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Research Article

BOX-PCR-based identification of bacterial species belonging to *Pseudomonas syringae* - *P. viridiflava* group

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Abstract

The phenotypic characteristics and genetic fingerprints of a collection of 120 bacterial strains, belonging to *Pseudomonas syringae sensu lato* group, *P. viridiflava* and reference bacteria were evaluated, with the aim of species identification. The numerical analysis of 119 nutritional characteristics did not show patterns that would help with identification. Regarding the genetic fingerprinting, the results of the present study supported the observation that BOX-PCR seems to be able to identify bacterial strains at species level. After numerical analyses of the bar-codes, all pathovars belonging to each one of the nine described genomospecies were clustered together at a distance of 0.72, and could be separated at genomic species level. Two *P. syringae* strains of unknown pathovars (CFBP 3650 and CFBP 3662) and the three *P. syringae* pv. *actinidiae* strains were grouped in two extra clusters and might eventually constitute two new species. This genomic species clustering was particularly evident for genomospecies 4, which gathered *P. syringae* pvs. *atropurpurea*, *coronafaciens*, *garçae, oryzae, porri, striafaciens*, and *zizaniae* at a noticeably low distance.

Key words: Pseudomonas syringae, phenotypic characters, genomospecies, BOX-PCR, bacterial identification.

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Introduction

Taxonomy of the large bacterial group *Pseudomonas syringae* (LOPAT I of Lelliott *et al.*, 1966) and *P. viridiflava* is currently under revision, since nine genomospecies were described (Gardan *et al.*, 1999). Many of the causal agents of plant bacterial diseases belong to this group of bacteria, which are either designated as species or as pathovars, *i.e.* infra-specific subdivision for strains specifically linked to a given host plant.

Identification of bacteria was traditionally performed by phenotypic descriptions, but this approach had some limits. Thus, not all genomospecies can be reliably distinguished by techniques other than quantitative DNA-DNA hybridization, which is not suitable for routine diagnosis. Alternative specific genomic fingerprints have been proposed as diagnostic tools (Versalovic *et al.*, 1994; Rademaker and Bruijn, 1997) by means of amplification of interspersed repetitive DNA sequences present in bacterial genomes, referred to as rep-PCR (Rademaker and Bruijn, 1997) or by amplification of random sequences by arbitrary primers, RAPD (Williams *et al.*, 1990*)*. One of these methods appeared interesting for the delineation of species (Onfroy *et al.*, 1999), subspecies (Louws *et al.*, 1998) or pathovars (Louws *et al.*, 1994) for instance. BOX-PCR, independent from the other rep-PCR techniques, has revealed the possibility of delineating *P. syringae* genomospecies (Marques *et al.*, 2000), as well as for typing *Aeromonas* spp. strains (Tacao *et al.*, 2005) and for identification of races and biovars of *Ralstonia solanacearum* (Galal *et al.*, 2003). The technique has also been used to investigate bacterial inoculum sources (Greco *et al.*, 2004), as a tool for unequivocal identification of strains belonging to a unique pathovar (El Tassa *et al.*, 1999) or to define new species, as a part of a polyphasic approach (Catara *et al.*, 2002).

At present, bacterial species discrimination is based on quantitative DNA-DNA hybridization, as recommended by Wayne *et al.* (1987). In view of the absence of diverse discriminating tests to distinguish the genomospecies assigned by Gardan *et al.* (1999), the objective of this study was to compare nutritional characteristics and genomic fingerprints of all the pathovars of the *P. syringae* -

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P. viridiflava group, thus checking the hypothesis that BOX-PCR could be correlated with those species discriminations and evaluating its potential for use as a taxonomic tool.

Material and Methods

Bacterial strains

Phytopathogenic fluorescent pseudomonads belonging to the *Pseudomonas syringae* group (Palleroni, 1984) were obtained from the "Collection Française des Bactéries Phytopathogènes" (CFBP, Angers, France), and comprised 120 strains (Table 1): strains of *P. savastanoi* pv. *phaseolicola*, including representatives of the nine races of the bacteria, isolated from a wide range of hosts and geographical origins (Taylor *et al.*, 1996); representative strains of two other bacteria pathogenic to the bean (*P. syringae* pvs. *tabaci* and *syringae*); strains of the very closely related *P. savastanoi* pv. *glycinea*; strains of *P. syringae* pvs. *syringae* (CFBP 3388) and *actinidiae* because of their ability to produce phaseolotoxin (Tamura *et al.*, 1989; Tourte and Manceau, 1995); strains which are type strains of species and pathovars included in the large group *P. syringae-viridiflava* and two strains 3650 and 3662 received as *P. savastanoi* pv. *phaseolicola*, but which differed considerably and are listed separately as unknown pathovars. In the following text, the ternary nomenclature

Table 1 - Strains of *Pseudomonas syringae*[1] group, *P. viridiflava* and related species *P. savastanoi*, *P. ficuserectae*, *P. amygdali*, *P. tremae*, *P. meliae*, *P. avellanae* and *P. cannabina*, according to the genomospecies designation[2].

Genomospecies	Species and pathovars	CFBP[3] n.
1	*P. syringae* pv. *aceris*	2339 [pt]
	P. syringae pv. *aptata*	1617 [pt]
	P. syringae pv. *atrofaciens*	2213 [pt]
	P. syringae pv. *dysoxyli*	2356 [pt]
	P. syringae pv. *japonica*	2896
	P. syringae pv. *lapsa*	1731 [pt]
	P. syringae pv. *panici*	2345 [pt]
	P. syringae pv. *papulans*	1754 [pt]
	P. syringae pv. *pisi*	2105 [pt]
	P. syringae pv. *syringae*	1392[T], 3388*, 4886, 4887, 4888
2	*P. amygdali*	3340[T]
	P. ficuserectae	3224 [T]
	P. meliae	3225 [T]
	P. savastanoi pv. *glycinea*	2214 [pt], 3356, 3357, 3361
	P. savastanoi pv. *savastanoi*	1670[T]
	P. savastanoi pv. *phaseolicola*	1390 [pt], 3632, 3633, 3634, 3635, 3636, 3637, 3638, 3639, 3640, 3641, 3642, 3643, 3644, 3645, 3646, 3647, 3648, 3649, 3651, 3652, 3653, 3654, 3655, 3656, 3657, 3658, 3660, 3661, 3663, 4704, 4705, 4706, 4847, 4848, 4849, 4850, 4851, 4852, 4859, 4860
	P. syringae pv. *aesculi*	2894 [pt]
	P. syringae pv. *ciccaronei*	2342 [pt]
	P. syringae pv. *dendropanacis*	3226 [pt]
	P. syringae pv. *eriobotryae*	2343 [pt]
	P. syringae pv. *hibisci*	2895 [pt]
	P. syringae pv. *lachrymans*	1644
	P. syringae pv. *mellea*	2344 [pt]
	P. syringae pv. *mori*	1642 [pt]
	P. syringae pv. *morsprunorum*	2116
	P. syringae pv. *myricae*	2897 [pt]
	P. syringae pv. *photiniae*	2899 [pt]
	P. syringae pv. *sesami*	1671 [pt]
	P. syringae pv. *tabaci*	2106 [pt], 4854, 4855, 4856, 4857, 4858, 4861[5], 4862[5]
	P. syringae pv. *ulmi*	1407 [pt]

Table 1 (cont.)

Genomospecies	Species and pathovars	CFBP[3] n.
3	*P. syringae* pv. *antirrhini*	1620 [pt]
	P. syringae pv. *apii*	2103 [pt]
	P. syringae pv. *berberidis*	1727 [pt]
	P. syringae pv. *delphinii*	2215 [pt]
	P. syringae pv. *lachrymans*	2440 [pt]
	P. syringae pv. *maculicola*	1657 [pt]
	P. syringae pv. *morsprunorum*	2351 [pt]
	P. syringae pv. *passiflorae*	2346 [pt]
	P. syringae pv. *persicae*	1573 [pt]
	P. syringae pv. *philadelphii*	2898 [pt]
	P. syringae pv. *primulae*	4091
	P. syringae pv. *ribicola*	4068
	P. syringae pv. *tomato*	2212 [pt]
	P. syringae pv. *viburni*	1702 [pt]
4	*P. syringae* pv. *atropurpurea*	2340 [pt]
	P. syringae pv. *coronafaciens*	2216 [pt]
	P. syringae pv. *garçae*	1634 [pt]
	P. syringae pv. *oryzae*	3228 [pt]
	P. syringae pv. *porri*[4]	1908 [pt]
	P. syringae pv. *striafaciens*	1674 [pt], 1686
	P. syringae pv. *zizaniae*	4117 [T]
5	*P. tremae*	3229[T]
6	*P. syringae* pv. *primulae*	1660 [pt]
	P. syringae pv. *ribicola*	2348 [pt]
	P. viridiflava	2107[T]
7	*P. syringae* pv. *helianthi*	2067 [pt]
	P. syringae pv. *tagetis*	1694 [pt]
8	*P. avellanae*[4]	4060[T]
	P. syringae pv. *theae*	2353 [pt]
9	*P. cannabina*	2341[T]
Unknown genomospecies	*P. syringae* pv. *actinidiae*	4909 [pt], 4911, 5095
Unknown genomospecies and pathovar	*P. syringae*	3650, 3662

[1]Species designated after Young *et al.*, 1996. [2]Genomospecies designated after Gardan *et al.*, 1999. [3]"Collection Française de Bactéries Phytopathogènes". [4]Pathovar name not validated by Young *et al.*, 1996. [5]Unknown pathovar. [T]Species type strain. [pt]Pathotype strain. *Strain of *P. syr. syringae* producing phaseolotoxin (Tourte and Manceau, 1995).

will be designated by the abbreviation *P. syr. syringae* instead of *P. syringae* pv. *syringae*.

Nutritional characterization

Assimilation of 99 carbon sources (sugars, alcohols, amino acids and organic acids) was performed with the Biotype 100 system (BioMérieux, La Balme-les-Grottes, France). The strips were inoculated with Biotype Medium 1 as recommended by the manufacturer, and the results read visually at two, four and six days after incubation at 28 °C. In addition, 20 conventional biochemical tests were carried out: arginine dihydrolase, oxidase, gelatin, nitrate reduction, levan, fluorescence, hypersensitive reaction (HR) on tobacco, esculin, pectinolysis on calcium pectinate, Tween esterase, DNAse, polypectate hydrolysis at pH 5 and 8.3, and the utilization of sucrose, lactate, L(+)tartrate,

D(-)tartrate, erythritol, mannitol, and sorbitol in ARJ medium (Gardan *et al.*, 1999).

Bacterial cultures and genomic DNA preparation

Strains were grown at 26-28 °C on King's medium B for 24 h. From these cultures, cells were washed with sterile distilled water, and a suspension was prepared, which was adjusted to an $O.D._{560}$ of 0.2, corresponding to a bacterial cell suspension at 10^8 cfu mL^{-1}. Aliquots of 500 µL in 2 mL cryotubes were stored at -20 °C. For utilization, after liquefying the suspension at room temperature, cells were lysed for 10 min in a boiling water bath, and the cryotubes kept on ice before use.

BOX primer and BOX-PCR reaction

The 22-mer BOXA1R oligonucleotide (Bioprobe Systems/Quantum, France) was used to generate BOX-PCR profiles (Versalovic *et al.*, 1991; Martin *et al.*, 1992). Amplification reactions were performed in volumes of 25 µL, containing 2 µM of the single BOX primer, 200 µM each of dATP, dCTP, dGTP and dTTP (Bioprobe Systems/Quantum, France), PCR reaction buffer (10 mM TrisHCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% TritonX100 and 0.2 mg mL^{-1} bovine serum albumin), 1.5 units of *Taq* DNA polymerase (Appligene-Oncor, France) and, as template DNA, 5 µL of a bacterial cell suspension at 10^8 cfu mL^{-1}. Amplification was performed in an MJ Research, Inc. PTC-100 Thermal Cycler programmed for an initial denaturation step of 7 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 53 °C and 8 min at 65 °C with a final elongation step of 15 min at 65 °C. PCR amplification products were detected by electrophoresis of 12 µL aliquots through 1.4% agarose gels in Tris-borate-EDTA (TBE) buffer (Sambrook and Russell, 2001), which were stained with ethidium bromide (EtBr 1.25 mg/L), visualized under UV light, and printed image through Bio-Print (Vilber Lourmat, France). DNA standards (1-kb DNA ladder Gibco BRL) were included in each electrophoresis gel. All of the amplifications were performed at least twice in separate assays, to ensure the reproducibility of the patterns, and only bands common to the replicate amplifications were scored. DNA fingerprints of strains were first compared for similarity by visual inspection of band patterns. They were considered identical when all scored bands in each pattern had the same apparent migration distance, even if a slightly different molecular weight was assigned to the same band over two or three different electrophoreses. Variations in intensity were not taken as differences.

Following the visual inspection, the patterns of all of the isolates were analyzed more rigorously using the Bio-Profil software (Vilber Lourmat, France). Band sizes were assigned by direct comparison to concurrently run DNA standards (1 kb). This information was used to construct a matrix table where each isolate was matched with a notation +/-, where (+) represents the identical presence and position of a band in the fingerprints to be compared.

Data analysis

Dendrograms were established using TAXONUM, software developed by G. Hunault and L. Gardan (Faculté des Sciences d'Angers, Angers, France). Cluster analysis was carried out using the unweighted pair-group method with averages (UPGMA) with the complement of Jaccard's similarity coefficient (Sneath and Sokal, 1973). Each fragment was considered as a separate marker in pairwise comparisons.

The BOX fragments as well as the biochemical tests characteristic of each cluster were identified by assessing the amount of information provided by each fragment or character, obtained by calculating the diagnostic ability coefficient (DAC) (Descamps and Véron, 1981).

Results

Nutritional characteristics

All of the 120 bacterial strains (Table 1) fitted with the general characteristics of *P. syringae sensu lato* and *P. viridiflava.* They were obligate aerobes, presenting oxidative metabolism of glucose, and being positive for levan (except *P. viridiflava*^{T}, *P. syr. ribicola*^{pt} and *P. syr. primulae*^{pt}) and tobacco hypersensitivity and negative for oxidase and arginine tests, produced fluorescent pigment on King's medium B (except for *P. syr.* a*ctinidiae* strains, *P. sav. glycinea* strain 3356, and *P. sav. phaseolicola* strains 3653 and 4706).

The numerical analysis of 119 nutritional characteristics (data not shown) evidenced two clusters. The first cluster contained *P. sav. phaseolicola*, *P. sav. glycinea*, *P. syr. tabaci* (two strains), *P. syr. mori* and *P. syr. sesami*, all belonging to genomospecies 2, and was distinguishable by two substrates only: sorbitol and meso-tartrate. The second cluster comprised all of the other strains evaluated, *i.e.* all nine genomospecies including other strains of *P. syr. tabaci* and other pathovars of genomospecies 2.

Comparing BOX fingerprints of 120 *Pseudomonas* strains

The amplification of genomic DNA of 88 *Pseudomonas* strains, followed by gel electrophoresis of resulting PCR products, showed 12 to 22 bands for the whole set of strains, and a total of 133 discrete bands were scored, ranging in size from 220 bp to 3.6 kb. From the first list of 89 strains, we were not able to amplify *P. syr. theae* DNA. The data matrix showing presence or absence of these 133 bands was analyzed by Jaccard coefficient and UPGMA, and a dendrogram displaying the distances between the 88 strains is shown in Figure 1.

At a distance of 0.72, all pathovars belonging to each one of the nine genomospecies described by Gardan *et al.*

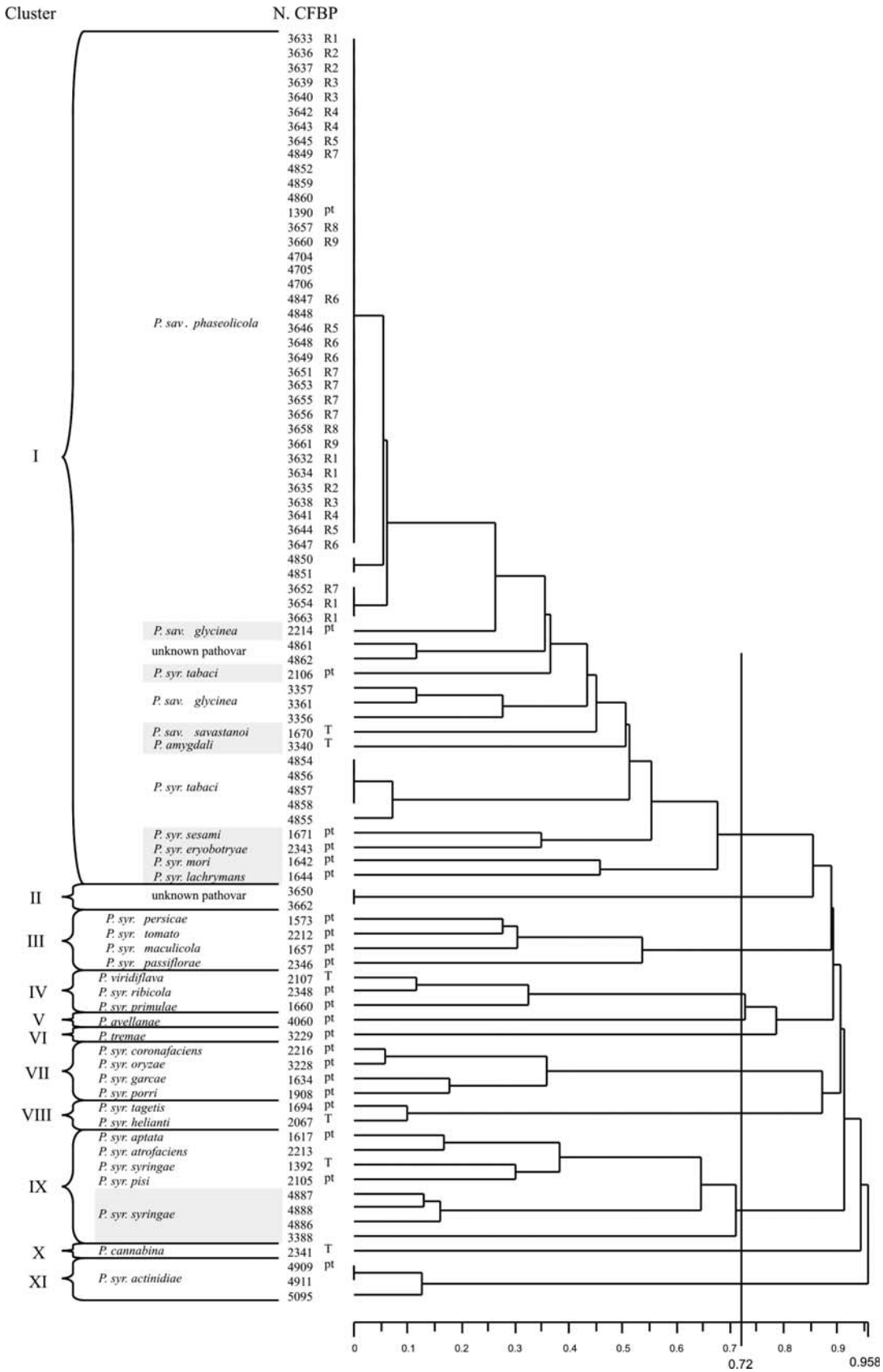

Figure 1 - Dendrogram obtained by comparison of BOX-PCR fingerprinting patterns from 88 bacterial strains belonging to *Pseudomonas savastanoi* species and *P. syringae* - *P. viridiflava* large group (UPGMA analysis, Jaccard coefficient). ^{T}: species type strain, ^{pt}: pathotype strain, R: race of *P. savastanoi* pv. *phaseolicola* as designated by Taylor *et al.* (1996) or race of *P. savastanoi* pv. *glycinea* as designated by Cross *et al.* (1966).

(1999) were clustered together. Cluster I included all of the strains of genomospecies 2. Into this group the great homogeneity of *P. sav. phaseolicola* strains obtained by BOX-PCR fingerprinting is illustrated in Figure 2, where only one different band is found and for only two strains. The eight remaining clusters, III, IV, V, VI, VII, VIII, IX and X corresponded to genomospecies 3, 6, 8, 5, 4, 7, 1 and 9, respectively (Gardan *et al.*, 1999). Inside cluster X, three additional strains of *P. syr. cannabina* were evaluated and showed the same fingerprint as CFPB 2341 (data not shown). The two *P. syringae* strains of unknown pathovars (3650 and 3662) and the three *P. syr. actinidiae* strains were grouped in clusters II and XI, respectively.

The second step of analyses originated from the results shown in Figure 2: the homogeneity of a given pathovar. In order to confirm the utility of BOX-PCR to identify the genomospecies, a second analysis was performed. From the total of 41 strains of *P. sav. phaseolicola,* only the type strain was maintained representing the pathovar, based on its fingerprint homogeneity. This analysis was performed upon 60 pathovar-type strains and one more strain of *P. syr. striafaciens*. A total of 61 strains were included in this step of analysis.

The dendrogram displaying the distance relationships between the strains is shown in Figure 3. At a distance of 0.73 seven clusters were shown (I to VII), where five of them (I, II, III, IV and VII) corresponded strictly to genomospecies 3, 1, 6, 9 and 4. The remaining two clusters (V and VI) clustered together at the mentioned distance, the genomospecies 2 and 5 (cluster V) and 7 and 8 (cluster VI). Despite this fact, when analyzing the two groups at a distance of 0.65 the four genomospecies are separated in tight groups: V.a at 0.65 clustered together all the pathovars of genomospecies 2, V.b clustered all the pathovars of genomospecies 5, VI.a clustered all the pathovars of genomospecies 7 and VI.b all the pathovars of genomospecies 8. Figure 4 shows this similarity in fingerprints between isolates from the same genomospecies, but from different pathovars and the great homogeneity inside genomospecies 4, clustered at a distance of 0.35.

The results of the present study support the observation that BOX-PCR seems to be able to identify bacterial strains at species level.

The two clusters (II and XI, Figure 1) not included into the first description of the genomospecies (Gardan *et al.*, 1999), might eventually constitute two new species.

The bands that most discriminated the ten clusters in the first study were selected by DAC analysis (Table 2).

Discussion

The data obtained from the analyses of phenotypic and genetic diversity of a collection of *P. syringae sensu lato* group, *P. viridiflava,* and reference bacteria showed that pathovars belonging to the genomospecies designed after Gardan *et al.* (1999) could be separated at species level by BOX-PCR pattern.

Although bacteria are traditionally identified by phenotypic descriptions, it is not possible to obtain from nutritional studies a battery of discriminating substrates to the genomospecies as related by Gardan *et al.* (1999), and confirmed in this study.

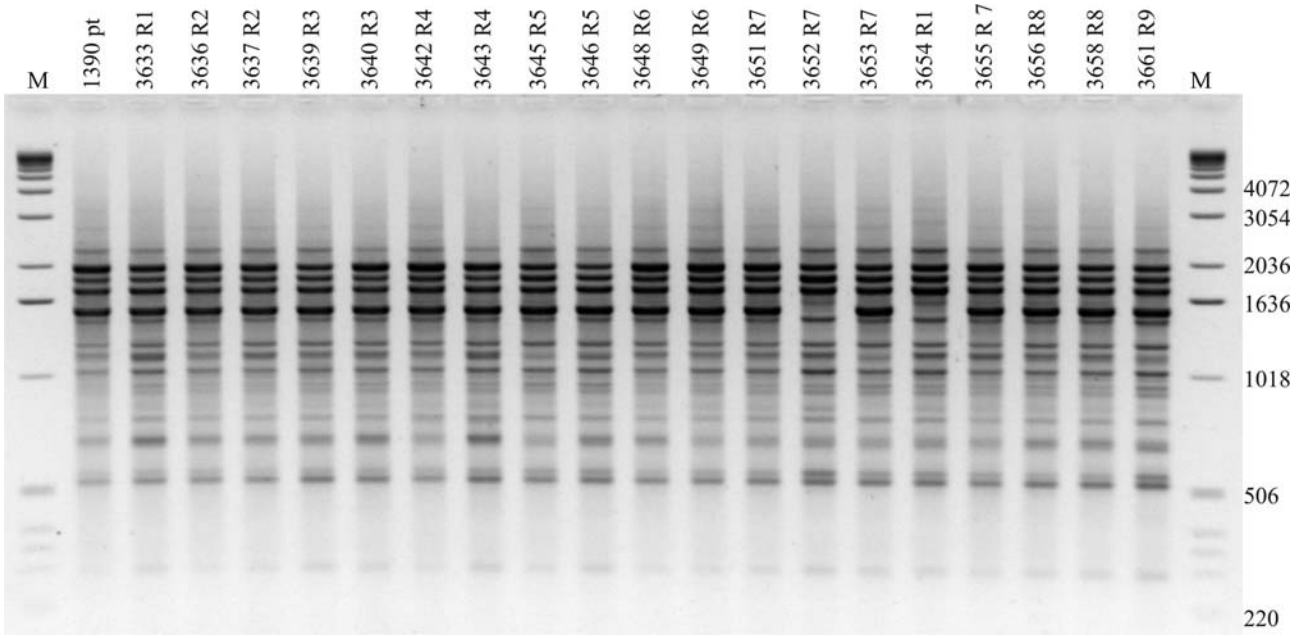

Figure 2 - Agarose gel electrophoresis of BOX-PCR fingerprinting patterns from genomic DNA of very homogeneous *Pseudomonas savastanoi* pv. *phaseolicola* strains, representing some of the nine races of the pathovar (indicated R1 to R9) and obtained from the "Collection Française des Bactéries Phytopathogènes". The molecular size marker is a 1-kb ladder (Gibco BRL Life Technologies Inc.) and the sizes are indicated in base pairs. Shown is a negative image of an ethidium bromide gel. ^{pt}: pathotype strain.

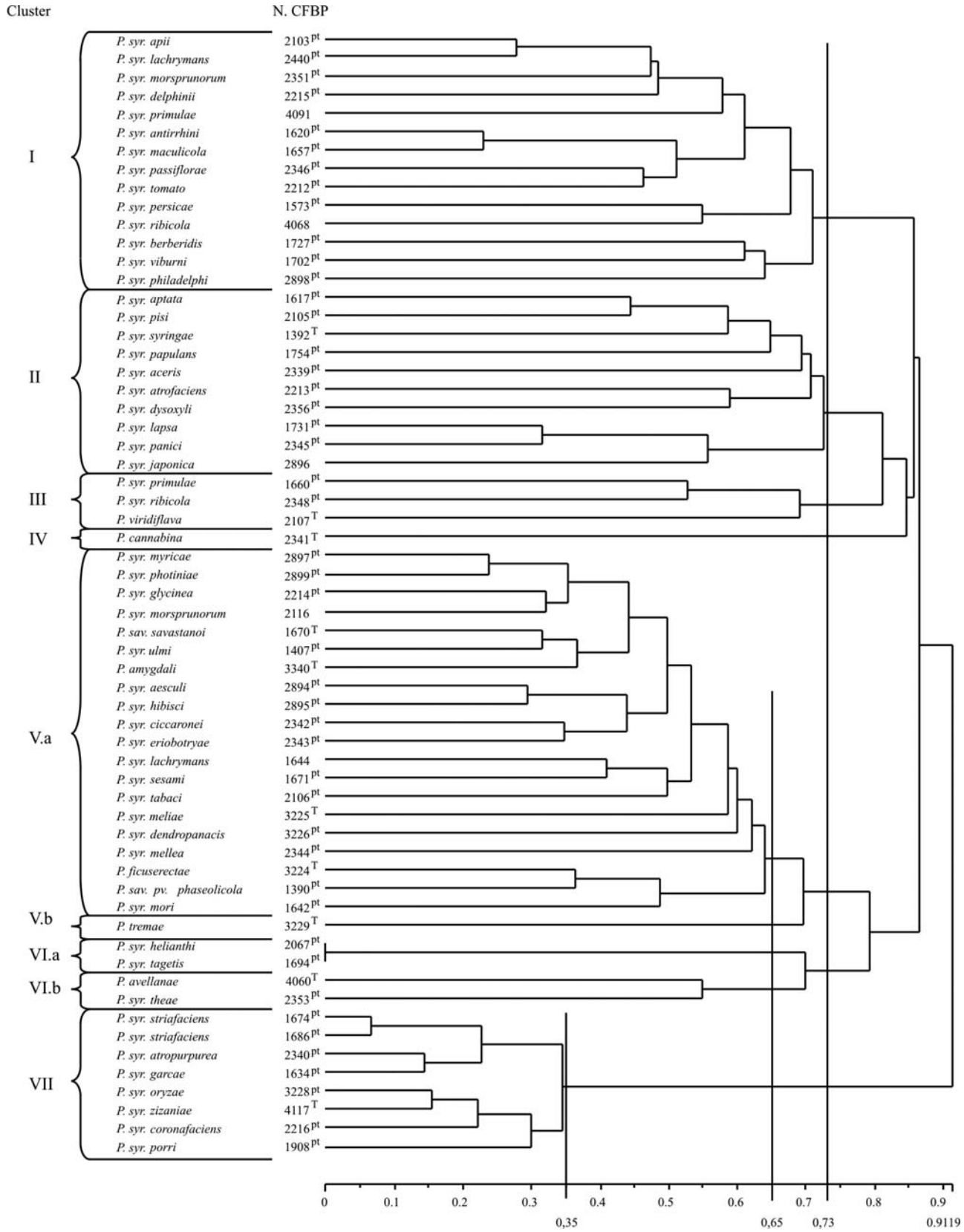

Figure 3 - Dendrogram obtained by comparison of BOX-PCR fingerprinting patterns from 61 bacterial type strains belonging to *Pseudomonas syringae* - *Pseudomonas viridiflava* large group (UPGMA analysis, Jaccard coefficient). Isolates obtained from the "Collection Française des Bactéries Phytopathogènes" (CFBP, Angers, France). ^{T}: species type strain, ^{pt}: pathotype strain.

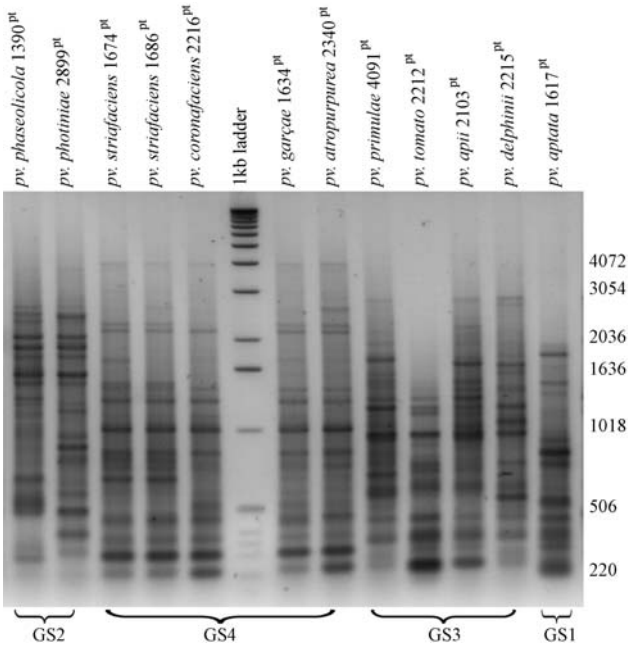

Figure 4 - Agarose gel electrophoresis of BOX-PCR fingerprinting patterns from genomic DNA of different pathovars belonging to *Pseudomonas syringae* (pathovars *photiniae*, *striafaciens*, *coronafaciens*, *garçae*, *atropurpurea*, *primulae*, *tomato*, *apii*, *delphinii* and *aptata*) and *P. savastanoi* pv. *phaseolicola*, which represent some of the nine known genomic species (GS), assigned after Gardan *et al.* (1999). Isolates obtained from the "Collection Française des Bactéries Phytopathogènes" (CFBP, Angers, France). The molecular size marker is a 1-kb ladder (Gibco BRL Life Technologies Inc.) and the sizes are indicated in base pairs. Shown is a negative image of an ethidium bromide gel. [pt]: pathotype strain.

A multiphasic approach has been proposed as being a reliable method of integrating different types of information, such as genotypic, phenotypic and phylogenetic data (Vandamme *et al.*, 1996). Methods of fingerprinting based on the analysis of the total genome may constitute a valuable complement (Rademaker and Bruijn, 1997).

Amplification of Box primer by PCR from DNA of 88 strains belonging to 31 pathovars of *P. syringae sensu lato* group and *P. viridiflava*, led to the establishment of patterns that allowed the distinction of 11 BOX clusters, where nine of them cut the nine genomospecies described inside those pseudomonads (Gardan *et al.*, 1999). When including 30 other strains representing the remaining species and pathovars for this first group, the genomospecies discrimination was confirmed, despite the necessity of cutting the dendrogram at different but close distances. Genomospecies 2 and 5 are very closely related, and the strain CFBP3229 of *P. syr. tremae,* originally included in genomospecies 5, could be clustered with genomospecies 2. A similar situation was found with genomospecies 7 and 8, whose strains could be separated at 0.65. In this study, *P. syr. avellanae* was clustered together with *P. syr. theae,* both corresponding to genomospecies 8. Using different molecular techniques, other authors found that the strains described by Gardan *et al.* (1999) as genomospecies 8 and 3 should be clustered together: Sarkar and Guttman (2004, utilizing multilocus sequencing typing, MLST), Inoue and Takikawa (2006, comparing the *hrpZ* and *hrpA* genes sequences). The present analysis, which used BOX-PCR fingerprinting, is capable of separating genomospecies 3 and

Table 2 - Discriminating BOX fragments, permitting distinction of clusters I to XI (Figure 1).

Group	Presence (+) or absence (-) of discriminating fragment														
	Number of strains	BOX-35	BOX-69	BOX-15	BOX-86	BOX-68	BOX-130	BOX-30	BOX-46	BOX-45	BOX-71	BOX-101	BOX-127	BOX-66	Genomospecies[1]
I[2]	37	+	+	-	-	-	-	-	-	-	-	-	-	-	2
II	2	-	+	-	-	-	-	-	-	+	-	-	-	-	?[3]
III	4	-	+	-	-	-	+	-	-	-	-	-	-	-	3
IV	3	-	-	-	+	-	-	-	-	-	-	-	-	+	6
V	1	-	-	-	-	-	+	+	-	-	+	-	-	+	8
VI	1	-	-	-	-	-	-	+	-	-	+	-	-	-	5
VII	4	-	+	-	+	-	-	+	+	-	-	-	+	-	4
VIII	2	-	-	+	+	-	+	-	+	-	+	+	-	-	7
IX	5	-	-	+	-	+	-	-	-	-	-	-	-	-	1
X	1	-	-	-	-	-	-	-	-	-	+	-	+	-	9
XI	3	-	-	-	-	-	-	-	-	+	-	+	-	-	?[4]

[1]Genomospecies assigned after Gardan *et al.* (1999). [2]Figure 2 contains the list of pathovars and strains of 11 clusters. [3]Strains CFBP 3650 and 3662, unknown genomic species and pathovar. [4]Strains of *Pseudomonas syringae* pv. *actinidiae*, unknown genomic species.

8. It also shows a remarkable homogeneity inside genomospecies 4, whose isolates are maintained together until a distance of 0.35.

Long after Louws *et al.* (1994), claimed BOX analysis could discriminate pathovars of *P. syringae*, it now appears that the authors were dealing with three different genomospecies: pv. *morsprunorum* (genomospecies 2), pv. *syringae* (genomospecies 1), and pv. *tomato* (genomospecies 3). According to Louws *et al.* (1995) BOX-PCR was able to distinguish strains A and B of *X. campestris* pv. *vesicatoria*, separated by DNA-DNA hybridization by Stall *et al.* (1994), and further named *X. axonopodis* pv. *vesicatoria* (ex A) and *X. vesicatoria* (ex B) (Vauterin *et al.*, 1995).

Regarding the two extra groups obtained in this study (clusters II and XI, Figure 1), they are not included in the nine known genomic species of Gardan *et al.* (1999), and might constitute two new species. Cluster II is composed of two bacterial strains isolated from beans (CFBP 3650 and 3662), for which hybridization rate with type strain of *P. sav. phaseolicola* (CFPB 1390 ^{pt}) was only 50% and 51%, respectively (data not shown). In addition, they did not possess the phaseolotoxin gene, their esterase isozyme patterns were very distinct and they were aesculine positive (Marques *et al.*, 2000). If there were no mistakes during the work, they might constitute another distinct bean pathogen belonging to the *P. syringae* group. Cluster XI of the dendrogram is composed of three strains of *P. syr. actinidiae* (CFPB 4909 the pathotype strain, 4911 and 5095). In order to define their inclusion as new genomospecies it would be necessary to provide quantitative DNA-DNA hybridization (Wayne *et al.*, 1987).

Quantitative DNA-DNA hybridization still constitutes the reference for the description of a new species, but it is not adapted to a routine base identification of bacteria. It seems very important to propose alternative techniques, which could reproduce identical results, in order to identify bacterial species.

Since our data basis is constituted from BOX-PCR profiles of 31 or 61 pathovars of *P. syringae* - *P. viridiflava*, new pathovars will be easily assigned to a known genomic species. Furthermore, considering the hypothesis that it may be possible to verify that discriminating bands detected by BOX-PCR technique could amplify specific DNA fragments of each genomic species, a new route would be offered to help identify the *P. syringae* group of plant bacteria at species level.

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