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Evidence for separate origins of the two *Pseudomonas avellanae* lineages

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Pseudomonas avellanae is the causal agent of hazelnut (*Corylus avellana*) decline, both in northern Greece and central Italy, and two lineages related to the geographical origins of the pathogen have previously been identified. Forty strains, obtained from all the areas where the disease has so far been observed, and representing six different subpopulations of the two lineages, were further assessed using insertion-sequence PCR genomic fingerprinting. The data previously obtained from repetitive-sequence PCR using ERIC and BOX primer sets and insertion-sequence PCR (IS50) were analysed using statistical methods, enabling genetic diversity and gene flow among the populations to be elucidated, as well as verifying the possible correlation between genetic diversity and geographical origin. The Mantel test performed with ERIC, BOX and IS50-PCR data revealed that the *P. avellanae* populations that are spatially distant from each other are also genetically dissimilar: gene flow estimates confirmed this. The present study supports the hypothesis that *P. avellanae* originated separately in Greece and Italy, and that the two lineages of the pathogen underwent separate local evolution.

Keywords: bacterial populations, *Corylus avellana*, gene flow, genetic diversity, hazelnut decline, repetitive-sequence PCR

Introduction

Pseudomonas avellanae is the causal agent of hazelnut (*Corylus avellana*) decline both in northern Greece and central Italy (Psallidas, 1987; Scortichini, 2002). Disease symptoms include the rapid wilting of branches and trees, which can be observed from spring to autumn. In some circumstances, longitudinal cankers are noticed along the trunk. *Pseudomonas avellanae* and two *Pseudomonas syringae* pathovars, *theae* and *actinidiae*, belong to genomospecies 8 according to Gardan *et al.* (1999) and Scortichini *et al.* (2002b). These genotypes can readily be distinguished from each other by amplified rDNA restriction analysis (ARDRA) using *Tru* 9I as the endonuclease and by repetitive-sequence PCR using BOX and ERIC primer sets, as well as by AFLP (Scortichini *et al.*, 2002b; Manceau & Brin, 2003).

When ARDRA analysis, which enables bacterial species to be distinguished at the species and/or subspecies level, was performed using nine different restriction endonucleases, it did not produce any differences in banding patterns of many *P. avellanae* strains from Greece and Italy (Scortichini *et al.*, 2002a). Moreover, sequencing of the 16S rDNA gene of strains from both countries also revealed a high degree of similarity (99·4%) between these two groups (Scortichini *et al.*, 2005).

In contrast, other phenotypic and genotypic techniques revealed some evident differences between the genotypes. Scortichini & Angelucci (1999) found that all strains isolated in Greece produced a fluorescent pigment under UV light on medium B (King *et al.*, 1954), whereas this pigment was quite faint for strains isolated in Italy, and disappeared after repeated transfer to bacterial culture media such as nutrient sucrose agar. Serotyping of *P. avellanae* using monoclonal antibodies raised against the O polysaccharides of the lipopolysaccharides in the bacterial cell wall clearly differentiated all strains isolated in Greece from those obtained in Italy (Ovod *et al.*, 1999). Further evidence for strain differentiation came from the assessment performed with repetitive-sequence PCR in which banding patterns obtained using ERIC and BOX primer sets clearly differentiated strains from Greece from strains from Italy (Scortichini *et al.*, 1998, 2002a). In addition, plasmid analysis revealed evident differences in number and size of plasmids between strains from the two areas (Janse *et al.*, 1996). From these data, it appears that *P. avellanae* consists of two different lineages. However, the overall genetic structure of the species, analysed by multilocus enzyme electrophoresis, was clonal and, in the resulting dendrogram, the group of Greek strains was clearly distinct from that of strains isolated in Italy

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(Scortichini *et al*., 2003). The aggressiveness to hazelnut of both groups is similar, and is remarkably high (Scortichini *et al*., 2002a).

Hazelnut decline has been observed in both Greece and Italy since the late 1970s, and epidemics are more severe on subacid soils (Scortichini, 2002). Hazelnut cultivars utilized in the two areas are different. In northern Greece, cv. Palaz was introduced from Turkey at the end of 1960s. Remarkably, *P. avellanae* has not yet been recorded in Turkey despite extensive hazelnut cultivation. In central Italy, cvs Tonda Gentile Romana and Nocchione are locally adapted and have been cultivated for thousands of years, and cv. Palaz is completely unknown.

The phenotypic and genetic differences observed between *P. avellanae* strains from Greece and Italy, taken together with the very different histories of hazelnut cultivation in the two countries, lead to the hypothesis of separate origins of the two *P. avellanae* lineages. To test this hypothesis, 40 representative *P. avellanae* strains from both countries were analysed with another molecular technique, insertion-sequence PCR, to assess the genomic structure of the bacteria at the strain level. Then, in order to elucidate the origins of the strains, data obtained from the present and previous studies (Scortichini *et al*., 1998, 2002a) were analysed using statistical methods to verify the correlation between strain genetic diversity and geographical distance between areas from where strains were originally isolated.

Materials and methods

Bacterial strains and growth conditions

For this study, 40 previously described *P. avellanae* strains (Scortichini *et al*., 1998, 2002a, 2002b, 2003), representing all the sites in northern Greece and central Italy from where the pathogen has been isolated to date, were selected (Table 1). The strains were routinely cultured on nutrient agar (Oxoid) with 5% sucrose (NSA) at 25–27°C.

Table 1 List of *Pseudomonas avellanae* strains used in this study

Strain[a]	Country	Province	Year of isolation
BPIC 631[(T)]	Greece	Drama	1976
BPIC 632	Greece	Drama	1976
BPIC 708	Greece	Drama	1987
BPIC 710	Greece	Drama	1987
BPIC 711	Greece	Drama	1987
BPIC 641	Greece	Kilkis	1976
BPIC 647	Greece	Kilkis	1976
BPIC 665	Greece	Kilkis	1976
BPIC 1077	Greece	Kilkis	1976
BPIC 1422	Greece	Kilkis	1976
BPIC 702	Greece	Katerini	1977
BPIC 703	Greece	Katerini	1977
BPIC 704	Greece	Katerini	1977
BPIC 706	Greece	Katerini	1977
BPIC 707	Greece	Katerini	1977
BPIC 714	Greece	Kavala	1987
BPIC 715	Greece	Kavala	1987
BPIC 716	Greece	Kavala	1987
BPIC 1435	Greece	Kavala	1990
BPIC 1436	Greece	Kavala	1990
ISPaVe 011	Italy	Rome	1992
ISPaVe 012	Italy	Rome	1991
ISPaVe 013	Italy	Rome	1992
ISPaVe 037	Italy	Rome	1992
ISPaVe 056	Italy	Rome	1993
ISPaVe 369	Italy	Rome	1993
ISPaVe 436	Italy	Rome	1995
ISPaVe 439	Italy	Rome	1995
ISPaVe 679	Italy	Rome	1996
ISPaVe 680	Italy	Rome	1996
ISPaVe 041	Italy	Viterbo	1992
ISPaVe 042	Italy	Viterbo	1992
ISPaVe 038	Italy	Viterbo	1993
ISPaVe 039	Italy	Viterbo	1993
ISPaVe 040	Italy	Viterbo	1993
ISF 2059	Italy	Viterbo	1994
ISF 683	Italy	Viterbo	1996
ISF TGR1	Italy	Viterbo	2002
ISF TGR2	Italy	Viterbo	2003
ISF TGR3	Italy	Viterbo	2003

[a] (T), Type strain; BPIC, Culture Collection of Benaki Phytopathological Institute, Kiphissia-Athens, Greece; ISF, Culture Collection of the Istituto Sperimentale per la Frutticoltura, Roma, Italy; ISPaVe, Culture Collection of the Istituto Sperimentale per la Patologia Vegetale, Roma, Italy.

DNA extraction and insertion-sequence PCR

To prepare total genomic DNA, a modification of the technique of Smith *et al*. (1995) was used. For each strain, a loopful (diameter *c*. 3 mm) of a single colony that had been grown for 24 h on NSA at 25–27°C was suspended in sterile saline (0·85% NaCl in distilled water) and centrifuged at 12 000 ***g*** for 2 min. The supernatant was discarded and the pellet suspended in bidistilled, filtered, sterilized water up to an optical density corresponding to $1–2 \times 10^8$ CFU mL^{-1}. The suspension was placed in boiling water for 10 min and then stored at –20°C for the PCR experiments. Primer IS50 (5′-CAGGACGCTACTTGTGT-3′), complement to the insertion sequence IS50 of Tn5 (Ullrich *et al*., 1993), was used for strain typing. The PCR reaction mixture of 30 μL contained 50 ng genomic DNA, 3 μL 10 × PCR reaction buffer, 3 mM $MgCl_2$, 0·4 mM dNTP, 50 pmol of the IS50 primer, and 1·5 U *Taq* DNA polymerase (Promega). The mixtures were overlaid with 50 μL mineral oil. The PCR amplification conditions were as follows: an initial denaturation cycle of 95°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 38°C for 1 min and extension at 72°C for 3·5 min; and a final extension step of 72°C for 10 min. After the reactions, 15 μL of the amplification products were separated on 8% polyacrylamide gel in 1 × Tris–borate–EDTA (TBE) buffer. Gels were stained with ethidium bromide and photographed under UV light with a Polaroid film type 52. The IS50-PCR amplifications were performed in duplicate.

Data analysis

ERIC and BOX repetitive PCR fragments obtained from previous studies (Scortichini *et al*., 1998, 2002a) and IS50-PCR fragments were scored as putative loci, with the presence/absence of the fragment indicating allele/no allele. Such molecular markers provided a high level of discrimination between bacterial strains and were selectively neutral, enabling the bacterial population structure to be studied (Gurtler & Mayall, 2001). The data sets were compiled as a matrix of strains and molecular fragments. For a more refined calculation, the strains were subdivided into populations according to the province from which they were originally isolated (Table 1). A total of six populations were computed. Standard population genetics statistics were performed using the POPGENE population genetic software (ver. 1·32, Molecular Biology and Biotechnology Center, University of Alberta, Edmonton, Canada). Mean genetic diversity estimates within and among the populations were calculated using the following indexes: (i) Nei's mean genetic diversity, h, calculated as $h = (1 - \sum p_i)^2$, where p_i is the frequency of allele i at the locus (Nei, 1973); (ii) Nei's original measure of genetic identity and genetic distance, G_{ST}, enabling the proportion of total genetic diversity attributable to population differentiation to be estimated (Nei, 1972); (iii) Nei's unbiased measures of genetic identity and distance, enabling between-group genetic distances to be estimated using the frequency of each band in each group (Nei, 1978); and (iv) gene-flow estimate independent of population size, N_m, calculated as: $N_m = 0{\cdot}5(1 - G_{ST})/G_{ST}$ (McDermott & McDonald, 1993). Genetic distance phenograms were generated according to the UPGMA method and the neighbour-joining (NJ) clustering algorithm (Saitou & Nei, 1987) using POPGENE. Genetic distance matrices obtained from ERIC and BOX repetitive PCR and IS50 PCR data, and Nei's original measure of genetic identity and genetic distance, were compared with the geographical distances between the *P. avellanae* populations according to the Mantel test (Mantel, 1967) using XLSTAT software (Addinson, New York). This test computes the linear correlation between two proximity matrices to reveal whether environmental variables are intercorrelated among themselves. The statistical significance of the resulting correlation coefficient was tested by performing 10 000 random permutations of the data set and calculating the proportion yielding values that were equal to or greater than the observed coefficient of correlation. The correlation was tested at $P = 0{\cdot}05$ according to Pearson's coefficient.

Results

Geographical genetic structure of *P. avellanae*

A total of 18 ERIC, 14 BOX and 28 IS50-PCR products were scored for all 40 *P. avellanae* strains. Both repetitive-sequence and IS50 PCR gave a high level of discrimination between populations of strains isolated from northern Greece and central Italy by showing some fragments unique to particular populations. A representative gel of IS50-PCR is shown in Fig. 1. The mean values of Nei's genetic diversity, h, for ERIC and BOX repetitive PCR and IS50-PCR data for the six *P. avellanae* populations are reported in Table 2. Genetic diversity values were generally greater for the strains isolated in central Italy. The highest values were observed with BOX-PCR data (0·16) for the strains obtained from Rome, and with IS50-PCR data (0·14) for the strains isolated from Viterbo province. In northern Greece, the strains from Kilkis, when assessed with BOX-PCR, showed the highest genetic diversity (0·07).

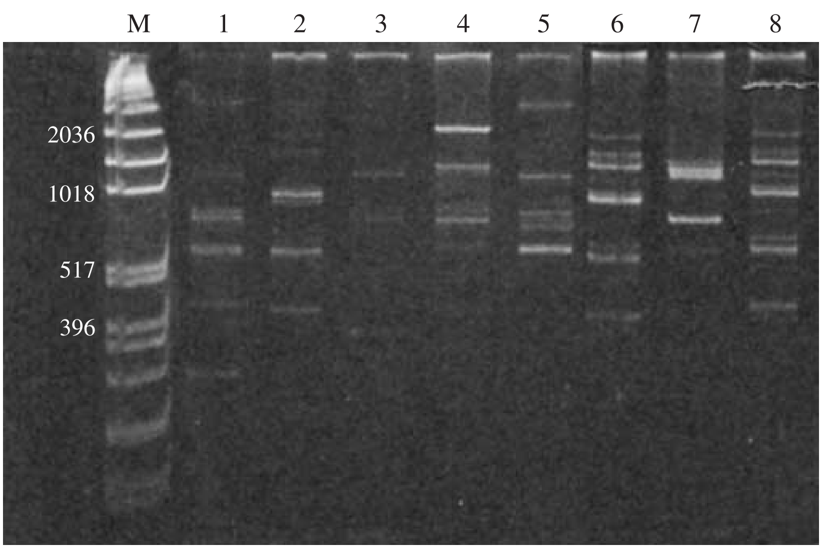

Figure 1 PCR fingerprint patterns from genomic DNA of representative *Pseudomonas avellanae* strains from Greece and Italy using primer IS50. Lanes: 1, *P. avellanae* ISPaVe 011; 2, ISF TGR1; 3, ISPaVe 037; 4, ISPaVe 013; 5, ISF 2059; 6, BPIC 631; 7, BPIC 714; 8, BPIC 710; M, molecular weight marker.

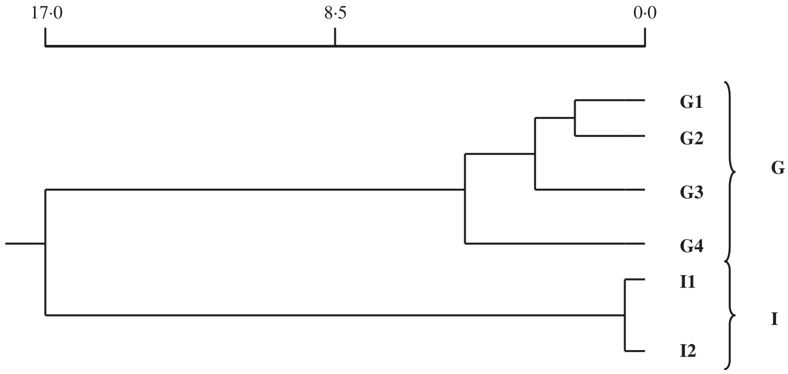

Figure 2 UPGMA dendrogram derived using the neighbour-joining algorithm based on Nei's genetic distance (Nei, 1972) between *Pseudomonas avellanae* populations of northern Greece (G) and central Italy (I) based on ERIC-PCR data. Provinces: G1, Drama; G2, Kilkis; G3, Katerini; G4, Kavala; I1, Viterbo; I2, Rome.

Table 2 Mean values of Nei's genetic diversity, *h*, for ERIC-PCR, BOX-PCR and IS50-PCR data in *Pseudomonas avellanae* populations from Greece and Italy

	Drama	Kilkis	Katerini	Kavala	Viterbo	Rome
ERIC-PCR	0·000	0·000	0·045	0·045	0·000	0·042
BOX-PCR	0·035	0·071	0·035	0·035	0·107	0·160
IS50-PCR	0·035	0·035	0·035	0·017	0·142	0·084

Table 3 Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) among *Pseudomonas avellanae* strains from Greece and Italy based on ERIC-PCR, BOX-PCR and IS50-PCR data

Drama	Kilkis	Katerini	Kavala	Viterbo	Rome
ERIC					
****	0·909	0·984	0·984	0·727	0·709
0·095	****	0·984	0·891	0·636	0·616
0·015	0·015	****	0·967	0·703	0·684
0·015	0·115	0·032	****	0·797	0·780
0·318	0·452	0·351	0·226	****	0·988
0·343	0·483	0·379	0·248	0·011	****
BOX					
****	0·884	0·900	0·975	0·781	0·718
0·122	****	0·884	0·808	0·721	0·679
0·105	0·122	****	0·900	0·879	0·831
0·025	0·213	0·105	****	0·781	0·718
0·246	0·326	0·129	0·246	****	0·992
0·330	0·387	0·184	0·330	0·007	****
IS50					
****	0·825	0·900	0·611	0·502	0·519
0·192	****	0·750	0·740	0·606	0·568
0·105	0·287	****	0·685	0·478	0·491
0·492	0·300	0·378	****	0·658	0·694
0·687	0·500	0·736	0·418	****	0·915
0·654	0·565	0·711	0·365	0·088	****

Pairwise comparison of the six populations according to Nei's coefficient of gene differentiation (G_{ST}) is shown in Table 3. Data obtained from ERIC and BOX repetitive PCR were similar, and indicated a strong similarity between the populations from Rome and Viterbo in central Italy (0·988 and 0·992, respectively). The measure of genetic identity among the four populations from northern Greece was also high, varying from 0·891 to 0·984 with ERIC and from 0·808 to 0·975 with BOX. The genetic distances between the populations from northern Greece and central Italy ranged from 0·226 to 0·452 for ERIC-PCR data and from 0·129 to 0·387 for BOX-PCR data. IS50-PCR data revealed a similar, but lower, genetic identity among the different populations (Table 3). The NJ dendrogram derived from the analysis of Nei's genetic identity and genetic distance based on ERIC-PCR data is shown in Fig. 2. The NJ dendrogram from BOX-PCR and IS50-PCR provided the same distribution of populations (data not shown). The dendrograms clearly revealed that the *P. avellanae* strains from Greece and Italy clustered separately. The four subpopulations from northern Greece are collectively indicated by G, and the two subpopulations from central Italy by I (Fig. 2). The comparison of Nei's gene differentiation among population matrices and the geographical distance between the sites in northern Greece and central Italy from where the *P. avellanae* strains were originally isolated revealed a positive and significant correlation between the two data sets after 10 000 random permutations using either ERIC and BOX-PCR or IS50-PCR data and the Mantel test (Fig. 3). The value of the Mantel product-moment correlation coefficient was always positive ($r = 0{\cdot}89$ for ERIC; $r = 0{\cdot}73$ for BOX; $r = 0{\cdot}74$ for IS50) and significantly different from 0 at $P = 0{\cdot}05$. Such data indicate that *P. avellanae* populations that are spatially distant from each other are also genetically dissimilar in terms of genetic differentiation. The gene-flow estimate, N_m, was always <1 (0·07 for ERIC-PCR; 0·26 for BOX-PCR; 0·10 for IS50-PCR), indicating separate differentiation of the two *P. avellanae* lineages G and I.

Discussion

The highly aggressive pseudomonad strains associated with hazelnut decline in northern Greece and central Italy belong to the same species, *P. avellanae* (Scortichini *et al*., 2002ab). However, when the strains from both countries were analysed using techniques that differentiate bacteria at strain level (Louws *et al*., 1994; Rademaker *et al*., 2000),

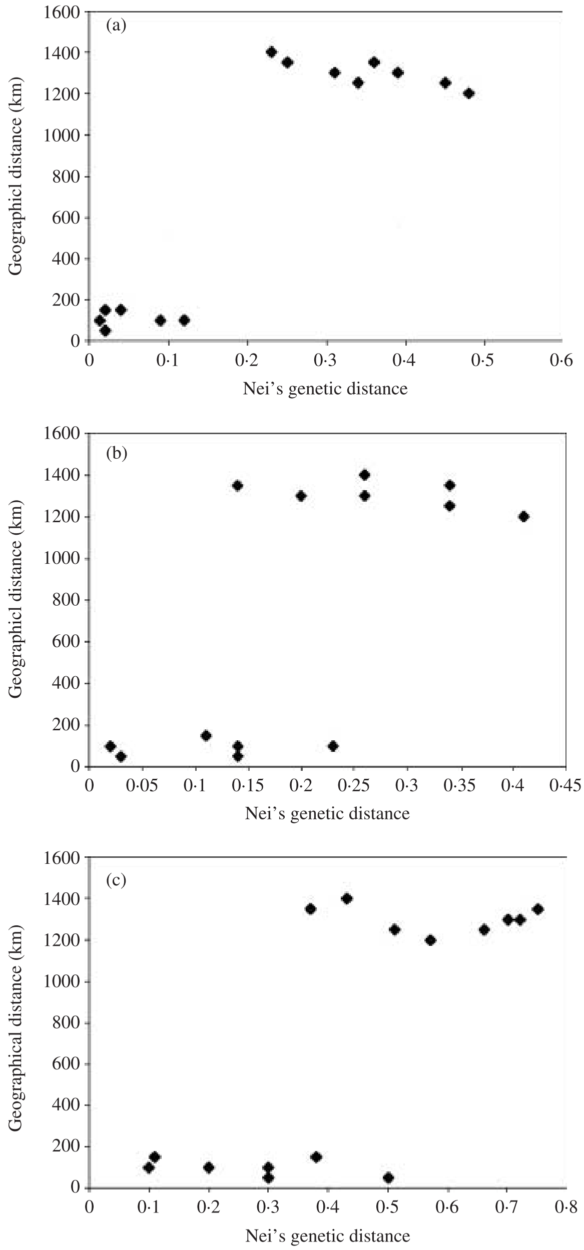

Figure 3 Plots of geographical distances and Nei's genetic distance derived using the Mantel test from ERIC-PCR (a); BOX-PCR (b); and IS50-PCR (c) data (Table 3) for four *Pseudomonas avellanae* populations obtained from Greece and two isolated in Italy. The distribution of correlations from 10 000 permuted samples indicates a significant isolation by distance between the two *P. avellanae* lineages ($P = 0{\cdot}05$). The strains from Italy appear in the upper part of the plots. The points represent the linear correlation observed within the two *P. avellanae* lineages.

in association with statistical procedures enabling genetic diversity to be correlated with geographical origin, two different *P. avellanae* lineages were found, strongly related to the areas where they were originally isolated. They are designated here as G from Greece and I from Italy. The NJ dendrograms derived from ERIC and BOX repetitive PCR and IS50-PCR data, and the corresponding matrices computed using Nei's coefficient of gene differentiation among the six populations of *P. avellanae* that were determined on the basis of geographical origin, G_{ST}, clearly support the existence of two lineages.

In addition, the Mantel test, performed to assess whether the geographical distances provided in one matrix were correlated with the genetic distances of a second matrix derived from ERIC, BOX and IS50-PCR data, clearly showed that the geographically distant populations were also dissimilar in terms of genetic diversity. Thus, although all pseudomonads inciting hazelnut decline belong to *P. avellanae*, the genetic differences found in the two lineages using neutral markers are probably strongly related to the geographical origins of the strains. The data obtained from the estimates of gene flow, N_m, confirm this hypothesis. For all three markers the values obtained were always <1, indicating differentiation of the strains locally, not influenced by the possible gene flow arriving from other populations of the pathogen (McDermott & McDonald, 1993). The data corroborate data previously obtained from both phenotypic and genotypic assessments of the same *P. avellanae* populations, which established the existence of the two lineages (Janse *et al.*, 1996; Scortichini *et al.*, 1998, 2002b; Ovod *et al.*, 1999; Scortichini & Angelucci, 1999). Different lineages within pathovars of other phytopathogenic *Pseudomonas* species have also been found recently (Oguiza *et al.*, 2004).

The different histories of hazelnut cultivation in Greece and Italy support the hypothesis of separate origins for the two *P. avellanae* lineages. The cultivars used are different, and there has been no exchange of propagative material between the two countries. In Greece, cv. Palaz was obtained from Turkey, where the disease still appears to be absent. The sole common link is the sudden appearance of the disease in areas characterized by the presence of subacidic soils (pH < 5·0). In Viterbo Province (central Italy), where hazelnut cultivation spans over 20 000 ha almost continuously, the first foci of hazelnut decline were observed in orchards growing on volcanic soils with subacidic pH. Even now, after more than 30 years of epidemics and using the same locally propagated cultivars, there are areas where the pH is higher, with no apparent presence of the pathogen (Scortichini, 2002). Similarly, in Greece the disease is present only in northern districts, despite the cultivation of the same cultivar in the south. Subacidic soils are also present in northern Greece where the bacterium has been isolated (Psallidas, 1987).

The almost contemporary description of the disease and isolation of the pathogen in two distant areas, and the similarity of the two bacterial lineages adapted to the same host plant, might be explained in terms of evolution through mutation events occurring in a pre-existing, endophytic strain(s). The modification(s) could have been triggered by the occurrence of stress such as very acidic soils. The new environment inside the tree might have represented a new 'adaptive landscape' for the bacteria (Elena & Lenski, 2003).

A moderately acidic environment can normally be tolerated by the bacterial cell, but can be lethal if combined with the presence of weak organic acids (Foster, 1999, 2000). In such a selective environment, a mutation conferring even a small advantage to the cell might have increased in frequency quite rapidly (Orr, 1998), and the spread of a new pathogenic clone(s) in the hazelnut orchards of northern Greece and central Italy could have subsequently led to local differentiation of the pathogen (Korona *et al*., 1994). The recent outbreaks of the disease and its ecological fitness (repetitive epidemics and spread) might also explain the clonal structure of *P. avellanae* as a whole (Scortichini *et al*., 2003). This hypothesis deserves more in-depth study.

Finally, the 'lateral gene transfer' of advantageous genomic traits deriving from other taxa (Ochman *et al*., 2000); the occurrence of 'periodic selection' (Levin, 1981) within the two lineages and the effects of possible 'selective sweeps' on the composition of the lineages (Majewski & Cohan, 1999); as well as the presence of 'mutator genotype(s)' (Moxon *et al*., 1994; Taddei *et al*., 1997; Tanaka *et al*., 2003) deserve further investigation to clarify the local adaptation of the pathogen.

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