syringae* pv. *theae*, *P. syringae* pv. *actinidiae*).

Table 4. Nutritional tests performed with strains of genomospecies 8 (*Pseudomonas avellanae*, *P. syringae* pv. *theae* and *P. syringae.* pv. *actinidiae*).

	P. avellanae	*P.s.* pv. *theae*	*P. s.* pv. *actinidiae*		
			Japan 1989	Italy 1994	Italy 2008-2009
Sorbitol	-	+	+	+	+
Erythritol	-	-	-	-	-
Glycerol	+	+	+	-	+
D(+)-trehalose	-	-	-	-	-
D(+)-mannose	+	+	+	+	+
Mannitol	+	+	+	+	+
L-rhamnose	-	-	-	-	-
L-alanine	+	+	+	+	+
D-fructose	+	+	+	+	+
Melibiose	-	-	-	-	-
Maltose	-	-	-	-	-
Dulcitol	-	-	-	-	-
D(-)-ribose	+	+	+	+	+
D(+)-sucrose	+	+	+	+	+
Myoinositol	+	+	+	+	+
D(+)-glucose	+	+	+	+	+

Comparison of 16S rDNA gene and nutritional tests. The comparison of the 16S rDNA gene (1,434 bp) sequences of eight isolates of *P. avellanae*, *P. s.* pv. *theae* and *P. s*. pv. *actinidiae,* including all the type-strains, disclosed some slight but consistent differences (Table 3). In particular, GAT was found for *P. s*. pv. *actinidiae* and ATC for *P. avellanae* and *P. s.* pv. *theae* in position 461-463. Other single-base differences were present at positions 1025, 1126 and 1128 (Table 3). Also the assessment of nutritional tests revealed some differences within the genomospecies 8 (Table 4). In fact, *P. avellanae* was unable to utilize sorbitol, contrary to *P. s.* pv. *theae* and *P. s*. pv. *actinidiae*. In addition, the *P. s*. pv. *actinidiae* strains of the recent epidemics in Italy utilized glycerol, contrary to the strains isolated in 1992. All the other nutritional features herein assessed were identical.

DISCUSSION

This work has shown that bacterial strains belonging to genomospecies 8 represent a tight cluster within the genomospecies grouping suggested by Gardan *et al*. (1999). In fact, MLST analysis revealed that: (i) all strains of this genomospecies cluster distantly from *P. s.* pv. *tomato* DC3000 (genomospecies 3) which, based on DNA-DNA hybridization and ribotyping, is the closest genomospecies (Gardan *et al.*, 1999); (ii) *P. s*. pv. *theae* and *P. s*. pv. *actinidiae* are genetically more closely related to one another than to *P. avellanae,* which also show a higher genetic variability. Interestingly, MLST analysis disclosed consistent differences among the *P. s.* pv. *actinidiae* strains isolated from different bacterial canker outbreaks in Japan and Italy, thus confirming that the current severe epidemic registered in Italy is caused by different populations of the pathogen (Ferrante and Scortichini, 2010; Marcelletti and Scortichini, 2011). Some phenotypic features can also to distinguish *P. avellanae* from *P. s*. pv. *theae* and *P. s*. pv. *actinidiae* since *P. avellanae* does not utilize sorbitol, contrary to the other genomospecies 8 members. Moreover, the 16S rDNA gene sequences revealed only one difference within genomospecies 8 at position 461-463, i.e. GAT for *P. s*. pv. *actinidiae* and ATC for *P. avellanae* and *P. s.* pv. *theae*. However, whether this difference is enough to differentiate these pathogens using detection techniques targeting such gene remains to be established.

The study of 38 effector protein genes revealed interesting differences among the strains of genomospecies 8. In fact, there were nine effector protein genes displayed by all 35 bacterial strains tested. However, each pathogen displayed a set of distinctive effector genes that putatively could be linked to the restricted pathogenicity showed by the members of this genomospecies. Moreover, *P. s.* pv. *actinidiae* strains of past epidemics in Japan and central Italy showed two effector genes that are absent in the strains of the current epidemics in Italy. These, however, have two effector genes (*hopA1* and *hopH1*) which do not occur in the other strains. Such data would suggest that effector genes might be involved also in the differential pathogenicity of diverse populations of the same pathogen. The role(s) played by such effector genes when acting singly or in association would seem an interesting area of research to further elucidate the molecular interaction of this genomospecies with their host plants.

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