Variability of the 16S–23S rRNA gene internal transcribed spacer in *Pseudomonas avellanae* strains

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Abstract

The 16S–23S rRNA gene internal transcribed spacer region (ITS1) from 34 strains of *Pseudomonas avellanae* and some strains of *Pseudomonas syringae* pathovars was amplified and assessed by restriction fragment length polymorphism (RFLP) using 10 restriction enzymes. In addition, the ITS1 region of four representative *P. avellanae* strains was sequenced and compared by the neighbour-joining algorithm with that of *P. syringae* pathovars. Two main groups of *P. avellanae* strains were observed that did not correlate with their origin. The ITS1 region sequencing revealed a high similarity with the *P. syringae* complex. One group of *P. avellanae* strains showed high similarity to *P. s.* pv. *actinidiae* and *P. s.* pv. *tomato*; another group showed similarity with *P. s.* pv. *tabaci* and *P. s.* pv. *glycinea*. Two strains clustered with *P. s.* pv. *pisi*. The difficulties to unambiguously classify the strains associated with hazelnut decline in Greece and Italy are discussed.

Introduction

On the basis of restricted pathogenicity and by means of discriminative biochemical and nutritional tests, the causative agent of hazelnut (*Corylus avellana* L.) bacterial canker and decline in northern Greece and central Italy was placed within the *Pseudomonas syringae* complex, as pathovar *avellanae* (Psallidas, 1993; Scortichini & Tropiano, 1994). Subsequently, based on 16S rRNA gene sequencing, whole-cell protein and fatty acid analysis, this pathogen was found to be significantly different from the other *P. syringae* pathovars tested and from other pseudomonads, and it was elevated to species level (Janse *et al*., 1996). Further studies performed using DNA–DNA hybridization, amplified ribosomal DNA restriction analysis (ARDRA) and amplified fragment length polymorphism (AFLP) analysis and a higher number of *P. syringae* pathovars strains highlighted not only the distinctiveness of *Pseudomonas avellanae* from the majority of *P. syringae* pathovars but also the high similarity with some of them, namely *P. s.* pv. *theae* and *P. s.* pv. *actinidiae* (Gardan *et al*., 1999; Scortichini *et al*., 2002a; Manceau & Brin, 2003). However, in ARDRA analysis, the restriction enzyme Tru9I differentiated *P. avellanae* from the other two *P. syringae* pathovars (Scortichini *et al*., 2002a).

Other studies performed using techniques to differentiate the bacteria at strain level such as repetitive-sequence PCR (rep-PCR), insertion-sequence PCR and multilocus enzyme electrophoresis revealed two distinct lineages within *P. avellanae*, which correlated with the geographic areas of hazelnut decline (i.e. Greece and Italy) (Scortichini *et al*., 2002b, 2003, 2006). Multilocus sequence typing (MLST) analysis performed by sequencing *rpo*D, *gyr*B, *glt*A and *gap*A genes, confirmed the distinctiveness of the two lineages and demonstrated that such strains are embedded within two of the five *P. syringae* phylogroups (Wang *et al*., 2007). Clearly, depending on the kind of technique used to assess genetic diversity at different levels, *P. avellanae* can be viewed as a distinct species or as a member of the *P. syringae* complex.

To further assess the genetic variability of this bacterium, the 16S–23S rRNA gene internal transcribed spacer (ITS1) region was investigated. This spacer region exhibits a high degree of sequence and length variation that can be exploited to compare closely related bacterial strains (Naimi *et al*., 1997; Guasp *et al*., 2000; Iteman *et al*., 2000). In particular, the ITS1 region alternates parts of conserved and hypervariable sequences allowing the characterization of strains at the subspecies or strain level (Garcia-Martinez *et al*., 2002). A limited number of studies on the ITS1 region concern phytopathogenic pseudomonads (Manceau &

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Horvais, 1997; Sawada *et al.*, 1997; Catara *et al.*, 2002; Milyutina *et al.*, 2004; Kong *et al.*, 2005) and further insights into *P. avellanae* diversity might be useful.

The main aims of this study were to increase the knowledge of the diversity of the ITS1 region of *P. avellanae* also in comparison with other phytopathogenic pseudomonads, to verify by this assessment the genetic relationships among the *P. avellanae* strains isolated from two different geographic areas, and to compare the results herein obtained with other techniques differentiating the bacteria at the species or strain level.

Materials and methods

Bacterial strains

The *P. avellanae* and *P. syringae* pathovars strains used for comparative purposes are listed in Table 1. All *P. avellanae* strains obtained from diseased hazelnut specimens collected in Greece and Italy were previously assessed by different molecular techniques (Scortichini *et al.*, 2002a, b, 2003, 2006). The strains were routinely cultured on nutrient agar

Table 1. List of *Pseudomonas* strains assessed by ITS1 region amplification and subsequent RFLP

Species-Pathovar	Strain	Origin-Year	ITS1 pattern	ITS1-RFLP
Pseudomonas avellanae	BPIC 631[T]	Greece-1976	A	A
	BPIC 632	Greece-1976	A	A
	BPIC 640	Greece-1976	A	A
	BPIC 641	Greece-1976	A	A
	BPIC 649	Greece-1976	A	A
	BPIC 659	Greece-1976	A	A
	BPIC 707	Greece-1977	A	A
	BPIC 715	Greece-1987	A	A
	BPIC 1077	Greece-1987	A	A
	BPIC 1422	Greece-1987	A	A
	BPIC 1436	Greece-1990	A	A
	ISPaVe 041	Italy-1992	A	A
	ISPaVe 042	Italy-1992	A	A
	ISPaVe 369	Italy-1995	A	A
	ISPaVe 436	Italy-1993	A	A
	ISF 1266	Italy-2004	A	A
	ISF 1267	Italy-2004	A	A
	ISF H1	Italy-2002	A	A
	ISF H2	Italy-2003	A	A
	ISF H3	Italy-2003	A	A
	ISF H4	Italy-2004	A	A
	ISPaVe 011	Italy-1991	B	B
	ISPaVe 012	Italy-1991	B	B
	ISPaVe 013	Italy-1992	B	B
	ISPaVe 2056	Italy-1994	B	B
	ISPaVe 2057	Italy-1994	B	B
	ISPaVe 2058	Italy-1994	B	B
	ISPaVe 2059	Italy-1994	B	B
	BPIC 665	Greece-1976	B	B
	BPIC 710	Greece-1987	B	B
	BPIC 714	Greece-1987	B	B
	ISF 3720	Italy-2004	B	B
	ISPaVe 037	Italy-1992	C	C
	ISPaVe 439	Italy-1995	C	C
P. syringae pv. *syringae*	NCPPB 3869	Italy-1992	D	D
P. s. pv. *actinidiae*	KW 1	Japan-1984	E	E
P. s. pv. *aptata*	PD195	The Netherlands-1987	F	F
P. s. pv. *tabaci*	BPIC 451	Greece-1975	G	G
P. s. pv. *phaseolicola*	1448A	Ethiopia-1985	H	H

BPIC, Culture Collection of Benaki Phytopathological Institute, Kiphissia-Athens, Greece; ISF, Culture Collection of C.R.A.-Istituto Sperimentale per la Frutticoltura, Roma, Italy; ISPaVe, Culture Collection of C.R.A.-Istituto Sperimentale per la Patologia Vegetale, Roma, Italy; NCPPB: National Collection of Plant Pathogenic Bacteria, CSL, York, UK; PD, Culture Collection of Plant Protection Service, Wageningen, the Netherlands; T, type-strain. KW 1, as received from Dr M. Goto.

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(Oxoid) supplemented with 3% of sucrose (mNSA) at 24–26 °C.

DNA extraction and PCR amplification

For DNA preparation, a loopful of *c.* 3 mm in diameter was taken from single colonies grown for 24 h at 24–26 °C on mNSA, suspended in sterile saline (0.85% of NaCl in distilled water) and centrifuged at 12 000 ***g*** for 2 min. After the supernatant was discarded, the pellet was suspended in bidistilled, filtered water to an OD corresponding to $1–2 \times 10^7$ CFU mL^{-1}. The suspension was placed in boiling water for 10 min and then stored at − 20 °C. Primers were chosen for PCR amplification of the 16S–23S rRNA gene intergenic spacer (ITS1): D21 (5′-AGC CGT AGG GGA ACC TGC GG-3′) and D22 (5′-TGA CTG CCA AGG CAT CCA CC-3′) (Manceau & Horvais, 1997). PCR was performed as follows: 3 mM $MgCl_2$, 0.25 mM of each dNTP, 20 pmol of each primer, 0.02 U *Taq* DNA-polymerase (Promega) in a final volume of 50 μL.

Amplifications were performed in an Eppendorf Mastercycle programmable thermal controller with the following steps: an initial denaturation at 94 °C for 2 min, 36 cycles of 94 °C for 1 min, 60 °C for 2 min, 72 °C for 1 min, a final extension at 72 °C for 2 min, and a final soak at 4 °C (Manceau & Horvais, 1997). Ten microliters of each amplified DNA fragment were run in a horizontal 3% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer. Gels were stained with ethidium bromide, visualized under a UV transilluminator (Spectroline) and photographed with a Kodak Gel Logic Imaging System apparatus. Each run was performed in duplicate.

Restriction analysis of amplified ITS1 fragments

Ninety microliters of each amplified DNA fragment were purified with the Wizard SV Gel and PCR clean up system (Promega), and an aliquot of 10 μL each was digested with the following enzymes: AluI, BfaI, Bsh1236I, HaeIII, HhaI, MseI, MspI, Sau3AI, TaqI and Tsp509I (Promega). Digestions were performed as described by the manufacturers and the resulting fragments were run in a horizontal 3% agarose gel in 1X TAE buffer. Each run was performed in duplicate.

Cloning and DNA sequencing

The PCR-amplified ITS1 DNA fragments of about 550 bp of *P. avellanae* BPIC 631 and BPIC 710, isolated in Greece, as well as ISPaVe 011 and ISPaVe 439, isolated in Italy, were purified from 1.5% agarose gel with the Wizard SV Gel and PCR clean-up system, as described by the manufacturer (Promega) and ligated in pGem-T easy vector (Promega). Ligated plasmids were transformed into competent *Escherichia coli* strain TOP10 F′ cells (Invitrogen) and the bacteria spread onto Luria–Bertani (LB) agar plates containing 50 μg mL^{-1} ampicillin, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 μg mL^{-1} X-gal. Plasmid DNA isolated from 15 white colonies was purified and digested according to Sambrook *et al.* (1989), and digested with EcoRI to verify the presence of the inserts. These clones were sequenced with primers D21 and D22 (Manceau & Horvais, 1997). DNA fragments were prepared for sequencing using a Qiagen Plasmid Midi kit according to the instructions supplied by the manufacturer (Qiagen). All the sequences were determined at Primm sequencing service. The ITS1 sequences of *P. avellanae* were aligned manually with other ITS1 sequences of *P. syringae* pathovars obtained from GenBank. MEGA 3.1 package was used for building a dendrogram from aligned sequences based on neighbour-joining (NJ) algorithm and for calculating the Bootstrap values (1000 replicates) (Kumar *et al*., 2004).

Nucleotide accession numbers

The ITS1 sequence data were submitted to the EMBL/GenBank Database under the following accession numbers: *P. avellanae* BPIC631, AM489489; *P. avellanae* ISPaVe 011, AM489490; *P. avellanae* BPIC710, AM489491; *P. avellanae* ISPaVe 439, AM489492.

Results

The primers D21 and D22 amplified the 16S–23S rRNA gene spacer region for all the *P. avellanae* and *P. syringae* pathovars tested. After electrophoresis, all *P. avellanae* strains gave two bands, which enabled the differentiation of strains into three groups (Table 1). The *P. syringae* pathovars utilized for comparison showed different banding patterns. A representative gel is shown in Fig. 1. The first group of *P. avellanae* strains, including the type-strain, showed two bands of about 550 and 650 bp (group A); another group yielded two bands of about 550 and 580 bp (group B). Remarkably, groups A and B include strains isolated both in Greece and in Italy. Only two strains, namely ISPaVe 037 and ISPaVe 439, both isolated in Italy, showed two bands of about 450 and 570 bp (group C) (see also Table 1). These findings indicate the presence of two ITS1 spacer regions in the genomes of these strains. When the ITS1-PCR products were digested, the sum of the sizes of restricted fragments in all cases did not agree with the sum of the sizes of undigested ITS1 amplification products. One representative gel obtained with MspI restriction enzyme is shown in Fig. 2. This confirms the presence of more than one type of ITS1 sequence in *P. avellanae* strains. All the restriction enzymes tested grouped the *P. avellanae* strains into the same three groups.

The length of the ITS1 regions of the four *P. avellanae* strains ranged from 532 to 548 bp from the 3′ end of the

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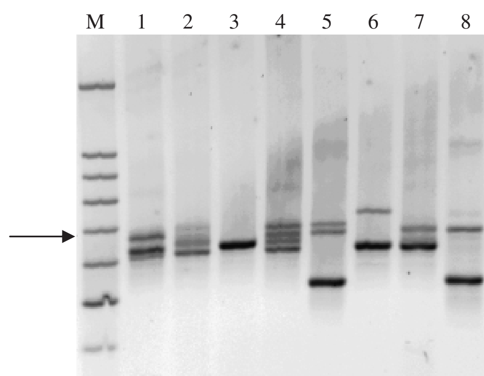

Fig. 1. Agarose gel electrophoresis of PCR-amplified ITS1 spacer region obtained with primers D21 and D22. The arrow indicates 600 bp. M: molecular size marker, 100-bp ladder (Promega). Lane 1, *Pseudomonas syringae* pv. *tabaci* BPIC 451; lane 2, *P. s.* pv. *actinidiae* KW 1; lane 3, *P. s.* pv. *aptata* PD 195; lane 4, *P. s.* pv. *syringae* NCPPB 3869; lane 5, *P. s.* pv. *phaseolicola* 1448A; lane 6, *P. avellanae* BPIC 631; lane 7, *P. avellanae* ISPaVe 011; lane 8, *P. avellanae* ISPaVe 439.

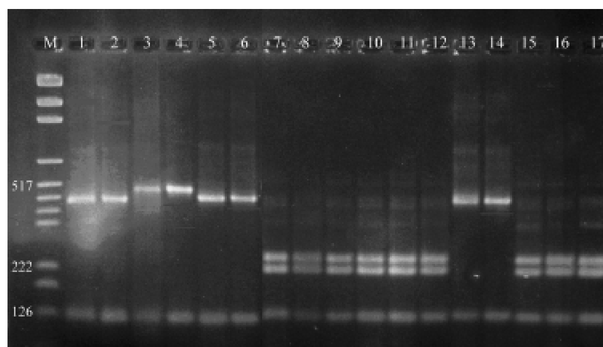

Fig. 2. Agarose gel electrophoresis of PCR-amplified ITS1 fragments of *Pseudomonas avellanae* strains digested with MspI. M: molecular size marker, pGem (Promega). Lane 1, ISPaVe 011; lane 2, ISPaVe 013; lane 3, ISPaVe 037; lane 4, ISPaVe 439; lane 5, ISPaVe 2059; lane 6, ISPaVe 2058; lane 7, ISPaVe 041; lane 8, ISPaVe 042; lane 9, BPIC 641; lane 10, BPIC 649; lane 11, BPIC 631; lane 12, BPIC 641; lane 13: BPIC 665; lane 14, BPIC 710; lane 15, BPIC 715; lane 16, BPIC 1422; lane 17, BPIC 1077.

ribosome-binding site to the 5′ end of the 23S rRNA gene. These lengths are in agreement with other *P. syringae* pathovars strains previously analysed in other studies. The nucleotide sequences were aligned and the 3′ end of 16S rRNA gene and the 5′ end of 23S rRNA gene were deduced and compared with other *P. syringae* pathovars using GenBank accessions. Two tRNA genes, $tRNA^{Ile}$ and $tRNA^{Ala}$, were identified and their nucleotide sequences were identical in the four *P. avellanae* strains studied and resulted the same as for the *P. syringae* pathovars compared here (data not shown).

Variable regions were found by sequence comparison with the ITS1 regions of *P. syringae* pathovars. At positions 116–120 bp of the spacer region, a fragment showing variability enabling the differentiation of *P. avellanae* BPIC 631, *P. s.* pv. *tomato* DC 3000, *P. s.* pv. *anthirrini* 4030, *P. s.* pv. *maculicola* 10, *P. s.* pv. *actinidiae* 9617, as well as *P. avellanae* ISPaVe 439, *P. s. phaseolicola* 19034, *P. s.* pv. *papulans* 4048 and *P. s.* pv. *pisi* 2452 from all the other phytopathogenic pseudomonads was found. Other fragments showing relevant variability were pointed out at positions 203–206, 223–224, 245–247 bp and within the Box B fragment. The region between the sequence of Box A and the 23S rRNA gene is conserved showing only single base substitutions. In particular, at position 532 bp an A instead of a G aids the discrimination of *P. avellanae* ISPaVe 011 and *P. avellanae* BPIC 710.

A dendrogram of relationships among *P. avellanae*, *P. syringae* pathovars and *Pseudomonas coronafaciens* inferred on the basis of ITS1 region sequencing and NJ algorithm is shown in Fig. 3. *Pseudomonas avellanae* BPIC 631 is included in a subcluster together with *P. s.* pv. *tomato* DC 3000, *P. s.* pv. *anthirrini* 4030, *P. s.* pv. *maculicola* 10, and *P. s.* pv. *actinidiae* 9617. *Pseudomonas avellanae* ISPaVe 011, isolated in Italy, is included in another cluster together with *P. syringae* pathovars *tabaci* 2863 and *glycinea* 8727. *Pseudomonas avellanae* BPIC 710 showed a high similarity with this cluster, whereas *P. avellanae* ISPaVe 439 was similar to *P. syringae* pathovars *phaseolicola* 19034, *papulans* 4048 and *pisi* 2452.

Discussion

This study showed that the ITS1 region structure of *P. avellanae* is similar to that of *P. syringae*. The length, the tRNA genes and the genetic sequence itself show high level of similarity to those of other pseudomonads belonging to the *P. syringae* complex. In addition, a certain degree of variability among the different *P. avellanae* strains tested, as in the case of *P. syringae* (Milyutina *et al.*, 2004), was also ascertained but no grouping according to the geographic origin of the strains was evident as in the cases of previous rep-PCR and MLST analysis (Scortichini *et al.*, 2006; Wang *et al.*, 2007). In fact, upon ITS1-RFLP and the NJ clustering of the ITS1 region sequences, most of the strains isolated from both Greece and Italy clustered in different groups, namely A and B. The variability found in certain fragments of the ITS1 region of *P. avellanae* strains confirms once more the mosaic structure of such a region characterized by conserved and variable blocks of sequences that can vary upon recombination events and/or horizontal transfers (Milyutina *et al.*, 2004). The cause of the variability found in *P. avellanae* strains is not known and deserves further investigation. By contrast, other phytopathogenic pseudomonads such as *P. s.* pv. *tomato* and *P. s.* pv. *tagetis* show no or very little variability by ITS1-RFLP and ITS1 sequencing analysis, respectively (Manceau & Horvais, 1997; Kong *et al.*,

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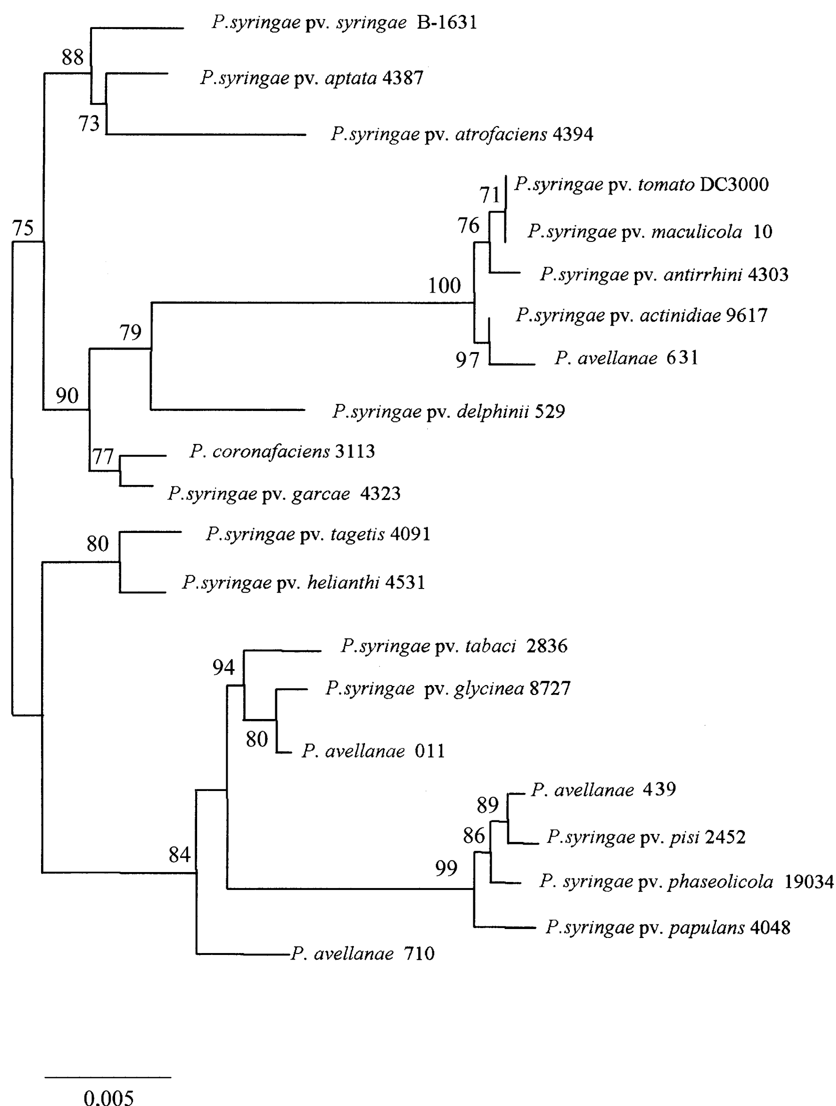

Fig. 3. Dendrogram based on the 16S–23S rRNA gene intergenic spacer region sequences of *Pseudomonas syringae* pathovars, *P. avellanae* and *P. coronafaciens* strains obtained using the neighbour-joining algorithm. The percentages of bootstrap replicates, based on 1000 bootstrap samples, are placed at the tree nodes. The bar scale indicates the rate of substitution per base.

2005). ITS1 spacer region characterization either by RFLP or by complete sequencing confirms its discriminatory capability at the strain level, whereas ARDRA analysis is more appropriate for grouping the strains at a higher level (Cho & Tiedje, 2000).

The dendrogram inferred using ITS1 region sequences of some *P. syringae* pathovars and *P. coronafaciens* partly reflects the genomospecies structure of the *P. syringae* complex inferred by DNA–DNA hybridization (Gardan *et al.*, 1999). Interestingly, *P. avellanae* type-strain BPIC 631 was highly similar to *P. s.* pv. *actinidiae* as previously observed by means of AFLP and rep-PCR analysis (Scortichini *et al.*, 2002a; Manceau & Brin, 2003). This strain belonging to the genomospecies 8, according to Gardan *et al.* (1999), showed also a relevant similarity with strains of the genomospecies 3, *P. s.* pv. *tomato*, *P. s.* pv. *maculicola* and *P. s.* pv. *anthirrini* as previously observed using DNA–DNA hybridization and repetitive PCR (Gardan *et al.*, 1999; Scortichini *et al.*, 2002a). Other *P. avellanae* strains not assessed by DNA–DNA hybridization analysis were clustered into another group with *P. s.* pv. *tabaci* and *P. s.* pv. *glycinea*, which belong to the genomospecies 2 *sensu* Gardan *et al.* (1999). *Pseudomonas avellanae* 439, isolated in Italy, showed remarkable similarity with *P. s.* pv. *pisi* (genomospecies 1). This finding was highlighted by means of MLST analysis and gene genealogies indicating that this strain diverged before the diversification of other strains belonging to the same clade and may have originated from an ancestral pea pathogen (Wang *et al.*, 2007).

This study also confirms and points out that the strains associated with bacterial canker and the decline of hazelnut in Greece and Italy are difficult to unambiguously

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classify. Techniques aimed to assess the variability of the 16S rRNA gene, widely used to infer taxonomic relationships among bacterial species, revealed a substantial differentiation of *P. avellanae* from the *P. syringae* complex (Janse *et al*., 1996; Catara *et al*., 2002; Scortichini *et al*., 2002a), with the hypervariable region of this gene showing high similarity with that of *Pseudomonas viridiflava* and *Pseudomonas amygdali* (Janse *et al*., 1996; Catara *et al*., 2002). In addition, an important phenotypic trait characterizing all the strains causing hazelnut decline is their inability to grow on nutrient agar, whereas the other *P. syringae* pathovars clearly show this feature (Psallidas, 1993; Scortichini & Tropiano, 1994). By contrast, other powerful discriminatory techniques, such as DNA–DNA hybridization, MLST and AFLP analysis and the result of this study on the ITS1 spacer region characterization, revealed a high similarity of the strains causing hazelnut decline with plant pathogenic bacteria classified within the *P. syringae* complex (Gardan *et al*., 1999; Manceau & Brin, 2003; Wang *et al*., 2007). It would seem that a mosaic genome characterizes the strains associated with hazelnut decline and that, depending on which feature is investigated, a different view and classification is obtained. Given the present scenario, such strains can be named as either *P. avellanae* or *P. syringae* pv. *avellanae*.

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