Microbiology (2003), **149**, 000–000 DOI 10.1099/mic.0.26380-0

Clonal population structure of *Pseudomonas avellanae* strains of different origin based on multilocus enzyme electrophoresis

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Received 31 March 2003
Revised 26 June 2003
Accepted 30 June 2003

To assess the genetic diversity and genetic relationships of *Pseudomonas avellanae*, the causative agent of hazelnut decline, a total of 102 strains, obtained from central Italy (provinces of Viterbo and Rome) and northern Greece, were studied using multilocus enzyme electrophoresis (MLEE). Their allelic variation in 10 loci was determined. All loci were polymorphic and 53 electrophoretic types (ETs) were identified from the total sample. The mean genetic diversity (*H*) was 0·65 and this value ranged from 0·37 for the least polymorphic to 0·82 for the most polymorphic locus. The dendrogram originated from MLEE data indicated two main groups of ETs, A and B. The groups do not appear to be correlated to the geographic origin of the strains, although all the ETs from northern Greece clustered into subgroup B1. *Pseudomonas syringae* pv. *actinidiae* and *P. syringae* pv. *theae*, included in the analysis as outgroups, clustered apart. The index of association (I_A) for *P. avellanae* was 0·90. The I_A values were always significantly different from zero for the population subsets studied and no epidemic structure was found. These results would indicate that the population structure of *P. avellanae* is clonal either in northern Greece or in central Italy. The recent outbreaks of the bacterium in new areas of hazelnut cultivation would explain the current clonal structure that is persisting over decades.

INTRODUCTION

Pseudomonas avellanae was first isolated in 1976 in northern Greece from hazelnut (*Corylus avellana* L.) (Psallidas & Panagopoulos, 1979) and named as *Pseudomonas syringae* pv. *avellanae*. Further evidence revealed by fatty acid methyl ester analysis, whole-cell protein analysis and from sequence comparison of the 16S rRNA gene changed the designation to *P. avellanae* (Janse *et al.*, 1996). In 1999, based on DNA-relatedness studies of *P. syringae* (hereafter abbreviated to *P. s.*) pathovars and related phytopathogenic pseudomonads, *P. avellanae* was placed in genomospecies 8, together with *P. s.* pv. *theae* (Gardan *et al.*, 1999). Subsequently, it has been shown that this genomospecies also includes *P. s.* pv. *actinidiae* (Scortichini *et al.*, 2002a). *P. avellanae* is a Gram-negative, non spore-forming rod, motile by means of 1–4 polar flagella, producing fluorescent pigments on King's medium B (King *et al.*, 1954), and circular, dome-shaped, glistening semi-translucent, butyrous, radially striated colonies on nutrient agar with 5 % sucrose (Janse *et al.*, 1996). This phytopathogen is the causative agent of bacterial canker and the decline of the hazelnut and it has severely damaged cultivation of the hazelnut in northern Greece (Psallidas, 1987) and central Italy (Scortichini, 2002). *P. avellanae* strains have *hrpW* and *hrpL* genes encoding the hairpin proteins involved in eliciting the hypersensitivity reaction in leaf tissues (Loreti *et al.*, 2001).

Genetic variability among this bacterium was ascertained by plasmid analysis (Janse *et al.*, 1996) and repetitive PCR, using enterobacterial repetitive intergenic consensus (ERIC), BOX A subunit of the BOX element of *Streptococcus pneumoniae* (BOX) and repetitive extragenic palindromic (REP) primer sets, and *P. avellanae* strains from northern Greece are clearly differentiated from those isolated in central Italy by two PCR products of approximately 300 and 800 bp using ERIC primers (Scortichini *et al.*, 1998, 2002b). Primers based on the 16S rRNA gene sequence (Scortichini & Marchesi, 2001) or on the *hrpW* gene sequence (Loreti & Gallelli, 2002) can be used for the rapid detection of this pathogen.

Where genetic variability within *P. avellanae* has been studied, nothing is known about the population structure in terms of genetic diversity and linkage disequilibrium. To study these fundamental aspects, we have used the

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Abbreviations: MLEE, multilocus enzyme electrophoresis; ERIC, enterobacterial repetitive intergenic consensus; ET, electrophoretic type; BOX, BOX A subunit of the BOX element of *Streptococcus pneumoniae*; REP, repetitive extragenic palindromic.

multilocus enzyme electrophoresis (MLEE) method combined with statistical treatment of the data. MLEE analysis enables investigation of whether the alleles at different loci within a population of bacteria are randomly associated, as in the case of linkage equilibrium, or whether a significant association exists between the alleles, as for linkage disequilibrium (Maynard Smith *et al.*, 1993). Several bacterial species, mainly of medical (Whittam *et al.*, 1983; Caugant *et al.*, 1987; Johnson *et al.*, 1994; Farfan *et al.*, 2000) or environmental (Wise *et al.*, 1995; Rius *et al.*, 2001) interest, have been investigated by means of MLEE. Such studies have shown that the population structure of the species studied can vary from clonal to panmictic, with cases of epidemic structure (Maynard Smith *et al.*, 2000). Concerning bacterial plant pathogens, there has only been one study performed with MLEE (Denny *et al.*, 1988) and information about these bacteria is lacking.

The objective of this study was to extend our knowledge on the genetic structure of *P. avellanae* strains isolated from hazelnut showing symptoms of decline in Greece and Italy by using MLEE. Similar to medical or environmental bacteria (Maynard-Smith *et al.*, 2000), the assessment of population structure of phytopathogenic bacteria is important for understanding their epidemiology as well as for checking the persistence of particular clones in time and space. Such studies, by revealing the genetic composition of the pathogen, can also contribute to more appropriate detection and control of the disease. The results of our analysis would indicate a clonal structure for the entire population of *P. avellanae* as well as for the population subsets studied. Some clones have persisted over decades both in northern Greece and in central Italy.

METHODS

Bacterial strains. A total of 102 *P. avellanae* strains associated with hazelnut decline were analysed by MLEE (Table 1). Isolates were obtained from hazelnut specimens showing symptoms of bacterial decline by using nutrient agar (Oxoid) with 5 % sucrose (NSA) as bacterial culture medium. Their identification was achieved by using well established techniques in a polyphasic identification procedure (Vandamme *et al.*, 1996). In particular, all isolates, after the preliminary screening based on biochemical, physiological and nutritional tests (Psallidas & Panagopoulos, 1979; Janse *et al.*, 1996; Scortichini *et al.*, 2002a), were compared with the type strain and representative strains of the species obtained from Greece and Italy by using SDS-PAGE of whole-cell protein extracts (Janse *et al.*, 1996) as well as repetitive PCR with ERIC, BOX and REP primer sets (Scortichini *et al.*, 1998, 2000, 2002a). *P. s.* pv. *syringae*, and *P. s.* pv. *theae* and *P. s.* pv. *actinidiae* strains, were also assessed for comparison purposes. *P. avellanae* field isolates showed protein patterns and repetitive PCR genomic fingerprinting clustering apart from the *P. s.* pathovars (Scortichini *et al.*, 2002a). In addition, all strains were assessed in terms of phenotypic and pathogenic diversity (Scortichini *et al.*, 2002b). The strains were routinely cultured on NSA. Strains from Greece were kindly provided by Dr P. G. Psallidas (Benaki Phytopathological Institute, Kiphissia–Athens, Greece). As outgroups of *P. avellanae*, strains of *P. s.* pv. *theae* and *P. s.* pv. *actinidiae*, belonging to genomospecies 8 *sensu* Gardan *et al.* (1999), were also assessed by using MLEE.

Preparation of lysates for electrophoresis. Loopfuls of pure culture, grown on NSA for 48 h at 25–27 °C, were suspended in 1·5 ml sterile buffer solution (10 mM Tris, 1 mM EDTA, 0·5 mM NADP, pH 6·8; SBS). After centrifugation (7000 ***g*** for 15 min at 5 °C), the pellet was suspended in 300 µl of a solution containing 70 % SBS, 30 % sterile double-distilled water and 0·4 % lysozyme (Sigma). The tubes were incubated at 37 °C for 1 h. Then they were centrifuged (7000 ***g*** for 15 min at 6 °C) and the supernatant fluid was dispensed in sterile Eppendorf tubes and stored immediately at −70 °C until used. Protein concentration was measured by using the method of Lowry with bovine serum albumin, using DC Protein Assay (Bio-Rad) as standard. To verify that the lack of enzymic activity was due to the true absence of the cell extract, other sets of lysates were obtained by using sonication. In this case, cell suspensions in SBS were disrupted by sonication in an ice-water bath for four 30 s cycles with a Bronson Ultrasonic Sonifier model 250, kindly provided by the Department of Biochemistry, Faculty of Biological Sciences, University of Rome 'La Sapienza', using setting 3 and duty cycle 50 % as described by Rius *et al.* (2001). Afterwards, cell debris was removed by centrifugation (8000 ***g*** for 10 min at 5 °C). The supernatant was dispensed in sterile Eppendorf tubes and stored at −70 °C until used.

Electrophoresis and specific enzyme staining. Non-denaturing vertical PAGE (Mini Protean; Bio-Rad) was used for all enzymes. The acrylamide concentration in the gels depended on the enzyme studied (6 % for continuous polyacrylamide gels and 10 %/8 % or 8 %/5 % for discontinuous polyacrylamide gels). Tris/HCl (0·8 M, pH 8·8) buffer was used in continuous gels and 0·125 M Tris/HCl (pH 6·8) stacking buffer and 0·4 M Tris/HCl (pH 8·8) resolving buffer were used in discontinuous gels. Tris/glycine (0·19 M, pH 8·3) buffer was used for the electrode compartment. A constant voltage, depending on the acrylamide concentration in the gel, was applied until the bromophenol blue band reached the bottom of the gel. All strains were run twice. The following 10 enzymes, representing a sample of structural genes of the bacterial genome and showing polymorphism (Selander *et al.*, 1986; Denny *et al.*, 1988; Rius *et al.*, 2001), were assayed: isocitrate dehydrogenase, shikimic dehydrogenase, superoxide dismutase, 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, esterase, leucine aminopeptidase, glutamate dehydrogenase-NADP, phosphoglucose isomerase and phosphoglucomutase. The staining of the gel to reveal specific enzyme activity was carried out following the method described by Selander *et al.* (1986). For each enzyme, distinct mobility variants were designated as electromorphs and numbered in order of decreasing anodal migration. Displacement of the electromorphs was expressed in terms of relative electrophoretic mobility with respect to the bromophenol blue band. Absence of enzyme activity was attributed to a null allele and designated as 0. Distinct combinations of alleles over the 10 loci assayed were named as electrophoretic types (ETs).

Data treatment. Electromorphs and ETs were equated with alleles and allelic combinations, respectively, for statistical analysis. Computer programs written and kindly provided by Professor T. S. Whittam (Department of Microbiology and Molecular Genetics, Michigan State University, USA) were used to analyse the data for ET designation, genetic diversity calculations and ET clustering. The genetic diversity (h) for a locus was calculated according to Nei (1978). The probability that two isolates differ at the j locus is $h_j = n(1-\sigma X_i^2)/(n-1)$, where X_i is the frequency of the i allele at the j locus and n is the number of individuals in the sample (Maynard-Smith *et al.*, 1993). Mean genetic diversity (H) is the arithmetic mean of h over all the loci examined. Clustering of data obtained by MLEE was performed by using the unweighted pair-group method with arithmetic averages (UPGMA). Distance is measured as the proportion of mismatched loci between pairs of ETs. The cophenetic correlation was

Table 1. Characteristics of the bacterial strains used in this study and their allele profiles at each locus

IDH, Isocitrate dehydrogenase; SKH, shikimic dehydrogenase; GD2, glutamate dehydrogenase-NADP; 6PG, 6-phosphogluconate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; EST, esterase; LAP, leucine aminopeptidase; SOD, superoxide dismutase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase.

ET	No. of strains	Reference strain*	IDH	SKH	GD2	6PG	G6P	EST	LAP	SOD	PGI	PGM	Country/ Province	Year of isolation
1	1	ISPaVe 42	0	0	5	4	1	6	4	3	6	2	Italy/VT	1992
2	6	ISPaVe 39	2	1	5	1	2	6	6	3	3	2	Italy/VT	1993
		ISPaVe 689	2	1	5	1	2	6	6	3	3	2	Italy/VT	1996
		ISF SVT2	2	1	5	1	2	6	6	3	3	2	Italy/VT	1998
		ISF SVT3	2	1	5	1	2	6	6	3	3	2	Italy/VT	1998
		ISF SVT4	2	1	5	1	2	6	6	3	3	2	Italy/VT	1998
		ISPaVe 369	2	1	5	1	2	6	6	3	3	2	Italy/RM	1995
3	1	ISPaVe 40	1	0	6	0	2	3	0	3	3	3	Italy/VT	1993
4	1	ISPaVe 2056	2	0	5	4	2	3	5	3	1	3	Italy/VT	1994
5	1	ISPaVe 2057	2	1	4	2	1	3	5	3	5	3	Italy/VT	1994
6	1	ISPaVe 2058	2	1	5	1	3	3	3	3	6	3	Italy/VT	1994
7	1	ISPaVe 2059	0	1	3	1	1	6	0	3	1	2	Italy/VT	1994
8	2	ISPaVe 683	1	1	2	1	2	3	0	3	0	2	Italy/VT	1996
		ISF Rad 4	1	1	2	1	2	3	0	3	0	2	Italy/VT	1998
9	2	ISPaVe 690	0	1	2	1	1	6	4	3	3	2	Italy/VT	1996
		ISF Rm1	0	1	2	1	1	6	4	3	3	2	Italy/RM	2000
10	2	ISPaVe 691	2	0	0	2	1	3	0	3	3	1	Italy/VT	1996
		ISF Rm2	2	0	0	2	1	3	0	3	3	1	Italy/RM	2000
11	1	ISF Bar 1	1	2	1	2	2	3	5	3	3	3	Italy/VT	1998
12	5	ISF Bar 6	1	2	0	2	1	3	5	3	3	3	Italy/VT	1998
		ISF Bar 2	1	2	0	2	1	3	5	3	3	3	Italy/VT	1998
		ISF Bar 3	1	2	0	2	1	3	5	3	3	3	Italy/VT	1998
		ISF Bar 4	1	2	0	2	1	3	5	3	3	3	Italy/VT	1998
		ISF Bar 5	1	2	0	2	1	3	5	3	3	3	Italy/VT	1998
13	1	ISF Bar 7	2	1	4	2	1	3	5	5	3	3	Italy/VT	1998
14	1	ISF Bar 9	1	1	4	2	0	3	5	5	3	3	Italy/VT	1998
15	5	ISF Bar 11	1	1	4	2	0	3	5	3	2	3	Italy/VT	1998
		ISF Bar 10	1	1	4	2	0	3	5	3	2	3	Italy/VT	1998
		ISF Bar 12	1	1	4	2	0	3	5	3	2	3	Italy/VT	1998
		ISF Bar 15	1	1	4	2	0	3	5	3	2	3	Italy/VT	2000
		ISF Bar 18	1	1	4	2	0	3	5	3	2	3	Italy/VT	2000
16	1	ISF SVT 5	1	1	4	1	3	6	0	3	3	2	Italy/VT	1998
17	1	ISF SVT 7	2	1	4	1	2	6	0	3	3	2	Italy/VT	1998
18	1	ISF SVT 8	0	1	4	1	3	6	0	3	6	2	Italy/VT	1998
19	1	ISF SVT 9	2	1	6	4	2	6	0	3	6	2	Italy/VT	1998
20	1	ISF SVT 10	0	1	4	4	2	6	0	3	6	2	Italy/VT	1998
21	1	ISF SVT 12	2	1	4	1	2	6	0	3	1	2	Italy/VT	1998
22	2	ISF Noc 1	1	2	2	2	2	3	5	5	3	3	Italy/VT	1998
		ISF Noc 3	1	2	2	2	2	3	5	5	3	3	Italy/VT	1998
23	2	ISF Noc 2	1	2	2	2	2	3	5	5	5	3	Italy/VT	1998
		ISF Rm 3	1	2	2	2	2	3	5	5	5	3	Italy/RM	2000
24	2	ISF Noc 4	1	2	2	2	2	3	5	3	5	0	Italy/VT	1998
		ISF Rm 4	1	2	2	2	2	3	5	3	5	0	Italy/RM	2000
25	2	ISF SCR 1	0	0	6	2	0	3	5	2	3	2	Italy/VT	1998
		ISF SCR 5	0	0	6	2	0	3	5	2	3	2	Italy/VT	1998
26	1	ISF SCR 3	0	2	3	1	2	3	0	3	1	2	Italy/VT	1998
27	1	ISF Rad 5	1	2	4	2	3	3	6	4	0	3	Italy/VT	1998
28	2	ISF Rad 6	2	1	2	1	2	3	0	3	0	2	Italy/VT	1998
		ISF Rad 13	2	1	2	1	2	3	0	3	0	2	Italy/VT	1999

Table 1. cont.

ET	No. of strains	Reference strain*	IDH	SKH	GD2	6PG	G6P	EST	LAP	SOD	PGI	PGM	Country/ Province	Year of isolation
29	2	ISF Rad 7	0	2	0	2	0	3	4	4	2	3	Italy/VT	1998
		ISF Rad 14	0	2	0	2	0	3	4	4	2	3	Italy/VT	1999
30	2	ISF Rad 8	1	2	4	2	3	3	5	4	2	3	Italy/VT	1998
		ISF Rad 15	1	2	4	2	3	3	5	4	2	3	Italy/VT	1999
31	2	ISF Rad 10	2	1	2	1	0	3	0	3	3	3	Italy/VT	1998
		ISF Rad 16	2	1	2	1	0	3	0	3	3	3	Italy/VT	1999
32	1	ISF T4	1	2	6	2	2	3	2	5	5	3	Italy/VT	1998
33	1	ISF T7	1	2	0	2	1	3	5	5	3	3	Italy/VT	1998
34	2	ISF Vet 2	1	0	0	2	2	3	4	2	2	2	Italy/VT	1999
		ISF Rm 5	1	0	0	2	2	3	4	2	2	2	Italy/RM	2000
35	4	ISF Vet 5	3	0	0	2	2	3	0	3	1	0	Italy/VT	1999
		ISF Vet 4	3	0	0	2	2	3	0	3	1	0	Italy/VT	1999
		ISF Vet 6	3	0	0	2	2	3	0	3	1	0	Italy/VT	1999
		ISF Vet 7	3	0	0	2	2	3	0	3	1	0	Italy/VT	1999
36	5	BPIC 631^T	3	5	3	1	1	3	5	2	3	2	Greece	1976
		BPIC 708	3	5	3	1	1	3	5	2	3	2	Greece	1987
		BPIC 710	3	5	3	1	1	3	5	2	3	2	Greece	1987
		BPIC 707	3	5	3	1	1	3	5	2	3	2	Greece	1977
		BPIC 1436	3	5	3	1	1	3	5	2	3	2	Greece	1990
37	5	BPIC 647	2	3	3	1	1	3	5	2	3	2	Greece	1976
		BPIC 640	2	3	3	1	1	3	5	2	3	2	Greece	1976
		BPIC 649	2	3	3	1	1	3	5	2	3	2	Greece	1976
		BPIC 659	2	3	3	1	1	3	5	2	3	2	Greece	1976
		BPIC 634	2	3	3	1	1	3	5	2	3	2	Greece	1976
38	1	BPIC 665	2	3	3	1	1	3	5	2	3	3	Greece	1976
39	1	BPIC Fl 3	2	1	3	2	0	3	5	1	3	2	Greece	1976
40	1	BPIC 1078	2	2	3	1	0	3	0	0	3	2	Greece	1986
41	1	BPIC 1077	2	3	3	1	2	3	0	2	3	2	Greece	1987
42	5	BPIC 703	3	3	3	1	1	3	0	2	3	2	Greece	1977
		BPIC 1079	3	3	3	1	1	3	0	2	3	2	Greece	1986
		BPIC 1435	3	3	3	1	1	3	0	2	3	2	Greece	1990
		BPIC 1423	3	3	3	1	1	3	0	2	3	2	Greece	1987
		BPIC 716	3	3	3	1	1	3	0	2	3	2	Greece	1987
43	1	BPIC 714	2	0	3	2	0	3	0	2	3	2	Greece	1987
44	3	ISPaVe 11	2	6	6	2	2	3	6	3	0	1	Italy/RM	1991
		ISF Rm 6	2	6	6	2	2	3	6	3	0	1	Italy/RM	2000
		ISF Rm 7	2	6	6	2	2	3	6	3	0	1	Italy/RM	2000
45	3	ISPaVe 12	3	5	5	4	2	6	6	3	1	2	Italy/RM	1991
		ISF Rm 8	3	5	5	4	2	6	6	3	1	2	Italy/RM	2000
		ISF Rm 9	3	5	5	4	2	6	6	3	1	2	Italy/RM	2000
46	3	ISPaVe 13	2	6	6	2	2	3	6	3	0	1	Italy/RM	1992
		ISF Rm 10	2	6	6	2	2	3	6	3	0	1	Italy/RM	2000
		ISF Rm 11	2	6	6	2	2	3	6	3	0	1	Italy/RM	2000
47	1	ISPaVe 36	3	1	3	1	2	6	4	3	0	2	Italy/RM	1992
48	1	ISPaVe 37	2	6	6	1	2	3	4	2	1	1	Italy/RM	1992
49	1	ISPaVe 436	0	0	3	4	2	6	0	3	0	2	Italy/RM	1995
50	1	ISPaVe 439	2	6	6	1	2	3	4	3	0	3	Italy/RM	1995
51	1	ISF Rm SO1	0	0	0	0	1	3	0	3	3	4	Italy/RM	1997
52	3	ISF Vel 2	1	2	0	2	3	3	6	3	3	3	Italy/RM	1998
		ISF Vel 3	1	2	0	2	3	3	6	3	3	3	Italy/RM	1998
		ISF Vel 5	1	2	0	2	3	3	6	3	3	3	Italy/RM	1998
53	2	ISF Vel 6	0	0	5	1	0	7	0	3	6	2	Italy/RM	1998
		ISF Vel 4	0	0	5	1	0	7	0	3	6	2	Italy/RM	1998

Table 1. cont.

ET	No. of strains	Reference strain*	IDH	SKH	GD2	6PG	G6P	EST	LAP	SOD	PGI	PGM	Country/ Province	Year of isolation
54	2	*P. s. theae* 2598^T	2	7	8	4	3	3	0	0	5	5	Japan	1970
		P. s. theae 4096	2	7	8	4	3	3	0	0	5	5	Japan	1970
55	3	*P. s. actinidiae* 3729^T	1	6	5	1	4	3	0	0	5	4	Japan	1984
		P. s. actinidiae 3740	1	6	5	1	4	3	0	0	5	4	Japan	1984
		P. s. actinidiae 3871	1	6	5	1	4	3	0	0	5	4	Italy	1992

*ISPaVe, Culture Collection of the Istituto Sperimentale per la Patologia Vegetale, Roma, Italy; ISF, Culture Collection of the Istituto Sperimentale per la Frutticoltura, Roma, Italy; BPIC, Culture Collection of the Benaki Phytopathological Institute, Kiphissia–Athens, Greece; VT, Viterbo province; RM, Rome province. The strains *P. s.* pv. *theae* 2598 and *P. s.* pv. *actinidiae* 3729, 3740 and 3871 were obtained from the National Collection of Plant-pathogenic bacteria, York, UK; *P. s.* pv. *theae* 4096 was obtained from the Collection Française de Bactéries Phytopathogènes, Angers, France.

calculated using NTSYS-PC version 1.80 (Rohlf, 1993). Multilocus linkage disequilibrium was estimated on the basis of distribution of allelic mismatches between pairs of bacterial strains among all the loci examined. The ratio of the observed variance in mismatches (V_O) to the expected variance at linkage equilibrium (V_E) provides a measure of multilocus linkage disequilibrium that can be expressed as the index of association $(I_A)=(V_O/V_E)-1$ (Brown *et al.*, 1980; Maynard Smith *et al.*, 1993). For populations in linkage equilibrium, $V_O=V_E$, and I_A is not significantly different from zero, whereas values of I_A greater than zero indicate that recombination has been rare or absent. To test if I_A differed significantly from its expected value of zero, the ETLINK program version 3.0, kindly provided by Professor T. S. Whittam, was used. The maximum variance obtained was compared to that expected in a freely recombining population (linkage equilibrium) by using 1·000 randomization of the dataset ($P=0·001$). The null hypothesis of a random association of alleles (i.e. the population is at linkage equilibrium) was rejected if this probability was smaller than the selected significance level. In addition, to test whether the population structure of *P. avellanae* and subsets was epidemic, *sensu* Maynard Smith *et al.* (1993), the I_A value was also calculated by taking into consideration only the ETs. For multilocus linkage disequilibrium analysis, the 102 *P. avellanae* strains were also studied as three population subsets according to their geographic origin. The three population subsets were composed as follows: 20 strains from northern Greece, 57 strains from central Italy (province of Viterbo) and 25 strains from central Italy (province of Rome). This last group of strains was isolated from one single hazelnut orchard established using propagative plant material obtained from the province of Viterbo. A G_T statistic was used to compare the mean genetic diversity of the three *P. avellanae* population subsets (Sokal & Rohlf, 1981).

RESULTS

ETs and genetic diversity

From a collection of 102 *P. avellanae* strains associated with hazelnut decline in central Italy and northern Greece, 53 ETs were identified. Table 1 summarizes their allele profiles. The most common ET was ET_{12} which includes six strains. All the enzyme loci were polymorphic and the number of alleles ranged from three (esterase) to seven (glutamate dehydrogenase-NADP). The mean number of alleles was 5·1. The mean genetic diversity (H) in the sample was 0·65. The genetic diversity ranged from 0·37 for the least polymorphic locus to 0·82 for the most polymorphic (Table 2). The highest genetic diversity was found in the strains obtained

Table 2. Allele frequencies and genetic diversities at 10 enzyme loci in 53 ETs of *P. avellanae*

For definition of abbreviations, see Table 1. Mean genetic diversity (H) = 0·5.

Enzyme locus	Maximum no. of alleles	Frequency of each allele								*h*
		1	2	3	4	5	6	7	0	
IDH	4	0·171	0·494	0·148					0·187	0·68
SKH	6	0·259	0·174	0·168		0·069	0·111		0·219	0·82
GD2	7	0·010	0·067	0·408	0·105	0·121	0·149		0·140	0·77
6PG	4	0·503	0·366		0·094				0·037	0·61
G6P	4	0·271	0·454	0·075					0·200	0·68
EST	3			0·766			0·206	0·028	0·37	
LAP	6		0·010	0·010	0·121	0·290	0·158		0·411	0·72
SOD	6	0·042	0·297	0·534	0·029	0·056			0·042	0·63
PGI	6	0·131	0·048	0·550		0·038	0·075		0·167	0·65
PGM	5	0·065	0·564	0·296	0·028				0·047	0·59

from the provinces of Rome and Viterbo (0·65 and 0·64, respectively). A comparison of genetic diversity between different sample subsets is shown in Table 3. Significant differences were detected in mean genetic diversity among the three different population subsets. In fact, the population from northern Greece ($H=0·36$) is different ($P<0·05$) from the two populations isolated in central Italy ($H=0·64$ for the population of Viterbo and $H=0·65$ for the population of Rome).

Occurrence of null alleles

Thirty-two of 102 *P. avellanae* strains (31·3 %) studied showed activity for all ten enzymes studied. However, at least one enzyme activity was not detected in the cell lysates prepared from the remaining 70 strains (Table 1). Of these, 40 strains lacked detectable activity for one enzyme, 13 lacked detectable activity for two enzymes, nine for three enzymes, seven for four enzymes and one for five enzymes. Null alleles were verified by measuring the protein content of the cell lysates, ranging from 1·1 to 2·5 mg ml^{-1}. In addition, lysates obtained from high cell-density preparations never yielded detectable activity for the target enzyme. Similarly, when lysates obtained from sonication were run, no activity was scored. When the same strains showing null alleles for some enzyme were stained for other enzymic activities, they revealed activity. There was no relationship between the presence or absence of detectable activity and the origin of the strains. All population subsets studied exhibited detectable activity for all ten enzymes studied.

Genetic relationships among multilocus genotypes

The genetic relationship among the 53 ETs of *P. avellanae* and the ETs of *P. s.* pv. *actinidiae* and *P. s.* pv. *theae* is shown in Fig. 1. The cophenetic correlation coefficient of the total sample was $R=0·79$. The shortest genetic distance observed between ETs (0·10) corresponds to a single locus difference. At a genetic distance of 0·71, two main groups of *P. avellanae* ETs were found, namely A and B. Group A included 18 ETs and contained strains isolated in central Italy, from the provinces of Viterbo or Rome. Group B contained the remaining 35 ETs. This group, in turn, can be divided into two subgroups, B1 and B2 (Fig. 1). Subgroup B1 contained all the strains isolated from northern Greece and some ETs from central Italy; subgroup B2 contained strains isolated from the provinces of Viterbo and Rome. *P. s.* pv. *actinidiae* and *P. s.* pv. *theae* clustered apart from the *P. avellanae* ETs, showing 97 % genetic distance.

Linkage disequilibrium analysis

The complete set of strains and subsets of populations were analysed for multilocus linkage disequilibrium (Table 3). The distribution of allele mismatches among 53 ETs of *P. avellanae* is shown in Fig. 2. The *P. avellanae* population studied here as a whole presents a distribution typical for a clonal species (i.e. bimodal distribution of mismatches)

Table 3. Multilocus linkage disequilibrium analysis of *P. avellanae* and population subsets

Strain group	No. of strains	No. of ETs	Mean no. of alleles per locus	Mean genetic diversity (H)	V_O	V_E	95 % confidence limit of V_E	P	$I_A \pm SD$
P. avellanae	102	53	5·1	0·65	4·08	2·14	1·5–2·8	0·001	0·90±0·15
Italy/Viterbo	57	35	4·4	0·64	4·87	2·24	1·4–3·0	0·001	1·17±0·19
Italy/Rome	25	16	3·8	0·65	6·27	2·00	0·6–3·5	0·001	2·00±0·35
Greece	20	8	2·2	0·36	5·34	1·78	0·5–3·0	0·001	2·00±0·34
All *P. avellanae* except ETs:									
22, 23, 37, 38	92	49	5·1	0·65	3·9	2·1	1·4–2·8	0·001	0·85±0·15
22, 23, 37, 38, 11, 12, 13, 14, 27, 30, 36, 42, 44, 46	64	41	5·0	0·63	3·7	2·1	1·3–2·9	0·001	0·71±0·18
22, 23, 37, 38, 11, 12, 13, 14, 27, 30, 36, 42, 44, 46, 8, 28, 16, 18	58	37	5·0	0·64	3·7	2·1	1·3–3·0	0·001	0·72±0·19

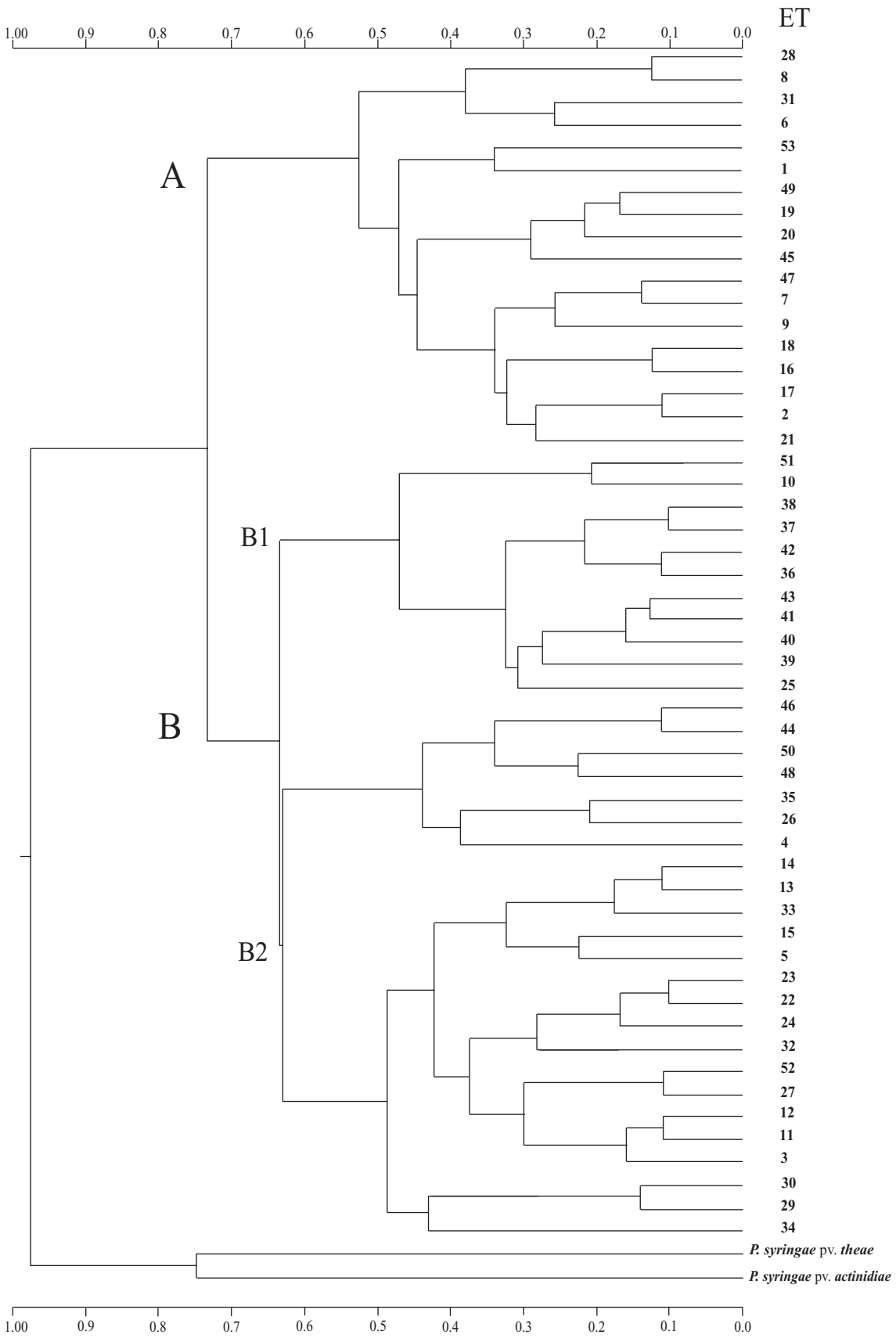

Fig. 1. Dendrogram constructed by the UPGMA method, showing the genetic relationships among 53 ETs of *P. avellanae* strains. The scale indicates the genetic distance.

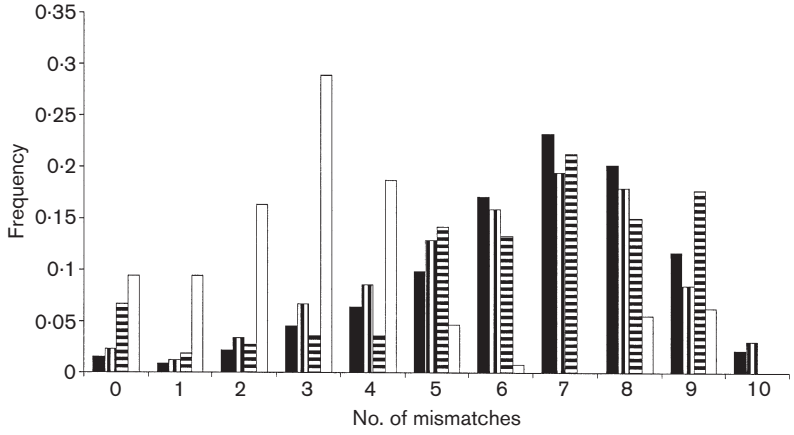

Fig. 2. Allele mismatch distribution among *P. avellanae* (black bars) and the three population subsets studied: vertical shading, Italy, Viterbo; horizontal shading, Italy, Rome; white bars, Greece.

(Whittam, 1992). When we analysed the allele mismatch distribution of the three population subsets, we again found distributions typical of clonal species, although the population from Greece showed a distinct distribution (Fig. 2). The I_A value for the 102 *P. avellanae* strains studied was $0{\cdot}90 \pm 0{\cdot}15$ which differs significantly ($P < 0{\cdot}001$) from zero, thus indicating a significant level of linkage disequilibrium. When we considered the different populations according to the geographic origin of the strains, we found that the strains isolated in Italy from Roma and Viterbo showed I_A values of $2{\cdot}00 \pm 0{\cdot}35$ and $1{\cdot}17 \pm 0{\cdot}19$, respectively, whereas the population from northern Greece showed a value of $2{\cdot}00 \pm 0{\cdot}34$. These values were significantly different from zero and indicate the clonal structure of the populations. To verify the robustness of the linkage disequilibrium analysis, we carried out repetitive calculations of I_A in subsets of the total population analysed in which the most closely related ET pairs up to a genetic distance of 0·15 were eliminated stepwise. Table 3 shows the I_A values for these subsets along with their significance. Also in these cases, all values of I_A exceeded the value expected in a corresponding population at linkage equilibrium (V_E). Finally, the I_A values calculated by using only the 53 ETs again showed a clonal structure of *P. avellanae* and subsets. In fact, the I_A value for *P. avellanae* was $0{\cdot}83 \pm 0{\cdot}18$, whereas I_A values for the three population subsets was $1{\cdot}1 \pm 0{\cdot}22$, $2{\cdot}1 \pm 0{\cdot}39$ and $2{\cdot}6 \pm 0{\cdot}48$ for the ETs of Viterbo, Rome and northern Greece, respectively.

DISCUSSION

In recent years data estimates of the genetic diversity and of the frequency of recombination in bacterial populations have been obtained by using MLEE (Maynard Smith *et al.*, 2000). The study of allelic variation in *P. avellanae* inferred by MLEE has yielded new insights into the genetic diversity and population structure of this phytopathogen. The level of genetic diversity found ($H = 0{\cdot}65$) is rather high. Other studies concerning pseudomonads reported either lower values, such as 0·47 for *P. s.* pv. *syringae* (Denny *et al.*, 1988) or higher values, such as 0·87 for *Pseudomonas stutzeri* (Rius *et al.*, 2001). When we analysed the three population subsets, we observed that the mean genetic diversity of the strains isolated in northern Greece was the lowest ($H = 0{\cdot}36$), whereas this value was higher and very similar (0·64 and 0·65) for the two populations of central Italy.

Previous studies on genetic diversity in *P. avellanae* based on the assessment of short interspersed elements of the bacterial genome were carried out by using repetitive PCR with ERIC, BOX and REP primer sets. These studies also revealed differences between the populations found in Greece and Italy. In fact, ERIC primer sets clearly indicated that all the strains isolated in Greece give two PCR products of 300 and 800 bp that have never been found in the strains isolated in Italy (Scortichini *et al.*, 1998; 2002b). It is interesting to note that the value of *H* found here is rather high for a bacterial species that appears to be strictly associated only to a single host plant, namely *C. avellana*. Until now, *P. avellanae* has never been isolated from environments (i.e. soil, water, alternative host plants) other than its host plant. Studies are under way to verify the possible presence of this phytopathogen in other ecological niches. However, it is worth noting that *P. avellanae*, in either Greece or Italy, is more virulent in hazelnut orchards established on very acidic soils ($pH < 4{\cdot}5$). Stress conditions occurring on plants could select different bacterial genotypes able to subsequently propagate in a clonal manner.

This study demonstrated a high frequency of occurrence of null alleles. A high frequency of occurrence of null alleles has already been reported for *P. stutzeri* (Rius *et al.*, 2001) as well as for *Helicobacter pylori* (Go *et al.*, 1996), other species with a high genetic diversity. Hypotheses to explain this result, such as the occurrence of enzyme-inactivating mutations or the absence of the structural gene, have still to be verified.

The dendrogram obtained in the present study shows no clear relationship between the geographic origin of the strains and their grouping. The value of the cophenetic correlation obtained ($R = 0{\cdot}79$) falls into the range (0·74–0·90) of most frequently occurring cophenetic correlation (Sneath & Sokal, 1973). The strains from central Italy are distributed in both groups A and B. However, all 20 strains from Greece clustered into subgroup B1, thus indicating

again the diversity of this population from the others. It is also interesting to note that no single ET includes strains obtained from both Greece and Italy. The dendrogram based on repetitive PCR and UPGMA analysis indicated two distinct groups of strains, related at 90 % similarity, that could be separated according to their geographic origin, namely central Italy and northern Greece (Scortichini *et al.*, 2002b). *P. s.* pv. *actinidiae* and *P. s.* pv. *theae* clustered apart from *P. avellanae*, thus indicating again that differences exist among these taxa.

The linkage disequilibrium analyses clearly indicated that *P. avellanae* is a clonal species when analysed at population subsets level also. The I_A value for all *P. avellanae* strains was $0{\cdot}90 \pm 0{\cdot}15$ ($P < 0{\cdot}001$). The I_A values were always significantly different from zero for all the population subsets studied. In addition, the stepwise elimination of the most closely related ET pairs revealed that the remaining ETs were also in linkage disequilibrium (Table 3). In addition, when only the ETs were analysed, the corresponding I_A value again indicated a clonal structure, *sensu* Maynard-Smith *et al.* (1993). Similar results were obtained by Denny *et al.* (1988) with other phytopathogens, such as *P. s.* pv. *syringae* and *P. s.* pv. *tomato*. Other examples of clonal species are found in environmental bacteria such as *P. stutzeri* (Rius *et al.*, 2001) as well as in bacteria of medical and veterinary interest such as *Salmonella* sp. (Selander *et al.*, 1990), *Haemophilus influenzae* (Maynard Smith *et al.*, 1993) and *Mycobacterium intracellulare* (Feizabadi *et al.*, 1997).

The clonal structure showed by *P. avellanae* and its genetic diversity found here and in previous studies can be explained by hypothesizing the possible occurrence of adaptive mutations conferring selective advantages to the clones during the colonization and adaptation of ecological niches (Cohan, 1994). Since *P. avellanae* did not show gene recombination, its variability might be explained, alternatively, by means of sequential evolution (Levin & Bergstrom, 2000). The clonal structure also indicates that horizontal gene transfer and recombination processes, possibly occurring also in *P. avellanae*, are not currently sufficient to disrupt the allelic association of the ten loci studied here. Alternatively, selection for an epistatic combination of alleles might maintain linkage disequilibrium in the presence of frequent recombination (Souza *et al.*, 1992). Like in other pseudomonads (i.e. *P. stutzeri*) (Rius *et al.*, 2001), the *P. avellanae* clones can be genetically different, but the phenotypic resemblance is sufficient to identify the species (Scortichini *et al.*, 2002a). However, *P. stutzeri*, a species adapted to soil and water, shows considerable genetic diversity and consists of at least eight genomovars as inferred by DNA–DNA hybridization studies (Rossellò *et al.*, 1991), whereas *P. avellanae*, a species colonizing a single host plant, shows very slight variability when assessed by DNA–DNA hybridization (Gardan *et al.*, 1999). The clonality of *P. avellanae* can also be explained by remembering the recent outbreaks of hazelnut bacterial decline in Greece or in Italy. In fact, the disease was first observed in Greece in 1976 on young hazelnut plantations in sites where this crop had never been cultivated before (Psallidas & Panagopulos, 1979). Similarly, in central Italy, the first foci of decline were observed in new areas of hazelnut cultivation in 1977 (Scortichini, 2002). Afterwards, the disease spread quite rapidly in the two areas, destroying thousands of trees. It is interesting to note that the exchange of hazelnut propagative material between Italy and Greece never occurred. Pathogenicity tests performed with strains of the three population subsets studied revealed their high level of virulence (Scortichini *et al.*, 2002b). Probably, these highly virulent clonal complexes persist over decades.

Finally, we stress the divergence of the *P. avellanae* population subsets in the context of ecotypic adaptation. In fact, the strains isolated in central Italy either from Viterbo or Rome, are very similar to each other and many ETs contain strains from the two provinces. In fact, the hazelnut orchard in the province of Rome was established with propagative material taken from Viterbo. The population subsets of northern Greece appear to be different from these. As in the case of another plant-associated bacterium, *Rhizobium leguminosarum* bv. *phaseoli* (Souza *et al.*, 1992), geographic separation might contribute to the allele diversity and would appear as an important factor for differential adaptation of bacterial phytopathogens to the same host plant cultivated in different areas.

ACKNOWLEDGEMENTS

The authors wish to thank Professor T. S. Whittam (Michigan State University, Department of Microbiology and Molecular Genetics, East Lansing, USA) for providing the software and Dr P. G. Psallidas (Benaki Phytopathological Institute, Athens, Greece) for providing *P. avellanae* strains from Greece. We also thank two anonymous reviewers for their helpful comments and suggestions.

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