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Genetic relatedness among *Pseudomonas avellanae*, *P. syringae* pv. *theae* and *P.s.* pv. *actinidiae*, and their identification

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Abstract

A total of 37 strains of *Pseudomonas avellanae*, *P. syringae* pv. *theae* and *P.s.* pv. *actinidiae*, including pathotype and reference strains, obtained from all the countries where these pathogens have been reported, were compared by means of ARDRA, repetitive PCR using ERIC, BOX and REP primer sets, whole-cell protein analysis, biochemical and nutritional tests, and pathogenicity tests. *P. syringae* pathovar type strains representing six genomospecies *sensu* Gardan et al. (1999), were also included for comparison in UPGMA cluster analysis of repetitive PCR data and SDS-PAGE of protein extracts. Among the 12 endonucleases used in ARDRA, only *Tru* 9I differentiated *P. avellanae* from *P.s.* pv. *theae* and *P.s.* pv. *actinidiae*. UPGMA cluster analysis of repetitive PCR genomic fingerprints showed 65% similarity between *P.s.* pv. *theae* and *P. avellanae* and 50% between the latter species and *P.s.* pv. *actinidiae.* Strains of *P.s.* pv. *actinidiae* could be grouped according to their geographic origin. Similar results were obtained with SDS-PAGE cluster analysis. PCR amplification using primers PAV 1 and PAV 22 that were developed to detect *P. avellanae* in apparently healthy and visibly infected hazelnut specimens yielded a band of 762 bp from all strains of *P. avellanae*, *P.s.* pv. *theae* and *P.s.* pv. *actinidiae*. All strains lacked the *syrB* gene. Based on these data, we suggest that *P.s.* pv. *actinidiae* should be included in the genomospecies 8 together with *P. avellanae* and *P.s.* pv. *theae*. Selected biochemical and nutritional tests could differentiate these groups of strains. Pathogenicity tests clearly indicated that each group is specifically pathogenic only on the host plant species from which it was originally isolated.

Introduction

Genetic and phenotypic fingerprinting revealed relatedness among some phytopathogenic pseudomonads, namely *Pseudomonas avellanae* (Psallidas) Janse et al., *P. syringae* pv. *theae* (Hori) Young et al., and *P.s.* pv. *actinidiae* Takikawa et al., isolated respectively from *Corylus avellana*, *Thea sinensis* and *Camellia sinensis*, and *Actinidia deliciosa*. According to Gardan et al. (1999), *P. avellanae* and *P.s.* pv. *theae* both belong to genomospecies 8 but *P.s.* pv. *actinidiae* strains were not included in their work. On the other hand, Sawada et al. (1999) sequenced pathogenicity-related genes and placed *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* together with other *P. syringae* pathovars (i.e. *tomato, maculicola*, *lachrymans, morsprunorum* and one *syringae* strain isolated from *Citrus iyo*) in group 1 of three distinct monophyletic groups. In this study *P. avellanae* was not included and *P.s.* pv. *theae* and *actinidiae* are very closely-related. *P. avellanae*, the causative agent of hazelnut bacterial canker and decline, has been reported in Greece (Psallidas and Panagopoulos, 1979) and Italy (Scortichini and Tropiano, 1994). *P.s.* pv. *theae* causes bacterial shoot blight of tea and camellia and it has been isolated in Japan (Takikawa et al., 1988) and China (Bradbury, 1986), whereas *P.s.* pv. *actinidiae*, causal agent of bacterial canker of kiwifruit, has been isolated in Japan (Takikawa et al., 1989), Italy (Scortichini, 1994) and South Korea

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(S.-J. Ko, pers. commun.). In many countries, the different hosts of these bacterial pathogens are cultivated in the same area and, at least in central Italy, hazelnut and kiwifruit are often cultivated on the same farm. The aim of this study was to assess the genetic and phenotypic relatedness among the different pathogens with restricted host specificity and to provide criteria for their identification.

Materials and methods

Bacteria and growth conditions

P. avellanae, *P.s.* pv. *actinidiae*, *P.s.* pv. *theae* as well as *P. syringae* pathovars and *P. viridiflava* strains used in this study are listed in Table 1. All strains were routinely cultured on nutrient glucose agar (nutrient agar supplemented with 1% glucose) (NGA), at 25–27 °C.

Amplified ribosomal DNA-restriction analysis (ARDRA)

P. avellanae, *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* strains were analysed by ARDRA, as described by Vannechoutte et al. (1992). The DNA coding for the 16S rRNA was amplified with primers PO (5′-GAGAGTTTGATCCTGGCTCAG) and P6 (5′-CTACGGCTACCTTGTTACGA) synthesized by Eurogentech (Seraing, Belgium) (Grifoni et al., 1995). For total genomic DNA preparation, a modification of the techniques developed by Smith et al. (1994) was used (Scortichini et al., 1998). The PCR mixture contained 6 µl of lysed cell suspension in a final reaction volume of 50 µl containing Promega *Taq* buffer (1.5 mM $MgCl_2$), 200 µM of each deoxynucleoside triphosphate, 36 pmol of each primer, and 1.5 U of *Taq* DNA polymerase (Promega, Madison, WI, USA). The mixture was overlaid with 50 µl of mineral oil. The reaction mixtures were incubated on an MJ Research (Watertown, MS, USA) PTC 100 programmable thermal controller following the amplification procedure described by Picard et al. (2000). Two microliters of each amplification mixture were analysed by agarose (1.0%, w/v) gel electrophoresis in 0.5 × Tris–Borate–EDTA (TBE) buffer containing 0.5 µg of ethidium bromide per ml, at 5.0 V cm^{-1}. A 5-µl aliquot of each PCR mixture containing approximately 1.5 µg of amplified 16S rDNA was digested with the following restriction endonucleases (Boehringer Mannheim, Monza, Italy): *Cfo* I, *Dra* I, *Eco* RI, *Hae* III, *Hin* fI, *Mbo* I, *Msp* I, *Mva* I, *Pst* I, *Rsa* I, *Taq* I and *Tru* 9I, as per the manufacturer's instructions. The reaction products were analysed by agarose (2.5%, w/v) gel electrophoresis in 0.5 × TBE buffer containing ethidium bromide at 0.5 µg ml^{-1}.

Repetitive-PCR genomic fingerprinting

Genomic fingerprinting and similarity were assessed by repetitive PCR and UPGMA cluster analysis for all strains listed in Table 1. For DNA preparation, the method of Smith et al. (1994) was used and the repetitive-PCR method was that of Louws et al. (1994).

Table 1. List of strains used in this study. The genomospecies number *sensu* Gardan et al. (1999) is given in brackets

Strain	Host	Country	Year of isolation
Pseudomonas avellanae (genomospecies 8)			
NCPPB 3487^T = BPIC 631	*C. avellana*	Greece	1976
NCPPB 3488 = BPIC Fl3	*C. avellana*	Greece	1976
NCPPB 3489 = BPIC 640	*C. avellana*	Greece	1976
NCPPB 3490 = BPIC 665	*C. avellana*	Greece	1976
NCPPB 3491 = BPIC 703	*C. avellana*	Greece	1977
BPIC 632	*C. avellana*	Greece	1976
BPIC 647	*C. avellana*	Greece	1976
BPIC 649	*C. avellana*	Greece	1976
BPIC 707	*C. avellana*	Greece	1977
BPIC 708	*C. avellana*	Greece	1987
BPIC 714	*C. avellana*	Greece	1987
BPIC 1077	*C. avellana*	Greece	1987
BPIC 1436	*C. avellana*	Greece	1990
NCPPB 3873 = ISPaVe 011	*C. avellana*	Italy	1991

Table 1. (*Continued*)

Strain	Host	Country	Year of isolation
NCPPB 3875 = ISPaVe 012	*C. avellana*	Italy	1992
ISPaVe 040	*C. avellana*	Italy	1993
ISPaVe 056	*C. avellana*	Italy	1994
ISPaVe 2059	*C. avellana*	Italy	1994
ISPaVe 439	*C. avellana*	Italy	1995
ISF T4	*C. avellana*	Italy	1998
ISF T7	*C. avellana*	Italy	1998
ISF V2	*C. avellana*	Italy	1999
ISF C1	*C. avellana*	Italy	2000
P. syringae pv. *theae* (genomospecies 8)			
NCPPB 2598^{T}	*T. sinensis*	Japan	1970
CFBP 4096	*C. sinensis*	Japan	1970
CFBP 4097	*C. sinensis*	Japan	NK
P. syringae pv. *actinidiae* (genomospecies unknown)			
NCPPB 3739^{T} = KW 11	*A. deliciosa*	Japan	1984
NCPPB 3740 = KW 30	*A. deliciosa*	Japan	1984
KW 1	*A. deliciosa*	Japan	1984
KW 429	*A. deliciosa*	Japan	1984
NCPPB 3871	*A. deliciosa*	Italy	1992
NCPPB 3873	*A. deliciosa*	Italy	1992
PD 2020	*A. deliciosa*	Italy	1992
PD 2515	*A. deliciosa*	Italy	1992
PD 2516	*A. deliciosa*	Italy	1992
KACC 10592 = KI 9911	*A. deliciosa*	South Korea	1999
KACC 10754 = KI 97148	*A. deliciosa*	South Korea	1997
P. syringae pv. *eriobotryae* (genomospecies 2)			
NCPPB 2331^{T}	*Eriobotrya japonica*	U.S.A.	1970
P. syringae pv. *helianthi* (genomospecies 7)			
NCPPB 2460^{T}	*Helianthus annuus*	Ethiopia	1961
P. syringae pv. *morsprunorum* (genomospecies 2)			
NCPPB 2787	*Prunus avium*	Greece	1961
P.s. pv. *persicae* (genomospecies 3)			
NCPPB 2761^{T}	*Prunus persica*	France	1974
P. syringae pv. *syringae* (genomospecies 1)			
NCPPB 281^{T}	*Syringa vulgaris*	United Kingdom	1950
NCPPB 1087	*Prunus avium*	Hungary	1958
NCPPB 1092	*Prunus armeniaca*	New Zealand	1951
NCPPB 1093	*Prunus armeniaca*	New Zealand	1951
NCPPB 3869	*Laurus nobilis*	Italy	1992
P. syringae pv. *tomato* (genomospecies 3)			
NCPPB 1106^{T}	*Lycopersicon esculentum*	United Kingdom	1960
P. syringae pv. *ulmi* (genomospecies 2)			
NCPPB 632^{T}	*Ulmus* sp.	Yugoslavia	1958
P. viridiflava (genomospecies 6)			
BPIC 394	*Solanum melongena*	Greece	1972

NCPPB: National Collection of Plant Pathogenic Bacteria, York, United Kingdom; BPIC: Benaki Phytopathological Institute Collection, Kiphissia-Athens, Greece; PD: Plant Protection Service Culture Collection, Wageningen, The Netherlands; CFBP: Collection Francaise de Bactéries Phytopathogènes, Angers, France; KACC: Korean Agricultural Culture Collection, Chonnam, South Korea; ISPaVe: Istituto Sperimentale per la Patologia Vegetale Culture Collection, Roma, Italy; ISF: Istituto Sperimentale per la Frutticoltura Culture Collection, Roma, Italy; NK: Not known.
^{T}: Type-strain.

The ERIC, BOX and REP primer sets were synthesized by Eurogentech (Seraing, Belgium). The PCR amplifications were performed in duplicate on a MJ Research PTC 100 programmable thermal controller following the procedures described elsewhere (Scortichini et al., 1998). Gels were analysed by the method of Smith et al. (1995) and involved scoring bands common to both amplifications. For each primer and for each strain, bands were scored as present (i.e. 1) or absent (i.e. 0) and the readings were entered in a computer file as a binary matrix. Similarity coefficient for all pairwise combinations were determined using Dice's coefficients (1945) and clustered by unweighted paired-group using aritmethic averages (UPGMA) by means of NTSYS software, version 1.80.

Whole-cell protein analysis

Soluble whole-cell protein extracts were collected for each strain according to Janse et al.(1996). SDS-PAGE electrophoresis was performed in duplicate on a Bio Rad Mini Protean apparatus, in a 12% (w/v) polyacrylamide gel, run vertically (40 mA constant current, 4 °C) until the bromophenol blue tracking dye had migrated to the bottom of the gel. For cluster analysis, the method previously described for the PCR genomic fingerprinting was followed.

Assessment with specific primers

P. avellanae, *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* strains were assessed for the production of a PCR product of 762 bp with primers developed for the detection of *P. avellanae* in apparently healthy and visibly infected hazelnut specimens (Scortichini and Marchesi, 2001). The following primers were used: PAV 1, targeting position 264–289 of the *P. avellanae* 16S rRNA gene, 5′-GGCGACGATCCGTAACTGGTCTGAGA-3′ and P 22, targeting position from 997 to 1025 of the same gene, 5′-TTCCCGAAGGCACTCCTCT ATCTCTAAAG-3′. DNA extraction and PCR was performed as for the repetitive PCR described above. Amplified PCR products were resolved by electrophoresis of 9 µl of reaction mixture on 1% agarose gel in 0.5 × TBE buffer, at 4 V cm^{-1} for over 2 h. DNA fragments were stained in 0.5 µg ml^{-1} ethidium bromide, visualized under a UV transilluminator and photographed with a Polaroid film type 55. In addition, the strains were tested for the presence of *syrB* gene encoding the production of cyclic lipodepsinonapeptides. For this purpose, primers B1 and B2, amplifying a band of 752 bp were used (Sorensen et al., 1998). The *P.s.* pv. *syringae* strains listed in Table 1 were used as controls.

Biochemical tests and carbon compounds utilization

The following biochemical tests were performed on *P. avellanae*, *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* strains using the methods described by Lelliott and Stead (1987): levan production, presence of oxidase, potato soft rot, presence of arginine dihydrolase, tobacco hypersensitivity (LOPAT test). The production of fluorescent pigments was tested on King et al.'s medium B (KB) (King et al., 1954) and CSGA medium (Luisetti et al., 1972). In addition, the following tests were performed: growth on nutrient agar (NA), gelatin liquefaction, hydrolysis of casein, starch, aesculin and arbutin, production of tyrosinase, lypolisis of Tween 80, presence of urease, utilizations of glucose, glycerol, adonitol, arabinose, D-xylose, DL-tartaric acid, dulcitol, inositol, cellobiose, L-histidine, L-arginine, L-tyrosine and trigonelline. The basal medium of Ayers et al. (1919) was used and organic acids were supplemented at 0.1% (w/v), whereas carbohydrates and the other carbon compounds were used at 0.2% (w/v). Growth on the surface of the agar was considered as indicative of substrate utilization.

Pathogenicity tests

To test host specificity, inoculation experiments were performed with selected strains. The following type and reference strains were chosen: *P. avellanae* NCPPB 3487 and NCPPB 3873, *P.s.* pv. *theae* NCPPB 2598 and CFBP 4096, *P.s.* pv. *actinidiae* NCPPB 3739 and NCPPB 3871. The strains were grown for 48 h on NGA medium, at 25–27 °C. Bacterial suspensions were photometrically adjusted to an optical density corresponding to 1–2 × 10^6 cfu ml^{-1}. Each strain was inoculated on to *C. avellana* cultivar 'Tonda Gentile Romana' and 'Nocchione', *A. deliciosa* cultivar 'Hayward' and *C. sinensis* cultivar 'Perfection'. Inoculations were carried out in autumn or spring using pot-cultivated plants in open-field conditions following standardized procedures. With respect to *C. avellana*, inoculations were done in early autumn, by inoculating one-year-old twigs with approximately 1000 cfu per leaf scars (Scortichini and Tropiano, 1994; Scortichini and Lazzari, 1996). For each strain, ten leaf scars were

inoculated. For kiwifruit, the technique adopted by Serizawa and Ichikawa (1993) was followed. In spring, when sprouting of the new leaves was observed, the strains were inoculated by puncturing either the leaf blade and the main vein or by spraying the bacterial suspensions on to the leaf surface. Ten leaves were inoculated with each strain. *C. sinensis* was inoculated in the spring by puncturing the new leaves and by wounding the twig and placing 10 µl of the suspension into the wounds. For each strain, ten leaves and ten twigs were inoculated. The appearance of symptoms were checked over the spring that followed the inoculation. Re-isolations were performed when infection occurred.

Results

ARDRA

The rDNA-based primers supported the amplification of 16S rDNA from all strains tested. PCR products contained a single band of about 1500 bp. All the amplified 16S rDNAs were digested with each of the 12 restriction endonucleases used but depending on the restriction endonuclease, one to five restriction fragments were observed. However, only with *Tru* 9I was it possible to discriminate between *P. avellanae* and the two *P. syringae* pathovars (Figure 1). All of the other endonucleases gave the same restriction patterns for all strains tested.

Repetitive PCR

All strains listed in Table 1 were assessed with ERIC, BOX and REP primers in repetitive PCR experiments. All primer sets gave reproducible genomic PCR profiles consisting of bands ranging in size from approximately 100 bp to 3 kb. For UPGMA analysis a total of 51 reproducible clearly resolved bands were scored: 24 for ERIC primers, 17 for BOX primer and 10 for REP primers. ERIC and BOX primer sets were more discriminative than REP in differentiating the strains. With ERIC primers, distinct DNA polymorphism was observed in the region between 100 and 1600 bp; whereas with BOX and REP primers, polymorphism was evident in the region between 200 and 2300 bp. Representative REP-PCR genomic fingerprints are shown in Figure 2. Separate cluster analysis of the three data sets gave similar results. Cluster analysis by means of UPGMA of the combined data sets from

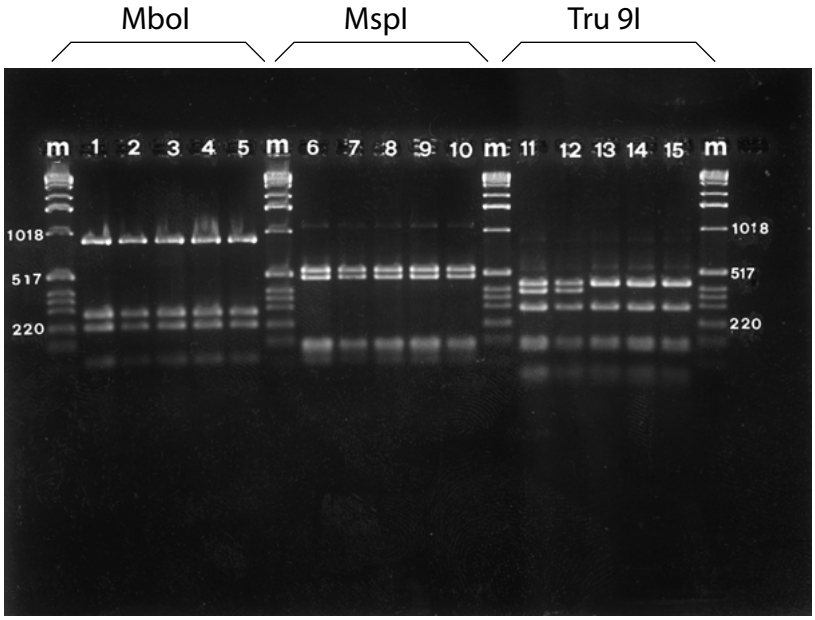

Figure 1. ARDRA banding patterns of amplified 16S rDNA using *Mbo* I, *Msp* I and *Tru* 9I restriction endonucleases of *P. avellanae* (lanes 1, 6, 11: NCPPB 3487^T; lanes 2, 7, 12: NCPPB 3875), *P. syringae* pv. *theae* (lanes 3, 8, 13: NCPPB 2598^T) and *P.s.* pv. *actinidiae* (lanes 4, 9, 14: NCPPB 3739^T; lanes 5, 10, 15: NCPPB 3871). *P. avellanae* can be differentiated from *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* by using *Tru* 9I.

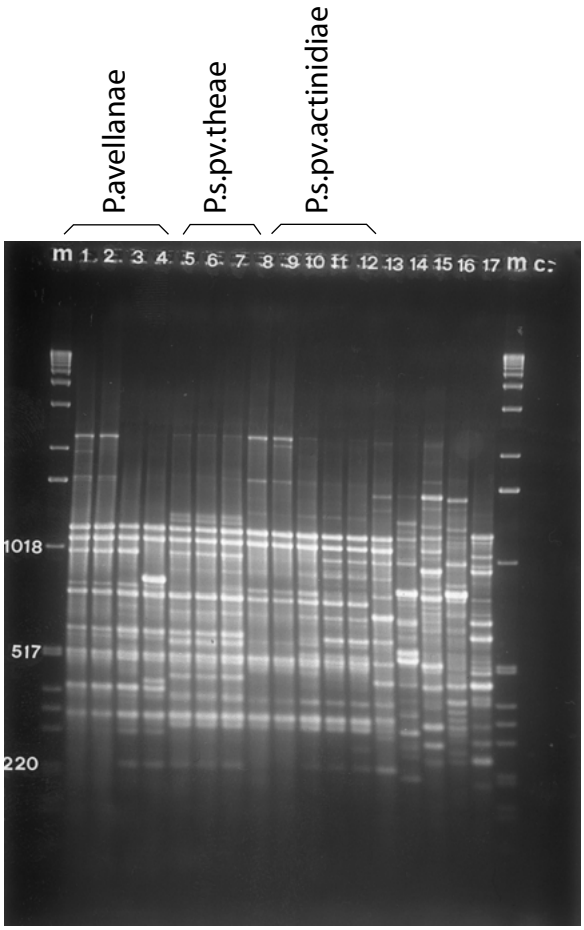

Figure 2. Repetitive PCR fingerprinting patterns from genomic DNA of *P. avellanae*, *P. syringae* pv. *theae*, *P.s.* pv. *actinidiae* and other phytopatogenic fluorescent pseudomonad strains, obtained by using BOX primer set. m: molecular size marker (1-kb ladder; Gibco BRL); the sizes are indicated in base pairs. Lane 1: *P. avellanae* ISF C1; lane 2: *P. avellanae* NCPPB 3875; lane 3: *P. avellanae* ISPaVe 056; lane 4: *P. avellanae* NCPPB 3487^T; lane 5: *P.s.* pv. *theae* NCPPB 2598^T; lane 6: *P.s.* pv. *theae* CFBP 4096; lane 7: *P.s.* pv. *theae* CFBP 4097; lane 8: *P.s.* pv. *actinidiae* NCPPB 3739^T; lane 9: *P.s.* pv. *actinidiae* NCPPB 3740; lane 10: *P.s.* pv. *actinidiae* KW 1; lane 11: *P.s.* pv. *actinidiae* KACC 10754; lane 12: *P.s.* pv. *actinidiae* KACC 10592; lane 13: *P.s.* pv. *tomato* NCPPB 1106^T; lane 14: *P.s.* pv. *ulmi* NCPPB 632^T; lane 15: *P.s.* pv. *syringae* NCPPB 3869; lane 16: *P.s.* pv. *helianthi* NCPPB 2460^T; lane 17: *P. viridiflava* BPIC 394. C: negative control.

ERIC, BOX and REP-PCR experiments revealed that among all *P. syringae* pathovars strains tested, *P.s.* pv. *actinidiae* strains showed a similarity of 50% with strains belonging to genomospecies 8 (i.e. *P. avellanae* and *P.s.* pv. *theae*). In addition, the strains could be grouped according to their geographical origin with strains from Japan, South Korea and Italy showing distinct profiles (Figure 3). However, all strains isolated from the same country showed similar profiles to one another. *P.s.* pv. *theae* strains appear to have a genomic fingerprint of 65% similarity with *P. avellanae*. This latter species is clearly composed by two different populations, one from northern Greece and the other from central Italy. Among the other *P. syringae* pathovar strains, *P.s.* pv. *tomato* NCPPB 1106 was the closest to genomospecies 8, showing 25% similarity in the pattern profile, whereas all the other *P. syringae* strains showed less than 20% similarity (Figure 3).

Whole-cell protein analysis

Representative bands between 21.5 and 66.2 kDa were selected from protein electrophoretograms for UPGMA analysis. Similarly to repetitive PCR, UPGMA cluster analysis showed that *P. avellanae* is closely related to *P.s.* pv. *theae* (i.e. similarity 70%) and to *P.s.* pv. *theae* (i.e. 60% of similarity, data not shown). The other *P. syringae* strains clustered apart with *P.s.* pv. *tomato* the closest showing 30% similarity. Whole-cell protein analysis, however, did not discriminate among *P.s.* pv. *actinidiae* strains isolated from Japan, South Korea and Italy. Indeed, all strains showed the same profile.

Assessment with specific primers

All *P. avellanae*. *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* strains tested by PCR amplification with primers P 1 and P 22, produced a product of 762 bp (Figure 4). None of the *P. avellanae*, *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* strains yielded a PCR product of 752 bp, indicating the absence of the *syrB* gene, whereas all *P.s.* pv. *syringae* strains tested showed the specific band (data not shown).

Biochemical tests and carbon compound utilization

All *P. avellanae*, *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* strains were levan-positive, oxidase-negative, potato soft rot-negative, arginine dehydrolase-negative, tobacco positive (LOPAT group Ia). In addition, all strains utilized glucose and glycerol and did not utilize dulcitol, adonitol, cellobiose, salicine and L-histidine. Some tests clearly differentiated *P. avellanae* and *P.s.* pv. *theae* from *P.s.* pv. *actinidiae* (Table 2).

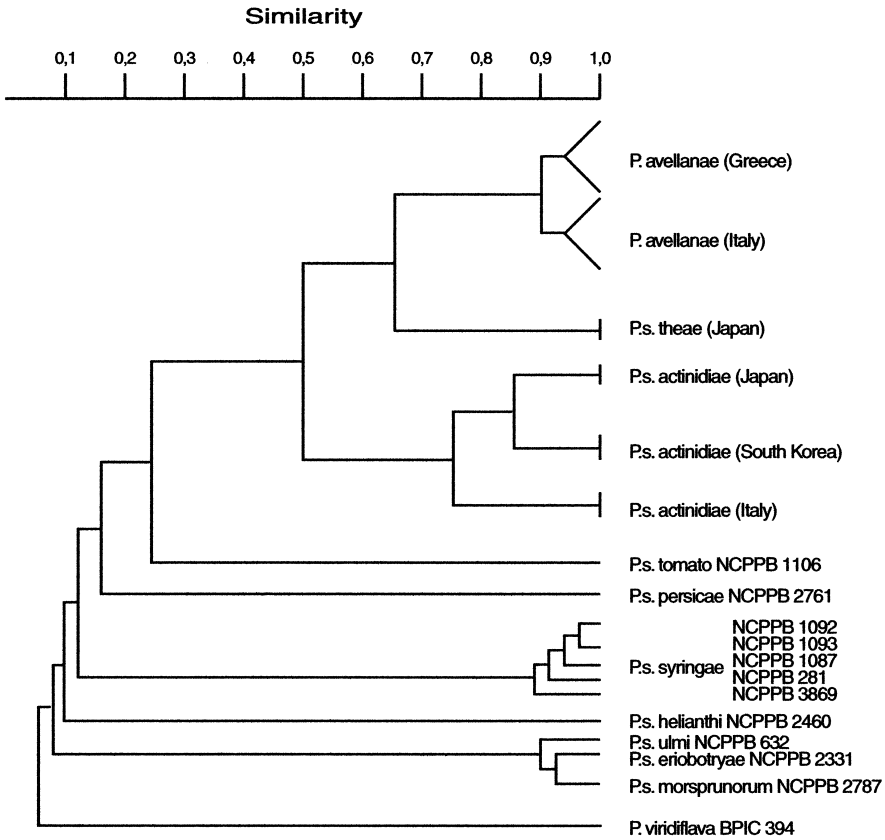

Figure 3. Dendrogram of relationships among *P. avellanae*, *P. syringae* pv. *theae*, *P.s.* pv. *actinidiae* and other fluorescent phytopathogenic pseudomonads. A similarity matrix was produced from analysis of repetitive PCR data obtained using ERIC, BOX and REP primer sets. Cluster analysis was performed by UPGMA on matrix calculated with the Dice's coefficients.

Pathogenicity tests

Inoculation tests clearly indicated that each group of strains was specifically pathogenic only on the host plant from which it was isolated. *P. avellanae* strains, incited twig dieback only on *C. avellana* and no symptoms were observed on Camellia and kiwifruit six months after inoculation. All *P.s.* pv. *actinidiae* strains induced leaf spot and wilting on *A. deliciosa*, whereas the other strains incited an evident hypersensitivity reaction (i.e. shot hole around the site of inoculation in case of puncturing). *P.s.* pv. *theae* strains induced wilting of the new leaves and necrosis around the wounds along the twigs of *C. sinensis*, whereas the other strains did not incite any visible symptoms. In every case, all of the inoculations carried out with the homologous pathogen were successful. Re-isolations from infected plants yielded strains with the same characteristics as the inoculated strains.

Discussion

This study shows that *P. avellanae*, *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* are genetically closely-related pseudomonads which exhibit host specificity. Among the 12 endonucleases tested in ARDRA, only one restriction enzyme, *Tru* 9I, separated *P. avellanae* from *P.s.* pv. *theae* and *P.s.* pv. *actinidiae*. In addition, repetitve PCR using ERIC, BOX and REP primer sets and whole-cell protein analysis revealed similarity in pattern profiles. With repetitive-PCR and UPGMA cluster analysis, *P. avellanae* and *P.s.* pv. *theae* strains showed 65% similarity and *P.s.* pv. *actinidiae* 50% similarity to *P. avellanae*. In addition, all the *P. syringae* pathovars strains tested had distinctive genomic and protein fingerprint patterns and clustered apart. Our data are in agreement with those reported by Gardan et al. (1999) concerning genomospecies 8 that includes only *P. avellanae* and *P.s.* pv.

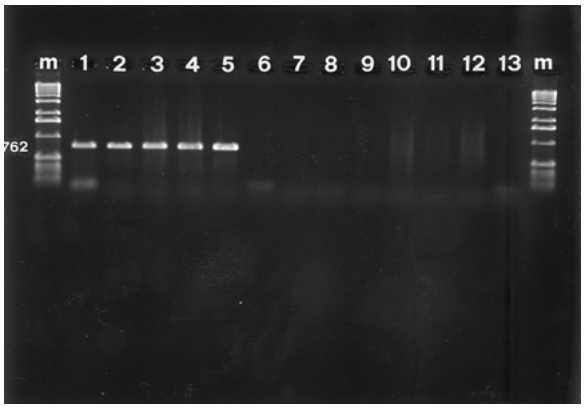

Figure 4. Gel electrophoresis of PCR products obtained from pure cultures of *P. avellanae* (lane 1: NCPPB 3487^T), *P. syringae* pv. *theae* (lane 2: NCPPB 2598^T; lane 3: CFBP 4096) and *P.s.* pv. *actinidiae* (lane 4: NCPPB 3739^T; lane 5: PD 2020) with primers PAV 1 and PAV 22. Lane 6: *P.s.* pv. *syringae* NCPPB 281^T; lane 7: *P.s.* pv. *syringae* NCPPB 1097; lane 8: *P.s.* pv. *eriobotryae* NCPPB 2331^T; lane 9: *P.s.* pv. *helianthi* NCPPB 2460^T; lane 10: *P.s.* pv. *ulmi* NCPPB 632^T; lane 11: *P.s.* pv. *morsprunorum* NCPPB 2787; lane 12: *P.s.* pv. *persicae* NCPPB 2761^T; lane 13: *P. viridiflava* BPIC 394.

Table 2. Biochemical and nutritional tests differentiating *P. avellanae*, *P. syringae* pv. *theae* and *P.s.* pv. *actinidiae* strains

	P. avellanae	*P.s.* pv. *theae*	*P.s.* pv. *actinidiae*
Fluorescence on KB	+[a]	−	−
Fluorescence on CSGA	+[b]	+	−[d]
Growth on NA	−	+	+[e]
Gelatin liquifaction	−	+	−
Casein hydrolisis	−	+	+
Arbutin hydrolisis	−	−	−[f]
Esculin hydrolisis	−	−	−[f]
Tyrosinase	−	−[c]	−
Tween 80	−[b]	−	−
Utilization of			
Arabinose	+	+	−
DL-tartaric acid	+	−	−
D-xylose	+	+	−
L-arginine	−	+	+
L-tyrosine	−	−	+
Trigonelline	−	+	−

[a]The fluorescence disappears after several tranfers on media especially with strains from central Italy.
[b]The strains from central Italy are negative.
[c]One strain studied by Takikawa et al. (1988) is positive.
[d]The strains from South Korea are positive.
[e]The strains from South Korea are negative.
[f]The strains from central Italy are positive.

theae with DNA-hybridization values ranging from 66 to 76% similarity. In a different study not including *P. avellanae* and *P.s.* pv. *theae*, Marques et al. (2000) found that three *P.s.* pv. *actinidiae* strains defined as unknown genomospecies, including the pathotype strain NCPPB 3739, are clearly distinct from *P.s.* pv. *syringae* strains when assessed by means of repetitive-PCR using BOX primer, thus confirming the data obtained in the present study. This was also demonstrated by means of fatty acid and whole-cell protein analysis (Janse and Scortichini, 1996).

Confirmation of the relatedness among the three pseudomonads was obtained by PCR using primers were used to detect *P. avellanae* in visibly and latently infected hazelnut specimens (Scortichini and Marchesi, 2001). The primers were designed by choosing the 16S rRNA sequences that differentiate *P. avellanae* from *P.s.* pv. *syringae* type-strain NCPPB 281 The primers were tested on several strains of *P. syringae* pathovars, namely pv. *morsprunorum*, pv. *persicae* and pv. *syringae*, and we never obtained a PCR product of 762 bp. In the present study, however, we found that the primers amplified all *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* strains tested indicating their similarity. When a comparison was made between the sequences of primer 22 (i.e. positions 997–1025 of 16S rRNA gene of *P. avellanae*) and the sequences of the same region of 16S rRNA gene of *P.s.* pv. *theae* (i.e. EMBL GenBank, accession AB001450) and *P.s.* pv. *actinidiae* (i.e. EMBL GenBank, accession AB001439), were recorded only one difference in the base sequence, namely the position 1025 (i.e. A for *P. avellanae* and G for *P.s.* pv. *theae* and *P.s.* pv. *actinidiae*). All strains lacked the *syrB* gene, thus indicating a substantial difference from *P.s.* pv. *syringae*. Another indirect confirmation of the genetic relatedness of the strains was obtained by Sawada et al. (1999) who performed a phylogenetic analysis of 19 *P. syringae* pathovars by sequencing four genes, *gyr* B, *rpo* D, *hrp* L and *hrp* S. In their study, *P. avellanae* was not included but *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* were placed in the same group 1 and were the most closely related strains of the group. Based on the data obtained in the present study and also from the results published by other researchers, we propose to include *P. avellanae*, *P.s.* pv. *theae* and *P.s.* pv. *actinidiae* in genomospecies 8.

The present study also indicates that the strains belonging to *P.s.* pv. *actinidiae* differ according to their geographic origin. In fact, the strains isolated from Japan, South Korea and Italy showed very similar profiles with distinctive polymorphism based

on repetitive-PCR and whole-cell protein analysis. However, all strains isolated in the same country showed a unique profile. By contrast, *P.s.* pv. *theae* strains (i.e. one isolated from *T. sinensis* and two from *C. sinensis*) showed the same profile with both techniques, whereas *P. avellanae* strains from northern Greece were different (i.e. 90% of similarity) from the strains obtained from central Italy (Scortichini et al., 1998; present study).

Gardan et al. (1999) also stated that genomospecies 3 and 8 are not clearly distinguished by ribotyping using *Sma* I and *Hin* cII and *P.s.* pv. *tomato* type strain NCPPB 1106 was recommended as the reference strain for enomospecies 3. Interestingly, by using this strain as an outgroup strain in the repetitive-PCR and UPGMA analysis, we also found that it is the most closely related strain to the genomospecies 8 (i.e. *P. avellanae* and *P.s.* pv. *theae*).

When artificially inoculated into plants, each group of strains incited disease symptoms only in the plant species from which they were originally isolated. *P. avellanae* strains did not cause symptoms in kiwifruit and *C. sinensis*, *P.s.* pv. *theae* did not incite infections in hazelnut or kiwifruit, and *P.s.* pv. *actinidiae* did not affect hazelnut and *C. sinensis*. These data confirm that these pseudomonads are host specific.

Phenotypic traits can distinguish these pseudomonads and aid their identification, especially in areas where the crops may harbour overlapping populations (i.e. eastern, central and western Asia, southern Europe). For this purpose, the key-biochemical tests reported in Table 2 are useful and take into account not only the data obtained in the present study but also the published data (Psallidas and Panagopoulos, 1979; Psallidas, 1993; Takikawa et al., 1988; 1989). Growing isolates on culture media such as NA, KB and CSGA can give important information for the discrimination of the isolates. We found variability among the strains in fluorescent pigment production on KB and CSGA which also occur in the fluorescent *Pseudomonas* group (Palleroni, 1984). For example, *P. avellanae* strains isolated in central Italy, change from slight fluorescence on KB when freshly isolated to the absence of fluorescence on both KB and CSGA (Scortichini and Angelucci, 1999; present study).

Data from a combination of genetic and phenotypic techniques as well as pathogenicity tests (i.e. polyphasic approach), suggest that the three groups of pseudomonads, although exhibiting host specificity, should be included in the same genomospecies 8 *sensu* Gardan et al. (1999) with *P. avellanae* as a distinct species.

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