Definition of Plant-Pathogenic *Pseudomonas* Genomospecies of the *Pseudomonas syringae* Complex Through Multiple Comparative Approaches

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ABSTRACT

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A total of 34 phytopathogenic strain genomes belonging to the *Pseudomonas syringae* species complex and related species, including many pathotype strains, were assessed using average nucleotide identity (ANI) analysis. Their taxonomic relationships were consistently confirmed by the tetranucleotide frequency correlation coefficient (TETRA) values, multilocus sequence typing analysis (MLSA) performed with seven housekeeping genes, using both maximum likelihood and Bayesian methods, and split consensus network analyses. The ANI, MLSA, and split consensus analyses provided consistent and identical results. We confirmed the occurrence of the well-demarcated genomospecies inferred *sensu* Gardan et al. using DNA-DNA hybridization and ribotyping analyses. However, some *P. syringae* strains of the pathovars *morsprunorum* and *lachrymans* were placed in different genomospecies in

our analyses. Genomospecies 1, 2, 4, 6, and 9 resulted well demarcated, whereas strains of genomospecies 3 and 8 had ANI values between 95 and 96% in some cases, confirming that this threshold reveals very closely related species that might represent cases of splitting entities or the convergence of different species to the same ecological niche. This study confirms the robustness of the combination of genomic and phylogenetic approaches in revealing taxonomic relationships among closely related bacterial strains and provides the basis for a further reliable demarcation of the phytopathogenic *Pseudomonas* species. Within each species, the pathovars might represent distinct ecological units. The possibility of performing extensive and standardized host range and phenotypic tests with many strains of different pathovars can assist phyto bacteriologists for better determining the boundaries of these ecological units.

Additional keywords: genome comparison, polyphasic taxonomy.

The genus *Pseudomonas* includes metabolically versatile species capable of living as parasites and/or saprophytes in the most diverse terrestrial habitats, including plants (38). Some *Pseudomonas* spp. damage certain cultivated and wild plants and mushrooms. Twenty-one plant-pathogenic *Pseudomonas* spp. have been described (5,6,22). Among these, *P. syringae* shows a composite ecology of species populations. In fact, this species currently encompasses 57 different pathovars (5), known as the “*P. syringae* complex,” capable of causing disease to herbaceous and woody crops belonging to both mono- and dicotyledonous plant species. A complex is defined as a single species composed of distinct populations capable of infecting a limited number of host plants (13,53). Such distinct populations can show limited or incomplete reproductive isolation resulting in poorly defined species boundaries. The pathovar (i.e., pathogenic variety) concept was introduced in the 1980s to clarify phytosanitary legislation. This concept enables the identification and naming of diverse plant diseases of bacterial origin to aid the development of appropriate detection techniques and/or quarantine protocols. Pathovars are defined as “strains or set of strains with the same or similar characteristics, differentiated at intrasubspecific level from

other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts” (14). Recent studies suggest that pathovar-host specificity is mainly due to the presence of typical repertoires of the type III secretion system effector proteins, which allow the pathovar to overcome host plant defense mechanisms (33).

Although the pathovar has no formal standing within the International Code of Nomenclature of Bacteria (29), which provides rules up to the subspecies level, it is recognized as an effective biological entity (15) and its existence is regulated under the auspices of the International Society of Plant Pathology. In addition, this specific term was initially considered to be a temporary categorization (14) because at that time few plant-pathogenic bacterial species could be differentiated by phenotypic tests as required by the Approved List of Bacterial Names (50). A comprehensive attempt to clarify the taxonomic relationships within the pathovars of the *P. syringae* complex was performed by Gardan et al. (16) using DNA-DNA hybridization (DDH) and ribotyping analyses. The authors assessed 48 pathovars of the complex and eight related species and proposed nine discrete genomospecies. Each genomospecies comprises strains, including the type-strain, exhibiting at least 70% DNA-DNA homology. However, according to the Bacteriological Code, a genomospecies should be named formally only when differentiating phenotypic characteristics are available (58). As Gardan et al. (16) could not provide reliable phenotypic traits (e.g., carbon source assimilation) to clearly distinguish all nine genomospecies, they could officially describe only two species (*P. cannabina* and *P. tremiae*). Consequently, most genomospecies still need to be formally described.

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The definition of a bacterial species is difficult. Currently, DDH, 16S rRNA gene sequence analysis, and multilocus sequence typing analysis (MLSA) are the techniques commonly utilized to genetically determine the bacterial species, with MLSA providing a good combination of reliability and repeatability (1) (see also Plant Associated and Environmental Microbes Database; www.pamdb.org/). As far as phytopathogenic pseudomonads are concerned, MLSA studies provided fundamental insights into their phylogenetic and taxonomic relationships (4,8,9,36,43).

However, each of these techniques has some limitations, such as the impossibility of assembling cumulative databases based on DDH and its reliability only for closely related species or sub-species, the low variability and conservative nature of 16S rRNA genes not permitting sufficient resolution to infer clear taxonomic relationships, and the occurrence of multiple 16S rRNA genes and the putative bias in the selection of the housekeeping genes for MLSA (41,42). Recently, the average nucleotide identity (ANI) analysis of conserved and shared genes between two bacterial strains based on pairwise genome comparisons has been proposed as a new standard for the definition of prokaryotic species (27). The bacterial genome assessment inferred using ANI well represented the degree of evolutionary distance between the compared genomes, and an ANI value of 94% was proposed to replace the classic DDH value of 70% for species demarcation (27). A more extensive study confirmed the reliability of such an analysis and noted a slightly narrower boundary of 95% identity for the consistent substitution of the DDH value of 70% (20). In confirming the robustness of the ANI analysis, it established the species demarcation boundary at a value of 95 to 96% identity and suggested further confirmation by the assessment of the tetranucleotide frequency correlation coefficient (TETRA) value (41). However, the combination of MLSA and genomic analysis, providing either a phylogenetic view or a pairwise genome comparison of all shared orthologous protein coding genes, could represent a robust approach for the reliable demarcation of bacterial species (11,17,42,45,51,52,54).

In this study using ANI and TETRA analyses, MLSA, both ML and Bayesian methods, and a consensus split network with seven housekeeping genes, we examined the currently available genomes of 34 plant-pathogenic strains of *P. syringae* pathovars and some related species representing seven of the nine genomospecies identified by Gardan et al. (16). Two genomospecies, genomospecies 5 (*P. tremae*) and 7 (*P. syringae* pv. *tagetis* and *P. syringae* pv. *helianthi*), could not be assessed because the genomes of these pathogens are not deposited in the databanks. The genomes of *P. fluorescens* A506 and *P. putida* UW4 were used as outgroups. We confirmed the existence of well-demarcated genomospecies within the *P. syringae* species complex in agreement with the DDH and ribotyping study of Gardan et al. (16). In addition, by revealing consistent ANI values between 95 and 96%, we highlighted the close relationship between genomospecies 3 and 8. The present analysis includes many pathotype strains of the *P. syringae* pathovar complex and thus provides the basis for a further reliable demarcation of plant-pathogenic *Pseudomonas* species through ANI/TETRA and MLSA analyses.

MATERIALS AND METHODS

Library preparation, genome sequencing, and assembly. For genomic sequencing, the DNA of *P. syringae* pv. *coryli* NCPPB 4273, pathotype-strain of the pathovar, was prepared as described elsewhere (36,46). Briefly, bacterial genomic DNA was extracted from 1 ml of overnight cultures grown in KB broth using a Wizard DNA purification kit (Promega Italia, Padova, Italy) following the manufacturer's instructions. DNA was measured and checked for quality using a NanoDrop (NanoDrop Products, Wilmington, DE). A total of 10 µg of DNA from each sample was fragmented by incubation for 70 min with 5 µl of dsDNA

Fragmentase (New England Biolabs, MA). The reaction was stopped with EDTA and purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). The eluate was end repaired using an End Repair kit (New England Biolabs) for 30 min at 20°C. The end-repaired DNA was A-tailed for 30 min at 37°C using a d-A Tailing kit (New England Biolabs). After purification using the MinElute purification kit (QIAGEN), the DNA was ligated using Quick T4 DNA ligase (New England Biolabs) to 500 pmol of Illumina adaptors that had been previously annealed by heating at 98°C for 3 min and then slowly cooling to 16°C in a thermocycler. After further purification using the MinElute purification kit (QIAGEN), 1 µl of each reaction was quantified by labeling with biotin, spotted on nitrocellulose after a serial dilution, and detected using an anti-biotin-AP conjugate (Roche Diagnostics, Monza, Italy) following manufacturer's instructions. Equal amounts of DNA from samples were pooled together and size fractionated by 2% MS-6 agarose (Conda, Madrid, Spain) gel electrophoresis in TAE buffer at 120 V for 60 min. Genomic DNA was sequenced using Illumina Genome Analyzer Iix (Illumina, San Diego, CA) at the Istituto di Genomica Applicata (Udine, Italy). In order to remove contaminants and adaptors, quality check of reads was performed using the extended randomized numerical alignEr (ERNE) filters, a string alignment package to provide tools to handle short reads (www.erne.sourceforge.net). Paired reads of 100 nts were assembled into contigs using the de novo (i.e., without using a reference genome) assembly option of the CLC genomic workbench (CLC-bio, Aarhus, Denmark) by setting the default parameters. Contig sequences were scanned for open reading frames by GLIMMER, version 3.02. (12) which had been previously trained on the complete genome sequences of *P. syringae* pv. *tomato* DC3000 (NC_004578.1, i.e., *Pto* DC3000), *P. syringae* pv. *phaseolicola* 1448A (NC_005773.3, i.e., *Pph* 1448A), and *P. syringae* pv. *syringae* B728a (NC_007005.1, i.e., *Psy* B728a). The putative proteins were annotated against the RefSeq database using a PERL script for recursive BLASTX searches (2) and MUMMER software (28).

Public sequence data. In addition to the newly sequenced *P. syringae* pv. *coryli* strain, the genome data from 35 *Pseudomonas* strains were downloaded from the National Center of Biotechnology Information (NCBI) and from DOE Joint Genome Institute/Integrated Microbial Genome (JGI/IMG-ER) GenBanks. These data included genome sequences of 31 *P. syringae* pathovars, and *P. avellanae*, *P. viridiflava*, and *P. cannabina* pv. *alisalensis* strains as well as of *P. fluorescens* A506 and *P. putida* UW4 as outgroups (Table 1).

ANI and TETRA analyses. To evaluate the taxonomic relationships of 34 strains of phytopathogenic *P. syringae* pathovars and related species, ANI and TETRA analyses were performed. The analyses of sequences for the determination of their relatedness according to ANI and TETRA were performed with the software JSpecies (41). The analysis regarded 34 genomes belonging to seven out of nine *P. syringae* genomospecies *sensu* Gardan et al. (16) (Table 1). Due to current absence of some genomes in database, strains of genomospecies 5 and 7 were not analyzed. In addition, *P. fluorescens* A506 and *P. putida* UW4 were included in the assessment as outgroups. ANI was calculated using the MUMmer algorithm implementation (i.e., ANIm) (25). TETRA was used as an alignment-free genomic similarity index as oligonucleotide frequencies carry a species-specific signal. The use of a tetranucleotide usage pattern has been shown to be a good compromise between signal strength and needed computational power (41). Pairwise comparison between genomes is performed by plotting the corresponding tetranucleotide frequency and then obtaining a regression line.

Taxonomy based on MLSA and consensus split networks. To further evaluate the taxonomic relationships of the 34 strains of *P. syringae* and related species, MLSA was also carried out.

P. fluorescens A506 and *P. putida* UW4 strains were used as outgroups. The maximum likelihood (ML) and Bayesian analyses were performed with nucleotides using seven housekeeping genes (*dnaQ*, *gltA*, *pheS*, *recA*, *rpoB*, *serS*, and *tyrS*), for a total of 3,003 nts. This set of genes was used to obtain a longer gene sequence to analyze than the classical set (i.e., *gapI*, *gltA*, *gyrB*, and *rpoD*). ML analysis was inferred with PhyML version 3.0 (21), with 1,000 bootstrap replicates, whereas for the Bayesian method we used MrBayes version 3.2.1, with 1,000,000 generations (<http://mrbayes.sourceforge.net>). To select the best fit model for both ML and Bayesian analyses, we used a phymtest procedure implemented in the R package APE (40). JC69 was used as best substitution model for both analyses. The robustness between the ML gene trees was tested by applying the Shimodaira-Hasegawa (SH) test (48) using the CONSEL program (49). The trees were visualized using FigTree software, version 1.1.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). In addition, a consensus split network tree for inferring unrooted phylogenetic networks, based on concatenation of 3,003 nucleotides from the 34 phytopathogenic pseudomonads and *P. fluorescens* A506 and *P. putida* UW4 strains as outgroups, was built using the neighbor-joining (NJ) algorithm with the Hamming distance method, obtained using the Splits-Tree4 software (23). Bootstrap analysis with 1,000 replications was performed by using the same software.

RESULTS

Genome sequence data. We generated a new sequence data from *P. syringae* pv. *coryli* NCPPB 4273, the reference pathotype-strain of the pathovar. The Illumina sequencing provided in total nearly 10 million 100-nt reads from the genomic DNA of the strain that passed the quality check. The genome size (nucleotides) of the strain was within the range of the previously sequenced and published *P. syringae* draft genomes (i.e., 6,096,328). The main genomic features of the draft genome are the following: number of reads, 6,398,721; number of contigs, 75; average contig size, 81,900; N50 (nt), 132,772; G+C content (%), 59.2; and genome coverage, 155. The sequence of the assembly was deposited in NCBI GenBank under the following accession number: *P. syringae* pv. *coryli* NCPPB 4273=AWQP000000000.

ANI and TETRA analyses. The newly sequenced *P. syringae* pv. *coryli* NCPPB 4273 genome, together with those of 33 phytopathogenic *P. syringae* pathovars and related *Pseudomonas* spp. strains, representative of seven of the nine genomospecies described by Gardan et al. (16) after their DDH and ribotyping assessment were cross compared to reveal their total similarities in terms of the amount of sequence identity. Two *Pseudomonas* spp., namely *P. fluorescens* A506 and *P. putida* UW4, were used as outgroups. The ANI value comparisons and calculations are shown in Figure 1 and Supplemental Table 1, and the corresponding TETRA

Fig. 1. Comparison of the average nucleotide identity values (i.e., percentage) between each genome of 34 strains of the *Pseudomonas syringae* species complex and related species (Table 1) as retrieved by pairwise alignment with JSpecies (36). The columns and the rows are ordered according to the genomospecies clustering inferred by DNA-DNA hybridization analysis by Gardan et al. (16). The comparison shows higher similarities as the colors get darker.

values are shown in Supplemental Table 2. The ANI and TETRA analyses, in many cases, revealed a consistent demarcation among the strains of the *P. syringae* pathovars and related species, thereby allowing the circumscription of putative species boundaries. In fact, the strains of genomospecies 2 *sensu* Gardan et al. (16), namely *P. syringae* pathovars *aesculi* (2250), *glycinea* (race 4), *mori* (MAFF 301020), *phaseolicola* (1448A), *tabaci* (ATCC 11528), and *P. savastanoi* pv. *savastanoi* (NCPBP 3335) had ANI values between 97.74 and 99.45% in any reciprocal comparison and lower than 90% compared with the other *P. syringae* and outgroup strains. *P. syringae* pv. *oryzae* 1_6 (genomospecies 4), *P. viridiflava* UASWS0038 (genomospecies 6), and *P. cannabina* pv. *alisalensis* BS91 (genomospecies 9) had ANI values lower than 89% when compared with all other *Pseudomonas* strains.

The strains included in genomospecies 1 by the DDH and ribotyping analyses of Gardan et al. (16) and other studies (34,46) and belonging to the *P. syringae* pv. *syringae sensu strictu* (16) had ANI values lower than 90% when compared with the strains of the other genomospecies and a genomic similarity ranging between 96 and 99% for intra-genomospecies comparison. However, it should be noted that the *P. syringae* pathovars *syringae* (B728a) and *aceris* (MAFF 302273) strains had ANI values between 95 and 96% when compared with the other strains of genomospecies 1, revealing a lower genomic similarity that should be further investigated. The strains belonging to genomospecies 3 (*P. syringae* pv. *lachrymans* MAFF 302278, *P. syringae* pv. *tomato* DC3000, T 1, TK40, Max13, and NCPBP 1108) and genomospecies 8 (*P. avellanae* BPIC 631, *P. syringae* pv. *theae* NCPBP 2598, *P. syringae* pv. *actinidiae* NCPBP 3739, NCPBP 3871, CRAFRU 8.43, LV-11, and LV-17) had very high ANI

values in the intra-genomospecies reciprocal comparison (Fig. 1). In addition, *P. syringae* pv. *morsprunorum* MAFF 302280 had ANI values higher than 96.3% when compared with the strains of genomospecies 8; therefore, it should be included in this genomospecies. However, the strains of genomospecies 3 and 8, in many cases, had ANI values between 95 and 96% when they were reciprocally compared, thus revealing a very close taxonomic and phylogenetic relationship between these two genomospecies. The TETRA analysis always confirmed these results, with values for the genomospecies demarcation always higher than 0.99 in any reciprocal comparison. This analysis was intended to verify and confirm whether an alignment-free genomic feature can be used to circumscribe bacterial species (41). *P. fluorescens* A506 and *P. putida* UW4, used as outgroups, always had ANI values lower than 86% in any reciprocal comparison with the phytopathogenic pseudomonads.

MLSA and consensus split network analyses. A phylogenetic tree of the concatenated DNA sequences based on the ML method of the seven housekeeping genes, namely *dnaQ*, *gltA*, *pheS*, *recA*, *rpoB*, *serS*, and *tyrS* (a total of 3,003 nucleotides) in 34 phytopathogenic pseudomonads, representative of seven genomospecies *sensu* Gardan et al. (16) and *P. fluorescens* and *P. putida* used as outgroups, is shown in Figure 2. The robustness of the tree was strongly supported by the SH test (Table 2). The dendrogram consistently confirmed the distinctiveness of the seven genomospecies inferred by DDH and ribotyping by Gardan et al. (16) and the present ANI assessment. In addition, the tree confirmed the close relationships between the strains of genomospecies 3 and 8 inferred by the ANI and TETRA analyses. Similar to the ANI and TETRA analyses, MLSA also confirmed *P. syringae*

TABLE 1. List of *Pseudomonas syringae* pathovars and related species strain genomes used in this study for genomic and phylogenetic analyses^a

Strain	Strain code	Strain acronym	Accession number	Genomospecies	MLSA
<i>P. syringae</i> pv. <i>syringae</i> B728a	Psy B728a	Psy1	NC_007005	1	1
<i>P. syringae</i> pv. <i>syringae</i> CRAFRU11	Psy CRAFRU11	Psy2	ATSU00000000	1	1
<i>P. syringae</i> pv. <i>syringae</i> CRAFRU12	Psy CRAFRU12	Psy3	ATSV00000000	1	1
<i>P. syringae</i> pv. <i>avellanae</i> CRAPAV013	Psav CRAPAV013	Psav	AKCJ00000000	1	1
<i>P. syringae</i> pv. <i>aceris</i> MAFF302273 PT	Psac M302273	Psac	AEA000000000	1	1
<i>P. syringae</i> pv. <i>aptata</i> DSM50252	Ptt DSM50252	Ptt	AEAN000000000	1	1
<i>P. syringae</i> pv. <i>coryli</i> NCPBP4273 PT	Psc NCPBP4273	Psc	AWQP00000000	1	1
<i>P. syringae</i> pv. <i>panici</i> LMG2367 PT	Psp LMG2367	Psp	ALAC000000000	1	1
<i>P. syringae</i> pv. <i>pisi</i> 1704B	Ppi 1704B	Ppi	AEAI000000000	1	1
<i>P. syringae</i> pv. <i>japonica</i> MAFF301072 PT	Psj M301072	Psj	AEAH000000000	1	1
<i>P. syringae</i> pv. <i>aesculi</i> 2250	Pae 2250	Pae	ACXT000000000	2	2
<i>P. syringae</i> pv. <i>glycinea</i> race4	Pgy Race4	Pgy	ADWY000000000	2	2
<i>P. syringae</i> pv. <i>mori</i> MAFF301020	Pmo M301020	Pmo	AEAG000000000	2	2
<i>P. syringae</i> pv. <i>phaseolicola</i> 1448A	Pph 1448A	Pph	NC_005773, NC_007274, NC_007275	2	2
<i>P. savastanoi</i> pv. <i>savastanoi</i> NCPBP3335	Psv NCPBP3335	Psv	ADMI02000000	2	2
<i>P. syringae</i> pv. <i>tabaci</i> ATCC11528	Pstab ATCC11528	Pstab	ACHU000000000	2	2
<i>P. syringae</i> pv. <i>lachrymans</i> MAFF302278 PT	Pla M302278	Pla	AEAM000000000	/	3
<i>P. syringae</i> pv. <i>tomato</i> DC3000	Pto DC3000	Pto1	NC_004578, NC_004632, NC_004633	3	3
<i>P. syringae</i> pv. <i>tomato</i> T1	Pto T1	Pto2	ABSM000000000	3	3
<i>P. syringae</i> pv. <i>tomato</i> TK40	Pto TK40	Pto3	ADFY000000000	3	3
<i>P. syringae</i> pv. <i>tomato</i> Max13	Pto Max13	Pto4	ADFZ000000000	3	3
<i>P. syringae</i> pv. <i>tomato</i> NCPBP1108	Pto NCPBP1108	Pto5	ADGA000000000	3	3
<i>P. syringae</i> pv. <i>oryzae</i> 1_6	Por 1_6	Por	ABZR000000000	4	4
<i>P. viridiflava</i> UASWS0038	Pvir UASWS0038	Pvir	AMQP000000000	6	6
<i>P. avellanae</i> BPIC631 T/PT	Pave BPIC631	Pave	ATDK000000000	8	8
<i>P. syringae</i> pv. <i>actinidiae</i> NCPBP3739 PT	Pan NCPBP3739	Pan1	AFTH000000000	8	8
<i>P. syringae</i> pv. <i>actinidiae</i> NCPBP3871	Pan NCPBP3871	Pan2	AFTF000000000	8	8
<i>P. syringae</i> pv. <i>actinidiae</i> CRAFRU8.43	Pan CRAFRU8.43	Pan3	AFTG000000000	8	8
<i>P. syringae</i> pv. <i>actinidiae</i> MAFF302091	Pan M302091	Pan4	AEAL000000000	8	8
<i>P. syringae</i> pv. <i>actinidiae</i> LV-11	Pan AOJU01LV	Pan5	AOJU000000000	8	8
<i>P. syringae</i> pv. <i>actinidiae</i> LV-17	Pan AOKF01LV	Pan6	AOKF000000000	8	8
<i>P. syringae</i> pv. <i>morsprunorum</i> M302280 PT	Pmp M302280	Pmp	AEAE000000000	8	8
<i>P. syringae</i> pv. <i>theae</i> NCPBP2598 PT	Pth NCPBP2598	Pth	ATDJ000000000	8	8
<i>P. cannabina</i> pv. <i>alisalensis</i> BS91 PT	Pcal BS91	Pcal	ID 2516653056	9	9
<i>P. putida</i> UW4	Ppu UW4	Ppu	NC_019670	Outgroup	
<i>P. fluorescens</i> A506	Pf A506	Pf	NC_017911	Outgroup	

^a The genomospecies *sensu* Gardan et al. (16) are also indicated. *P. fluorescens* A506 and *P. putida* UW4 are included as outgroups. T: type strain of the species; PT: pathotype strain of the pathovar. MLSA, multilocus sequence typing analysis.

pv. *lachrymans* MAFF 302278 as a member of genomospecies 3 and *P. syringae* pv. *morsprunorum* MAFF 302280 as a member of genomospecies 8. The representative strains of genomospecies 4 (*P. syringae* pv. *oryzae* 1_6), 6 (*P. viridiflava* UASWS0038), and 9 (*P. cannabina* pv. *alisalensis* BS91) clustered separately from the other groups. Finally, the two outgroups *P. fluorescens* A506 and *P. putida* UW4 clustered far apart. The dendrogram built on with the Bayesian method provided the same strain genomes clustering as ML (Fig. 3). Both trees were supported by high bootstrap values. The consensus-split network, built for inferring unrooted phylogenetic networks and based on the concatenated alignment of the 3,003 nucleotides of the seven housekeeping genes, provided a significant and identical phylogenetic analysis compared with the ML tree and was supported by high bootstrap values (Figs. 4 and 5).

DISCUSSION

This study confirms the robustness of the combination of genomic and phylogenetic approaches in revealing taxonomic relationships among closely related bacterial strains for the definition of species (42,51,52). We applied the ANI/TETRA analyses jointly with MLSA, using both ML and Bayesian methods, and split consensus network analyses for seven housekeeping genes in 34 strains belonging to the plant-pathogenic *P. syringae* complex and related species, and we confirmed the occurrence of putative species generally reflecting the classification of Gardan et al. (16), which was inferred through DDH and ribotyping analyses. Our analyses included several pathotype strains of the *P. syringae* pathovars and the type strain of *P. avellanae*, providing additional value to this assessment. The joint utilization of genomic data and phylogenetic analyses is very useful for providing new opportunities to infer bacterial species using a robust, reliable, and inexpensive approach (42,51,52). In addition to the seminal studies proposing and confirming ANI analysis as a valuable method for the demarcation of bacterial species (20,27,41), assessments of bacterial taxa using this approach that led to the redefinition of bacterial species have been reported for *Acinetobacter* spp. (10), *Neisseria* spp. (3), and *P. avellanae* (46). The present study con-

firms that ANI/TETRA analyses combined with MLSA and consensus network can substitute for DDH to provide a reliable definition of bacterial species of the *P. syringae* complex. Our study highlights the clear distinctiveness of the strains of genomospecies 1, 2, 4, 6, and 9. According to Gardan et al. (16), genomospecies 1 includes *P. syringae* pv. *syringae* strains *sensu stricto* and some other *P. syringae* pathovars, and their DDH values ranged between 71 and 90%. The present study, taking into account strains other than those assessed by DDH and ribotyping, confirmed these conclusions. Within the group, however, there were some cases of strain comparison with ANI values between 95 and 96%, such as *P. syringae* pv. *aceris* MAFF 302273 and *P. syringae* pv. *syringae* B728a. MLSA trees and split consensus network analyses confirmed the distinctiveness of genomospecies 1 from the other genomic clusters. *P. syringae* pv. *syringae*, as revealed by the DDH assessment, is a heterogeneous group of strains characterized by their unrestricted pathogenicity and genetic heterogeneity (32,47,59), consequently, genomic variability should be expected. In contrast, the strains of genomospecies 2 used in this work formed tight and homogeneous clusters with ANI values consistently greater than 97.5%, and *P. syringae* pv. *oryzae* 1_6, (genomospecies 4), *P. viridiflava* UASWS0038 (genomospecies 6), and *P. cannabina* pv. *alisalensis* BS91 (genomospecies 9) were well demarcated from all other genomospecies.

TABLE 2. Shimodaira-Hasegawa (SH) test for the congruence of phylogenetic trees inferred with maximum likelihood analysis and performed on 15 bifurcating trees^a

Gene	SH
<i>dnaQ</i>	1.000
<i>gltA</i>	0.998
<i>pheS</i>	0.992
<i>recA</i>	0.999
<i>rpoB</i>	0.939
<i>serS</i>	1.000
<i>tyrS</i>	0.998
Concatenated	0.982

^a All values are significant at $\alpha = 0.05$.

Fig. 2. Phylogenetic relationships among 34 strains of the *Pseudomonas syringae* species complex and related species as obtained with multilocus sequence typing analysis using 3,003 concatenated nucleotides of seven housekeeping genes (*dnaQ*, *gltA*, *pheS*, *recA*, *rpoB*, *serS*, and *tyrS*) with bootstrap values greater than 65% (1,000 replicates) shown at the nodes. The phylogenetic tree was built with the maximum likelihood (ML) method and with JC69 as the best substitution model using the PHYLIP package. *P. fluorescens* A506 (Pf) and *P. putida* UW4 (Ppu) were included as outgroups. G: genomospecies number *sensu* Gardan et al. (16).

To establish the reliability and threshold accuracy of ANI analysis for robust species demarcation, a boundary of 95 to 96% was proposed (27,41). The present study confirms that such a threshold reveals the occurrence of very closely related species

that may represent cases of splitting populations or the convergence of different species to the same ecological niche. In fact, we found that some phytopathogenic *Pseudomonas* strains of genomospecies 3 and 8 had ANI values between 95 and 96%. The

Fig. 3. Phylogenetic relationships among 34 strains of the *Pseudomonas syringae* species complex and related species as obtained with multilocus sequence typing analysis using 3,003 concatenated nucleotides of seven housekeeping genes (*dnaQ*, *gltA*, *pheS*, *recA*, *rpoB*, *serS*, and *tyrS*) inferred with the Bayesian method. Bootstrap values greater than 65% (1,000,000 generations) are shown at the nodes. The interior node values of the tree are clade credibility values based on the posterior credibility values produced by MrBayes. G, genomospecies number *sensu* Gardan et al. (16).

Fig. 4. Split consensus network of the concatenated 3,003 nucleotides of seven housekeeping genes (*dnaQ*, *gltA*, *pheS*, *recA*, *rpoB*, *serS*, and *tyrS*) for the 34 strains of the *Pseudomonas syringae* species complex and related species. Bootstrap values are shown at the main nodes. The split consensus network confirmed the results obtained with average nucleotide identity and multilocus sequence typing analysis analyses. G: genomospecies number *sensu* Gardan et al. (16).

close relationships between genomospecies 3 and 8 had been revealed previously by ribotyping analysis (16) of three strains of genomospecies 3 which were included in genomospecies 8, and several restriction fragments were observed to be common to all strains of both genomospecies. The close relationship between the genomospecies 3 and 8 was also demonstrated by *hrp* gene and *gyrB* and *rpoD* genes analysis (24,44). Further studies are necessary to elucidate the evolutionary trajectories of these two groups of phytopathogens that infect both woody and herbaceous host plant species, leading to their current taxonomic similarity. A case of convergent evolution to the same host, namely *Corylus avellana*, was found for *Pseudomonas* strains of genomospecies 1 (i.e., *P. syringae* pv. *avellanae* and *P. syringae* pv. *coryli*) and 8 (*P. avellanae*) (46,56). We also confirmed that *P. syringae* pv. *morsprunorum* MAFF 302280 is a member of genomospecies 8 (46). Notably, Gardan et al. (16) included the pathotype strain of *P. syringae* pv. *morsprunorum* (CFBP 2351=NCPPB 2995=LMG 5075t2=MAFF 302280) in their study and classified it as a member of genomospecies 3, even though the ribotyping analysis clearly showed its very close relationship to the strains of genomospecies 8 and with *P. syringae* pv. *theae* in particular. Young et al. (60) claimed that CFBP 2351 is unsuitable as a pathotype strain for this pathovar, and Gardan et al. (16) stated that it is not pathogenic. Consequently, Gardan et al. (16) and Bull et al. (5) proposed CFBP 2116, which was included in genomospecies 2, as a possible reference pathotype strain for the pathovar *morsprunorum*. Unfortunately, genomic data are not available for this strain. It should be added that, primarily based on biochemical tests (30), *P. syringae* pv. *morsprunorum* is, in turn, subdivided into two races, namely race 1 and race 2. The strains of the two races appear quite homogenous and are thus reliably identified and distinguished according to biochemical and physiological tests (55) as well as by repetitive-sequence polymerase chain reaction (PCR) typing, MLST, and 16S rRNA gene sequence analyses (18,25,31). According to these criteria, *P. syringae* pv. *morsprunorum* MAFF 302280 belongs to race 2, similar to other

pathogenic strains (18,31), whereas there are no race indications for CFBP 2116 even though it should, presumably, belong to race 1. Currently, genomic data for the strain comparisons of race 1 are not available, but the possibility that members of this race form a distinct species from the strains of race 2 (i.e., genomospecies 8) appears reasonable (7,46). Additional analyses of other strains of both races 1 and 2 using a phylogenomic approach can contribute to the clarification of the existence of different *Pseudomonas* species that have pathogenically converged to the same host plants, namely *Prunus avium*, *Prunus cerasus*, and *Prunus domestica*. This study also confirms that the strains isolated from *Actinidia deliciosa* and *A. chinensis* in New Zealand, the so called "less virulent" *P. syringae* pv. *actinidiae* strains LV 11 and LV 17, should be included within the *P. avellanae* species (46). In fact, the close relationships between these strains and members of genomospecies 8, particularly *P. syringae* pv. *theae*, have been also demonstrated by other MLSA and genome comparisons (8,37).

The placement of *P. syringae* pv. *lachrymans* into the genomospecies scheme proposed by Gardan et al. (16) raises some questions. In their DDH and ribotyping analyses, these authors assessed two strains, CFBP 1644 and CFBP 2440, which were placed into genomospecies 2 and 3, respectively. Neither is the pathotype strain of the pathovar. The present study assessed the pathotype strain of the pathovar, CFBP 2104=NCPPB 1436=MAFF 302278, which was included within genomospecies 2. The pathovar *lachrymans* is heterogeneous in terms of both virulence and molecular features as inferred by PCR-restriction fragment length polymorphism of the internal transcribed spacer 1 (ITS1) region and amplification of DNA fragments surrounding rare restriction sites (35,39). This pathovar is presumed to represent a case of pathogenic convergence of different species to the same host plants. Similarly, additional strain genome assessments using the approach of the present study could provide novel insights into the taxonomic and phylogenetic relationships of the *P. syringae* pvs. *morsprunorum* and *lachrymans*.

Fig. 5. Particular of the split consensus network of strains of the *Pseudomonas syringae* species complex and related species shown in Figure 3. The bootstrap values are shown at each node. **A**, genomospecies 8; **B**, genomospecies 2; **C**, genomospecies 3; and **D**, genomospecies 1. Table 1 provides the strain codes.

This study provides the basis for a further reliable species demarcation within the *P. syringae* species complex and contributes to the definition of more robust genomic and taxonomic relationships among the different pathovars in each genomospecies. Further analyses, possibly taking into account all the missing type and pathotype strains of the complex, would provide additional refinement of the current species demarcation (42). Another fundamental requirement for bacterial species definition and identification is their phenotypic demarcation (54). Similar to Gardan et al. (16), we are currently unable to provide a reliable phenotypic trait or technique to identify the genomospecies here inferred by the genomic and phylogenetic approaches. Modern approaches such as MALDI-TOF and high-field cyclotron Fourier transform mass spectrometry techniques can provide robust phenotypic assessments (42).

Concerning the composition of phytopathogenic bacterial species as revealed by the present study, a possible scenario might be the occurrence, within one single species, of some or many different pathovars, differentiated mainly by their accessory genome elements coding for virulence or environmental fitness traits and enabling them to colonize the host plant(s) and cause disease. Each single pathovar of a species may represent a distinct biological unit currently adapted to a well-defined niche(s), (i.e., the host plant(s) and the linked environment). Within this context, both the phylogenomic (51,52) and ecological-evolutionary (26, 57) species concepts could be pertinent for explaining the relevant biological diversity and adaptive capability of bacterial phytopathogens, even though the diversity of each pathovar within a species should require the confirmation of its ecological distinctness through phenotypic characterization (19,42). The possibility of performing extensive and standardized host range and phenotypic tests with many strains of different pathovars can possibly assist phytobacteriologists for better determining the boundaries of these ecological units.

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