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Genetic diversity, presence of the *syrB* gene, host preference and virulence of *Pseudomonas syringae* pv. *syringae* strains from woody and herbaceous host plants

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A total of 101 *Pseudomonas syringae* pv. *syringae* strains, obtained from international culture collections or isolated from diseased tissues of herbaceous and woody plant species, were assessed by repetitive PCR using the BOX primer, and for the presence of the *syrB* gene. Representative strains were also tested for pathogenicity to lilac, pear, peach, corn and bean, as well as for virulence to lemon and zucchini fruits. The unweighted pair-group method using arithmethic averages analysis (UPGMA) of genomic fingerprints revealed 17 different patterns which grouped into three major clusters, A, B and C. Most of the strains (52·4%) were included in patterns 1–4 of group A. These patterns comprised strains obtained from either herbaceous or woody species, and showed four fragments of similar mobility. Genetic variability was ascertained for strains isolated from apple, pear, apricot, *Citrus* spp. and cereals. No clear relationship was observed between host plant and bacterial genomic fingerprint. Variability was also observed in pathogenicity and virulence tests. The inoculation of pear leaves discriminated strains isolated from pear as well as the very aggressive strains, whereas inoculation of lilac, peach and corn did not discriminate the host plant from which the strains were originally isolated. Lemon fruit inoculation proved very effective for *P. syringae* pv. *syringae* virulence assessment. The *syrB* gene was present in almost all strains.

Keywords: genomic fingerprinting, *Pseudomonas syringae*, repetitive PCR, virulence, *syrB*

Introduction

The concept of the pathovar, introduced by Young *et al.* (1978) and Dye *et al.* (1980), was thought to provide a nomenclature at the subspecies level for plant pathogenic bacteria that could help plant pathologists in identifying the causative agents of typical diseases, and to provide a formal nomenclature for plant quarantine legislation. Pathovars are circumscribed on the basis of distinct host ranges that, in many cases, are very restricted, and on their capacity to cause characteristic symptoms. In addition, biochemical and nutritional tests can also aid this differentiation (Young & Triggs, 1994). However, *Pseudomonas syringae* pv. *syringae*, a pathovar capable of causing disease on more than 200 different plant species (Bradbury, 1986; Young, 1991), seems quite distant from the definition. Some studies stress that within such a pathovar some strains appear specifically pathogenic only to certain host plants. Host specificity appears to be evident for *P. syringae* pv. *syringae* strains infecting grasses (Gross & De Vay, 1977) and beans (Cheng *et al.*, 1989). However, the assessment of host specificity and virulence of strains – either for identification or characterization purposes – requires standardized procedures, as different responses can be obtained by adopting different techniques (Young, 1991), and some plant species appear more suitable than others for defining the virulence of the strains (Endert & Ritchie, 1984; Yessad-Carreau *et al.*, 1994; Little *et al.*, 1998). In addition, some groups of strains show a genetic diversity that appears to be related to the host plant from which they were originally isolated (Little *et al.*, 1998) or to the area from which they were obtained (Gonzalez *et al.*, 2000). The production of cyclic lipodepsinonapeptides such as syringomycin, that are mycotoxic metabolites produced by most strains of many *P. syringae* pv. *syringae* (Mo & Gross, 1991), can be detected using PCR and specific primers to amplify the *syrB* gene encoding these metabolites (Sorensen *et al.*, 1998). This analysis can greatly assist identification and

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characterization, as the toxins are considered the main virulence factor of *P. syringae* pv. *syringae* (Mo & Gross, 1991). Consequently, a combination of techniques enable the study of the genetic relatedness between strains and reveal host specificity and virulence, as well as ability to produce toxic compounds. The objectives of this study were to investigate the genetic relatedness of *P. syringae* pv. *syringae* strains isolated from woody and herbaceous plant species using repetitive PCR (Louws *et al.*, 1994) and unweighted pair-group method using arithmethic averages analysis (UPGMA) cluster analysis, and to determine whether host preference exists among these strains. Finally, virulence and the possible presence of the *syrB* gene were also assessed.

Materials and methods

Bacterial strains

The *P. syringae* pv. *syringae* strains used in this study are listed in Table 1. The strains obtained from international culture collections or bacterial plant disease laboratories were revived from lyophilized ampoules and subsequently routinely cultured on nutrient agar (NA; Oxoid, Basingstoke, UK) at 25–27°C.

Isolation of *P. syringae* pv. *syringae* from diseased plants

From 1996 to 2000, samples of plant tissues presumably infected by *P. syringae* pv. *syringae*, received from different areas of cultivation in Italy, were collected. Samples were obtained from apple, pear, hawthorn, apricot, orange, lemon, lilac, kiwifruit, olive, chestnut, hazelnut, magnolia, bean, pepper and cauliflower (Table 1). Small pieces of tissue taken from the margins of lesions were crushed in sterile saline (SS: 0·85% NaCl in distilled water). Serial tenfold dilutions were also prepared. Aliquots of 0·1 mL were spread on medium B (King *et al.*, 1954). The plates were incubated for 2–3 days at 25–27°C, and fluorescent colonies were purified on NA and assayed by LOPAT tests (Lelliott *et al.*, 1966). With the isolates belonging to LOPAT group IA, the biochemical confirmation tests for *P. syringae* pv. *syringae* proposed by Young (1991) were performed by following the methods described by Lelliott & Stead (1987). Subsequently, representative strains were tested for pathogenicity and virulence, presence of the *syrB* gene and genomic fingerprinting using the BOX primer (Louws *et al.*, 1994).

DNA preparation and repetitive PCR

For total genomic DNA preparation, the technique of Smith *et al.* (1995) was used. A single colony of each strain grown for 48 h on NA was suspended in SS and centrifuged at 12 000 ***g*** for 2 min. After discarding the supernatant, the pellet was suspended in SS at an optical density corresponding to $1–2 \times 10^8$ cfu mL^{-1}. The suspension was heated in boiling water for 10 min, then stored at –20°C. The repetitive PCR (rep-PCR) method used was that of Louws *et al.* (1994). The BOX primer was synthesized by Eurogentec (Seraing, Belgium). Amplification was performed in an MJ Research (Watertown, MS, USA) PTC programmable thermal controller in 25 μL reaction volumes containing 200 μM deoxynucleoside triphosphate, 2 mM $MgCl_2$, primers at 60 pmol, 1·0 U *Taq* polymerase and 4 μL template DNA preparation. The PCR mixture was overlaid with 25 μL mineral oil. Thermal cycling was carried out as described by Louws *et al.* (1994): an initial denaturation cycle at 95°C for 7 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension cycle at 65°C for 8 min, a single final extension cycle at 65°C for 16 min, and final soak at 4°C. The PCR amplifications were performed in duplicate. PCR products were separated by gel electrophoresis on 1·5% agarose (Seakem LE, Rockland, ME, USA) in 1 × TAE buffer, at 5 V cm^{-1} over 5 h, stained with ethidium bromide, visualized under a UV transilluminator Spectroline (Spectronic Corporation, Westburg, NY, USA) and photographed with a Polaroid film type 55.

Data analysis

Banding patterns obtained after PCR were normalized using GELCOMPAR II version 1·01 (Applied Maths, Kortrik, Belgium) and compared using Dice's coefficients (Dice, 1945) S_D: $S_D = [2A/(B + C)]$, where A is the number of fragments common to both patterns, B is the number of fragments in one pattern, and C is the number of fragments present in the other pattern. Cluster analysis was performed according to the unweighted pair-group-average method (UPGMA) using NTSYS software PC-version 1·80 (Exeter Software, New York, NY, USA) (Rohlf, 1993). Phenograms were constructed with the tree display option (TREE). A cophenetic value matrix was calculated using the COPH option and compared with the original similarity matrix using the MXCOMP option to test the goodness-of-fit of the cluster analysis.

Detection of the *syrB* gene

To detect the possible presence in the strains of the *syrB* gene coding for the production of cyclic lipodepsinonapeptides, we used the primers B1 (5′-CTTTCC-GTGGTCTTGATGAGG-3′) and B2 (5′-TCGATTTT-GCCGTGATGAGTC-3′), amplifying a 752 bp band, and the PCR amplification procedure described by Sorensen *et al.* (1998).

Plant inoculation

To verify a possible host preference amongst the strains and to assess their virulence, artificial inoculations to different plant species were performed on the basis of previous studies aiming to characterize the pathogenic behaviour of *P. syringae* pv. *syringae* strains (Gross & De Vay, 1977; Endert & Ritchie, 1984; Cheng *et al.*, 1989; Young, 1991; Yessad *et al.*, 1992; Yessad-Carreau *et al.*,

Table 1 List of *Pseudomonas syringae* pv. *syringae* strains used in this study

Strain of *P. syringae* pv. *syringae*	Origin and year of isolation (if known)
Malus pumila	ISF M1, ISF M2, ISF M3, ISF M4, ISF M5 (Italy-Latium, 1996)
	ISF M6, ISF M7, ISF M8, ISF M9, ISF M10, ISF M11, ISF M12, ISF M13 (Italy-Piedmont, 1996)
	PD 1176 (Netherlands, 1998)
	NCPPB 2775 (France, 1964)
	NCPPB 3010 (Poland, 1972)
Pyrus communis	ISF P1, ISF P2, ISF P3, ISF P4, ISF P5 (Italy-Latium, 1996)
	IVIA 93-3-4-K, IVIA 93-4-5N, IVIA 773-1 (Spain, 1993)
	BPIC 692 (Greece, 1965), BPIC 1405 (Greece, 1989)
	B-301 (UK)
Crataegus oxyacantha	ISF BS1, ISF BS2 (Italy-Piedmont, 1998)
Rosa communis	PD 1113 (Netherlands, 1998)
Prunus armeniaca	ISF AL1, ISF AL2, ISF AL3, ISF AL4, ISF AL5, ISF AL6 (Italy-Veneto, 1996)
	T-22-36, F 545 (France, 1992)
	NCPPB 1092, NCPPB 1093 (New Zealand, 1951)
	BPIC 989, BPIC 990 (Greece, 1992)
	IVIA AVO 2 (Spain, 1992)
	P46 (Portugal, 1992)
	NCPPB 2507 (Hungary, 1969)
Prunus avium	NCPPB 1087 (Hungary, 1958)
Prunus persica	B3A (USA-California)
Citrus sinensis	452/1, 452/2 (USA-California)
	BPIC 987 (Greece, 1982)
	SF AR1 (Italy-Latium, 1996)
Citrus reticulata	BPIC 244 (Greece, 1963) BPIC 988 (Greece, 1982)
	ISF MA1, ISF MA2 (Italy-Latium, 1996)
Citrus limon	427/1, 427/2 (USA-California)
	ISF L1 (Italy-Latium, 1996)
Citrus medica	BPIC 177 (Greece, 1967)
Syringa vulgaris	NCPPB 281^{T} (UK, 1950)
	PD 346 (Netherlands, 1982)
	SY12 (Japan)
	ISF LL1 (Italy-Latium, 1999)
Actinidia chinensis	PD 2768, PD 2774 (USA-California, 1990)
	ISF AC1, ISF AC2 (Italy-Latium, 1997)
Olea europea	ISF O1 (Italy-Latium, 1998)
Castanea sativa	ISF CA1 (Italy-Latium, 1998)
Corylus avellana	ISF NOC1, ISF NOC2, ISF NOC3, ISF NOC4 (Italy-Latium, 2000)
Persea americana	NCPPB 191 (Israel, 1946)
Laurus nobilis	NCPPB 3869 (Italy-Umbria, 1992)
Betula sp.	NCPPB 1565 (New Zealand, 1954)
Magnolia grandiflora	ISF MG1, ISF MG2 (Italy-Latium, 1998)
Abelmoschus esculentus	NCPPB 1676 (Kenya, 1965)
Triticum aestivum	PD 2631, PD 2632, PD 2633 (Germany)
	SD 202 (USA-South Dakota)
	SD 246 (USA-California)
	PSS 61 (USA-Delaware)
Hordeum vulgare	PD 2618 (USA)
	475-A, 475-B (USA-California)
Zea mays	384-4 (USA-California)
	PS4A/1 (USA-Nebraska)
Sorghum vulgare	392 (USA-California)
Panicum miliaceum	B 359 (Japan)
Setaria italica	397-1 (USA-California)
Phaseolus vulgaris	Y 37 (USA)
	ISF F1 (Italy-Latium, 1997)
Capsicum annuum	ISF PP1 (Italy-Latium, 1998)
	ISF PP2 (Italy-Latium, 1999)
Saccharum officinarum	SC1 (Japan)
Brassica oleracea	ISF CAV1 (Italy-Latium, 1998)
Beta vulgaris	366 (USA-California)
Allium porrum	NCPPB 2694 (New Zealand, 1973)

NCPPB: National Collection Plant Pathogenic Bacteria, York, UK.
PD: Plant Protection Service, Wageningen, Netherlands.
BPIC: Benaki Phytopathological Institute, Kiphissia-Athens, Greece.
ISF: Istituto Sperimentale per la Frutticoltura, Roma, Italy.
IVIA: Instituto Valenciano de Investigaciones Agrarias, Moncada-Valencia, Spain.
^{T}Type strain.
The strains from USA and Japan were isolated by Drs D. Gross and J. De Vay (University of California, Davis, USA) and were obtained via Professor N.S. Iacobellis (Università della Basilicata, Potenza, Italy).

1994). For each inoculation, a bacterial suspension of $1–2 \times 10^7$ cfu mL^{-1} was used, obtained from 48-h-old colonies grown on NA. With lilac, peach and pear, the inoculations were carried out at the beginning of spring, in open-air conditions, with air temperature not exceeding 20°C, using pot-cultivated plants, whereas excised bean pods, lemon and zucchini fruits and corn leaves were inoculated at room temperature (20–23°C) and maintained for 48 h after the inoculation in a humid chamber. Inoculations were repeated twice. Lilac (*Syringa vulgaris* cv. Sensation) and pear (*Pyrus communis* cv. Conference) leaves were inoculated by pricking the leaf lamina (in four different places) and the petiole of fully expanded leaves with a sterile needle, and placing 10 μL of the suspension on each wound. Ten days after inoculation, pathogenic reactions were recorded as positive if the petiole and leaf darkened and the lamina showed a progressive wilting. For each strain, five leaves and petioles were inoculated. Two-year-old peach (*Prunus persica*) seedlings were inoculated in either the leaves or stems. Leaf laminae were inoculated as previously described for lilac. In addition, the stems were longitudinally wounded (1 cm) using a sterile scalpel, and 0·1 mL of the suspension was placed onto the wound with a micropipette. Pathogenic reactions were recorded 10 days after inoculation. The leaves were scored as positive if wilting was present. Concerning stem inoculation, the presence of a progressive necrotic lesion was observed upwards and downwards from the wound. For each strain, five leaves and five stems were inoculated. Corn (*Zea mays* cv. Santos) seedlings were inoculated in fully expanded leaves as described for lilac. For each strain, five leaves were inoculated. The pathogenic reaction was checked 7 days after inoculation. Bean (*Phaseolus vulgaris* cv. Corallo) pods, lemon (*Citrus lemon*) and zucchini (*Cucurbita pepo* ssp. *pepo* cv. President) fruits were first surface-sterilized with chlorine water, then, after rinsing with sterile distilled water, they were inoculated by puncturing the surface with a sterile needle and placing 10 μL of the bacterial suspension onto the wound. Pathogenic reactions were assessed 7 days after inoculation. Ten sites involving two fruits of each host were inoculated with each strain.

Results

Strain isolation and identification

Forty-three putative *P. syringae* pv. *syringae* isolates were obtained from apple, pear, hawthorn, apricot, orange, lemon, lilac, kiwifruit, olive, chestnut, hazelnut, magnolia, bean, pepper and cauliflower. All isolates were positive for levan production and hypersensitivity response on tobacco leaves, and negative for the presence of oxidase and arginine dihydrolase and for potato rotting (LOPAT group IA). In addition, they hydrolysed aesculin and arbutin, liquefied gelatin, utilized inositol, quinate and sorbitol but not anthranilate, homoserine or L(+)-tartrate, and did not produce tyrosinase. The isolates were identified as putative *P. syringae* pv. *syringae* according to Young (1991), and were further assessed for pathogenicity, virulence, presence of the *syrB* gene and genomic fingerprints.

BOX analysis

DNA fingerprints of 101 isolates, obtained from international culture collections or recently isolated in Italy from woody and herbaceous species, were determined using rep-PCR with the BOX primer. Reproducible genomic PCR profiles consisted of bands ranging in size from ≈200–1600 bp. For the UPGMA analysis a total of 21 reproducible, clearly resolved bands were scored. A cophenetic value of >0·91 was determined for the similarity matrix, indicating a high goodness-of-fit for the cluster analysis. The analysis revealed that the *P. syringae* pv. *syringae* isolates studied could be differentiated into 17 different patterns. On the basis of similarity level, the patterns could be clustered in turn into three main groups: A–C. Representative BOX patterns are shown in Figs 1 and 2; the pattern composition and grouping of the strains are summarized in Table 2 and Fig. 3.

Group A included patterns 1–8 and a distinct Hazelnut pattern typically shown by the isolates from *Corylus avellana*. The overall similarity level of the group was around 70%. Group B included patterns 9–11 as well as distinguished patterns exhibited by isolates from peach, kiwifruit and bean. The Peach pattern showed 50% similarity with group A, whereas the other patterns showed around 40% similarity. Group C included three deviating isolates classified as *P. syringae* pv. *syringae*, showing approximately 15% similarity with the other two groups.

The majority of the strains (52·4%) clustered into patterns 1–4 (Table 2). These patterns were characterized by the presence of four clearly reproducible bands ranging in size between 850 and 1600 bp (Figs 1 and 2), and included isolates from either woody or herbaceous host plants. Most of the isolates studied were from plant species frequently damaged by *P. syringae* pv. *syringae*, such as apple, pear, apricot and *Citrus* spp. Pattern 5 included the *P. syringae* pv. *syringae* type strain NCPPB 281 and two other isolates, PD 346 from *S. vulgaris* and NCPPB 1087 from *Prunus avium*. This pattern mainly differed from patterns 1–4 in the absence of the 1600 bp band. Pattern 6 included five isolates from herbaceous species and the isolate from *Olea europea*. This pattern did not show two of the four typical bands of patterns 1–4 (Fig. 1), whereas patterns 7 and 8 showed only one of these four bands (Fig. 1). Group B included patterns 9–11, which were quite dissimilar from the first nine and, in addition, a few distinctive patterns shown by the isolates from peach, kiwifruit and bean (Fig. 1). Pattern 11 included isolates from host plants frequently damaged by *P. syringae* pv. *syringae*, such as apple, pear and apricot. Group C included deviating isolates from *Betula* sp., *Hordeum vulgare* and *Allium porrum*.

It is worth noting that variability among isolates from the same plant species did occur. The 16 isolates from apple clustered into four different patterns, as did the 15 isolates from apricot, whereas the 11 isolates from pear

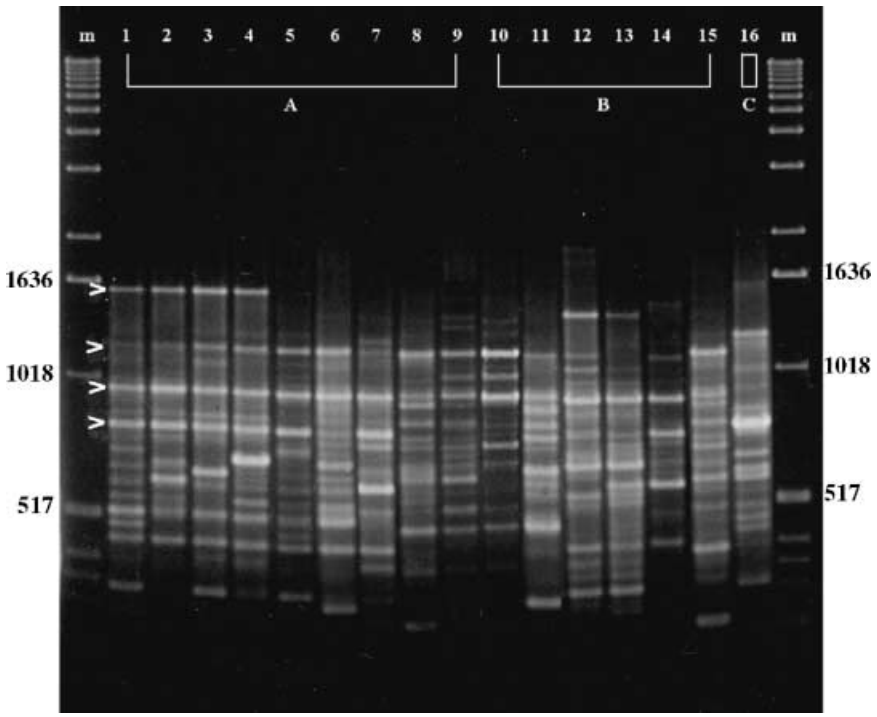

Figure 1 BOX fingerprints of *Pseudomonas syringae* pv. *syringae* strains isolated from herbaceous and woody host plants showing strain variability within the pathovar. Arrows on the left indicate the four fragments common to 53 of the 101 strains tested (patterns 1–4). The patterns are clustered into groups A, B and C (Fig. 3). Lane m, molecular size marker (1 kb ladder, Gibco-BRL); lane 1, BPIC 244 (*Citrus reticulata*, pattern 1); lane 2, 452/2 (*Citrus sinensis*, pattern 2); lane 3, BPIC 989 (*Prunus armeniaca*, pattern 3); lane 4, PD 2632 (*Triticum aestivum*, pattern 4); lane 5, NCPPB 281^{T} (*Syringa vulgaris*, pattern 5); lane 6, ISF NOC 1 (*Corylus avellana*, pattern H); lane 7, ISF O1 (*Olea europea*, pattern 6); lane 8, ISF M1 (*Malus pumila*, pattern 7); lane 9, ISF AL 5 (*P. armeniaca*, pattern 8); lane 10, B3A (*P. persica*, pattern P); lane 11, ISF AC1 (*Actinidia chinensis*, pattern KW-It); lane 12, PSS 61 (*T. aestivum*, pattern 9); lane 13, 452/1 (*C. sinensis*, pattern 10); lane 14, ISF P3 (*Pyrus communis*, pattern 11); lane 15, ISF F1 (*Phaseolus vulgaris*, pattern B); lane 16, PD 2618 (*Hordeum vulgare*, pattern 12). ^{T}*P. syringae.* pv. *syringae* pathotype strain; H, Hazelnut pattern; P, Peach pattern; KW-It, Kiwifruit-Italy pattern; B, Bean pattern.

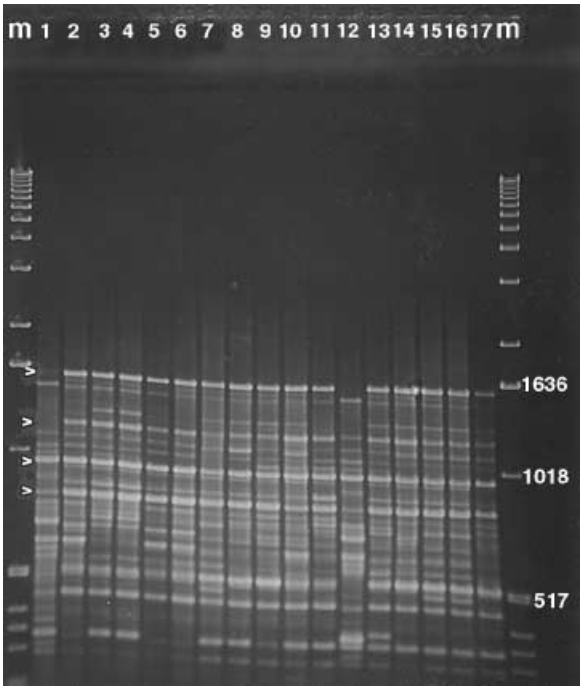

Figure 2 BOX fingerprints of *Pseudomonas syringae* pv. *syringae* strains showing variability among the strains obtained from *Citrus* spp., but also similarity among strains isolated from different species. Arrows on the left indicate the four fragments common to 53 of the 101 strains tested (patterns 1–4). Lane m, molecular size marker (1 kb ladder; Gibco-BRL); lane 1, 452/1 (*C. sinensis*-California (USA), pattern 10); lane 2, 452/2 (*C. sinensis*-California (USA), pattern 2); lane 3, ISF AR 1 (*C. sinensis*-Italy, pattern 1); lane 4, BPIC 987 (*C. sinensis*-Greece, pattern 1); lane 5, 427/1 (*C. limon*-California (USA), pattern 2); lane 6, 427/2 (*C. limon*-California (USA), pattern 2); lane 7, PD 2633 (*Triticum aestivum*-Germany, pattern 1); lane 8, ISF BS1 (*Crataegus oxyacantha*-Italy, pattern 1); lane 9, BPIC 990 (*Prunus armeniaca*-Greece, pattern 1); lane 10, ISF AL 6 (*P. armeniaca*-Italy, pattern 3); lane 11, ISF MG 1 (*Magnolia grandiflora*-Italy, pattern 1), lane 12, ISF MG 2 (*M. grandiflora*-Italy, pattern 10); lane 13, BPIC 177 (*C. medica*-Greece, pattern 4); lane 14, ISF MA 1 (*C. reticulata*-Italy, pattern 1); lane 15, ISF MA 2 (*C. reticulata*-Italy, pattern 3); lane 16, BPIC 988 (*C. reticulata*-Greece, pattern 3); lane 17, ISF PP1 (*Capsicum annuum*-Italy, pattern 1).

Table 2 Number of strains of *Pseudomonas syringae* pv. *syringae* generating 1 of 17 BOX genomic fingerprinting patterns

	Number of strains with BOX pattern																	
Host	1	2	3	4	5	6	7	8	Haz[a]	9	10	11	P[b]	KwCa[c]	KwI[d]	Bean	12	Total
Malus pumila		8		3			3					2						16
Pyrus communis	4						4					3						11
Crataegus oxyacantha	2																	2
Rosa communis												1						1
Prunus armeniaca	4	6	4					1										15
Prunus avium					1													1
Prunus persica													1					1
Citrus sinensis	2	1									1							4
Citrus reticulata	2		2															4
Citrus limon	1	2																3
Citrus medica				1														1
Syringa vulgaris		2			2													4
Actinidia chinensis														2	2			4
Olea europea						1												1
Castanea sativa	1																	1
Corylus avellana									4									4
Persea americana										1								1
Laurus nobilis	1																	1
Betula sp.																	1	1
Magnolia grandiflora	1										1							2
Triticum aestivum	3			2						1								6
Hordeum vulgare	2																1	3
Zea mays		1				1												2
Sorghum vulgare		1																1
Panicum miliaceum						1												1
Setaria italica						1												1
Phaseolus vulgaris																2		2
Capsicum annuum	1					1												2
Saccharum officinarum								1										1
Brassica oleracea						1												1
Beta vulgaris	1																	1
Allium porrum																	1	1
Abelmoschus esculentus											1							1
Total	**25**	**21**	**6**	**6**	**3**	**6**	**7**	**2**	**4**	**2**	**3**	**6**	**1**	**2**	**2**	**2**	**3**	**101**

[a]Hazelnut. [b]Peach. [c]Kiwifruit-California. [d]Kiwifruit-Italy.

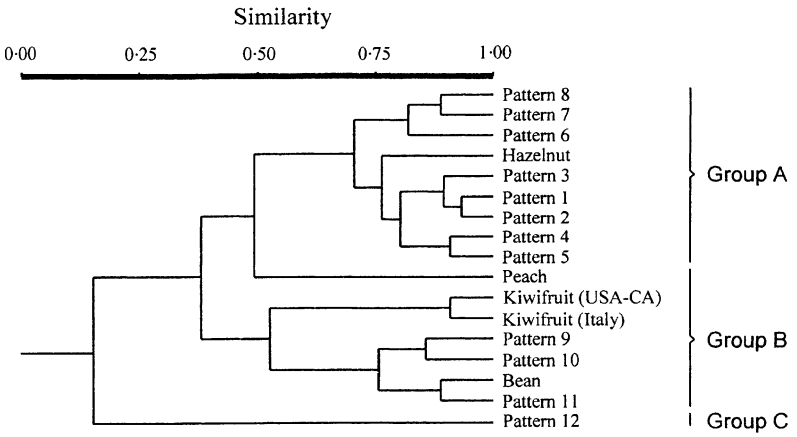

Figure 3 Dendrogram of genetic relatedness of the BOX fingerprint patterns generated by 101 strains of *Pseudomonas syringae* pv. *syringae*. Cluster analysis was performed using Dice's coefficients. The scale at the top indicates the degree of genetic relatedness between strains.

were grouped into three different patterns. In these cases, strains belonging to either group A or B were found. Variability was also detected in isolates obtained from *Citrus* spp. and cereals, although the similarity between the patterns was high, as in the case of the isolates belonging to patterns 1 and 2 from orange and lemon (Fig. 2). By contrast, isolates obtained from different plant species, locations and years sometimes showed identical genomic fingerprinting, such as ISF CA1 and ISF PP2, from *Castanea sativa* and *Capsicum annuum* in 1998 and 1999, respectively. However, no relationship between strains and geographical area could be ascertained, except for the four isolates obtained from kiwifruit which exhibited two similar but distinct patterns according to the area of isolation, California (USA) or central Italy.

Host preference, virulence and detection of the *syrB* gene

Forty-five strains representative of the 17 BOX-PCR fingerprint patterns were assayed in pathogenicity and virulence tests. The results are summarized in Table 3. Within group A, the strains belonging to the first four patterns, with the exception of the strains isolated from cereals, did not show host preference. All strains caused progressive lesions on lilac, pear, corn and peach, although to different extents, with the exception of the strains from cereals, which were unable to infect pear leaves. Strains with patterns 1–4 were also the most virulent towards lemon and zucchini fruits. In particular, isolates ISF P2 from pear and NCPPB 3869 from laurel induced severe symptoms on all the host plants tested. All strains with such patterns tested positive for the detection of the *syrB* gene and, with the exception of four isolates (BPIC 989, 382-4, 392 and PS4A) which did not cause apparent symptoms, all strains induced reddish or greyish necrotic lesions on bean pods. Strains with patterns 5 and 6 were unable to incite symptoms on pear, and were weakly virulent on lemon and zucchini. The type strain, NCPPB 281, did not infect pear or corn. These strains had the *syrB* gene and induced a necrotic lesion on bean pods. Strains with pattern 7 were able to cause symptoms on all plant tests and were highly virulent on lemon fruits, whereas no lesions were caused on zucchini fruits. Interestingly, ISF M1, obtained from apple, was the sole strain of group A capable of inducing a water-soaked lesion on bean pods. Strains with pattern 8 had the *syrB* gene but did not infect any of the plants tested, with the exception of corn, but in contrast were virulent on lemon fruits. The isolate with the Hazelnut pattern infected all test plants except pear and appeared highly virulent on both lemon and zucchini fruits.

The strains belonging to group B exhibited more restricted pathogenicity and were less virulent than the strains of group A (Table 3). In particular, isolates from Peach and Kiwifruit patterns and those with pattern 9 did not infect pear and were weakly virulent. The isolates from bean incited a water-soaked lesion on bean pods. Two strains, NCPPB 191 from avocado (pattern 9) and ISF M62 from magnolia (pattern 10), did not have the *syrB* gene, and NCPPB 191 did not cause infection on any of the plants tested.

All the strains belonging to group C (pattern 12) did not have the *syrB* gene and did not cause significant symptoms on the tested plants, although they were virulent on zucchini fruits. All the other strains listed in Table 1 that were not utilized for pathogenicity and virulence tests (Table 3) tested positive for the presence of the *syrB* gene.

Discussion

This study showed a genetic diversity among *P. syringae* pv. *syringae* isolates obtained from different woody and herbaceous host plants. Diversity was also observed among isolates from the same host plant as well as among isolates from the same site, at the same time and from the same host. Repetitive PCR performed with the BOX primer enabled differentiation of 17 patterns that clustered into three groups, A, B and C, on UPGMA. Patterns 1–4 included the majority (52·4%) of the isolates studied and comprised strains obtained from 18 of the 33 plant species. Similarly to Little *et al.* (1998), who used ERIC primer sets, the present study found that the most represented patterns shared four PCR products of similar mobility. By contrast, with the exception of a few distinguished patterns, which could be associated with the host plant of origin, no clear-cut relationship was found between host plant and bacterial genomic fingerprint. The associations found here were based on few isolates, and a larger number will be necessary to confirm these possible relationships. The strains from hazelnut, isolated from 1-year-old seedlings in a nursery, appeared genetically different from the *P. syringae* pv. *syringae* strains obtained from adult trees in orchards (Scortichini *et al.*, 2002). Isolates from apple, pear, apricot, *Citrus* spp. and wheat (hosts from which *P. syringae* pv. *syringae* is frequently isolated worldwide) exhibited three or four different patterns that, in some cases (such as apple, pear, orange and wheat) clustered into two different groups. By contrast, two strains from different years and locations and from different host plants (chestnut and pepper) showed an identical genomic fingerprint.

The *P. syringae* pv. *syringae* pathotype strain, NCPPB 281, clustered in pattern 5 which comprised only three isolates and lacked one of the four bands common to the first four patterns. The diversity of the type strain has already been pointed out in other studies. In a comparison of 111 *P. syringae* pv. *syringae* strains based on numerical analysis of phenotypic features, Roos & Hattingh (1987) found that the type strain deviated from the most representative groups of strains, which were mainly obtained from deciduous fruit trees grown in South Africa. Subsequently, Gardan *et al.* (1991), in a similar study with other strains, pointed out that the type strain was distantly related to phenon 14, which included 93 out of 108 strains. Similarly, Young (1991) found the type strain aberrant for seven out of 30 biochemical tests performed. This study confirmed that the genomic fingerprint of this strain is different from the majority of the strains studied

Table 3 Pathogenicity, virulence tests and presence of the *syrB* gene of *Pseudomonas syringae* pv. *syringae* strains

Pattern	Lilac	Pear	Corn	Peach	Lemon	Zucchini	Bean	*syrB*
Pattern 1								
P. communis ISF P2	+++	+++	+++	+	+++	+	NL	+
C. oxyacantha ISF BS1	+	+++	+	+++	+++	+	NL	+
P. armeniaca BPIC989	+	+	+	+	+++	+	–	+
C. sinensis ISF AR 2	+	+	+++	+	+++	–	NL	+
C. reticulata BPIC 988	+++	+	+++	+	+++	–	NL	+
L. nobilis NCPPB 3869	+++	+++	+++	+++	+++	+	NL	+
M. grandiflora ISFMG1	+	+	+++	+	+++	+	NL	+
T. aestivum PD 2633	+	–	+	+	+	–	NL	+
H. vulgare 475-A	+	–	+++	–	+	–	NL	+
Pattern 2								
M. pumila ISF M9	+	+	+++	+	+++	+++	NL	+
P. armeniaca BPIC 990	+	+	+	+	+++	+	NL	+
C. sinensis BPIC 987	+++	+	+++	+	+++	–	NL	+
C. limon 427/1	+	+	+++	+	+++	+	NL	+
S. vulgaris ISF LL1	+++	+	–	+++	+	+++	NL	+
Z. mays 382-4	+	–	+++	+	+++	–	–	+
S. vulgare 392	–	–	+	+	+	–	–	+
Pattern 3								
P. armeniaca F 545	+	+	+	+	+++	+	NL	+
C. reticulata ISF MA1	+++	+	+++	+	+++	+	NL	+
Pattern 4								
M. pumila ISF M5	+	+	+	+	+++	+	NL	+
C. medica BPIC 177	+	+	+++	+	+++	–	NL	+
T. aestivum PD 2631	+	–	+	+	+	–	NL	+
Pattern 5								
P. avium NCPPB 1087	+	–	+	–	+	+	NL	+
S. vulgaris NCPPB 281	+	–	+	+	+	+	NL	+
Pattern 6								
O. europea ISF O1	+	–	+	+	+	–	NL	+
Z. mays PS4A/1	+	–	+	–	–	–	–	+
C. annuum ISF PP1	+	–	+	–	–	–	NL	+
B. oleracea ISF CAV1	+	–	+	–	–	–	NL	+
Pattern 7								
M. pumila ISF M1	+	+	+++	+	+	–	WS	+
P. communis IVIA773-I	+++	+++	+++	+	+	–	NL	+
Pattern 8								
P. armeniaca ISF AL5	–	–	–	–	+	–	NL	+
S. officinarum SC1	–	–	–	–	+	–	NL	+
Hazelnut								
C. avellana ISF NOC1	+	–	+	+	+++	+++	NL	+
Pattern 9								
P. americana NCPPB191	–	–	–	–	+	–	–	–
T. aestivum PSS 61	+	–	+	–	+	–	NL	+
Pattern 10								
C. sinensis 452/1	+	+	–	+	+++	–	NL	+
M. grandiflora ISFMG2	–	–	+	–	+	–	NL	–
Pattern 11								
M. pumila PD 1176	+	+	+++	–	+++	+	NL	+
P. communis ISF P3	+	+++	+++	+	+++	+	NL	+
Peach								
P. persica B3A	+	–	+	+++	+	+	NL	+
Kiwifruit (CA)								
A. chinensis PD2768	–	–	+	+	+	–	–	+
Kiwifruit (I)								
A. chinensis ISF AC1	+	–	–	–	+	–	NL	+
Bean								
P. vulgaris ISF F1	+	–	+++	+++	+++	+++	WS	+
Pattern 12								
Betula sp. NCPPB1565	–	–	–	–	–	+	NL	–
H. vulgare PD 2618	–	–	+	–	–	+	NL	–
A. porrum NCPPB2694	–	–	–	–	–	+	NL	–

+, Progressive lesions on more than 80% of inoculated sites on leaves and on more than 50% of inoculated sites in fruits.
+++, Progressive lesions on more than 80% of inoculated sites on leaves and leaf wilting of more than 50% of inoculated leaves of lilac, pear and corn, or presence of progressive necrotic lesion around the wound on peach stem, within 7 days after inoculation. Progressive necrotic lesions on more than 80% of inoculated sites on fruits.
–, No sign of progressive lesions on more than 80% of inoculated sites on leaves and bean pods.
NL, development of necrotic lesions.
WS, development of water-soaked tissue around site of inoculation.

here, although NCPPB 281 cannot be considered as unrepresentative of the pathovar. As lilac is a minor cultivated plant species, the strains obtained from this ornamental and used in comparative studies are few, and usually not sufficiently representative of the range of variability. However, these phenomena deserve further investigation. On the other hand, the strains of group C showed genomic fingerprints deviating from the other two groups, and their current identification as *P. syringae* pv. *syringae* seems questionable.

Pathogenicity tests on lilac, pear, corn and peach indicated that the strains with patterns 1–4 and 7 were capable of infecting and causing extensive lesions on most of the host plants tested. The inoculation of lilac leaves and petioles was recommended to identify putative *P. syringae* pv. *syringae* isolates (Young, 1991), as with environmental conditions suitable for disease expression, only *P. syringae* pv. *syringae* isolates are capable of inducing infection. That particular study, however, was performed with only 12 strains, including five isolates from lilac. When more isolates were assessed, Yessad-Carreau *et al.* (1994) found that 29 out of 81 isolates, including two from lilac, failed to cause infection. The present study found that lilac leaves were infected by most of the isolates tested, although some did not incite any symptoms. Lilac leaf and petiole inoculation appears to be an important, but not decisive, pathogenicity test to confirm *P. syringae* pv. *syringae* identification. On pear, only a few isolates were capable of inciting progressive lesions on more than 80% of inoculated sites on leaves and leaf wilting of more than 50% of the inoculated leaves. Apart from the isolates from pear, one from hawthorn and another from laurel were very aggressive. The inoculation of different pear organs can lead to a variable evaluation of the pathogenicity of *P. syringae* pv. *syringae* isolates. Pear fruitlets and vegetative shoots support growth and pathogenic activity of *P. syringae* pv. *syringae* isolates from several host plants (Panagopoulos, 1967; Roos & Hattingh, 1987; Burkowicz & Rudolph, 1994), whereas leaves are more discriminating of isolates mainly obtained from pear (Yessad-Carreau *et al.*, 1994) as well as highly aggressive isolates from other hosts (present study). It is interesting to note that all the isolates from cereals were unable to cause any sign of infection on pear leaves. The inoculation of corn and peach led to similar results, and indicated again that isolates clustering in patterns 1–4 were the most pathogenic. Such plant tests appear to be insufficiently discriminatory for assessing the possible host specificity of putative *P. syringae* pv. *syringae* isolates.

Virulence assessment used lemon and zucchini fruits. Lemon fruits appeared quite suitable for testing virulence, as isolates with all BOX fingerprint patterns (with the exception of the Bean pattern) incited the typical black necrotic lesion. The lemon test also enabled differentiation of the deviating strains of group C which did not induce any symptoms. The same strains, in contrast, caused lesions on zucchini fruits. In a study carried out with 319 *P. syringae* pv. *syringae* strains from pear, Panagopoulos (1967) found that lemon fruit inoculation revealed the virulence of all the strains except three. The present study confirms this finding, and such a test seems to be fundamental to assessing the virulence of putative *P. syringae* pv. *syringae* strains. On bean pods, only isolates from *P. vulgaris* and one from apple induced a water-soaked lesion, whereas all other strains tested caused dry, reddish or greyish, necrotic lesions. These results confirm the observations of Yessad-Carreau *et al.* (1994).

The *syrB* gene was detected in most of the isolates tested. However, two isolates from group B, NCPPB 191 from avocado and ISF MG 2 from magnolia, as well as those from group C, did not have the *syrB* gene. Syringomycin is one of the major virulence factors of *P. syringae* pv. *syringae* (Mo & Gross, 1991). However, it has been shown that not all strains produce such compounds (Gross & De Vay, 1977; Zeller *et al.*, 1997).

Finally, this study confirms what was said by Bradbury (1986) about the so-called pathovar *syringae*, which appears 'as a complicated mosaic of slightly different taxa from a large number of different host plants'. It could be added that *P. syringae* pv. *syringae*, as the numerous populations forming this pathovar are still called, represents a good example of genetic and pathogenic variability. In such a case, the term 'pathovar' as originally proposed and explained does not seem the most appropriate. Further assessments aiming to elucidate the genetic variability of such a complex and to circumscribe the possible host specificity of certain strains would be particularly useful to clarify the relationships among the populations. These studies could contribute to cluster groups of strains within *P. syringae* pv. *syringae*, as was done recently for the other pathovars of *P. syringae* and for the related pseudomonads (Gardan *et al.*, 1999).

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References

Bradbury JF, 1986. *Guide to Plant Pathogenic Bacteria*. Kew, UK: International Mycological Institute.

Burkowicz A, Rudolph K, 1994. Evaluation of pathogenicity and of cultural and biochemical tests for identification of *Pseudomonas syringae* pathovars *syringae*, *morsprunorum* and *persicae* from fruit trees. *Journal of Phytopathology* **141**, 59–76.

Cheng GY, Legard DE, Hunter JE, Burr TJ, 1989. Modified bean pod assay to detect strains of *Pseudomonas syringae* pv. *syringae* that cause bacterial brown spot of snap beans. *Plant Disease* **73**, 419–23.

Dice LR, 1945. Measurement of the amount of ecological association between species. *Ecology* **26**, 297–302.

Dye DW, Bradbury JF, Goto M, Hayward AC, Lelliott RA, Schroth MN, 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Review of Plant Pathology* **59**, 163–8.

Endert E, Ritchie DF, 1984. Detection of pathogenicity, measurement of virulence, and determination of strain variation in *Pseudomonas syringae* pv. *syringae*. *Plant Disease* **68**, 677–80.

Gardan L, Cottin S, Bollet C, Hunault G, 1991. Phenotypic heterogeneity of *Pseudomonas syringae* van Hall. *Research in Microbiology* **142**, 995–1003.

Gardan L, Shafik H, Belouin S, Brosch R, Grimont F, Grimont PAD, 1999. DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (*ex* Sutic and Dowson 1959). *International Journal of Systematic Bacteriology* **49**, 469–78.

Gonzalez AJ, Landeras E, Mendoza MC, 2000. Pathovars of *Pseudomonas syringae* causing bacterial brown spot and halo blight in *Phaseoulus vulgaris* L. are distinguishable by ribotyping. *Applied and Environmental Microbiology* **66**, 850–4.

Gross DC, De Vay JE, 1977. Population dynamics and pathogenicity of *Pseudomonas syringae* in maize and cowpea in relation to the *in vitro* production of syringomycin. *Phytopathology* **67**, 474–83.

King EO, Raney MK, Ward DE, 1954. Two simple media for the demonstration of pyocianin and fluorescin. *Journal of Laboratory and Clinical Medicine* **44**, 301–7.

Lelliott RA, Stead DE, 1987. Methods for the diagnosis of bacterial diseases of plants. In: Preece TF, ed. *Methods in Plant Pathology*, Vol. 2. Oxford, UK: Blackwell Scientific Publications.

Lelliott RA, Billing E, Hayward AC, 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. *Journal of Applied Bacteriology* **29**, 470–89.

Little EL, Bostock RM, Kirkpatrick BC, 1998. Genetic characterization of *Pseudomonas syringae* pv. *syringae* strains from stone fruits in California. *Applied and Environmental Microbiolology* **64**, 3818–23.

Louws FJ, Fulbright DW, Stephens CT, De Bruijn FJ, 1994. Specific genomic fingerprinting of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* **60**, 2285–96.

Mo Y-Y, Gross DC, 1991. Plant signal molecules activate the *syrB* gene, which is required for syringomycin production by *Pseudomonas syringae* pv. *syringae*. *Journal of Bacteriology* **173**, 5784–92.

Panagopoulos CG, 1967. Comparison of *Pseudomonas syringae* isolates from citrus and pear trees. *Proceedings of the 2nd International Conference on Plant Pathogenic Bacteria, Lisbon, 17–21 January 1967*, Abstract No. 12.

Rohlf FJ, 1993. *NTSYS-PC. Numerical Taxonomy and Multivariate Analysis System*, *Version* 1·80. New York, USA: Exeter Software.

Roos IMM, Hattingh MJ, 1987. Pathogenicity and numerical analysis of phenotypic features of *Pseudomonas syringae* strains isolated from deciduous fruit trees. *Phytopathology* **77**, 900–8.

Scortichini M, Marchesi U, Rossi MP, Di Prospero P, 2002. Bacteria associated with hazelnut (*Corylus avellana* L.) decline are of two groups: *Pseudomonas avellanae* and strains resembling *P. syringae* pv. *syringae*. *Applied and Environmental Microbiology* **68**, 476–84.

Smith JJ, Offord LC, Holderness M, Saddler GS, 1995. Genetic diversity of *Burkholderia solanacearum* (synonym: *Pseudomonas solanacearum*) race 3 in Kenya. *Applied and Environmental Microbiology* **61**, 4263–8.

Sorensen KN, Kim K-H, Takemoto JY, 1998. PCR detection of cyclic lipodepsinonapeptide-producing *Pseudomonas syringae* pv. *syringae* and similarity of strains. *Applied and Environmental Microbiology* **61**, 226–30.

Yessad S, Manceau C, Luisetti J, 1992. A detached leaf assay to evaluate virulence and pathogenicity of strains of *Pseudomonas syringae* pv. *syringae* on pear. *Plant Disease* **76**, 370–3.

Yessad-Carreau S, Manceau C, Luisetti J, 1994. Occurrence of specific reactions induced by *Pseudomonas syringae* pv. *syringae* on bean pods, lilac and pear plants. *Plant Pathology* **43**, 528–36.

Young JM, 1991. Pathogenicity and identification of the lilac pathogen, *Pseudomonas syringae* van Hall 1902. *Annals of Applied Biology* **118**, 283–98.

Young JM, Triggs CM, 1994. Evaluation of determinative tests for pathovars of *Pseudomonas syringae* van Hall 1902. *Journal of Applied Bacteriology* **77**, 195–207.

Young JM, Dye DW, Bradbury JF, Panagopoulos CG, Robbs CF, 1978. A proposed nomenclature and classification for plant pathogenic bacteria. *New Zealand Journal of Agricultural Research* **21**, 153–77.

Zeller W, Xie X-L, Bereswill S, Geider K, 1997. Taxonomy and virulence of bacterial blight (*Pseudomonas syringae* pv. *syringae*) from pome fruit trees. In: Rudolph K, Burr TJ, Mansfield JW, Stead DE, Vivian A, von Kietzell J, eds. Pseudomonas syringae *Pathovars and Related Pathogens*. Dordrecht, the Netherlands: Kluwer Academic, 465–9.