FURTHER MOLECULAR CHARACTERIZATION OF *PSEUDOMONAS SYRINGAE* pv. *CORYLI*

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SUMMARY

The aim of this paper was to further investigate some molecular features of *Pseudomonas syringae* pv. *coryli*, the causal agent of hazelnut bacterial twig dieback, and to assess its genetic relationship with some other phytopathogenic pseudomonads of various genomospecies (*sensu* Gardan *et al.*, 1999). The characterization was done by cloning and sequencing some key genes utilized in taxonomical studies and/or in plant-pathogen interaction such as 16S rDNA, *hrpL*, as well as the effector genes of the pathogenicity island and the phaseolotoxin *tox* gene cluster of *Pseudomonas syringae* pv. *phaseolicola.* Analysis of *hrpL* and 16S rDNA genes with Mega 3.1 software and the neighbor-joining clustering algorithm showed a relationship of *P. syringae* pv. *coryli* with pseudomonads of genomospecies 1, suggesting its inclusion into this group. Investigation of effector and phaseolotoxin genes showed the inconsistent presence of these genes in some *P. syringae* pv. *coryli* strains. Neither the effectors nor the PCR product related to the *tox* gene cluster of *P. syringae* pv. *phaseolicola* were amplified from the majority of the strains. However, one strain showed the presence of a homologous of *avrPphD*, and another contained either this effector (*avrPphD*) or the 1.9 kb fragment of the phaseolotoxin *tox* gene cluster.

Key words: 16S rDNA, *hrpL*, effector genes, genomospecies, sequencing.

INTRODUCTION

Pseudomonas syringae pv. *coryli* the causal agent of twig dieback of hazelnut (*Corylus avellana L.*), has been recorded so far only from Italy and Germany (Scortichini *et al.*, 2005; Poschenrieder *et al.*, 2006; Cirvilleri *et al.*, 2007). As shown by analysis of whole cell fatty acids, ERIC-PCR, partial *hrpL* and partial 16S rDNA gene sequencing (Scortichini *et al.*, 2005), this pathogen clearly clusters into a group distinct from *Pseudomonas avellanae* and from other *P. syringae* pathovars.

To further assess its relationships with strains belonging to different plant bacterial genomospecies (*sensu* Gardan *et al.*, 1999), phylogenetic trees were constructed using sequences of the entire 16S rDNA and *hrpL* genes, as well as of those of HrpL deduced amino acid sequences. These genes have been used to study the genetic relationship among *P. syringae* spp. (Cournoyer *et al.*, 1996; Sawada *et al.*, 1999; Manceau and Brin, 2003). In addition, since effector genes seem to play a role in the fitness and pathogenicity of phytopathogenic bacteria (Chang *et al.*, 2000; Chen *et al.*, 2000), the presence of effector gene homologues of the pathogenicity island of *Pseudomonas syringae* pv. *phaseolicola* (= *Pph*-PAI) (Jackson *et al.*, 1999) was investigated in *P. syringae* pv. *coryli* strains. So far, the occurrence of these effector genes has been studied in *P. syringae* pv. *tomato*, some pathovars of *Pseudomonas savastanoi* (*nerii, glycinea, savastanoi, fraxini*) (Jackson *et al.*, 2002), and *P. avellanae* (Jackson *et al.*, 2002; Loreti *et al.*, 2003). These studies have shown that not all the *Pph*-PAI homologue genes are present in the genome of the bacterial strains surveyed (Jackson *et al.*, 2002; Loreti *et al.*, 2003).

The two main objectives of this study were to assess (i) the relationship of *P. syringae* pv. *coryli* with some pseudomonad strains of different genomospecies and (ii) the occurrence of several effector and phaseotoxin *tox* genes in *P. syringae* pv. *coryli.*

MATERIALS AND METHODS

Bacterial cultures and growth conditions. All *P. syringae* pv. *coryli* and other pseudomonad strains were routinely cultured on nutrient agar (Oxoid, Basingstoke, UK) supplemented with 1% glucose (NGA) at 26-28°C. Stock cultures were lyophilised and regenerated on NSA medium, at 26-28°C. *P. syringae* pv. *coryli* strains isolated from Piedmont (northern Italy) were previously deposited at the NCPPB [National Collection of Plant Pathogenic Bacteria, Csl, York, UK (NCPPB 4273^T, NCPPB 4274)] and at the ISPaVe [Culture Collection of CRA-Centro di Ricerca per la Pa-

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Table 1. Bacterial gene sequences (*hrpL* and 16S rDNA) used in this study.

Bacterial species	Strain designation[1]	Authors or reference	Accession number	
			hrpL	16S
P. syringae pv. *coryli*	NCPPB 4273^{T}	Loreti and Scortichini	AM231726	AJ889841
	NCPPB 4274	Loreti	AM231730^{TP}	_
	DPP 48	Loreti	AM231731^{TP}	_
	ISPaVe 598	Loreti and Scortichini	AM231729	AM399035^{TP}
	DPP 51	Loreti	AM231728	_
	ISPaVe 595	Loreti and Scortichini	AM231727^{TP}	AM399034^{TP}
P. syringae pv. *syringae*	B 728a	Feil *et al.*	CP000075	NC_007005
	61	Xiao *et al.*	U03854	_
	NCPPB 3869	Loreti and Scortichini	AM400987^{TP}	AM399036^{TP}
P syringae pv. *coronafaciens*	LMG 13190^{T}	Moore	_	Z76660
P. syringae. pv. *P haseolicola*	1448A^{T}	Joardar *et al.*	CP000058	NC_005773
P. syringae pv. *pisi*	MAFF301208	Sawada	AB016389	_
P. syringae pv. *aptata*	MAFF302253^{T}	Sawada	AB016309	_
P. syringae. pv. *atrofaciens*	CFBP2213	Cournoyer and Prigent-Combaret	AF478342	_
P. syringae pv. *japonica*	MAFF301072^{T}	Sawada	AB016329	_
P. syringae pv. *glycinea*	MAFF302260^{T}	Sawada	AB016325	_
P. syringae pv. *tagetis*	LMG 5090	Songs *et al.*	DQ246442	_
	MAFF302271	Sawada	_	AB001449
P. syringae pv. *papulans*	CFBP 1754	Cournoyer and Prigent-Combaret	AF478343	_
P. syringae pv. *morsprunorum*	MAFF302280^{T}	Sawada	AB016361	AB001445
P. syringae pv. *eriobotryae*	MAFF302259	Sawada	AB016321	_____
P. syringae pv. *tomato*	DC3000	Buell *et al.*	NC-004578	NC_004578
P.syringae pv. *maculicola*	MAFF302264	Sawada	AB016341	AB001444
P. syringae pv. *theae*	PT1^{T}	Sawada	AB016413	AB001450
P. syringae pv *actinidiae*	NCPPB3739^{T}	Sawada	AB016305	AB001439
	NCPPB 3740	Scortichini	_	AJ889840^{TP}
P. avellanae	BPIC 631^{T}	Janse *et al.*	_	X95745
	BPIC 714	Scortichini	_	AJ889838^{TP}
	ISF 2059	Scortichini	_	AJ889839^{TP}
	NCPPB 3873	Loreti	AM400985*	_
	BPIC FL13	Loreti	AM400986*	_
P. amygdali	ATCC 33614	Anzai	_	D84007
P. cannabina	CFBP 2341^{T}	Behrendt *et al.*	_	AJ492827*
P. tremae	CFBP 6111^{T}	Behrendt *et al.*	_	AJ492826*
P. viridiflava	LP23.1a	Jakob *et al.*	AF508899	AY574908

* = partial sequence; ^{T} = type strain; ^{TP} = this paper

[1]ATCC: American Type Culture Collection, Manassas, VA, USA; BPIC: Benaki Phytopathological Institute Culture Collection, Kiphissia-Athens, Greece; CFBP: Collection Francaise de Bactéries Phytopathogénes, Angers, France; DPP: Culture Collection of Dipartimento di Protezione delle Piante, University of Sassari, Italy; ISF: Culture Collection of CRA, Centro di Ricerca per la Frutticoltura, Rome, Italy; ISPaVe: Culture Collection of CRA, Centro di Ricerca per la Patologia Vegetale, Rome, Italy; LMG: Culture Collection, University of Gent, Belgium; MAFF: Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan; NCPPB: National Collection of Plant Pathogenic Bacteria, York, UK

tologia Vegetale, Rome, Italy, (ISPaVe 595, ISPaVe 598)]. *P. syringae* pv. *coryli* strains from Sardinia (southern Italy) were obtained from the culture collection of the Dipartimento per la Protezione delle Piante, University of Sassari, Italy (DPP 48, DPP51).

16S rDNA and *hrpL* sequencing. The entire 16S rDNA gene of *P. syringae* pv. *coryli* ISPaVe 595, ISPaVe 598, *P. syringae.* pv. *syringae* NCPPB 3869, *P. syringae* pv. *actinidiae* NCPPB 3740, and *P. avellanae* BPIC 714, ISF 2059 was sequenced. Strains used for amplification and sequencing of the full length *hrpL* gene were: *P. syringae* pv. *syringae* NCPPB 3869, *P. syringae.* pv. *coryli* NCPPB 4274, ISPaVe 595, and DPP 48. Template preparation, PCR amplification of the 16S rDNA and *hrpL* genes, and nucleotide sequencing were according to Scortichini *et al.* (2005).

Full length 16S rDNA and *hrpL* sequences, previously obtained by Scortichini *et al.* (2005) for *P. avellanae* (strain BPIC 631 for 16S rDNA and strains NCPPB 3873, BPIC and FL13 for *hrpL*), for *P. syringae* pv. *coryli* (NCPPB 4273 and ISPaVe 598 for both genes and DPP 51 for *hrpL*) and sequence data of different phytopathogenic bacteria belonging to different genomospecies from GenBank database were included in the analyses and are reported in Table 1.

Detection of effector and phaseolotoxin genes. Strains investigated for the presence of effector genes by DNA amplification were: *P. syringae* pv. *coryli* strains NCPPB 4273, NCPPB 4274, ISPaVe 598, ISPaVe 595, DPP 48, and DPP 51. Strains *P. syringae* pv. *phaseolicola* 1448A and *P. avellanae* NCPPB 3873 were included as controls. The amount of purified bacterial genomic DNA was estimated comparing known standards in ethidium bromide stained 1% agarose gel. PCR reactions were conducted in a final volume of 50 µl containing 1x PCR buffer (Fermentas, Vilnius, Lithuania), 0.05 U/µl Pfu DNA polymerase (Fermentas, Vilnius, Lithuania), 0.2 mM dNTPs, 0.04 ng μl^{-1} BSA, 0.1 µM each primer, 25 ng genomic DNA. The thermal profile consisted of an initial hot-start step 70°C for 3 min and an initial denaturation step 94°C for 3min, followed by 30 cycles at 94°C (1 min), annealing at the appropriate temperature for each gene (1.5 min) and extension at 72°C (from 1.5 min to 2 min, depending on the gene length).

PCR primers for *virPphA*, ORF3, *avrPphC,* and *avrPphD,* gene amplification were obtained from R.W. Jackson (personal communication). Primers for ORF2: (ORF2-5':GTGAAGAAGAACAAACGCCCAGT; ORF2-3':TCAAATCGGCAGCCCTGC), ORF4 (ORF4-F:ATGGTGGGTATCAACAGAGCA; ORF4-R: TCACTCCACACGTGCTCTC) and *avrPtoB* (*AvrPtoB*-LF: GGAGAGGATCAGCATATG; *AvrPtoB*-LR: TCAGGGGACTATTCTAAAAG) were designed on published sequences.

Amplification of a 1.9 kb fragment of phaseolotoxin *tox* gene was as described by Prosen *et al.* (1993). All primers were synthesized by Invitrogen-Life Technologies (Paisley, UK). PCR reactions were analysed by electrophoresis in 1% agarose gel and stained with ethidium bromide. All amplicons were excised, eluted or directly purified with the NucleoSpin Extract II Kit (Macherey-Nagel, Duren, Germany). DNA sequencing was by GeneLab (Rome, Italy).

Sequence analysis. *hrpL* and 16S rDNA sequences were aligned with the corresponding homologous gene of different *Pseudomonas* species belonging to various genomospecies (*sensu* Gardan *et al.* 1999), shown in Table 1. Multiple alignment of nucleotide and amino acid sequences was done using ClustalW algorithm (Higgins and Sharp 1998). Phylogenetic analysis was with MEGA software, version 3.1 (Kumar *et al.*, 2004) and computed using Kimura's 2-parameter model (Kimura 1980) and the neighbour-joining (NJ) clustering algorithm (Saitou and Nei 1987). Bootstrap analyses were made to estimate the significance level of the NJ tree internal branches (Hedges, 1992).

RESULTS AND DISCUSSION

16S rDNA and *hrpL* genes sequencing. The molecular relationship between 16S rDNA gene sequences of *P. syringae* pv. *coryli*, *P. syringae* pathovars and other phytopathogenic pseudomonad are reported in Fig. 1, in which three main groups are represented. One group with 95% bootstrap (I) showed a pronounced progression of sub-grouping comprising sequences of different genomospecies. Strains of *P. syringae* pv. *coryli* clustered (90% bootstrap) in one subgroup, branched in two lineages, one including *P. syringae* pv. *coryli* ISPaVe 595, ISPaVe 598 and *P. syringae* pv. *pisi* (genomospecies 1) and the other the type strain *P. syringae* pv. *coryli* NCPPB 4273 together with *P. syringae* pv. *syringae* strains (NCPPB 3869 and B728a) of genomospecies 1. Strains of genomospecies 2 formed a separated lineage (62% bootstrap) that comprised also *P. tremae* and *P. coronafaciens* which, according to Gardan *et al.*(1999) belong to genomospecies 5 and 4, respectively.

Sequencing of full length 16S rDNA gene confirmed, as previously observed from partial 16S rDNA sequencing (Scortichini *et al.*, 2005), that *P. syringae* pv. *coryli* grouped separately from *P. avellanae* strains. This latter species clustered in a second well supported group (II, with 85% bootstrap value), with strains belonging to genomospecies 3 (*P. syringae* pv. *tomato* and *P. syringae* pv. *morsprunorum)* and 8 (*P. syringae* pv. *theae.)* Strains of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *maculicola* grouped in a third separate group (III). *P. viridiflava* (genomospecies 6), branched separately from all strains. However, further studies including a larger number of

strains and other techniques may provide a clearer picture on the relationships among the three main groups of *P. syringae* pathovars and related *Pseudomonas* species.

The neighbour-joining (NJ) tree based on the relationship between *hrpL* sequences is shown in Fig. 2 in which three main groups are represented. The first group (I) showed two subgroups. One subgroup was represented by *P. syringae* pv. *coryli* strains that branched, with a high bootstrap value (99%), with several *P. syringae* pathovars (*P. syringae* pv. *syringae, P. syringae* pv. *pisi, P. syringae* pv. *aptata, P. syringae* pv. *japonica, P. syringae* pv. *atrofaciens, P. syringae* pv. *papulans, P. syringae* pv. *aceris*) all belonging to genomospecies 1 *sensu* Gardan *et al*. (1999). Sequence homology ranged from 94 to 100%, with the highest values observed between strains of *P. syringae* pv. *coryli* (DPP 51 and DPP 48) and *P. syringae* pv. *syringae* NCPPB 3869, and between strains of *P. syringae* pv. *coryli* NCPPB 4273 and NCPPB 4274. Moreover, *P. syringae* pv. *coryli* strains NCPPB 4273, NCPPB 4274, ISPaVe 595 and

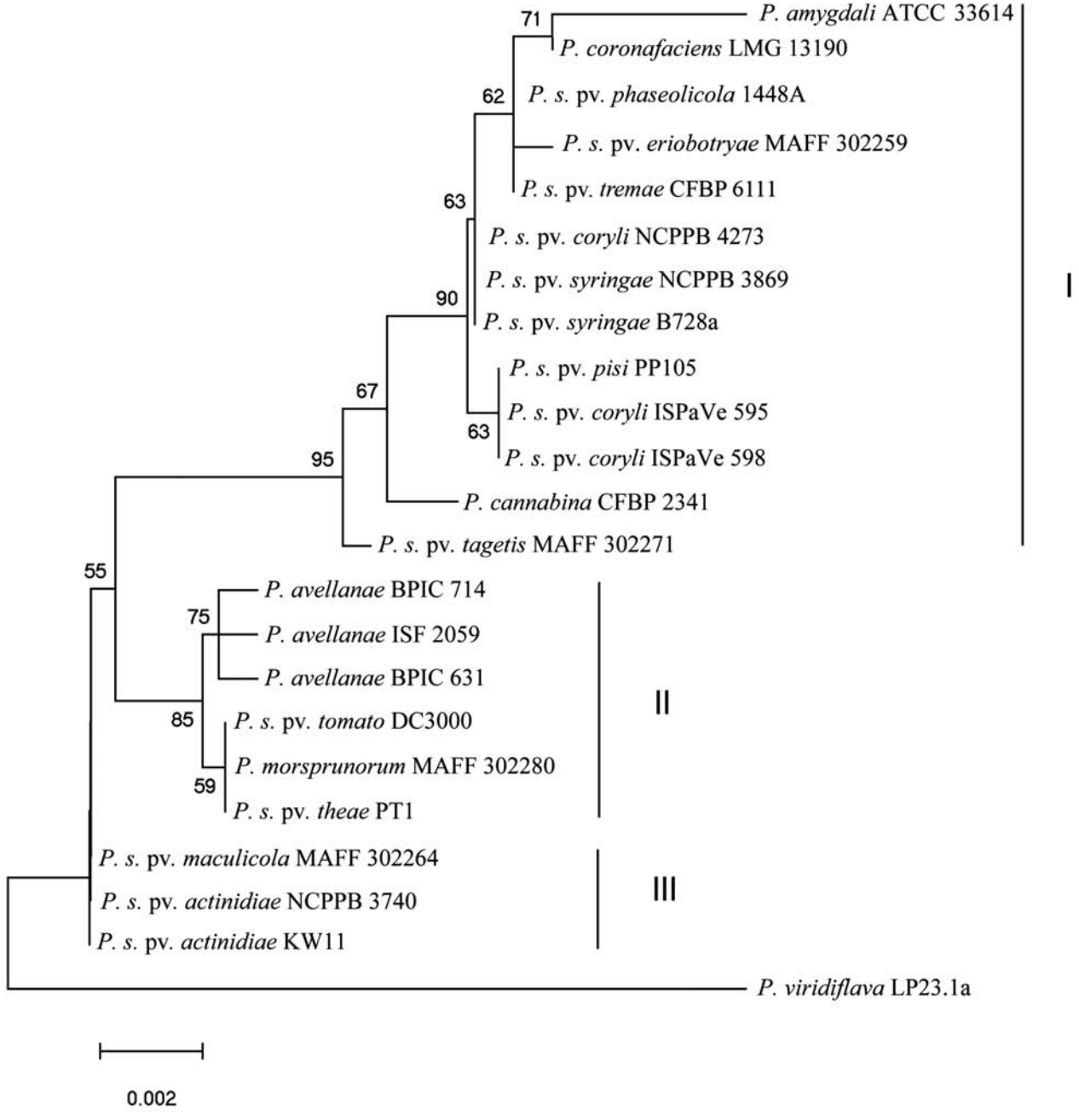

Fig. 1. Neighbour-joining tree based on the ClustalW alignment of the 16S rDNA sequences of different *Pseudomonas* species belonging to different genomospecies *sensu* Gardan *et al.* (1999). Bootstrap values (1000 replicates) are given for the branches with >50% support. The scale at the bottom indicates a genetic distance proportional to the number of substitutions per site.

ISPaVe 598 showed 94-96% homology with strains DPP 48, DPP 51 of the same species and with several *P. syringae* strains of genomospecies 1 (namely: *P. syringae.* pv. *papulans*, *P. syringae* pv. *syringae*, *P. syringae* pv. *pisi*, *P. syringae* pv. *apatata*, *P. syringae.* pv. *japonica*, *P. syringae.* pv. *atrofaciens, P. syringae* pv. *aceris*). *P. syringae* pathovars of genomospecies 2 (*P. syringae* pv. *myricae*, *P. syringae.* pv. *eriobotryae*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *glycinea*) branched, separately, in the second well supported (100% bootstrap) subgroup.

The full length *hrpL* sequence of *P. syringae* pv. *coryli* grouped separately from *P. avellanae* confirming the data obtained by Scortichini *et al.* (2005) from partial *hrpL* sequencing. Moreover, *P. avellanae, P. syringae* pv. *actinidiae* and *P. syringae* pv. *theae* grouped together in a single group (II) with 100% bootstrap value (Fig. 2) and with homology ranging between 97-99%. In particular, strains of genomospecies 3 and 8 formed two major branches. *P. avellanae* strains grouped together with high bootstrap value (99%); *P. syringae* pv. *tomato*, *P. syringae* pv. *maculicola and P. syringae.* pv. *morsprunorum* showed a progression of subgrouping that lead to a subgroup comprising *P. syringae* pv. *theae* and *P. syringae* pv. *actinidiae. P. syringae* pv. *tagetis* (genomospecies 7)

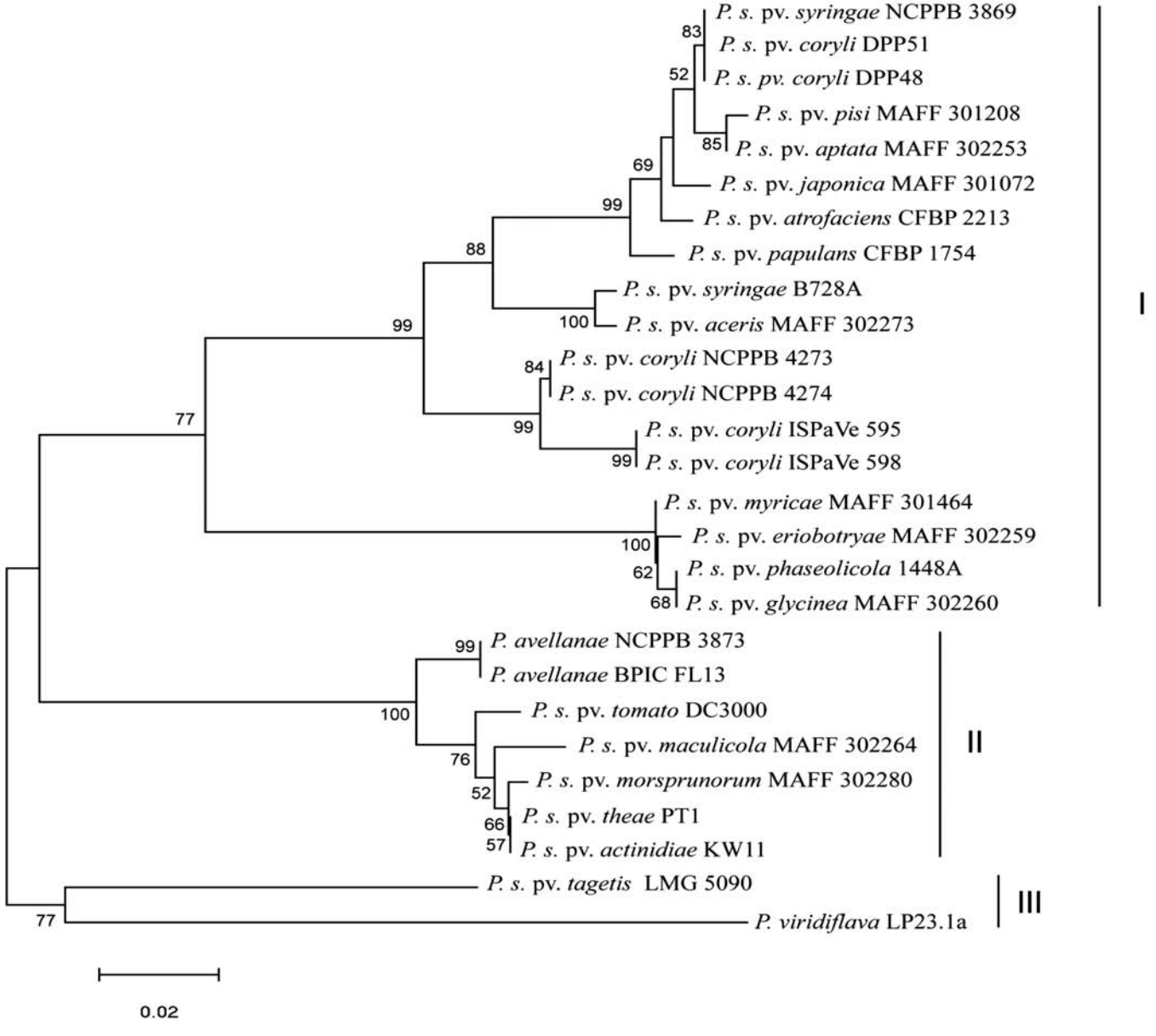

Fig. 2. Neighbour-joining tree based on the ClustalW alignment of the *hrpL* sequences of different *Pseudomonas* species, belonging to different genomospecies *sensu* Gardan *et al.* (1999). Bootstrap values (1000 replicates) are given for the branches with >50% support. The scale at the bottom indicates a genetic distance proportional to the number of substitutions *per* site.

and *P. viridiflava* (genomospecies 6) formed a separated well supported (77% bootstrap value) group (III).

The topology of NJ tree from the derived hrpL amino acid sequences was similar to that deduced from nucleotide sequences (data not shown).

In conclusion, the analysis based on sequencing of 16S rDNA and *hrpL* genes and the HrpL deduced amino acid sequences of representative strains of *P. syringae* pv. *coryli* revealed its relatedness with *Pseudomonas syringae* strains of genomospecies 1.

Since the *hrpL* gene is considered a good candidate for establishing phylogenetic relationship among phytopathogenic bacteria and it has been used to investigate genetic relationships of *P. syringae* spp. (Cournoyer *et al.*, 1996; Sawada *et al.*, 1999; Manceau and Brin, 2003), based on the data obtained in the present study we propose to include *P. syringae* pv. *coryli* in genomospecies 1.

Detection of effector genes. Homologues of the most representative genes of the *Pph*-PAI (*virPphA*, ORF2, ORF3, ORF4, *avrPphC*), and other effector genes (*avrPphD*, a*vrPtoB*) were checked for presence in *P. syringae* pv. *coryli* strains. These genes were chosen for their importance for the fitness and for the pathogenicity of model-phytopatogenic bacteria such as *P. syringae* pv. *phaseolicola* 1448A and *P. syringae* pv. *tomato* DC3000. Previous search of these effector genes in *P. syringae* pv. *tomato*, in *P. savastanoi* pathovars *nerii, glycinea, savastanoi, fraxini* (Jackson *et al.*, 2002) and in *P. avellanae* (Jackson *et al.*, 2002; Loreti *et al.*, 2003) showed that not all the *Pph*-PAI homologue genes were present in different strains. Some of the effector genes, such as *virPphA*, *avrPtoB* and *avrPphD* are more widely distributed among phytopathogenic bacteria (Jackson *et al.*, 2002; Boch *et al.*, 2002; Arnold *et al.*, 2001). Our investigation showed that the majority of *P. syringae* pv. *coryli* strains do not seem to contain any of these effectors, with the exception of DPP 51 and ISPaVe 598, which possessed an *avrPphD* homologue. Sequencing of the amplicons confirmed the identity of homologous genes. Nucleotide sequences of homologous effector genes were deposited in the GenBank database (Accession Nos: AM410553, AM410554). Despite their wide distribution, neither *virPphA* nor *avrPtoB* homologues were amplified from *P. syringae* pv. *coryli*. *virPphA* is known to act as a virulence gene (Jackson *et al.*, 1999) for *P. syringae* pv. *phaseolicola* and is present on the plasmid or on the chromosome of several *P. savastanoi* pathovars, but is absent also in *P. avellanae* (Jackson *et al.*, 2002) which, instead, contains a homologue of *avrPtoB* (Loreti *et al.*, 2003). Is known that AvrPtoB homologues from different *P. syringae* pathovars have conserved avirulence and virulence activities (Lin *et al.*, 2006), suggesting a similar virulence activity for these pathogens. Probably, the virulence of *P. syringae* pv. *coryli* should be controlled by other effectors. Nevertheless, it is possible that these genes may have sequence variations with respect to *P. syringae* pv. *phaseolicola* or *P. syringae* pv. *tomato,* thus making more difficult the amplification of homologues in *P. syringae* pv. *coryli*.

Detection of phaseolotoxin *tox* gene. Only the strain ISPaVe 598, among the *P. syringae* pv. *coryli* strains, apparently contains an homologue of the 1,9 fragment of phaseolotoxin *tox* gene. Phaseolotoxin, originally considered to be restricted to *P. syringae* pv. *phaseolicola* (Hatziloukas *et al.*, 1995), was also found to be produced by *P. syringae* pv. *actinidiae* (Tamura *et al.*, 1989). The other pathovars of *P. syringae* are not able to produce phaseolotoxin and also do not have the *argK* gene like some non-toxigenic strains of *P. syringae* pv. *phaseolicola* which do not carry the *argK-tox* gene cluster (Gonzalez *et al.*, 2003). However, Mastromarino *et al.* (2007), showed the presence of the 1,9 kb of the *tox* gene of *P. syringae* pv. *phaseolicola* (Prosen *et al.*, 1993) in *P. avellanae* strain NCPPB 3873 and assumed some interaction between *P. avellanae* and bean leaf. Moreover, another strain isolated from hazelnut in the Langhe area (northern Italy) together with the *P. syringae* pv. *coryli* strains was reported to have homologies with *P. syringae* pv. *phaseolicola* (Loreti *et al.*, 2003). Finally, this part of fragment of *tox* gene is present also in one strain of *P. syringae* pv. *coryli*, the ISPaVe 598 (Accession number: AM410903). Primers enabled the amplification of the region corresponding to ORF5 and ORF6 of the 1,9 kb fragment of the *tox* gene; the sequencing of 713 bp fragment of this region showed 100% homology with homologue sequences of *P. syringae* pv. *phaseolicola* (Accession Nos: U27310; DQ 141263; CP000058), *P. syringae* pv. *actinidiae* (Accession No: AB237163) and 99% with other homologue sequences of *P. syringae* pv. *phaseolicola* (Accession nos: L35553; AB237164) and *P. avellanae* BPIC 631 (Accession no: AM283541).

The search of homologous of gene involved in plant-pathogen interaction is a useful strategy to improve the knowledge of some genome traits of phytopathogenic bacteria infecting woody species (for instance *P. syringae* pv. *coryli*) for which studies on interaction with the host are more difficult than for bacteria infecting herbaceous hosts. For this purpose, further analyses will be performed using degenerated primers and also using primers for other effectors to better investigate the presence of effector genes in *Pseudomonas syringae* pv. *coryli*.

ACKNOWLEDGEMENTS

The authors thank Dr. Daniele De Simone for preparing the figures. Research partly financed by the Italian Ministry of Agricultural and Forestry Policies, project CO.RI.BIO "Ricerche sul nocciolo finalizzate all'ottenimento di produzioni biologiche di qualità". Publication no. 5.

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Received July 11, 2007
Accepted November 20, 2007