SHORT COMMUNICATION

TYPING OF *PSEUDOMONAS SYRINGAE* pv. *PISI* STRAINS BY FLUORESCENT AFLP FINGERPRINTING

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SUMMARY

Eighteen well-characterized *Pseudomonas syringae* pv. *pisi* strains, representative of the pathogen's seven races were investigated by fluorescent AFLP (fAFLP) fingerprinting and were clearly differentiated. Typing by fAFLP provided a distinct, differentiating profile for each of the strains. Cluster analysis of the data grouped the seven *P. syringae* pv. *pisi* races into two well-separated clusters, these showing a genetic similarity of 68%. Group I included all strains belonging to races 1, 3B, 4B, 5 and 7, and group II included the strains belonging to races 2, 3A, 4A, 6 and local strains of race 6. It was possible to distinguish strains of races 1 and 3B, 4A and 6, 1 and 5, as well as strains of races 3 and 6, indistinguishable by other previously used techniques. Analysis of the fAFLP electropherograms also allowed, in some cases, identification of group-specific and race-specific discriminative fragments.

Key words: fAFLP, molecular typing, *Pseudomonas syringae* pv. *pisi*.

Bacterial blight of pea (*Pisum sativum* L.), caused by *Pseudomonas syringae* pv. *pisi* (Sackett) Young, Dye and Wilkie (Sackett, 1916), is a seed-borne disease occurring in most pea growing areas all over the world, occasionally causing serious reduction in yield and seed quality following epidemics (Taylor *et al.*, 1972; Bradbury, 1986; Roberts *et al.*, 1995; Masuda and Nishiyama 2001).

The pathogen shows a well-defined race structure and seven races have been distinguished on the basis of their reaction on a set of eight differential cultivars of pea. The genetics of the cultivar-race combination have been explained in terms of a gene-for-gene relationship (Bevan *et al.*, 1995), and specific resistance genes in the host and avirulence genes in the pathogen result in race/cultivar incompatibility and induction of a hypersensitive reaction. Taylor *et al.* (1989) found widespread resistance in different commercial pea cultivars to races carrying different avirulence genes, whereas race 6 carries no avirulence genes and is able to attack all pea cultivars. The disease has been reported in Europe since 1985 (Stead and Pemberton, 1987; Schmit, 1991). The only indication of pea blight in Italy was provided by Cirulli and Ercolani (1969) who identified the pathogen (NCPPB 2222) later referred to as race 1. In 1996, *P. syringae* pv. *pisi* was isolated in several field crops in Sicily (Cirvilleri and Caldarera, 1997) and the strains were identified as race 6 (Cirvilleri *et al.*, 1998).

Identification and characterization of strains belonging to the different races of *P. syringae* pv. *pisi* is necessary to determine the choice of resistant cultivars in different growing areas and also to improve detection protocols and control measures. Various molecular techniques have been used to characterize strains and races of *P. syringae* pv. *pisi.* Two sets of specific primers enabling the amplification of fragments potentially unique to *P. syringae* pv. *pisi* allowed the separation of races into two phylogenetic groups, designated I (containing the races 1, 3B, 4B, 5 and 7) and II (containing races 2, 3A, 4A and 6), but no further difference between races was detected (Arnold *et al.*, 1996). In that study, the authors observed for the first time that a few strains of races 3 and 4 (group II) showed the DNA fragments of group I, and thus distinguished races 3A from 3B and 4A from 4B. The seven races were also separated into the same two groups I and II with PCR primers designed from the genes *avrPpiA1* and *avrPpiB1* (Catara *et al.*, 1998) and with primers based on highly conserved areas sequences upstream and downstream of *avrPpiA1* and *avrPpiB1* (Arnold *et al.*, 2001).

In both studies the profiles obtained did not differentiate between races 1 and 3B nor between races 4A and 6. Strains of the seven races of *P. syringae* pv. *pisi* were also analysed by repetitive-sequence PCR (rep-PCR) and clamped homogeneous electric field (CHEF) (Cirvilleri *et al.*, 1998) and the same two groups of races were detected. The first group included strains belonging to races 1, 5 and 7, the other group included strains of races 2, 3A, 4A and 6 (3B and 4B were not included in that study), plus 20 local strains isolated in Sicily and identified as race 6 (Cirvilleri *et al.*, 1998). Rep-PCR did

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not differentiate between strains and races within the two groups, whereas CHEF analysis delineated six different profiles, and distinguished strains of races 4A and 6; it was not possible to distinguish between strains of races 1 and 5 and between strains of races 3 and 6. Moreover, local strains of race 6 were identical and showed high similarity to the strains of race 3 and 6.

The objective of this study was to characterize and distinguish *P. syringae* pv. *pisi* strains belonging to the seven races by the fluorescent AFLP (fAFLP) technique to obtain a better insight on strain diversity in *P. syringae* pv. *pisi*. The strains used in this study (Table 1) included eighteen representative of the seven races of *P. syringae* pv. *pisi* and one *P. syringae* pv. *syringae* strain (NCPPB 3869) used as outgroup.

Genomic DNAs were extracted and purified using the DNA Purification Kit (Puregene, Gentra, Minneapolis, MN, USA), following the manufacturer's instructions. DNA templates were prepared using restriction enzymes *Eco*RI and *Mse*I and adaptor ligations were carried out as reported by Vos *et al.* (1995) and modified by Cirvilleri *et al.* (2006). The PCR mixtures consisted of 5 µl of ligated DNA, 1 µl of 5 µM *Mse*I-primer, 1 µl of 5 µM *Eco*RI-primer labeled at the 5' end with cy5 fluorophore, 1 µl of each of the deoxynucleoside triphosphates (10 mM), and 5 U of Taq polymerase. Enzymes, T4 DNA ligase, dNTPs and Taq polymerase were from Invitrogen-Life Technologies (Paisley, UK) and all primers and oligos were from MWG Biotech, Inc. (High Point, NC, USA). PCRs were performed in a DNA thermal cycler (GeneAmp PCR system 9600, Perkin Elmer, Norwalk, Connecticut, USA). The fAFLP products were separated with a CEQ 8000 Genetic Analysis System automated DNA sequencer (Beckman and Coulter, Fullerton, CA, USA) as previously described (Cirvilleri *et al.,* 2006).

Table 1. Strains of *Pseudomonas* used in this study.

Species	Strain	Race	fAFLP group	fAFLP subgroup	Collection number	Origin	Country
P. s. pv. *pisi*	299A (T)	1	I	1	NCPPB 3430	HRI, Wellesbourne, UK	New Zealand, 1970
"	4585	3B	I	2		HRI, Wellesbourne, UK	Australia, 1992
"	4578A	3B	I	2		HRI, Wellesbourne, UK	Australia
"	1812A	4B	I	2		HRI, Wellesbourne, UK	U.S.A.
"	4481	5	I	1		L. Gardan*	**
"	974B (T)	5	I	1	NCPPB 3433		U.S.A., 1978
"	1691	7	I	2		HRI, Wellesbourne, UK	Australia, 1976
"	4129	2	II			L. Gardan*	**
"	203	2	II			HRI, Wellesbourne, UK	New Zealand, 1969
"	870A (T)	3A	II	4	NCPPB 3496	HRI, Wellesbourne, UK	U.S.A., 1975
"	1380A	4A	II	4		HRI, Wellesbourne, UK	U.S.A., 1985
"	895A (T)	4A	II	4	NCPPB 3500	HRI, Wellesbourne, UK	U.S.A, 1975
"	1704B (T)	6	II	3	CFBP 2709	HRI, Wellesbourne, UK	France, 1986
"	1683	6	II	3		HRI, Wellesbourne, UK	Hungary, 1956
"	1688	6	II	4		HRI, Wellesbourne, UK	France, 1973
"	B2	6	II	4		PVCT	Italy, 1999
"	F2	6	II	4		PVCT	Italy, 1999
"	P2	6	II	3		PVCT	Italy, 1999
P. s. pv. *syringae*	ISPaVe015				NCPBB 3869	ISF, Rome, Italy	Italy, 1992

HRI = Horticulture Research International, Wellesbourne, UK
PVCT = Plant Pathology, University of Catania, Italy.
CFBP = Collection Francaise de Bacteries Phytopathogens, INRA, Angers, France.
NCPPB = National Collection of Plant Pathogenic Bacteria, Plant Pathology Laboratory, Harpenden, Hertsfordshire, UK.
T = Type strain
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**no information is available concerning the country of origin

The data were analyzed using CEQ 8000 analysis software. Electropherograms of all fAFLP profiles were visually inspected for polymorphisms, with the presence (1) or absence (0) of fragments from 60 to 700 bp scored in a binary matrix and stored in Microsoft Excel 2000. Cluster analysis was performed according to the unweighted pair-group method with average linkages (UPGMA) exporting the output files to the PHYLIP 3.6 software package (Felsenstein, 2004). Similarity coefficients were determined using Dice's coefficient (Dice, 1945). A dendrogram was constructed and its robustness was assessed by bootstrap analysis (1,000 repeated samplings) (Felsenstein, 1985).

Analysis by fAFLP of the 18 *P. syringae* pv. *pisi* strains gave 18 different fingerprints. The *Eco*RI/*Mse*I primers amplified a number of fragments ranging between 29 (strain 181.2.A race 4B) and 63 (strain F2 race 6). The peak heights, indicating the relative fluorescence of the fragments detected, did not vary between replicate runs with identical DNAs. The dendrogram of 18 the *P. syringae* pv. *pisi* strains derived from UPGMA analysis is shown in Fig. 1. Two well-separated main clusters were distinguished when using a 68% similarity index as cut-off: cluster I, including all strains belonging to races 1, 3B, 4B, 5 and 7, and cluster II, including strains belonging to races 2, 3A, 4A, 6 and local strains of race 6. The race clustering was in agreement with clusters previously delineated reported (Arnold *et al.*, 1996; 2001; Catara *et al.*, 1998; Cirvilleri *et al.*, 1998).

fAFLP analysis discriminated between clusters and between races included in the same cluster. Moreover, all strains within the same race were distinguished. In cluster I, subgroup 1 consisted of strains of races 1 and 5, and subgroup 2 contained strains of races 3B, 4B and 7.

In cluster II, strains of race 6 also clustered in two subgroups (3 and 4). fAFLP allowed discrimination between local strains of race 6, indistinguishable by CHEF analysis (Cirvilleri *et al.*, 1998). Race 6 local strain B2

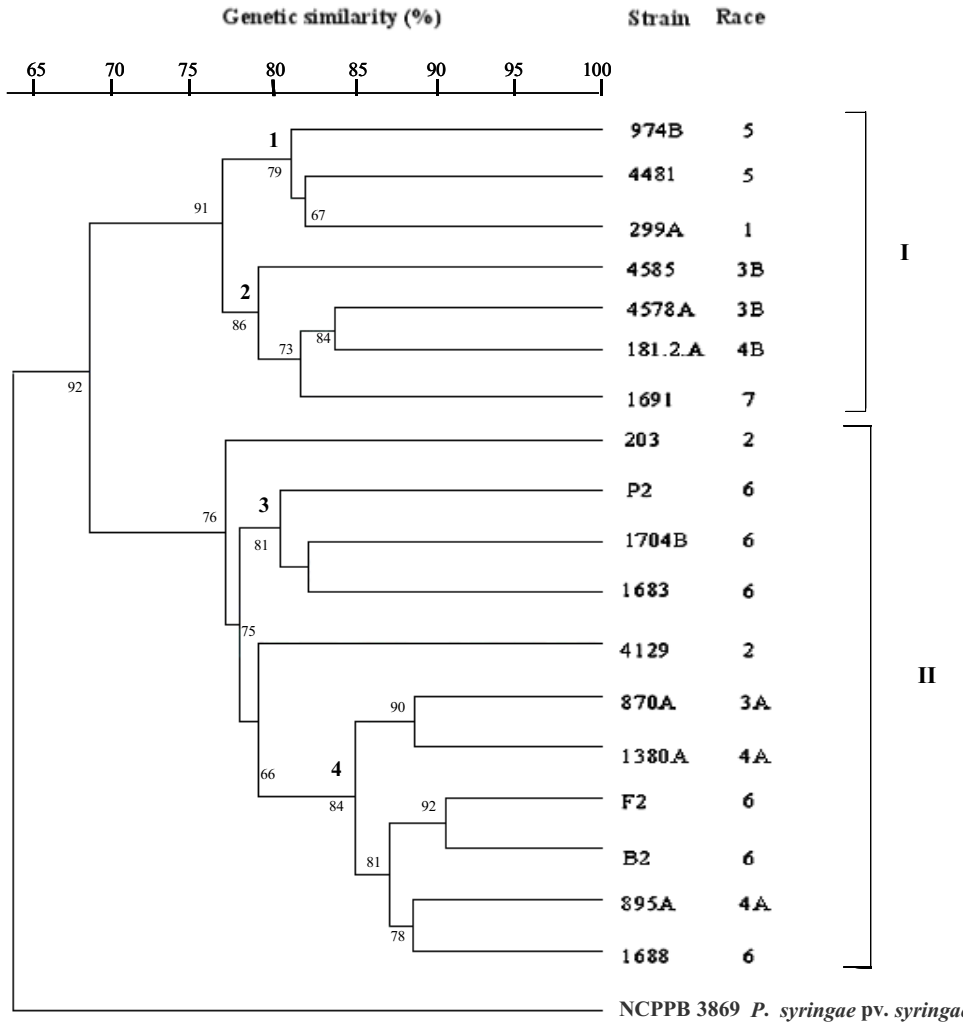

Fig. 1. Dendrogram of 18 *Pseudomonas syringae* pv. *pisi* strains representative of the seven races analyzed by fluorescent AFLP. Similarity between fingerprints was calculated by the unweighted pair-group method with average linkages (UPGMA) using Dice's coefficient, and the robustness of the tree was assessed by bootstrap analysis (1,000 repeated samplings). I and II are genomic groups. 1, 2, 3, and 4 are genomic subgroups. *P. syringae* pv. *syringae* strain NCPPB3869 was used as an outgroup.

clustered in subcluster 3, whereas race 6 local strains F2 and B2 clustered together in subcluster 4 and showed maximum similarity (92%). Race 2 strains 203 and 4129 did not fall into either of subgroups 3 and 4. fAFLP fingerprinting distinguished between strains of races 1 and 3B and between strains of races 4A and 6 (indistinguishable with previously used PCR analysis) and allowed discrimination between strains of races 1 and 5 and between strains of races 3 and 6 (indistinguishable with CHEF analysis). Bootstrap values show the robustness of the tree. The main group and subgroup nodes had moderate consistency in clustering and showed confidence at a maximum of 0.92.

Group-specific discriminative fragments were identified. All strains belonging to races of cluster-group II showed three specific fragments of 164, 177 and 295 bp, whereas no fragment was common to all strains belonging to cluster-group I (Table 2).

Race-specific discriminative fragments were also identified in strains of race 1, 5, 7 and 2 (Table 2).

In the present study, fAFLP analysis was used to characterize and differentiate representative strains of the seven races of *P. syringae* pv. *pisi*. Previously, multiplex PCRs with four pairs of primers, two designed from genes *avrPpiA1* and *avrPpiB1* (Catara *et al.*, 1998), allowed assignment of strains to their races using single-PCR amplification, but the profiles obtained did not differentiate between strains of races 1 and 3B, nor between strains of races 4A and 6. Specific primers designed from conserved regions flanking *avrPpiA1* from race 2 (Vivian *et al.*, 1989) and *avrPpiB1* from race 3A (Cournoyer *et al.*, 1995) led to identification of two novel genes: *avrPpiG*, present only in races of *P. syringae* pv. *pisi* belonging to group II, and *avrPpiC*, detected in a wide range of *P. syringae* pathovars (Arnold *et al.*, 2001). Moreover, two primers, DA72 and DA73, designed from highly conserved sequences upstream and downstream of *avrPpiA1* and *avrPpiB1* (Arnold *et al.*, 2001) were used to amplify fragments from each of the races of *P. syringae* pv. *pisi*. However, profiles thus obtained did not differentiate between strains of races 1 and 3B, nor between strains of races 4A and 6.

Since DNA fingerprinting methods used until now were not suitable for strain and race differentiation, *f*AFLP was assessed and appeared suitable for studying genetic polymorphism in this pathovar. In addition, we found that the discriminatory power of *f*AFLP was higher than that of CHEF (Cirvilleri *et al.*, 1998). Indeed, *f*AFLP revealed different profiles for the strains belonging to race 6 and for strains of races 1 and 5 and 3 and 6. Moreover, this technique differentiated strains belonging to races 1 and 3B and of strains belonging to races 4A and 6 that

Table 2. FAFLPs characteristic fragments pointed out by fAFLP analysis in *Pseudomonas syringae* pv. *pisi* strains.

Strains	Race	FAFLP group	Presence of fragments of the following size (bp)[a]													
			75	120	123	134	148	164	177	212	272	295	395	421	432	541
299A	1	I		+						+				+		+
4585	3B	I														
4578A	3B	I														
1812A	4B	I														
4481	5	I	+													
974B	5	I	+													
1691	7	I			+	+					+		+		+	
4129	2	II					+	+	+			+				
203	2	II					+	+	+			+				
870A	3A	II						+	+			+				
1380A	4A	II						+	+			+				
895A	4A	II						+	+			+				
1704B	6	II						+	+			+				
1683	6	II						+	+			+				
1688	6	II						+	+			+				
B2	6	II						+	+			+				
F2	6	II						+	+			+				
P2	6	II						+	+			+				

[a] +, differential fragments characteristically present in fAFLP profiles.

were indistinguishable with primers based on *avrPpiA1* and *avrPpiB1* (Catara *et al.*, 1998; Arnold *et al.*, 2001).

fAFLP analysis possesses several advantages over other techniques. It is inherently flexible, and its discriminatory power can be increased or decreased through the use of different selective primers, however it had not been shown that this is a good technique for phylogenetic and molecular analysis. Figure 1 now clearly reveals the variation that exists: strains of race 2 were distantly related, as well as strains of race 6. Increased resolution obtainable by using additional primer sets is probably needed to optimise the power of the technique. Moreover, in this study it was found that a dual restriction digest with *Eco*RI and *Mse*I was an excellent approach to generate information-rich fAFLP patterns from all *P. syringae* pv. *pisi* strains examined.

More strains will be needed for identifying, characterizing and differentiating the races and for phylogenetic analysis, although we have shown this to be a good technique for identifying strains, each of which produced a unique fingerprint.

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