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Genetic characterization by fluorescent AFLP of *Pseudomonas savastanoi* pv. *savastanoi* strains isolated from different host species

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The genetic diversity of 71 *Pseudomonas savastanoi* pv. *savastanoi* strains isolated from different host species and from diverse geographical regions was determined by fluorescent amplified fragment length polymorphism (f-AFLP) analysis. The study was carried out using three different selective primer combinations. Strains of *P. syringae* pv. *syringae*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *glycinea*, *P. syringae* pv. *tagetis* and *P. amygdali* were also included as outgroups. Based on cluster analysis of f-AFLP data, all *P. savastanoi* pv. *savastanoi* strains showed a high degree of similarity, grouping in a cluster and forming a taxon clearly separate from outgroup strains. AFLP analyses failed to support placing strains of *P. savastanoi* pv. *savastanoi*, *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *glycinea* in the same species. Strains of *P. savastanoi* pv. *savastanoi* formed subclusters that correlated with the host species. Strains identified within these subclusters were related to the geographical region where the strains were isolated. Strains of *P. savastanoi* pv. *savastanoi* from olive were divided into two subclusters. Strains from oleander were differentiated from those from ash and were divided into two additional subclusters, distinct from olive strains. Three strains isolated from jasmine showed a high level of similarity among them but, at a lower Dice similarity coefficient, were linked to a subcluster including olive strains. Finally, two strains isolated from privet were similar to strains from olive and were included in the same subcluster.

Keywords: host range, molecular ecology, Oleaceae, *Pseudomonas savastanoi*, taxonomy

Introduction

Pseudomonas savastanoi pv. *savastanoi* (*P. s.* pv. *savastanoi*) (Gardan *et al.*, 1992; Young *et al.*, 1996) [= *P. syringae* subsp. *savastanoi* Janse (1982)] is a plant pathogenic bacterium that causes hyperplastic symptoms on olive (*Olea europaea*), oleander (*Nerium oleander*), ash (*Fraxinus excelsior*) and on minor host plants such as privet (*Ligustrum japonicum*) and *Jasminum* spp. The disease caused on olive plants (known as 'olive knot disease') has been known since ancient times and occurs almost worldwide wherever the olive is grown. However, the classification of this bacterium is still unclear and controversial. The name *P. s.* pv. *savastanoi* includes an assemblage of strains regardless of the host range and physiological and pathological characteristics which differentiate strains isolated from different host species (Janse, 1982; Surico *et al.*, 1985; Surico & Iacobellis, 1992; Iacobellis *et al.*, 1998). Other studies have also shown differences among the various strains that were lumped into *P. s.* pv. *savastanoi*. Strains isolated from olive, oleander and ash have been included in different groups using analysis of whole cell fatty acids (Janse, 1991), low molecular weight restriction fragments (Mugnai *et al.*, 1994) and restriction fragment length polymorphism (RFLP) (Sisto *et al.*, 2002). On the basis of differences found between strains, the names *P. s.* pv. *nerii* and *P. s.* pv. *fraxini* were also reported as valid alternative names (Young *et al.*, 1996) for the strains isolated from oleander and ash, respectively. However, *P. s.* pv. *savastanoi* is the preferred name for strains isolated from both olive plants and other host plants of the Oleaceae.

On the basis of a numerical analysis and DNA hybridization data, Gardan *et al.* (1992) proposed elevating *P. syringae* subsp. *savastanoi* to species level as *P. savastanoi* sp. nov. and more recently, this species was included in a genomospecies distinct from that of *P. syringae sensu stricto* (Gardan *et al.*, 1999). However, the classification of *P. savastanoi* as a bacterial species distinct from *P. syringae* has recently been questioned (Sarkar & Guttman, 2004).

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Sarkar & Guttman (2004) pointed out that the two bacterial species should not be separated, based upon results of multilocus sequence typing (MLST) (Enright & Spratt, 1999), an approach based on diverse housekeeping genes and not simply related to host specificity.

The amplified fragment length polymorphism (AFLP) technique is a DNA fingerprinting method that can be used on any DNA, regardless of its origin or complexity. Its usefulness in characterizing bacterial populations was shown by Janssen *et al*. (1996), who showed the superior discriminative power of this technique in differentiating highly related bacterial strains. Thereafter, AFLP has been used in the genotyping of a number of bacterial species and strains, such as in the genera *Pseudomonas* (Clerc *et al*., 1998; Geornaras *et al*., 1999), *Bacillus* (Ticknor *et al*., 2001), *Ralstonia* (Yu *et al*., 2003), *Xanthomonas* (Schaad *et al*., 2005), and *Yersinia* (Fearnley *et al*., 2005). However, there have been no previous reports on the molecular characterization of strains of *P. s*. pv. *savastanoi* by AFLP fingerprinting.

This study evaluated the genetic diversity of *P. s*. pv. *savastanoi* strains to determine if there were differences that may be related to the host species and/or the geographical region where the strains were isolated. In addition, *P. s*. pv. *savastanoi* strains were compared with strains of *P. syringae* pv. *syringae*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *glycinea*, *P. syringae* pv. *tagetis*, and *P. amygdali* used as outgroups. This was done using fluorescent AFLP (f-AFLP), the latest version of the AFLP method which uses fluorescent dye-labelled primers (Savelkoul *et al*., 1999; Mortimer & Arnold, 2001).

Materials and methods

Bacterial strains, media, growth conditions and DNA extraction

Seventy-one *P. s*. pv. *savastanoi* strains isolated from olive (34 strains), oleander (20 strains), ash (12 strains), privet (two strains) and jasmine (three strains), were used in this study (Table 1). Ten out of the 34 strains from olive were atypical levan-positive strains isolated in central Italy (Iacobellis *et al*., 1993; Marchi *et al*., 2005). *Pseudomonas amygdali* NCPPB2610, *P. syringae* pv. *phaseolicola*

Table 1 Bacterial strains used in this study

Strain[a]	Host plant of isolation	Geographical region
Pseudomonas savastanoi pv. *savastanoi*		
ITM101, ITM105, PVBa206, PVBa207, PVBa223, PVBa224, PVBa225, PVBa227, PVBa229, PVBa230, ITM317, PVFi-Giar3	Olive	Southern Italy
PVFi1[b]*, PVFi2[b]*, PVFi3[b]*, PVFi5[b]*, PVFi6[b]*, PVFi8[b]*, PVFiA14[b]*, PVFiC8[b]*, PVFiC27[b]*, PVFi-T1*	Olive	Central Italy
ITM301, ITM302, ITM304	Olive	USA (California)
ITM901, ITM903, PVFi-DR2	Olive	Greece
ITM906, ITM907, ITM909, ITM913, ITM915, ITM916	Olive	Portugal
PVBa208–1, PVBa215	Privet	Southern Italy
ITM723, ITM724, ITM725	Jasmine	Greece
PVBa204, PVBa213, PVBa219, ITM310, ITM311, ITM313, ITM315, ITM401, ITM402, ITM404, ITM413, ITM510, ITM516, ITM519, ITM521	Oleander	Southern Italy
ITM601, ITM602	Oleander	Northern Italy
ITM305, ITM306	Oleander	USA (California)
NCPPB640	Oleander	Ex Yugoslavia
PVFiF1[b], PVFiF2[b], PVFiF3[b], PVFiF4[b]	Ash	Italy
NCPPB1006, NCPPB1464	Ash	United Kingdom
PD120, PD161, PD179	Ash	The Netherlands
CFBP1838, T5-1[c], PD532	Ash	France
P. amygdali		
NCPPB2610	Almond	Greece
P. syringae pv. *phaseolicola*		
NCPPB2571	Bean	United Kingdom
P. syringae pv. *syringae*		
NCPPB2268	Alfalfa	Ex Yugoslavia
P. syringae pv. *glycinea*		
NCPPB2753	Soybean	France
P. syringae pv. *tagetis*		
NCPPB2489	*Tagetes erecta*	United Kingdom

[a]CFBP, Collection Française de Bactéries Phytopathogènes, INRA, Angers, France; ITM, Culture collection of Istituto Tossine e Micotossine da Parassiti vegetali, C.N.R., Bari, Italy; NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK; PD, Culture collection of Plant Protection Service, Wageningen, the Netherlands; PVBa, Culture collection of Dipartimento di Patologia vegetale, Università degli Studi, Bari, Italy; PVFi, Culture collection of Dipartimento di Biotecnologie Agrarie-Patologia vegetale, Università degli Studi, Firenze, Italy.
[b]from G. Surico.
[c]from L. Gardan.
*Levan-positive strain.

NCPPB2571, *P. syringae* pv. *syringae* NCPPB2268, *P. syringae* pv. *glycinea* NCPPB2753 and *P. syringae* pv. *tagetis* NCPPB2489 were used as outgroup strains in AFLP analysis.

P. savastanoi and *P. syringae* strains were grown for 36 h at 26°C on 5% sucrose nutrient agar (SNA) (Nutrient Broth-DIFCO) or King's B (KB) agar (King *et al*., 1954). For liquid cultures, after growth on the above-mentioned media, bacteria were added to 20 mL of KB broth and incubated at 26°C on an orbital shaker (140 rpm) up to a bacterial concentration of about 10^8 cfu mL^{-1}. *Pseudomonas amygdali* was grown under the same conditions using medium 523 agar or broth (Kado *et al*., 1972). Bacterial DNA was extracted from liquid cultures by using the Puragene DNA Isolation Kit (Gentra System) according to manufacturer's instructions. Bacteria were preserved at −80°C, in a liquid medium supplemented with 20% glycerol.

16S rDNA sequence analysis and amplified 16S ribosomal DNA restriction analysis (ARDRA)

Amplification of 16S rDNA was performed using 40 ng of bacterial genomic DNA from each sample, according to the procedure of Di Cello *et al*. (1997), with slight modifications. The universal primers P0 and P6 used for 16S rDNA amplification (Caccamo *et al*., 1999) are located at the 5′ and 3′ ends of the 16S rRNA gene and their locations make possible the amplification of almost all the gene. Each 25 μL reaction mixture contained 10 mM Tris-HCl, 50 mM KCl, 1·5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 1 μM of each primer and 1 U of *Taq* DNA polymerase (Polymed). The reaction mixtures were first incubated for 1 min 30 s at 95°C, and then cycled for 35 cycles according to the following temperature profiles: 30 s at 95°C, 30 s at annealing temperature of 60°C for the first five cycles, 55°C for the next five cycles and 50°C for the last 25 cycles, then 4 min at 72°C, followed by a final extension for 10 min at 72°C.

Aliquots (3 μL) of the PCR products were analysed by agarose gel electrophoresis (1·2% w/v) in TAE buffer (Sambrook *et al*., 1989), stained with 0·5 μg mL^{-1} of ethidium bromide, and photographed under ultraviolet light. The length of the amplified DNA fragments was estimated by comparison with a 1 Kb Plus DNA Ladder (Invitrogen). The PCR products were then purified with a QIAquick gel extraction kit (Qiagen), and then used for both restriction analysis of amplified 16S rDNA (ARDRA) and sequencing.

For ARDRA experiments, 1 μg of each amplicon was digested with 3 units of the restriction enzyme *Alu*I (Invitrogen) in a total volume of 20 μL at 37°C for 3 h. The enzyme was then inactivated with treatment at 65°C for 10 min, after which the products of digestion were analysed by agarose gel electrophoresis (2·5% w/v) in TAE buffer.

Sequencing of the 16S rDNA PCR products was performed by using the universal primers P0, P5 and P6 (Caccamo *et al*., 1999) with the ABI PRISM BigDye Terminator Ready Reaction Kit V 3·1 (Applied Biosystems) and run on an Applied Biosystems ABI Prism 3100 Genetic Analyzer. 16S rRNA gene sequences were examined for similarity with deposited sequences using the BLAST algorithm (Altschul *et al*., 1997) and the distance tree option available through the National Institute of Health's National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/).

f-AFLP analysis

f-AFLP analysis was performed by using the AFLP Microbial Fingerprinting kit (Applied Biosystems) according to the manufacturer's recommendations. For each strain tested, approximately 10 ng of genomic DNA was digested with *Eco*RI and *Mse*I restriction endonucleases (New England Biolabs), and simultaneously ligated to *Eco*RI and *Mse*I site-specific adapters. A preselective PCR (72°C for 2 min; 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min) was carried out in a 20 μL (final volume) mixture using *Eco*RI + 0 and *Mse*I + 0 primers and PCR products of each reaction were diluted 20:1 with TE. For selective PCR, 1·5 μL of the resulting diluted PCR samples were amplified in a 10 μL (final volume) mixture using selective primers.

To determine which selective primer pairs yielded the highest quality of AFLP fingerprints, twelve selective primer pairs were initially tested on a sample of six *P. s*. pv. *savastanoi* strains; thereafter, PCR products of preselective amplifications from 76 strains (Table 1) were analyzed using the following combinations of *Eco*RI and *Mse*I adaptor-specific primers with base selection: *Eco*RI + A/*Mse*I + 0, *Eco*RI + G/*Mse*I + C and *Eco*RI + G /*Mse*I + T. The *Eco*RI primers were labelled with fluorescent dye (Applied Biosystems).

The PCR programme for selective AFLP amplification was as follow: one cycle of 94°C for 2 min and one cycle of 94°C for 20 s, 66°C for 30 s and 72°C for 2 min; this cycle was followed by nine cycles in which the annealing temperature was reduced by 1°C at each cycle from 65°C to 57°C. After that, 20 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 2 min were performed, followed by a final step at 60°C for 30 min. PCR amplifications were performed in a model 9700 Perkin-Elmer thermocycler (Applied Biosystems). After amplification, 1 μL of reaction product was mixed with 20 μL of formamide and 1 μL of GeneScan-500 (ROX) size standard (Applied Biosystems), ranging from 35 to 500 bp in size. The mixture was heated for 3 min at 95°C and cooled on ice. The amplification products were separated by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Fragments were sized by using ABI Genescan version 2·1 software (Applied Biosystems) and all the electropherograms were visually inspected and compared for peak detection with the same software. Peak height threshold was set at 50 fluorescent arbitrary units and any peak less than this value was not included in the analysis. To ascertain the reproducibility of AFLP fingerprints, f-AFLP analyses were repeated at least twice. The presence or

absence of a peak was scored as 1 or 0, respectively, and the binary matrix obtained in an Excel format was then used as input for the NTSYSpc cluster analysis software (version 2·1, Exeter Software). Cluster analysis was performed by using the Dice similarity coefficient (S_D) and the unweighted pair group method with averages (UPGMA) algorithm provided in the NTSYSpc software package.

Results

16S rDNA sequence analysis and ARDRA

A single band of about 1450 bp was obtained from all strains of *P. s.* pv. *savastanoi* isolated from olive, oleander and ash when amplifying and sequencing their 16S rDNA. Sequencing of the amplicons showed that strains from olive and oleander had only a few base changes that were not related to the species of host plant. By contrast, all the strains from ash showed a point substitution (A/G) at position 557, originating a sequence corresponding to an extra *Alu*I restriction site in addition to those common to strains from olive and oleander. ARDRA using *Alu*I confirmed that ash strains were very different from olive and oleander strains.

Sequences of the 16S rRNA gene from bacterial strains PVBa224 (from olive), ITM313 (from oleander) and PVFiF1 (from ash) were submitted to GenBank as accession numbers AM265392, AM265391 and AM265390, respectively. A BLAST analysis on these sequences verified that these strains belonged to the species *P. savastanoi*. In fact, the results obtained showed that all three sequences AM265390, AM265391 and AM265392 clustered together with other *P. s.* pv. *savastanoi* 16S rDNA sequences present in the databases.

AFLP analyses

Replicate AFLP experiments generated highly reproducible peak patterns. In total, 249 diverse AFLP peaks were scored in the set of *P. s.* pv. *savastanoi* strains within the range 50–500 bp, of which 180 (72%) were polymorphic. The average number of peaks generated per strain by primer pairs *Eco*RI + A/*Mse*I + 0, *Eco*RI + G/*Mse*I + C and *Eco*RI + G /*Mse*I + T was 57, 37 and 31, respectively.

All *P. s.* pv. *savastanoi* strains were linked at the S_D level of 0·84 and assigned to a single large cluster (I) (Fig. 1). They were also clearly distinguished from the outgroup strains which showed an S_D level lower than 0·52. Cluster I comprised subclusters IIa (S_D = 0·86), IIb (S_D = 0·89), IIc (S_D = 0·89) and IId (S_D = 0·90) (Fig. 1). All the strains isolated from ash constituted subcluster IId; subcluster IIb contained only strains from oleander. By contrast, strains isolated from olive plants were split between subclusters IIa and IIc. The three strains from jasmine showed a high level of similarity (S_D = 0·96) and were linked to subcluster IIc at the S_D level of 0·88.

Further subclusters seem to be mainly related to the geographical region where the strains were isolated. Subcluster IIa contained subclusters IIIa and IIIb. Subcluster IIIa included strains isolated in southern Italy from olive plants together with two strains isolated from privet. By contrast, strains from Portugal and California were grouped, but clearly distinguished, in subcluster IIIb. The majority of strains isolated from oleander originated from southern Italy; they formed subcluster IIIc and were differentiated from two strains isolated in California (ITM305, ITM306) and a strain isolated in the former Yugoslavia (NCPPB640). Seven strains isolated from olive plants in southern Italy were grouped in subcluster IIId together with two strains isolated in Greece. All the strains present in subcluster IIIe were characterized by the same geographical origin (central Italy) as well as by atypical phenotypic features (non-fluorescent and levan-positive). Finally, strains isolated from ash in Italy were clearly differentiated within subcluster IId from the other strains isolated in different geographical regions.

Discussion

AFLP analyses showed a high degree of similarity of all *P. s.* pv. *savastanoi* strains which formed a single taxon, clearly differentiating them from all outgroup strains. Since the outgroup strains in this study included *P. syringae* pv. *syringae* NCPPB2268, *P. syringae* pv. *tagetis* NCPPB2489, *P. amygdali* NCPPB2610, *P. syringae* pv. *phaseolicola* NCPPB2571 and *P. syringae* pv. *glycinea* NCPPB2753, the results support the classification of *P. savastanoi* as a species distinct from *P. syringae*. However, AFLP analyses failed to support placing strains of *P. s.* pv. *savastanoi*, *P. s.* pv. *phaseolicola* and *P. s.* pv. *glycinea* in the same species as previously proposed (Gardan *et al.*, 1992). Moreover, the results showed that AFLP fingerprints were useful for distinguishing *P. s.* pv. *savastani* from related bacterial species including *P. s.* pv. *syringae*, *P. s.* pv. *tagetis*, *P. s.* pv. *phaseolicola*, *P. s.* pv. *glycinea* and *P. amygdali*.

Although all *P. s.* pv. *savastanoi* strains were included in a single cluster, a number of subclusters were clearly identified by AFLP analyses. Previous studies, based on DNA-DNA hybridization (Gardan *et al.*, 1992, 1999) or on biochemical tests (Bella *et al.*, 2003), did not distinguish *P. s.* pv. *savastanoi* strains according to the host species. On the contrary, the findings here further support previous studies, based on DNA fingerprinting (Mugnai *et al.*, 1994; Sisto *et al.*, 2002), which revealed genetic differences related to the host species. Although sequencing of the 16S rRNA gene did not, as expected, discriminate between closely related strains isolated from olive and oleander, it did distinguish the above-mentioned strains from those isolated from ash.

Some clusters, based on AFLP analysis, correlated with the species of the host plant. All *P. s.* pv. *savastanoi* strains isolated from oleander or ash were grouped into two separate clusters. These results were consistent with phytopathological features which characterize and distinguish *P. s.* pv. *savastanoi* strains isolated from the above-mentioned host species (Surico *et al.*, 1985; Iacobellis *et al.*, 1998). In addition, these findings support

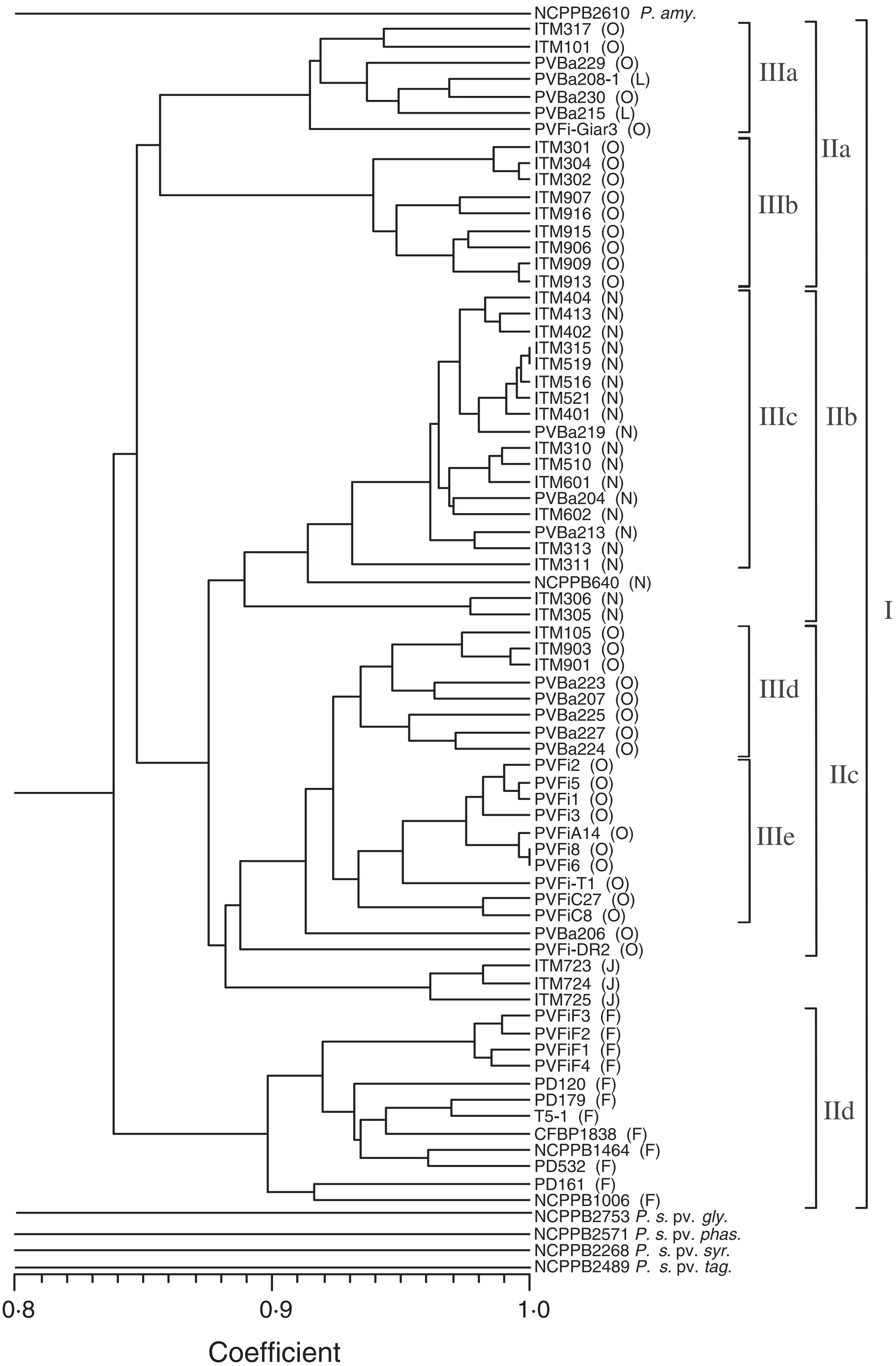

Figure 1 Dendrogram based on f-AFLP fingerprints of 71 *Pseudomonas savastanoi* pv. *savastanoi* strains and five outgroup strains. The dendrogram was constructed by using UPGMA and the DICE similarity coefficient. Outgroup strains are *P. amygdali* NCPPB2610, *P. syringae* pv. *phaseolicola* NCPPB2571, *P. syringae* pv. *syringae* NCPPB2268, *P. syringae* pv. *glycinea* NCPPB2753 and *P. syringae* pv. *tagetis* NCPPB2489. The clusters of strains and their designations are labelled to the right of the dendrogram. Cluster I includes all *P. s.* pv. *savastanoi* strains. The capital letters in brackets following the strain codes are the host plants *P. s.* pv. *savastanoi* strains were isolated from: O, *Olea europaea*; N, *Nerium oleander*; F, *Fraxinus excelsior*; L, *Ligustrum japonicum*; J, *Jasminum* spp.

the validity of both denominations *P. savastanoi* pv. *nerii* and *P. savastanoi* pv. *fraxini* (Young *et al*., 1996) which seem to reflect clear genetic differences. *Pseudomonas s.* pv. *savastanoi* strains from olive plants were separated into two clusters on the basis of AFLP fingerprints and seem to be more genetically heterogeneous than those from oleander and ash. In addition, one of those clusters included two strains isolated from privet together with olive strains. The high level of similarity of strains from privet with some strains isolated from olive is not unexpected because cross-pathogenicity tests indicated that strains from privet may cause disease on olive plants and vice versa (Janse, 1982). It is possible that strains isolated from olive and those from privet are not distinct bacterial populations and therefore are indistinguishable by AFLP analysis. However, additional privet strains should be examined before any conclusions are made. The position of strains from jasmine remains unclear; they grouped with olive strains but at a lower S_D level.

AFLP-based results of this study revealed that strains from the same geographical region are, in the majority of cases, genotypically more closely related to one another than those isolated from different geographical regions. However, the similarity between groups of strains isolated from different regions suggests the same genetic origin of these strains.

The results support the classification of atypical levan-positive strains isolated from olive plants as *P. savastanoi* (Iacobellis *et al*., 1993; Marchi *et al*., 2005). However, it remains to be established whether the grouping of all those strains into a single cluster is due to their geographical origin (central Italy) or to their phenotypic features.

The studies here clearly demonstrate the discriminative power of the AFLP technique in enabling a detailed analysis of the genetic variation between strains of *P. s.* pv. *savastanoi* both from different host species, and from diverse geographical locations. AFLP fingerprints could provide an excellent tool in epidemiological studies and provide useful markers for development of diagnostic PCR, or for identifying genes responsible for phenotypes.

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