Brenneria quercina and *Serratia* spp. isolated from Spanish oak trees: molecular characterization and development of PCR primers

C. Poza-Carrion , I. Aguilar , F. J. Gallego , Y Nunez-Moreno , E. G. Biosca , R. Gonzalez', M. M. Lopez and P. Rodriguez-Palenzuela

Departamento de Biotecnologla-UPM, E.T.S. Ingenieros Agronomos, 28040 Madrid; Institute Nacional de Investigacion y Tecnologfa Agraria yAlimentaria, Dpto. de Biotecnologfa, Ctra A-6 km 7.5, 28040 Madrid; Departamento de Genetica, Facultad de Biologfa, Universidad Complutense de Madrid, Av. Complutense s./n., 28040 Madrid; Departamento de Microbiologfa y Ecologfa, Universidad de Valencia, Av. Dr. Moliner 50, Burjassot 46100, Valencia; and[e] Institute Valenciano de Investigaciones Agrarias, Moncada 46113, Valencia, Spain

Brenneria quercina has been reported as one of the causal agents of oak decline in Spain. To investigate the bacterial variability of this pathogen from different Spanish oak forests, a collection of 38 bacterial isolates from seven geographic locations and from different oak species was analysed by sequencing 16S rDNA and rep-PCR fingerprinting. All Spanish isolates of *B. quercina* were grouped by rep-PCR into a homogenous cluster that differed significantly from *B. quercina* reference strains from California. 16S rDNA analysis revealed that 34 out of 38 isolates were *Brenneria.* However, four isolates belonged to the genus *Serratia,* suggesting that this bacterium could cause cankers in oak trees. The information obtained by rep-PCR fingerprint analysis was used to develop PCR primers for the sensitive and specific detection of *B. quercina* from infected plant tissues. Pathogenicity tests performed with *Brenneria* and *Serratia* isolates showed that both were able to grow and cause cankers in oak trees.

Keywords: drippy nut disease, *Quercus ilex, Quercus pyrenaica,* rep-PCR detection and diagnosis

Introduction

Brenneria quercina is the causal agent of drippy nut disease of oaks. Hildebrand & Schroth (1967) first described it in two *Quercus* species *(Q. agrifolia* and *Q. wislizenii)* in the interior valleys of central and northern California; drippy nut was characterized by the appearance of copious bacterial ooze from the acorns, which in most cases resulted in nut abortion. *Brenneria quercina* seemed to be restricted to the nut of these *Quercus* species in California until it was reported on *Q. pyrenaica* and *Q. ilex* in Spain (Soria *et al.,* 1997) as responsible for bleeding cankers in the bark and oozing from buds (Biosca *et al.,* 2003). Strains of *B. quercina* were isolated from oak species in different geographic areas within Spain and characterized phenotypically (Soria *et al.,* 1997; Biosca *et al.,* 2003). Relationships between *B. quercina* and other Enterobacteriaceae were investigated by comparison of nucleotide and peptide sequences and concatenated sequences (Hauben *et al.,* 1998a; Young & Park, 2007).

Little is known about the incidence of the disease caused by *B. quercina* and the genetic relationships among different isolates. 16S rDNA analysis is a widespread tool to determine the phylogenetic position of a group of isolates. A considerable part of the 16S rRNA gene is conserved in all bacterial genera, whereas a smaller part is variable; this enables genealogical distances to be estimated and phylogenies derived from them (Hauben *etal,* 1997; Kwon *et al,* 1997; Young *etal.,* 2004; Ribbeck-Busch *etal,* 2005; Young & Park, 2007). Using the 16S rDNA sequence, species of the *Brenneria* genus were grouped in a cluster and its position within the Enterobacteriaceae determined (Kwon *et al,* 1997; Hauben *etal,* 1998a; Sproer *etal,* 1999; Brown *etal,* 2000; Young & Park, 2007).

Several molecular methods are used to rapidly identify and classify pathogenic bacteria. Rep-PCR genomic fingerprinting with Enterobacteriaceae repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic (REP) primer sets were developed for this purpose (Stern *et al,* 1984; Hulton *etal,* 1991; Versalovic *et al,* 1991; Louws *et al,* 1994). Rep-PCR is a powerful, sensitive and reproducible technique to characterize, detect and identify pathogenic bacteria through their

genetic profiles (Dawson *et al.,* 2002; Barionovi *et al.,* 2006).

The objective of this work was to classify bacteria isolated from diseased oak trees from different sites in Spain using 16S rDNA analysis and rep-PCR analysis. A new PCR-based diagnostic test, based on the rep-PCR results for detection of *B. quercina* in plant tissues, is also reported.

Materials and methods

Microbiological methods

Sources of bacterial isolates used in this study are listed in Table 1. Isolations were made from infected material as previously described (Biosca *et al.,* 2003). Briefly, isolations were made on King's B (KB) medium (King *et al.,* 1954) plus cycloheximide (250 *jig* mL^{-1}), and after 48-72 h at 25°C, *Brenneria-like* colonies were subcultured. Isolates were kept at -80°C in Hogness freezing medium [final concentration: 3-6 mM K_2HP0_4, 1-3 mM KH_2P0_4,1 mM $MgS0_4$, 2 mM sodium citrate, 4-4% (v/v) glycerol]. Bacterial identification was performed using the Biolog system (Biolog Inc.) following the manufacturer's recommendations and the identification was completed according to Biosca *et al.* (2003). *Brenneria* and *Serratia* strains were cultivated in KB medium at 28°C.

Pathogenicity assays

Three isolates were selected for the pathogenicity test: strain N-78a from *Q. pyrenaica,* IVIA-1467a from *Q. pyrenaica* and N-77 from *Q. ilex;* 10 mM $MgCl_2$ was used as a negative control.

Four *Q. pyrenaica* (6-7 years old) and nine *Q. ilex* (2-3 years old) trees were inoculated per bacterial strain with a suspension containing 10^9 bacterial cells in 1 mL of 10 mM $MgCl_2$, applied to a small cavity in the trunk created with a cork borer (2 mm diameter). The trees were incubated in a greenhouse (28°C/70% RH) and 3 months after inoculation, the external bark from inoculated trees was removed from the canker; bacteria were isolated from this ooze and identified with the Biolog system.

ERIC and rep-PCR genotyping

Bacterial genomic DNA was extracted using the GenomicPrep ^{M} Cells and Tissue DNA Isolation Kit (Amersham Biosciences) following the manufacturer's instructions. PCR reactions were performed in a final volume of 25 *flh* with the following components: 15 ng DNA template, 50 pmol oligonucleotides (ERIC or REP), 1-25 mM dNTPs, 2 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 2-5 mM $MgCl_2$, and 2-5 *flh* of lOx concentrated stock of PCR buffer. The sequence of the primers used were: ERIC-a 5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC-b 5'-AAGTAAGTGACTGGGGTGAGCG-3' (Hulton *etal,* 1991); REP-a 5'-RCGYCTTATCMGCGGTAC-3' and REP-b 5'-NNNRCGYCGNCATCMGGC-3' (Stern *et al,* 1984). Each primer pair was 5'-labelled with one of the following fluorescent dyes: 6-FAM for ERIC and HEX for REP (Applera).

PCR reactions were performed in a Perkin Elmer 9600 thermocycler with the following conditions: initial denaturing (95°C, 7 min), 30 cycles of denaturing (94°C, 1 min), annealing (ERIC 52°C, 1 min; rep 44°C, 1 min) and extension (65°C, 8 min); and a final extension (65°C, 15 min) (Louws *etal,* 1994). Aliquots (2 *jih)* of PCR products were mixed with 12 *flh* of formamide and 0-5 *jiL* of red-DNA size standard (GENESCAN-500 ROX, Applera). Samples were denatured at 94°C for 3 min and separated by capillary electrophoresis at 15 kV for 30 min in an ABI Prism 310 DNA Sequencer (Applera). Results were analysed by using GENESCAN analysis software 3-1 (Applera). This system was used to obtain genetic profiles for each bacterial isolate. All experiments were repeated twice under the same conditions.

16S rDNA amplification and sequencing

16S rDNA PCR amplification of different isolates was performed following Hauben *et al.* (1997): total reaction volume was 50 *flh* and contained 2-5 ng of DNA template, 200 */1M* dNTPs, 2-5 mM $MgCl_2$, 2 U of AmpliTaq Gold DNA polymerase, 50 pmol of each oligonucleotide and 5 *flh* lOx concentrated stock of PCR buffer. The primers used were: 16F27 5'-AGAGTTTGATCMTGGCTCAG-3' and 16R1525 5'-TTCTGCAGTCTAGAAGGAGGTGWTCCAGCC-3' (Hauben *etal,* 1997). Amplification was performed in a Perkin Elmer 9600 thermocycler with an initial denaturing temperature of 95°C for 7 min, followed by 25 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 2 min), with a final extension of 68°C for 7 min (Hauben *et al,* 1997). The amplified fragment was purified and sequenced in a 377 DNA automated sequencer (Perkin Elmer). Sequence alignments were performed at the internet site of the National Center for Biotechnology Information (http://www4.ncbi.nlm.nih.gov) with the BLAST network service (Altschul *et al,* 1997). In addition to the former primers used for amplification, another primer pair was used to complete the DNA sequence: 16R1087 5'-CTCGTTGCGGGACTTAACCC-3' and 16F530 5'-TTCGTGCCAGCAGCCGCGG-3' (Hauben *etal, 1997).*

Data analyses

Genetic profiles obtained by ERIC and rep-PCR from capillary separation were combined and converted into a binary matrix on the basis of presence/absence. To estimate similarity between different isolates, the simple matching algorithm was used (Nei, 1972). A dendrogram was built showing similarity between isolates after applying the UPGMA method (Sneath & Sokal, 1973). The concordance of the similarity data obtained from the analyses of ERIC and REP primers was determined with

Table 1 Sources of field isolates and collection strains used in this study

Isolate[a]/Strain[a]	Original host	Geographic origin	Source[3]
Brenneria quercina field isolates:			
(IVIA-1994-14, IVIA-1994-15)*	*Quercus Hex* (acorn) *Q. Hex*	Madrid	"his study
(IVIA-1251-3, IVIA-1251-5C)*	(canker)	Madrid	
(IVIA-1442-2, IVIA-1442-8a)*	*Oilex* (canker)	Madrid	
(IVIA-1467-a, IVIA-1467-h)*	*Q. pyrenaica* (canker)	Madrid	
(IVIA-1618-a, IVIA-1618-e)*	*Q. ilex* (acorn)	Segovia	
(IVIA-1625-1, IVIA-1625-2)*	*Q. pyrenaica* (leaf bud)	Madrid	
(IVIA-1745-3, IVIA-1745-5)*	*O. ilex* (canker)	Albacete	
(IVIA-1915-5, IVIA-1915-14)*	*Q. ilex* (acorn)	Madrid	
(IVIA-1927-1, IVIA-1927-4)*	*Q. ilex* (acorn)	Madrid	
(IVIA-1934-1, IVIA-1934-2)*	*Q. ilex* (acorn)	Madrid	
(IVIA-1940-1, IVIA-1940-3)*	*Q. ilex* (acorn)	Segovia	
(IVIA-1958-1, IVIA-1959-1)*	*O. ilex* (canker)	Teruel	
(IVIA-1982-1, IVIA-1983-2)*	*O. ilex* (canker)	Alicante	
(IVIA-1986-6, IVIA-1986-7)*	*Q. ilex* (acorn)	Alicante	
(IVIA-1988-2b, IVIA-1988-2)*	*Q. ilex* (acorn)	Valencia	
(IVIA-1988-3b, IVIA-1988-3)*	*Q. ilex* (acorn)	Valencia	
N-76	*Q. ilex* (acorn)	Segovia	
N-77	*Q. ilex* (acorn)	Madrid	
Serratia spp. field isolates:			
(IVIA-1251-4, IVIA-1251-5)*	*O. ilex* (canker)	Madrid	
(N-78-a, N-78-b)*	*Q. pyrenaica* (canker)	Burgos	
Collection strains:			
Brenneria rubrifaciens NCPPB 2020	*Juglans regia*		MCPPB
Brenneria nigrifluens NCPPB 564	*J. regia*		MCPPB
[b] *Pantoea agglomerans* 1742-5	*Olea europaea*		"his study
"Erwinia herbicola NCPPB 2971	*Wisteria floribunda*		MCPPB
Pectobacterium carotovorum subsp. *atrosepticurn* SCRI 1001	*Solanum tuberosum*		3CRI
Pectobacterium carotovorum subsp. *carotovorum* 312	*S. tuberosum*		"his study
Erwinia amylovora NCPPB 595	*Pyrus communis*		MCPPB
Erwinia rhapontici 1093-1 a	*Allium cepa*		"his study
Dickey a dadanfl;AC4150	Multiple		
Erwinia iupinicoia 3299			
Erwinia mallotivora 1818	*Mallotus* sp.		
Pantoea stewartii 2994	*Zea mays*		
Brenneria salicis NCPPB 447	*Salix* sp.		MCPPB
Brenneria alni NCPPB 3934	*Alnus* sp.		MCPPB
Pseudomonas syringae pv. *tomato* DC 3000	*Lycopersicon esculentum*		A. Collmer
Pseudomonas syringae pv. *phaseolicola*	*Phaseolus* spp.		MCPPB
Pseudomonas syringae pv. *tabaci*	*Nicotian a* spp.		MCPPB
Brenneria quercina NCPPB 1852	*Quercus* sp.		MCPPB
Brenneria quercina NCPPB 1853	*Quercus* sp.		MCPPB
Brenneria quercina NCPPB 3077	*Quercus* sp.		MCPPB
Brenneria quercina CFBP 1266	*Quercus* sp.		CFBP
Serratia rubidaea 868 T			CECT
Escherichia coli DH5a			Hanahan (1983)

[a]IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain; NCPPB, National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, UK; CFBP, Collection Francaise de Bacteries Phytopathogenes, Angers, France; SCRI, Scottish Crop Research Institute, Invergowrie, Dundee, UK; CECT, Coleccion Espanola de Cultivos Tipo, Universidad de Valencia, Burjasot, Valencia, Spain.
"The preferred name for *Erwinia herbicola* is now *Pantoea agglomerans.*
*Both isolates obtained from identical geographic origin and host.

the Mantel test (Mantel, 1967). Similarity matrices and dendrograms were constructed using NTSYS-pc 2-0 (Exeter Software). To determine the degree of significance, a bootstrap was made using the TFPGA (tools for population genetic analysis) 1-3 software (Efron, 1982; Manly, 1997). 16S rDNA sequences were aligned and a similarity matrix was generated using the method described by Maidak *etal.* (1996). Dendrograms were obtained using the UPGMA method.

Development of PCR primers specific for *B. quercina*

The primer pair REP-a and REP-b with no 5'-label was used to specifically amplify bacterial DNA (Stern *et al.*, 1984) for the detection of *B. quercina* using the above described conditions. Amplified DNA products (10 *flh* for rep-PCR) were analysed by electrophoresis in 1-5% agarose gel containing a 0-75x dilution of the standard TAE buffer (Sambrook *et al.*, 1989) and ethidium bromide (1 *jig* mL^4) at 30 V for 20 h and were detected by UV transillumination. A 517-bp DNA fragment specific for *B. quercina* isolates (see Results) was cloned into the pGEM®-T Easy vector (Promega Corporation), according to the manufacturer's instructions. Two primers, REP-517-a and REP-517-b (sequences 5'-GTCAACATAACAGCGACTAC-3' and 5'-CACG-TATCGCATTGATTCTG-3', respectively) (Roche Diagnostics) were designed and synthesized to target the cloned *B. quercina* REP-amplified fragment.

Specific amplification of the 517-bp fragment was performed using this primer pair and 20 ng of *B. quercina* DNA template in a total reaction volume of 50 *flh*, containing 5 *flh* of lOx concentrated stock of PCR Gold Buffer with no $MgCl_2$ (Applied Biosystems), 5 *jlh* of 25 mM $MgCl_2$ solution (Applied Biosystems), 8 *flh* of 10 mM dNTP mix, 04 /iM primers and 2 U of AmpliTaq Gold ^{M}. Conditions for amplification were optimized and carried out as follows: an initial denaturation at 95°C for 7 min, after which 30 cycles of denaturation (30 s at 95°C), primer annealing (30 s at 60°C) and primer extension (40 s at 72°C) were performed, followed by a final extension at 72°C for 10 min (Hauben *et al.*, 1998b).

The sensitivity of this method was first estimated in bacterial suspensions and on natural samples of acorn oozes by preparing decimal dilutions (from 10^9 to 1 CFU mL^4) of *B. quercina* cultures in 10 mM $MgCl_2$ buffered saline. These dilutions were used for PCR and plated on KB medium to determine the cell concentration of *B. quercina.* One millilitre of each dilution was centrifuged (10 000 ***g*** for 10 min) and the bacterial pellet was resuspended in 1 mL of water. Aliquots (5 *flh)* were then heated for 5 min at 95°C, cooled on ice, and subjected to PCR. To test the validity of this system for the detection of *B. quercina* in infected oak leaves, 100 *flh* of decimal (from 10^9 to 1 CFU mL^4) *B. quercina* dilutions were injected into young *Quercus* leaves. These dilutions were plated on KB medium to determine the cell concentration of *B. quercina.* After 24 h, the samples were prepared to PCR following the method described by Llop *et al.* (1999). Negative controls with uninfected plants were included in every DNA extraction series.

Results

Field sampling

A total of 38 *Quercus* samples from several species of trees affected by oak decline were collected in different areas of Spain and bacteria were isolated from the samples (Table 1). Symptoms were found in bark, acorns and buds (Fig. 1). One of the most characteristic symptoms found were canker lesions in the bark of the infected trees. In acorns and buds, the most typical symptom was oozing of plant sap (Fig. 1), which in some cases resulted in acorn abortion (Fig. 1). In more than 90% of cases a single dominant bacterial type was isolated from the infected tissue. The homogeneity of the colonies was confirmed by analysing an appropriate number of colonies of each isolate with Biolog-GN microplates.

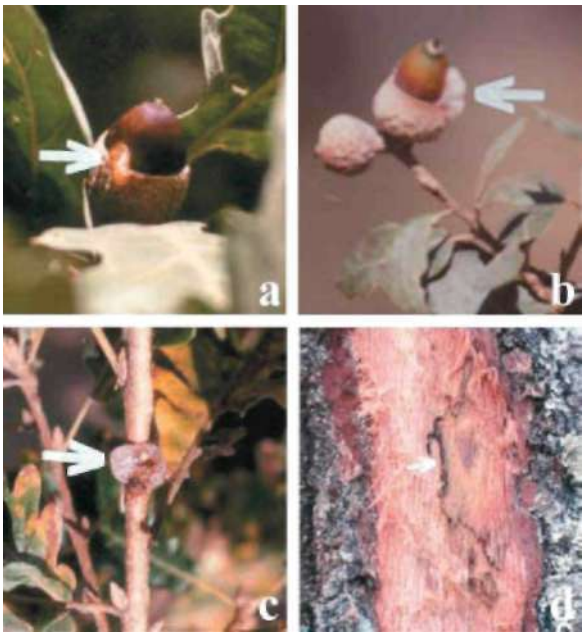


Figure 1 Symptoms of drippy nut disease in naturally infected of *Quercus pyrenaica.* Damage and/or exudates on the tissues are indicated with arrows, (a and b) Acorn cup with oozing typical of the disease, (c) leaf bud with oozing, (d) detail of a 'bleeding canker'.

Purified cultures of *Brenneria-like* colonies, isolated from infected trees, were identified by metabolic profile analysis using Biolog-GN microplates. The results obtained indicated that most isolates, and the corresponding *B. quercina* type strains, had a very similar metabolic profile (Table 2), confirming that the *Brenneria-Yike* isolates were in fact *B. quercina.* Exceptions were strains 1251-4, 1251-5, N-78a and N-78b, which showed similar colony morphology, but differed in the results of some of the tests and formed a homogenous group different from the others (Table 2), although this results was not easily confirmed. It should be noted that in a previous work with the Biolog system (Klingler *et al.*, 1992), when attempts were made to identify *Serratia* species, the system did not work properly. Similarly, when the group of isolates named as *Serratia* in the present study were characterized using 16S sequencing, the Biolog system was unable to identify them (data not shown).

Table 2 Metabolic profile characteristics of the Spanish isolates and reference strains of *Brennena quercina* and *Serratia* spp. using the Biolog-GN system

Carbon source	Isolate/strain[3]	Carbon source	Isolate/strain[3]
a-Cyclodextrin		Itaconic acid	
Dextrin		a-Keto Butyric Acid	
Glycogen		a-Keto Glutaric Acid	
Tween 40		a-Keto Valeric Acid	
Tween 80		D,L-Lactic Acid	
N-Acetyl-D-Galactosamine		Malonic Acid	
N-Acetyl-D-Glucosamine		Propionic Acid	
Adonitol		Quinic Acid	
L-Arabinose		o-Saccharic Acid	
o-Arabitol		Sebacic Acid	
o-Cellobiose		Succinic Acid	
i-erythritol		Bromosuccinic Acid	
o-Fructose		Succinamic Acid	
L-Fucose		Glucuronamide	
D-Galactose		L-Alaninamide	
Gentiobiose		o-Alanine	
a-o-Glucose		L-Alanine	
m-inositol		L-Alanyl-glycine	
a-o-Lactose		L-Asparagine	
Lactulose		L-Aspartic Acid	
Maltose		L-Glutamic Acid	
D-Mannitol		Glycil-L-Aspartic Acid	
o-Mannose		Glycil-L-Glutamic Acid	
o-Melibiose		L-Histidine	
B-Methyl-D-Glucoside		Hydroxy-L-Proline	
o-Psicose		L-Leucine	
o-Raffinose		L-Ornithine	
L-Rhamnose		L-Phenylalanine	
D-Sorbitol		L-Proline	
Sucrose		L-Pyroglutamic Acid	
o-Trehalose		o-Serine	
Turanose		L-Serine	
Xylitol		L-Threonine	
Pyruvic Acid Methyl Ester		D,L-Carnitine	
Succinic Acid Mono-Methyl-Ester		y-Amino Butyric Acid	
Acetic Acid		Urocanic Acid	
Cis-Aconitic-Acid		Inosine	
Citric Acid		Uridine	
Formic Acid		Thymidine	
D-Galactonic Acid Lactone		Phenyethyl-amine	
o-Galacturonic Acid		Putrescine	
o-Gluconic Acid		2-Aminoethanol	
D-Glucosaminic Acid		2,3-Butanediol	
D-Glucuronic Acid		Glycerol	
a-Hydroxybutyric Acid		D,L-a-Glycerol Phosphate	
B-Hydroxybutyric Acid		a-o-Glucose-1-Phosphate	
y-Hydroxybutyric Acid		D-Glucose-6-Phosphate	
p-Hydroxy Phenylacetic Acid			

[a]A, *B. quercina* NCPPB 1852; B, *B. quercina* Spanish isolates (summary of all data); C, 1251-4 and 1251-5 Spanish isolates (summary of all data); D, N-78a and N-78b Spanish isolates (summary of all data).
(+) positive in the test, (-) negative in the test and (=) variable in the test.

Pathogenicity assays

The symptoms typical of bacterial canker described by Biosca *et al.* (2003), were reproduced after inoculation of two *Quercus* species with bacterial isolates of *B. quercina* and *Serratia* spp. *Quercus Hex* and *Q. pyrenaica* trees were inoculated with two isolates identified as *B. quercina* (IVIA-1467a and N-77), one isolate

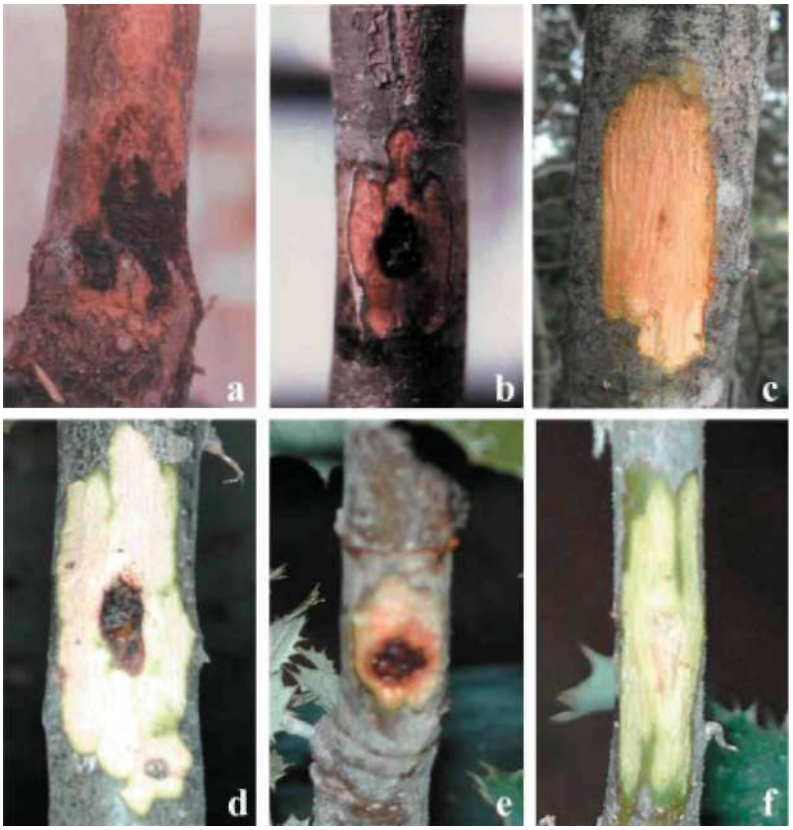


Figure 2 Symptoms 3 months after artificial inoculation of (a, b, c) *Quercus pyrenaica* and (d, e, f) *Q. ilex* with (a and d) *Brenneria quercina* isolate 1467-a *Q. ilex,* respectively, (b and e) *Serratia* isolate N-78-a or (c and f) 10 mM $MgCl_2$ as a negative control.

identified as a *Serratia* spp. (N-78-a) and with 10 mM $MgCl_2$ as a negative control (Fig. 2). Attempts to re-isolate the inoculated isolates from bark canker lesions were successful, with identity confirmed by the Biolog system.

Molecular characterization of field isolates

Molecular profiles from 42 isolates (38 field isolates and four *B. quercina* reference strains) were obtained by rep-PCR and gel electrophoresis (Fig. 5). In addition the bands were separated by capillary electrophoresis and these data were used for further analysis, as described in Materials and methods. To test the reproducibility of the system, the experiments were repeated using identical PCR conditions and DNA from different bacterial cultures obtained under the same experimental conditions. In all cases, the DNA banding pattern obtained was the same. The primer pairs used in this study gave a total of 144 distinct DNA fragments, ranging from 40 bp to 850 bp in length. The numbers of amplified fragments obtained with ERIC and REP primers were 84 and 60, respectively, UPGMA dendrograms produced using data from ERIC and REP primers were very similar; a positive correlation between the ERIC and REP datasets was confirmed using the Mantel test (^ERIC-REP = 0-813). Therefore, the results from the ERIC and REP systems were combined into a single matrix and a unique dendrogram generated (Fig. 3).

The dendrogram revealed that the *B. quercina* isolates could be differentiated into three groups: clusters I, II and III, whose branches were statistically significant according to the bootstrap test (Nei, 1972) (Fig. 3). Cluster I comprised most of the Spanish field isolates, cluster II included the *B. quercina* reference strains, and cluster III included isolates 1251-4, 1251-5, N-78a and N-78b. Isolates 1251-4 and 1251-5 were both obtained from *Q. ilex*, from the same focus in Madrid; N-78a and N-78b were obtained from *Q. pyrenaica*, from the same focus in Burgos. These four isolates showed a metabolic profile very different from all the other isolates (Table 2). Moreover, these four could be divided into two subgroups: one corresponding to 1251-4/1251-5, and the other including N-78a/N-78b. These four isolates from cluster III were genetically distant from the other field isolates and from *B. quercina* type strains (Fig. 3).

There was no correlation with host or region within Spain for isolates examined (Fig. 3).

Phylogenetic analyses based on 16S rDNA sequence

The 16S rDNA genes from one selected isolate of the *B. quercina* group (isolate 1940-1, cluster I; GenBank Ace. No. EF534571) and from two isolates of cluster III (1251-5, Ace. No. EF534570; and N-78b, Ace. No. EF534572) were amplified and sequenced. A dendrogram based on these sequences and the sequence data available in the Ribosomal Database Project (Larsen *et al.,* 1993) and

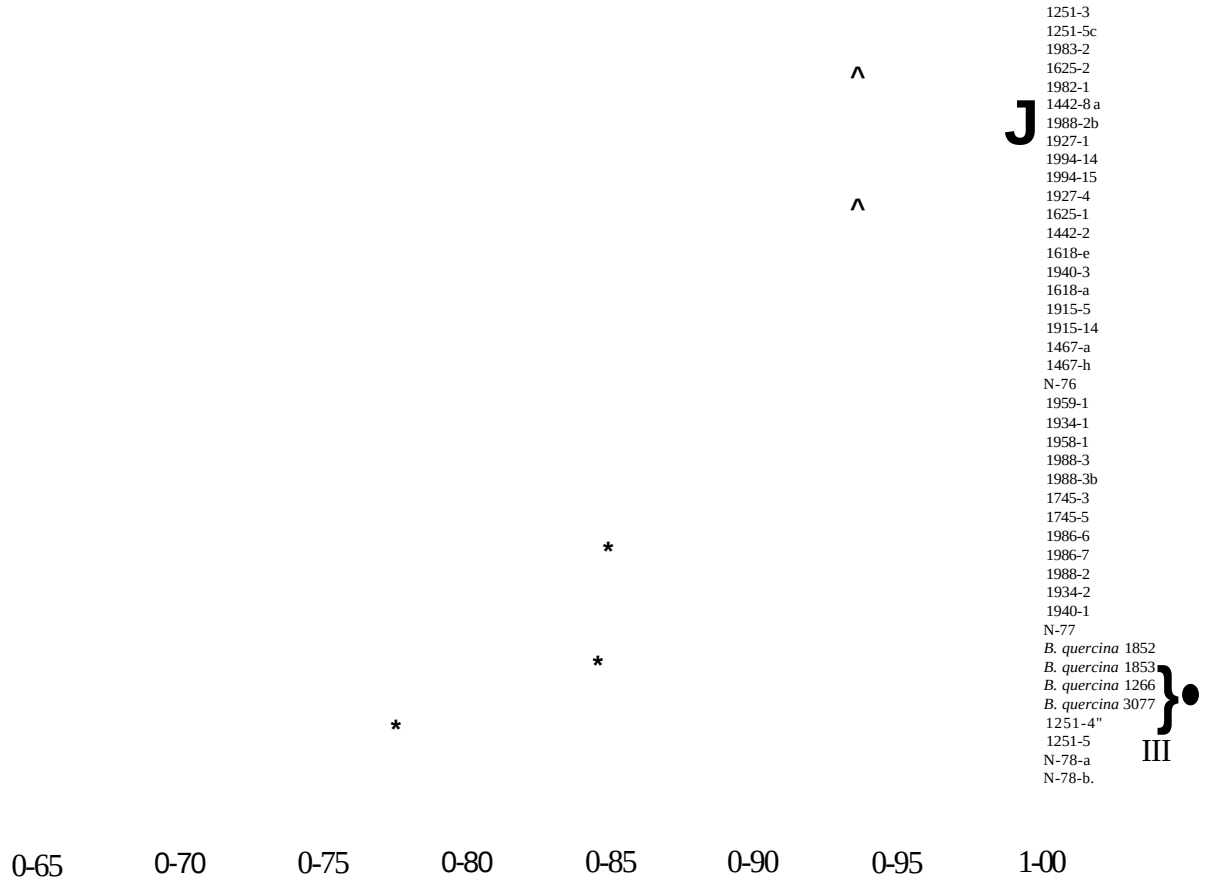


Figure 3 Similarity relationships between *Brenneria quercina* collection strains and field isolates. The dendrogram was obtained from a simple-matching similarity matrix generated from the rep-PCR (ERIC and REP PCR primers) system, clustered by UPGMA. The dendrogram combined REP and ERIC datasets. A similarity of 1 -0 indicates 100% identity between strains. An asterisk indicates that the node is statistically significant at the 95% level according to the bootstrap method. Group I: Spanish field isolates of *B. quercina;* group II: type-collection strains of *B. quercina* from California; group III: Spanish field isolates of *Serratia.*

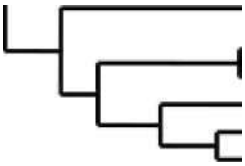
Hauben *et ah* (1998a) was built according to the UPGMA method, in order to determine the phylogenetic position of these three isolates within the enterobacterial group. Isolate 1940-1 showed 99-2% nucleotide similarity to *B. quercina* 16S rDNA, clustering in group III, whereas N-78b and 1251-5 showed 97-3 and *97-6%* similarity, respectively with 16S rDNA from *Serratia rubidaea* (DSM 4480 strain) (Fig. 4; Table 3).

Development of a specific PCR protocol for detection of *B. quercina*

Two DNA fragments of *c.* 500 bp obtained in rep-PCR fingerprints visualized by gel electrophoresis appeared in all *B. quercina* isolates, whereas they were not present in isolates of other bacteria, including *Serratia* (Fig. 5). These two fragments were cloned and sequenced; one was selected for further investigation. Primers REP-517-a and REP-517-b were designed for specific amplification of a 517-bp fragment (ace. no. EF534573). The amino acid sequence corresponding to the amplified fragment showed 86% identity with the DNA repair protein RecN from *Escherichia coli.*

To confirm the specificity of the primers, DNA from *57* isolates, comprising 34 field isolates of *B. quercina,* four field isolates of *Serratia* and 19 reference strains (Table 1) were tested by PCR using primers REP-517-a and REP-517-b. Spanish isolates of *B. quercina* from different geographical origins and recovered in different oak species and Californian isolates yielded a DNA fragment of 517 bp, whereas no band resulted from the amplification of the DNA of any of the following bacterial species: *E. coli, B. rubrifaciens, B. nigrifluens, B. salicis, B. alni, Pantoea agglomerans, P. stewartii, Erwinia herbicola (= Pantoea agglomerans), Pectobacterium carotovorum* ssp. *atrosepticum, P. carotovorum* ssp. *carotovorum, Erwinia amylovora, E. rhapontici, E. lupinicola, E. mallotivora, Dickeya dadantii* and *Pseudomonas syringae* (data not shown).

The sensitivity of this method was first estimated in bacterial suspensions; the amplification of the 517-bp band was detected on agarose gels down to a bacterial



En/ernhacler nimiprewurtdix
Emerobacter asteentgatus
En tertthacter di\aahenx
Pectobacrerium cypripedii ~'\
Dickeyit dmUilMl
Pectabaaerium e, odortferum
Pectobacterium c carotavoram
Pectobactcrittm c betavascaioriwt >- **<imup 11**
Peeiabacieriunt e, wastibiae
Peetttbacleriam c atra.yepticmti
Peaobaaerium caccicciditm ^
N-7Bb
[251-5
Serratia rubidaeti Grou|i *Serratiu*
Serratia marceseaa
Serratia odorifera
Pantoea s *indotogenes*
Pautaea x. xteKiirtii
Pantaea ananaih
PanSoea agglameratts IA1G 2(iiM **Croup IV**
Pantaea agglamerans l.Md 2S6S **J**
Envinht mallotivora ~-
Erwinia psidii
Eraiaia amyinvara
Erti'ia ia pyrijafiae
Emiaia rhapoiitici LAfG 2(188 *p*>- Croup I
Envia ia persk'mus
Lnviuia rhapootici 1 ,MC 26*1
*Ern'inia traeheipbila*_____.
(freitneria qttercina ~^
1*40-1
Brenneria aini
Rreittixriti nigriftiums >- (,n>u|i III
Sretttieriu paratiisiaca
Brenneria sidicis
Brenneria rubrifacletts

1%

Figure 4 UPGMA dendrogram depicting the estimated phylogenetic relationships among *Enterobactenaceae* strains and *Serratia* strains, based on pairwise comparisons of nearly complete 16S rDNA sequences. 16S rDNA sequences for strains determined in this study are shaded, the rest of the sequence was obtained from the Ribosomal Database Project (Larsen *etal.,* 1993) and from (Hauben *etai,* 1998a). The distance between two species was obtained by summing the lengths of the connecting horizontal branches using the bottom scale (% sequence divergence). Group I, II, III and IV represent the genera *Erwinia, Pectobacterium, Brenneria* and *Pantoea,* respectively (Hauben *etal.,* 1998a). See Table 3 for expanded names of Group II and IV species.

concentration of 10^2 CFU mL^4 (Fig. 6a). In natural samples of acorn ooze the detection limit obtained was 10^2-10^3 CFU mU^1 (Fig. 6b). This lower sensitivity may have resulted from the chemical complexity of acorn ooze compared with the pure bacterial culture sample. In infected plant material, the amplification of the 517-bp band was detected on agarose gels at all bacterial concentrations above 10^2 CFUmU^1 (Fig. 6c).

Discussion

The genetic diversity of bacteria associated with symptoms of drippy nut and bacterial canker disease in oak forests of the Spain was investigated. DNA profiles of 42 bacterial isolates (38 field isolates and four *B. quercina* reference strains) from different oak species and from several geographic locations were obtained by rep-PCR, which was combined with a capillary electrophoresis analysis system. A dendrogram differentiated three clusters: cluster I, in which almost all the Spanish isolates were located; cluster II, containing *B. quercina* type strains from California; and cluster III, which contained four Spanish field isolates.

Most of the Spanish isolates (except N-78a, N-78b, 1251-4 and 1251-5) were grouped into cluster I. No significant differences could be found between the branches in this group, indicating a lack of subgroups or subpopulations in the Spanish isolates group. The data from rep-PCR were in accord with those obtained by Biolog identification and 16S rDNA sequence analysis,

Table 3 Similarity values determined from pairwise comparisons among 16S rDNA gene sequences. Shaded grey dates indicate sequences obtained in the present work; remainder are from the Ribosomal Database Project (Larsen *etal.,* 1993) and from Hauben *etal.* (1998a)

^D ^D o

. g a s

a. a:

Enterobacter nimipressuralis	100																																			
Enterobacter cancerogenus	98-3	100																																		
Enterobacter dissolvens	98-1	98-6	100																																	
Pantoea stewartii ssp. *indologenes*	96-3	97-0	96-6	100																																
P.stewartii ssp. *stewartii*	96-4	97-1	96-6	99-5	100																															
Pantoea ananatis	96-3	97-1	96-1	98-1	98-3	100																														
Pantoea agglomerans LMG 2660	95-7	96-2	95-5	97-0	97-2	98-3	100																													
Pantoea agglomerans LMG 2565	95-8	96-2	95-6	96-9	97-2	98-3	99-3	1100																												
Erwinia amylovora	96-0	96-4	96-6	97-0	97-0	96-3	97-5	95-9	100																											
Erwinia rhapontici LMG 2688	96-1	96-7	96-7	96-1	96-4	95-8	95-7	95-8	97-7	1100																										
Erwinia persicinus	96-0	96-8	96-8	95-9	96-2	95-6	96-0	96-2	97-6	99-0	100																									
Erwinia rhapontici LMG 2691	96-0	96-8	96-8	95-9	96-2	95-6	96-0	96-2	97-6	99-0	100	100																								
Erwinia tracheiphila	95-5	95-5	96-1	95-4	95-4	95-6	94-9	95-1	95-2	94-7	94-5	94-5	100																							
Erwinia mallotivora	94-9	95-6	96-3	96-5	96-6	96-0	95-7	95-8	97-1	96-8	96-6	96-6	95-4	100																						
Erwinia psidii	95-2	96-0	96-4	96-8	96-8	96-1	96-0	95-6	96-4	96-0	95-7	95-7	96-0	97-6	100																					
Pectobacterium cypripedii	95-7	97-1	96-2	95-7	95-8	95-7	94-7	94-6	96-0	96-7	96-3	96-3	94-2	95-4	94-7	100																				
Dickeya dadantii	95-7	97-0	96-2	95-6	95-7	95-7	94-5	94-6	95-8	96-2	96-0	96-0	94-3	95-5	94-9	98-3	100																			
P'carotovorum ssp. *odoriferum*	96-8	96-9	96-4	95-5	95-7	95-3	95-2	95-2	95-0	95-3	95-2	95-2	94-9	95-0	94-8	3-3	96-8	100																		
P. carotovorum ssp. *carotovorum*	97-2	97-3	96-8	96-0	96-3	95-9	95-6	95-7	95-6	95-9	95-5	95-5	95-2	95-6	95-7	96-6	97-0	98-9	100																	
Pectobacterium cacticidum	95-3	95-5	95-0	93-9	94-0	93-8	94-7	94-4	93-9	94-0	94-8	94-8	93-4	93-5	93-6	95-1	95-5	96-3	96-5	100																
P. carotovorum ssp. *betavasculorum*	96-8	96-9	96-1	95-8	96-1	95-9	95-8	95-6	95-7	96-1	95-7	95-7	94-6	95-2	95-5	96-6	97-1	97-3	98-0	96-1	100															
P carotovorum ssp. *wasabiae*	96-5	96-9	96-1	95-1	95-4	95-1	95-5	95-7	95-5	96-5	96-8	96-8	94-5	95-3	94-4	97-1	97-5	97-8	98-1	97-2	98-1	100														
P. carotovorum ssp. *atrosepticum*	96-9	97-4	96-3	95-8	96-0	95-8	95-2	95-4	95-4	96-6	96-2	96-2	94-6	94-9	94-5	97-3	97-7	97-7	97-8	96-1	98-5	98-6	100													
Brenneria quercina	94-5	95-3	94-6	95-3	95-6	95-6	95-5	95-0	94-0	94-3	94-2	94-2	93-2	93-7	95-0	94-5	94-3	94-8	94-7	93-9	95-7	94-5	95-2	100												
Brenneria alni	93-9	94-7	93-9	94-0	94-0	93-6	93-4	93-3	93-5	94-5	94-4	94-3	93-0	94-2	93-7	94-8	95-2	94-2	94-5	94-4	94-5	95-3	95-1	94-3	100											
Brenneria nigrifluens	95-1	95-8	94-7	94-7	94-8	94-5	93-7	93-8	94-7	94-3	94-0	94-0	93-8	94-1	94-2	95-7	95-8	95-9	96-4	95-2	95-5	95-8	95-6	94-8	96-5	100										
Brenneria paradisiaca	93-5	94-8	94-1	94-0	94-1	94-0	93-7	93-3	93-8	94-1	94-1	94-1	92-5	94-0	93-8	96-3	97-3	94-5	94-5	93-7	95-8	95-1	95-5	95-0	94-6	95-0	100									
Brenneria salicis	93-7	95-0	93-8	93-7	93-7	94-1	92-9	92-9	94-0	94-6	94-6	94-6	91-7	93-7	92-6	96-1	96-3	94-7	95-0	94-7	94-6	95-5	95-7	94-6	96-0	96-4	95-8	100								
Brenneria rubritaciens	93-3	94-4	93-5	94-1	94-2	94-9	94-3	94-0	93-9	93-0	92-5	92-5	92-4	93-3	93-6	94-8	94-9	94-2	94-5	94-2	95-5	94-0	94-5	95-5	93-8	95-7	95-8	96-3	100							
N-78b	96-1	96-0	96-0	95-6	95-9	95-2	95-3	94-9	95-0	95-0	94-7	94-7	93-9	94-9	95-8	95-0	95-2	96-0	96-4	94-2	96-3	95-2	95-4	95-7	93-7	94-4	94-7	93-1	94-4	100						
1940-1	94-5	95-4	94-6	95-0	95-3	95-4	95-6	95-1	93-5	94-5	95-0	94-9	93-0	93-3	94-5	94-9	94-6	94-6	94-5	94-4	95-5	95-2	95-4	99-2	94-8	94-8	95-4	94-9	95-1	95-5	100					
Serratia rubidaea	95-2	95-7	95-7	95-6	95-5	95-6	95-2	94-7	94-2	94-0	94-0	93-9	93-1	94-2	95-2	94-9	94-8	95-4	95-6	94-0	95-4	94-2	94-7	96-7	93-7	93-7	94-3	93-9	95-0	97-3	96-5	100				
1251-5	96-0	96-0	96-3	95-7	95-6	95-2	94-7	94-2	95-0	94-7	94-8	94-8	93-5	94-8	95-4	95-2	95-4	95-7	95-9	94-0	96-0	95-0	95-5	95-6	93-9	94-0	95-1	93-7	94-0	98-4	95-3	97-6	100			
Erwinia pyritoliae	95-8	96-3	96-3	96-5	96-5	95-9	96-0	96-2	98-7	97-0	97-7	97-7	94-9	96-8	96-1	95-8	95-7	94-9	95-5	94-5	95-7	96-1	95-5	93-8	93-2	94-5	93-6	93-9	93-7	95-0	93-8	93-9	94-6	100		
Serratia marcescens	96-7	97-2	97-2	96-3	96-1	95-5	94-9	94-7	94-9	94-8	94-8	94-7	94-1	94-9	95-0	95-9	96-1	96-8	96-9	94-8	95-8	95-8	96-2	94-8	94-0	94-5	94-1	94-0	93-5	96-8	95-1	97-5	97-2	94-6	100	
Serratia odoritera	96-0	96-7	96-3	95-5	95-4	95-2	94-4	94-3	95-2	94-6	94-7	94-7	94-2	94-7	94-3	95-6	96-3	96-3	96-0	94-8	95-9	95-7	96-2	94-9	94-4	94-6	94-3	94-2	93-7	96-0	94-8	97-0	96-8	95-1	97-8	1

^*Pectobacterium.*

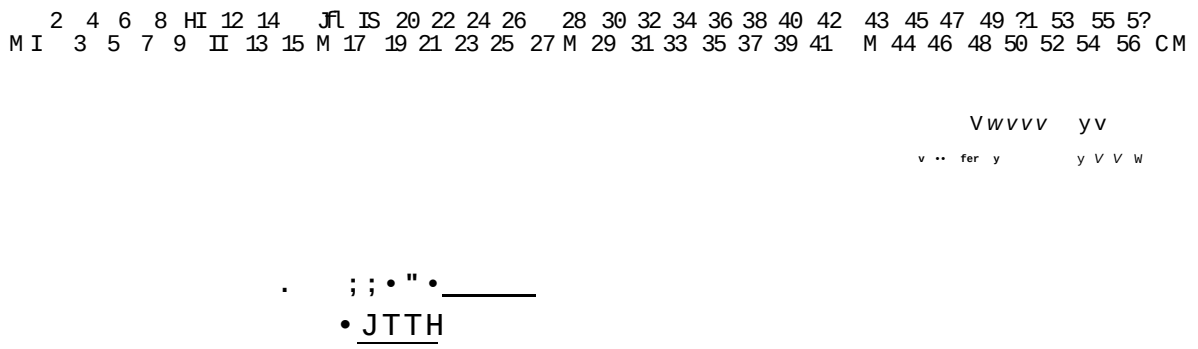


Figure 5 Rep-PCR fingerprint patterns of genomic DNA from field isolates and type collection strains. PCR product patterns were generated using the REP primers listed in the text. Lanes 1-15: type-collection strains: (1) *Erwinia herbicola* NCPPB 2971; (2) *Pantoea stewartii*2994; (3) *P. agglomerans* 1742-5; (4) £ *amylovora* NCPPB 595; (5) £ *rhapontici* 1093-1a; (6) £ *mallotivora* 1818; (7) *Dickeya dadantii* AC4150; (8) *Pectobacterium carotovorum* ssp. carotoi/orum312; (9) fi *carotovorum* ssp. *atrosepticum* SCRI 1001; (10) *Brenneria alni* NCPPB 3934; (11)0. *nigrifluens* NCPPB 564; (12) 0. sa/lc/s NCPPB 447; (13) 0. *rubrifaciens* NCPPB 2020; (14) £ *lupinicola* 3299; (15) fi *syringae* pv. tomato DC 3000. Lanes 16-57: field isolates and type-collection strains from 0. *quercina:* (16) 1251-4; (17) 1251-5; (18) 1251-3; (19) 1251-5c; (20) N-76; (21) N-77; (22) N-78a; (23) N-78b; (24) 0. *quercina* CFBP 1266; (25) 0. *quercina* 1852; (26) 0. *quercina* 1853; (27) 0. *quercina* 3077; (28) 1442-2; (29) 1442-8a; (30) 1467-a; (31) 1467-h; (32) 1618-a; (33) 1618-e; (34) 1625-1; (35) 1625-2; (36) 1745-3; (37) 1745-5; (38) 1915-5; (39) 1915-14; (40) 1927-1; (41) 1927-4; (42) 1934-1; (43) 1934-2; (44) 1940-1; (45) 1940-3; (46) 1958-1; (47) 1959-1; (48) 1982-1; (49) 1983-2; (50) 1986-6; (51) 1986-7; (52) 1988-2b; (53) 1988-2; (54) 1988-3b; (55) 1988-3; (56) 1994-14; (57) 1994-15. Lane C, negative control. Lane M, 100-bp DNA Ladder Plus molecular size marker GeneRulerTM (MBI Fermentas). The size of the two DNA fragments used as diagnostic tools (boxed) was 500-600 bp.

indicating that most of the Spanish isolates corresponded to *B. quercina.* Moreover, these *B. quercina* Spanish isolates were closely related to other *Brenneria* spp., such as *B. alni, B. nigrifluens* and *B. salicis,* whose natural hosts are woody plants (Hauben *et al.,* 1998a). Although the samples were collected in different geographic areas of Spain and from different oak species, no clear relation could be found between strains and geographic origins and/or natural hosts. The fact that *B. quercina* reference strains from California belonged to a different cluster than the *B. quercina* Spanish field strains, suggests a divergence between these groups of isolates but further data will be needed to investigate the origin of Spanish isolates of *B. quercina.*

Cluster III contained Spanish isolates N-78a, N-78b, 1251-4 and 1251-5 and was genetically distant from the *B. quercina* isolates in cluster I. 16S rDNA sequences from these strains were compared with previously reported data (Kwon *et al.,* 1997; Hauben *et al.,* 1998a). Isolates N-78b and 1251-5 showed 97-3 and 97-6% similarity, respectively, to the *S. rubidaea* type strain. When the other *B. quercina* field isolates were subjected to the same analysis, 99-2% similarity to *B. quercina* type strain was obtained. The 16S rDNA sequence analysis clearly showed that strains N-78a, N-78b, 1251-4 and 1251-5 belonged to the genus *Serratia.*

Certain pathogenic bacteria have a wide range of hosts and can be found in several niches. For example, *Fantoea agglomerans* (formerly *Erwinia herbicola/Enterobacter agglomerans)* is associated with plants as an epiphyte or a pathogen, but can also be found as an opportunistic pathogen in humans. *Serratia* sp. is also an opportunistic human pathogen that can be found in plants (Grimont *et al.,* 1977; Grimont & Grimont, 1984). *Serratia* species are phylogenetically close to the former *Erwinia* genus, which included *Brenneria* (Grimont *et al.,* 1977; Hauben *et al.,* 1998a; Sproer *et al.,* 1999). Different virulence systems have been described in *Serratia* which share common features with other bacterial phytopathogens, such as the *P. carotovorum hexA* gene, implicated in the regulation of pectolytic enzymes (Harris *et al.,* 1998) or the *S. marcescens rap* gene, homologous to the *Erwinia hor* genes, which has been implicated in the regulation of antibiotic production and exo-enzymes, which in turn are involved in pathogenicity (Thomson *et al.,* 1997).

Pathogenicity tests suggested that the Spanish *B. quercina* and *Serratia* isolates are able to survive and grow on oak trees, and to produce bark symptoms. Also, the fact that the studied isolates satisfied Koch's postulates supports the hypothesis that both bacteria are causal agents of oak disease. The pathogenicity of *Brenneria* is well described (Hildebrand & Schroth, 1967; Biosca *et al.,* 2003), whereas *Serratia* has not been previously reported as a plant pathogen. The data point to the possibility of the latter isolates being pathogens of trees, but confirmation between field symptoms and *Serratia* isolates it is still required. Alternatively *Serratia* spp. may be secondarily associated with infected oaks as a saprophyte and displaces *B. quercina* at later stages of the disease.

Additionally in this work, PCR primers have been developed for the specific detection of *B. quercina* in

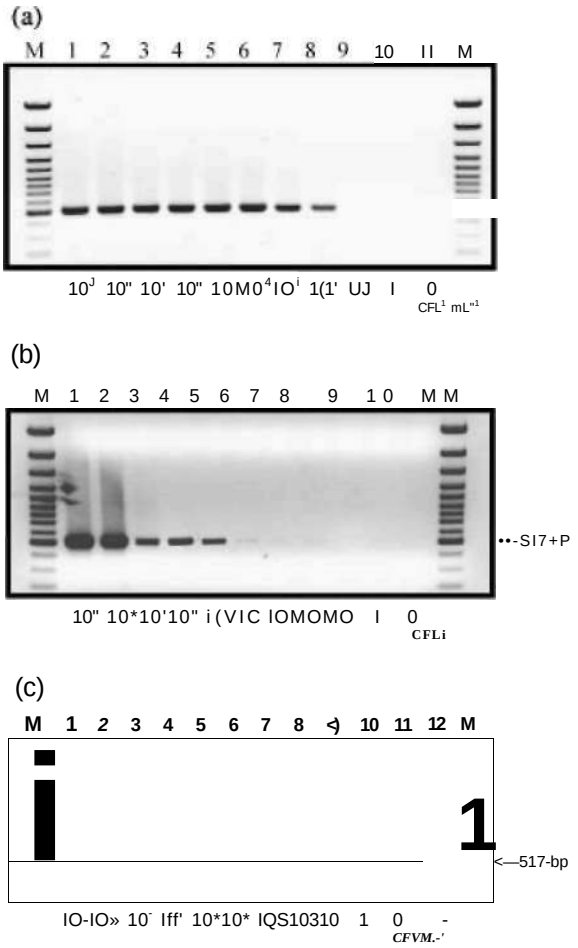


Figure 6 Sensitivity test for PCR-based detection of *Brenneria quercina* using primers REP-517-aand REP-517-b. (a) Sensitivity of the method in the analysis of pure culture samples of *B. quercina* field isolate 1940-1; lanes 1-10, PCR-amplified products obtained with decreasing concentrations of bacteria from 10^9 to 1 CFU ml_~$^{-1}$; lane 11, PCR-negative control; lane M, 100-bp DNA Ladder Plus molecular size marker, (b) Sensitivity of method in analysis of ooze from acorns; lanes 1-10, PCR-amplified products obtained with decreasing concentrations of bacteria from 10^9 to 1 CFU ml_~$^{-1}$; lane 11, PCR-negative control; laneM, 100-bp DNA Ladder Plus molecular size marker. The size of the diagnostic product (arrowed) was 517 bp. (c) Sensitivity of method in analysis of *Quercus pyrenaica* leaves inoculated with of *B. quercina* (field isolate 1940-1); lanes 1-10, PCR-amplified products obtained with decreasing concentrations of bacteria from 10^9 to 1 CFU mL~^{1} of plant extract; lane 11, PCR-negative control; lane 12, non-inoculated plant material; lane M, 100-bp DNA Ladder Plus molecular size marker.

liquid culture, acorn ooze and inoculated *Quercus* leaves. The specificity of these primers was demonstrated by absence of amplification of the unique fragment in 16 bacterial species phylogenetically related to *B. quercina* (including *E. coli),* although the possibility cannot be excluded that another bacterial field isolate could yield a positive result. Clearly, the specificity of the primers is not caused by the absence of the sequence corresponding to the *recN* gene, since this gene is widely conserved among the enteric bacteria. The data indicate that the sensitivity of the method is high, enabling the detection of *B. quercina* in concentrations as low as 10^2 CFU mL^{4}. This sensitivity is in line with other reports using PCR-based detection methods (Hauben *et al.*,1998b; Penyalver *et al.*, 2000). These are the first primers for the specific detection and identification of *B. quercina* and will be useful for the diagnosis of this pathogen in plant material showing symptoms and for the detection of its epiphytic and endophytic presence, which may improve knowledge of the epidemiology of this poorly studied bacterium.

Acknowledgements

We gratefully acknowledge Dr S. Soria (Patrimonio Nacional, Spain) for the generous supply strains and isolates supplied and Dr M. Milgroom (Cornell University, NY), Dr R. Raposo (INIA, Spain), Dr E. Lopez-Solanilla (ETSIA-UPM, Spain), Dr L. M. Rubio (University of California-Berkeley, CA) and Dr S. E. Lindow (University of California-Berkeley, CA) for the critical reading of this manuscript. We also thank Dr D. Garcia (Coleccion Espanola de Cultivos Tipo (CECT), Universidad de Valencia, Spain) for the type strains of the *Serratia* ssp. provided. We acknowledge Carlos Rojas and Joaquin Garcia-Guijarro for technical assistance. This work was financed by the Ministry of Education and Science, Project BIO-2005-07198 and by grant 1FD97-0911-C03-02 of the FEDER programme from the EU.

References

Altschul SF, Madden TL, Schaffer AA *et al.,* 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389-402.

Barionovi D, Giorgi S, Stoeger AR, Ruppitsch W, Scortichini M, 2006. Characterization of *Erwinia amylovora* strains from different host plants using repetitive-sequences PCR analysis, and restriction fragment length polymorphism and short-sequence DNA repeats of plasmid pEA29. *Journal of Applied Microbiology* 100, 1084-94.

Biosca EG, Gonzalez R, Lopez-Lopez MJ *et al.,* 2003. Isolation and characterization of *Brenneria quercina,* causal agent for bark canker and drippy nut of *Quercus* spp. in Spain. *Phytopathology* 93, 485-92.

Brown EW, Davis RM, Gouk C, van der Zwet T, 2000. Phylogenetic relationships of necrogenic *Erwinia* and *Brenneria* species as revealed by glyceraldehyde-3-phosphate dehydrogenase gene sequences. *International Journal of Systematic and Evolutionary Microbiology* 50, 2057-68.

Dawson SL, Fry JC, Dancer BN, 2002. A comparative evaluation of five typing techniques for determining the diversity of fluorescent pseudomonads. *Journal of Microbiological Methods* 50, 9-22.

Efron B, 1982. *The Jackknife, the Bootstrap and Other Resampling Plans.* Philadelphia, PA, USA: Society for Industrial and Applied Mathematics.

Grimont PA, Grimont F, De Rosnay HC, 1977. Taxonomy of the genus *Serratia. Journal of General Microbiology* 98, 39-66.

Grimont PAD, Grimont F, 1984. Genus VIII. *Serratia* Bizio 1823, 288AL. In: Krieg NR, ed. *Bergey's Manual of Systematic Bacteriology.* Baltimore, MD, USA: Williams & Wilkins, 477-84.

Hanahan D, 1983. Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* 166, 557-70.

Harris SJ, Shih YL, Bentley SD, Salmond GP, 1998. The *hexA* gene of *Erwinia carotovora* encodes a LysR homologue and regulates motility and the expression of multiple virulence determinants. *Molecular Microbiology* 28, 705-17.

Hauben L, Vauterin L, Swings J, Moore ERB, 1997. Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *International Journal of Systematic Bacteriology* 47, 328-35.

Hauben L, Moore RB, Vauterin L, Steenackers M, Mergaert J, 1998a. Phylogenetic position of phytopathogens within the *Enterobacteraceae. International Journal of Systematic and Applied Microbiology* 21, 384-97.

Hauben L, Steenackers M, Swings J, 1998b. PCR-based detection of the causal agent of watermark disease in willows *(Salix* spp.). *Applied and Environmental Microbiology* 64, 3966-71.

Hildebrand DC, Schroth MN, 1967. A new species of *Erwinia* causing the drippy nut disease of live oaks. *Phytopathology* 57,250-3.

Hulton CS, Higgins CF, Sharp PM, 1991. ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli, Salmonella typhimurium* and other *Enterobacteriaceae. Molecular Microbiology* 5, 825-34.

King EO, Ward MK, Rainey DE, 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* 44, 301-7.

Klingler JM, Stowe RP, Obenhuber DC, Groves TO, Mishra SK, Pierson DL, 1992. Evaluation of the Biolog automated microbial identification system. *Applied and Environmental Microbiology* 58, 2089-92.

Kwon SW, Go SJ, Kang HW, Ryu JC, Jo JK, 1997. Phylogenetic analysis of *Erwinia* species based on 16S rRNA gene sequences. *International Journal of Systematic Bacteriology* 47, 1061-7.

Larsen N, Olsen GJ, Maidak BL *et al,* 1993. The ribosomal database project. *Nucleic Acids Research* 21, 3021-3.

Llop P, Caruso P, Cubero J, Morente C, Lopez MM, 1999. A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. *Journal of Microbiology Methods* 37,23-31.

Louws FJ, Fulbright DW, Stephens CT, de Bruijn FJ, 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* 60, 2286-95.

Maidak BL, Olsen GJ, Larsen N, Overbeek R, McCaughey MJ, Woese CR, 1996. The ribosomal database project (RDP). *Nucleic Acids Research* 24, 82-5.

Manly BJF, 1997. *Randomization, Bootstrap, and MonteCarlo Methods in Biology,* 2nd edn. London, UK: Chapman & Hall.

Mantel N, 1967. Adaptation of Karber's method for estimating the exponential parameter from quantal data, and its relationship to birth, death, and branching processes. *Biometrics* 23, 739-46.

Nei M, 1972. Genetic distance between populations. *American Naturalist* 106,283-92.

Penyalver R, Garcia A, Ferrer A, Bertolini E, Lopez MM, 2000. Detection of *Pseudomonas savastanoi* pv. *savastanoi* in olive plants by enrichment and PCR. *Applied and Environmental Microbiology* 66, 2673-7.

Ribbeck-Busch K, Roder A, Hasse D *et al.,* 2005. A molecular biological protocol to distinguish potentially human pathogenic *Stenotrophomonas maltophilia* from plant-associated *Stenotrophomonas rhizophila. Environmental Microbiology 7,* 1853-8.

Sambrook J, Fritsch EF, Maniatis T, 1989. *Molecular Cloning: a Laboratory Manual,* 2nd edn. Cold Spring Harbor, New York, USA: Cold Spring Harbor Laboratory Press.

Sneath PHA, Sokal RR, 1973. *Numerical Taxonomy.* San Francisco, CA, USA: W H. Freeman.

Soria S, Lopez MM, Lopez Lopez MJ, 1997. Presencia, sintomatologia y danos de *Erwinia quercina* en Espana y su posible relacion con la 'seca de la encina'. *Ecologia* 11, 295-301.

Sproer C, Mendrock U, Swiderski J, Lang E, Stackebrandt E, 1999. The phylogenetic position of *Serratia, Buttiauxella* and some other genera of the family *Enterobacteriaceae. International Journal of Systematic Bacteriology* 49,1433-8.

Stern MJ, Ames GF, Smith NH, Robinson EC, Higgins CF, 1984. Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell* 37, 1015-26.

Thomson NR, Cox A, Bycroft BW, Stewart GSAB, Williams P, Salmond GPC, 1997. The Rap and Hor proteins of *Erwinia, Serratia* and *Yersinia:* a novel subgroup in a growing superfamily of proteins regulating diverse physiological processes in bacterial pathogens. *Molecular Microbiology* 26, 531-44.

Versalovic J, Koeuth T, Lupski JR, 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* 19, 6823-31.

Young JM, Park DC, 2007. Relationships of plant pathogenic enterobacteria based on partial *atpD, car A,* and *recA* as individual and concatenated nucleotide and peptide sequences. *Systematic and Applied Microbiology* 30, 343-54.

Young JM, Park DCh, Weir BS, 2004. Diversity of 16S rDNA sequences of *Rhizobium* spp. implications for species determinations. *FEMS Microbiology Letters* 238, 125-31.