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Diversity of the bacterial community in the surface soil of a pear orchard based on 16S rRNA gene analysis

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Summary. A cultivation-independent approach based on polymerase chain reaction (PCR)-amplified partial small subunit rRNA genes was used to characterize bacterial populations in the surface soil of a commercial pear orchard consisting of different pear cultivars during two consecutive growing seasons. *Pyrus communis* L. cvs *Blanquilla*, *Conference*, and *Williams* are among the most widely cultivated cultivars in Europe and account for the majority of pear production in Northeastern Spain. To assess the heterogeneity of the community structure in response to environmental variables and tree phenology, bacterial populations were examined using PCR-denaturing gradient gel electrophoresis (DGGE) followed by cluster analysis of the 16S ribosomal DNA profiles by means of the unweighted pair group method with arithmetic means. Similarity analysis of the band patterns failed to identify characteristic fingerprints associated with the pear cultivars. Both environmentally and biologically based principal-component analyses showed that the microbial communities changed significantly throughout the year depending on temperature and, to a lesser extent, on tree phenology and rainfall. Prominent DGGE bands were excised and sequenced to gain insight into the identities of the predominant bacterial populations. Most DGGE band sequences were related to bacterial phyla, such as Bacteroidetes, Cyanobacteria, Acidobacteria, Proteobacteria, Nitrospirae, and Gemmatimonadetes, previously associated with typical agronomic crop environments. [**Int Microbiol** 2010; 13(3):123-134]

Keywords: bacterial diversity · community structure · agricultural soils · fruit tree orchards · pear cultivars

Introduction

Soils have highly distinct microbial communities that reflect the interactions between many different selection factors, including the physical and chemical characteristics of the soil (e.g., soil texture, nutrient and organic matter content, and pH) and environmental factors, such as climate and vegetation [7,8,28]. Plant roots influence soil-borne microbial communities by several mechanisms, such as the secretion of specific organic compounds, competition for nutrients, and providing a solid surface for attachment [12,18,22]. Since root-released products can be highly specific for a plant species or even a particular cultivar, plants are thought to selectively enrich both their rhizospheres and the surrounding soil for microorganisms that are well adapted to the utilization of these released specific organic compounds [9]. Also, aerial plant parts that are incorporated within the soil after the growing season (e.g., annual leaf fall) may differ in their composition of sugars, organic acids, and phenolic compounds, all of which in turn are influenced by genetics, ontogenesis, and environmental conditions [1]. However, little is known about the effects of the seasonal supply of these carbon and energy sources on the microbial communities of the surface soil in fruit tree orchards.

This partial view of soil microbial communities has come about as a result of their enormous complexity and genetic diversity, and the fact that only a small portion (1–10%) of

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the microbial groups present in situ can be isolated from soil and characterized in axenic cultures [39]. DNA fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) are widely used to study the dynamics and structure of complex microbial communities [2,29,35]. These molecular approaches provide the potential for significant progress in our understanding of microbial diversity in natural ecosystems and offer a springboard for the application of genetic techniques to environmental microbiology [33,37]. However, both under- and overestimation of the real number of genotypes could occur due to the inherent methodological limitations of this PCR-dependent method [17]. Furthermore, only by coupling DGGE with sequencing it is possible to avoid incorrect interpretations of the profiles, which may result from the migration of sequences of different phylogenetic affiliations to the same gel position.

Bacterial diversity has been widely studied using DNA fingerprinting methods using soil from fields of different agricultural crops, but not from that of fruit tree orchards devoted to intensive farming. Major decomposers can be found among soil microbial communities and they are highly competitive in terms of consuming simple carbon compounds such as root exudates and fresh plant litter. Indeed, slight variations of leaf composition at the species or even cultivar level may lead to shifts in soil community composition. Furthermore, there is evidence that plants produce certain types of exudates to favor the growth of protective bacteria, and that bacteria adapt the environment for plant communities [44]. The aim of our study was to assess the diversity shifts in the surface soil bacterial communities associated with different pear cultivars over two consecutive growing seasons. *Pyrus communis* L. cvs. *Blanquilla*, *Conference*, and *Williams* are among the most widely cultivated cultivars in Europe and account for the majority of pear production in Northeastern Spain. Bacterial communities were characterized using PCR-DGGE analysis of 16S ribosomal DNA. Similarities in the microbial communities were studied through analysis of the DNA fingerprints and statistical treatment of the 16S ribosomal DNA band data. Finally, prominent bands, which presumably represent the predominant bacteria, were subjected to sequencing analysis in order to investigate the magnitude of seasonal changes in the bacterial community and the possible influence of pear cultivars on bacterial community composition.

Table 1. Physico-chemical characteristics of soil

Soil properties	Mean ± SD
pH (1:2 w/v H_2O)	8.27 ± 0.17
EC[a] (mS/cm)	1.00 ± 0.28
Apparent density (g/cm^3)	1.38 ± 0.08
Organic matter (%)	2.49 ± 0.81
Nitrogen[b] (%)	0.13 ± 0.05
C:N ratio	11.41 ± 0.77
$CaCO_3$	
total (%)	33.95 ± 3.50
active (%)	10.03 ± 1.86
Sodium (mg/kg)	175.69 ± 39.56
Potassium (mg/kg)	556.23 ± 54.25
Magnesium (mg/kg)	189.89 ± 71.09
Calcium (mg/kg)	1897.97 ± 364.99
Phosphorous (mg/kg)	34.90 ± 12.10
Chloride (mg/kg)	77.75 ± 6.74
Nitrate (mg/kg)	74.40 ± 17.36
Sulfate (mg/kg)	277. 68 ± 138.0

[a] EC: electrical conductivity.
[b] Nitrogen: ammoniacal and organic nitrogent content.

Materials and methods

Field site and sample collection. Soil samples were collected from a pear orchard cultivated using conventional farming methods and located in Epila, Zaragoza, Spain (1º 19′ 39″ W, 41º 36′ 9″ N). Pesticide treatments based on copper or contact fungicide sprays were applied to the trees under a standard schedule to control tree diseases. The tree phenology, pruning, and disease control treatments are indicated in Fig. 1 (see Results). The soil was characterized as loamy of alluvial origin, with 38.5% sand, 37.5% silt, and 24.0% clay. An overview of the soil properties is given in Table 1. Soil management was practically absent except for weed control (cutting), localized irrigation (6 l/m^2/d), and fertirrigation (200 mg/l of 20-10-20 N/P/K) applied directly to the tree bulb area. The field plot was structured in six rows of the same cultivar (either *Williams*, *Conference*, or *Blanquilla*),—all grafted on BA29 rootstock—which were delimited by two rows of pollinators (*Doyenne du Comice* cultivar). Within the experimental plot, two subplots for each cultivar were chosen for analysis: A, B (*Conference* cultivar); C, D (*Blaquilla* cultivar); and E, F (*Williams* cultivar).

Samples were collected on 20 March 2002, 11 June 2002, 26 May 2003, and 9 July 2003, that is, twice during each vegetation period, in order to reveal changes between two consecutive growing seasons and to compare the cyclic nature of the possible shifts. Additionally, during each vegetative period, samples were selected as a function of environmental variables (temperature and moisture). For each sampling time and plot, two independent soil samples were taken. Each one consisted of ten randomly selected samples taken from the corresponding trees, as close to the trunk as possible (approximately 5–10 cm from the tree trunk). The non-decomposed leaves and roots were removed and the top 5 cm of soil was collected. The samples were mixed and brought to the laboratory on ice (4ºC) and preserved at –20ºC until further processing. DNA was extracted within 24 h after sample collection. The surface soil horizons were sampled exclusively because this fraction of the sediment may be strongly influenced by various organic matter inputs from decaying plant material.

Weather variables were monitored with an automatic weather station located at the experimental orchards. Temperature and rainfall were monitored using a CR10X data-logger (Campbell Scientific, Leicester, UK) connected to a combined temperature (model HMP35AC) and rainfall (model ARG100) electronic sensor.

DNA extraction and amplification of the 16S rRNA genes. DNA from 250-mg surface soil samples was extracted using the Ultraclean Soil DNA Kit (MoBio Laboratories, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Extracted DNA was inspected on 1% agarose gels. 16S rRNA genes were amplified with the primer set 341F (*Escherichia coli* positions 341-357) with a GC clamp (40-nucleotide GC-rich sequence, 5′-CCT ACG GGA GGC AGC AG-3′) and 907RM (*E. coli* positions 926–907) (5′-CCG TCA ATT CMT TTG AGT TT-3′) specific for the domain *Bacteria*, as described by Schäfer and Muyzer [35]. PCR mixes were of a final volume of 50 µl and contained 5–50 ng of template DNA, 0.2 µM of each primer (341F with a GC clamp and 907RM), 200 µM of each dNTP, 5 µl of buffer (200 mM Tris-HCl pH: 8.4, 500 mM KCl), 2.5 µl W-1 1%, 1.5 µl of 50 mM $MgCl_2$, and 0.5 µl Taq DNA polymerase (5 U/µl, Invitrogen). Thermocycling was conducted in a minicycler TM instrument (MJ Research) and the reaction conditions were those described previously [35]. DNA was quantified electrophoretically using DNA molecular weight markers in amounts ranging from 10 to 200 ng (Invitrogen) on 2% agarose gels.

DGGE analysis of total community DNA. Five-hundred ng of PCR product was applied on a denaturing gradient gel. DGGE was carried out using a Bio-Rad DCode system, as described [35], in a 6% polyacrylamide gel with 20–80% denaturant gradient (100% denaturant contained 7M urea and 40% v/v deionized formamide). Electrophoresis was performed at 60ºC with a constant voltage of 100 V for 18 h. The gels were stained with ethidium bromide (0.5 µg/ml), then inspected under UV illumination and photographed. Prominent bands were excised from the gels, reamplified, and then purified using the PCR Clean up Kit (MoBio Laboratories) for subsequent sequencing.

Sequencing and phylogenetic analysis. Sequencing reactions were performed by Macrogen (South Korea) using the Big Dye Terminator v3.1 sequencing kit; reactions were run in an automatic capillary type ABI 3730XL analyzer-96. Sequences were first screened to detect potential chimeric artifacts using the CHIMERA_CHECK program from the Ribosome Database Project [27] and then compared to those deposited in the GenBank nucleotide database using the BLAST program [38]. Partial sequences were imported and aligned within the ARB software program, available at [http://www.arb-home.de]. The phylogenetic tree was generated on the basis of long (>1400 bp) 16S rRNA sequences using the neighbor-joining method and a mask corresponding to the nucleotide positions of the 16S rRNA of *E. coli*. Partial 16S rRNA gene sequences retrieved in this study were then added to the core trees using the ARB parsimony tool, which enables the addition of short sequences without changing the tree topology. The resulting tree was pruned to retain the closest relatives.

The 16S rRNA gene sequences determined in this study are shown in Table 2 (see Results) and are available at the EMBL database under accession numbers AM503967 through AM504020. Each band designation includes a code specifying its origin (ASZ, Agricultural Soil, Zaragoza, Spain) followed by a number indicating the order in which the sequence was isolated from the gel.

Data analysis. In accordance with previous work [35], a band in a denaturing gel was interpreted as representing a distinct sequence type or phylotype. Therefore, for cluster analysis, DGGE profiles were transformed into binary code, scoring each band position as 1 (present) or 0 (absent). Similarity matrices for all pair-wise combinations of DGGE profiles were constructed from the binary matrix using the Dice coefficient as a measure of proximity. Then, the distance matrices were used as data for hierarchical clustering by an unweighted pair group with mathematical averages (UPGMA) based on Euclidian distances. The number of bands and the relative abundance of specific bacterial groups from the different samples were treated statistically by two-way analysis of variance (ANOVA) and Levene's test for homogeneity of variance. Sampling time and cultivars were used as factors. Pair-wise multiple comparisons between all means were made using the Tukey test at $P < 0.05$. Principal-component analysis (PCA) was used to reveal relationships between biological and environmental variables. Bartlett's test for sphericity and the Kaiser-Meyer-Olkin (KMO) test of sampling adequacy were initially performed on the data and confirmed the appropriateness of conducting PCA. Data were handled using correlation on the basis of the standard Euclidean distance between samples to define their dissimilarity. Orthogonal varimax rotation with Kaiser normalization was subsequently performed to facilitate the interpretation. Only principal components with eigenvalues > 1 were retained for interpretation. These analyses were performed using the SPSS v11.0 software package. Bootstrap analysis (1000 replications) corresponding to cluster analysis was carried out using the DGGEStat program designed by Erik Van Hannen and available at [http://www.sb-roscoff.fr/marine_microbes/index.php?option=com_simpleboard&func=view&id=46].

Results

Phenological and environmental conditions. The data on tree development and the climatic parameters of temperature and rainfall are presented in Fig. 1. Pear trees vary slightly in their bloom period due to differences among cultivars but during the study period full bloom of the trees on the subplots occurred between late April and early May. *Blanquilla* bloomed first, followed by *Williams*, and then *Conference*. Harvest is also slightly variable, depending on climatological conditions, but during the study period the fruits were harvested in September, and in the same order as blooming. Leaf fall was complete by late November.

Mean monthly temperatures were maximum from June to August and minimum during December and January, for the two years of the study. Maximum rainfall was concentrated in late spring (May–June) and autumn (September–October). Globally, 2002 was drier (350 mm) than 2003 (497 mm). Therefore, sampling in March 2002 (coded as s-1) corresponded to relatively dry mild-cold conditions about five months from leaf fall, in June 2002 (s-2) to warm and wet conditions, in May 2003 (s-3) to a wet and mild temperature period, and in July 2003 (s-4) to a warm and dry period. Moreover, there was no rainfall either on the sampling days or on the days before or after.

Bacterial community structure. DNA extracted from soil samples was predominantly of high molecular weight and suitable for use as PCR templates. For the analysis, 16S rRNA gene fragments amplified from DNA extracted from the soil of *Blanquilla*, *Conference*, and *Williams* pear cultivars at each sampling time were compared by running the reaction products in parallel on the same denaturing gra-

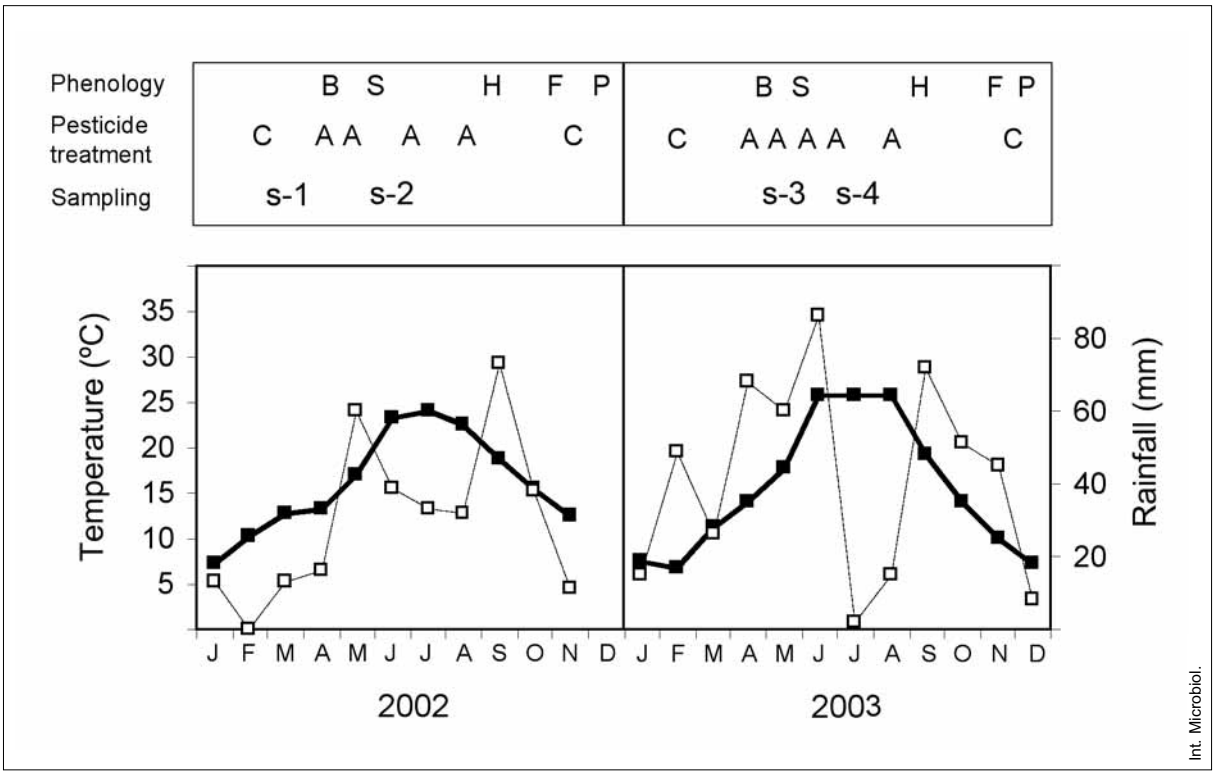


Fig. 1. (Upper panel) Phenology of the trees, pesticide treatments, and sampling schedule. The phenology of the trees corresponds to bloom (B), fruit set (S), harvest (H), leaf fall (F), and pruning (P). Pesticides sprayed on the trees were copper derivatives (C) and antifungals (A). Samplings were performed in March and June 2002 (s-1 and s-2), and in May and July 2003 (s-3 and s-4). (Lower panel) Monthly mean temperature and total rainfall in the pear orchard for the two years of the study (open squares, rainfall; closed squares, temperature).

dient gel (Fig. 2A). Samples from *Conference* cultivars (A-B) corresponding to s-2 were discarded because they repeatedly failed to amplify by PCR even when adjuvants to the PCR mix, such as DMSO (5–10%) and bovine serum albumin (0.1–0.8 µg/µl final concentration) were added. Repeated DGGE runs of the same PCR product as well as repeated PCR amplification of the same DNA extract followed by DGGE produced similar banding profiles, indicating a high degree of gel reproducibility. Furthermore, DGGE profiles corresponding to both independent replicates for each sampling time and plot showed a high degree of similarity (83–91%; data not shown).

DNA amplification produced 29–47 detectable bands of various intensities per sample that were displayed from approximately 30% to 65% denaturant. Overall, there were no statistically significant differences in ribotype richness, neither across the three pear cultivars nor over time ($p > 0.05$), with the exception of a sample from plot F corresponding to s-2 (2F).

Although the DGGE patterns exhibited many dissimilarities (Fig. 2A), a few common intensely stained bands were present in almost all of the lanes (i.e., bands 6, 8, 50, 52).

Cluster analysis. Clustering of the samples revealed that all profiles were about 75% similar (Fig. 2B). This comparison did not take into account shifts in band intensities, although direct visualization suggested evidence of changes in intensity over time. Moreover, samples from plot F corresponding to s-2 and s-4 sampling times were excluded from the analysis because of their high degree of divergence.

A comparison of the DNA fingerprints showed that similar communities were associated with the three different cultivars. The patterns in the occurrence of ribotypes revealed that there were only two specific bands for each cultivar, and that these were absent in the two other cultivars. However, only two of these bands were prominent (bands 6 and 26, specific for *Williams* and *Conference* cultivars, respectively) such that they could be recovered from the gel and sequenced (Table 2).

Regardless of the cultivars, they were differences between years and sampling times. Profiles from the year 2002 (1A–2E; 83% similarity) were clearly distinct from those of the year 2003 (3A to 4E) (Fig. 2B), while samples corresponding to 2003 were more heterogeneous. Profiles corresponding to 3B, 3D, and 3F were clearly separated from the S4 sampling time, while samples 3A, 3C, and 3E could be grouped together with

Table 2. Sequence analysis of excised DGGE bands from the agricultural soil studied

Band	Closest relative	Identity (%)	Accession no.	Phylogenetic affiliation
1, 4, 7, 10, 18, 48	*Leptolyngbya* sp.	91.4–99.4	AY239603	Cyanobacteria
2	Uncultured *Escherichia* sp.	94.0	EF662421	Gammaproteobacteria
3	*Pseudomonas rhizosphaerae*	95.7	AY866408	Gammaproteobacteria
5, 40	Uncultured bacterium	97.0–97.6	AY703467	Acidobacteria
6	*Prunus persica* chloroplast	98.4	DQ768222	Plant/chlorop.[a]
8, 19, 50	*Leptolyngbya* sp.	98.3–99.0	AJ639895	Cyanobacteria
9, 16, 51, 53	Uncultured diatom chloroplast	96.1–96.7	AY389874	Diatom/chlorop.[a]
11	Uncultured soil bacterium	97.0	AY493954	Bacteroidetes
12, 37	Uncultured bacterium	89.9–99.5	AF545645	Gemmatimonadetes
13	*Thauera* sp. R5	87.3	AB287434	Betaproteobacteria
14	*Navicula pelliculosa* chloroplast	91.4	FJ002220	Diatom/chlorop.[a]
15	Uncultured Bacteriodetes bacterium	95.0	AY921957	Bacteroidetes
17	Uncultured diatom chloroplast	91.8	AY168751	Diatom/chlorop.[a]
20, 23, 32, 33	Uncultured bacterium	90.3–95.7	AJ863255	Bacteroidetes
21	Uncultured *Nitrospira* sp.	94.3	DQ414437	Nitrospirae
22, 38	Uncultured bacterium	93.0–98.0	AY647378	Bacteroidetes
24	Uncultured soil bacterium	92.2	AY836600	Bacteroidetes
25	Uncultured bacterium	85.0	DQ270442	Acidobacteria
26	Uncultured bacterium	91.1	AY647886	Deltaproteobacteria
27	Uncultured bacterium	91.7	AF543363	Bacteroidetes
28	Uncultured *Nitrospira* sp.	90.0	EF074208	Nitrospirae
29	Uncultured *Nitrospira* sp.	99.4	DQ414438	Nitrospirae
30, 43	Uncultured *Nitrospira* sp.	98.1–99.6	EF074297	Nitrospirae
31, 46, 49	Uncultured Bacteriodetes bacterium	98.3–99.6	DQ004377	Bacteroidetes
34	Uncultured bacterium	94.3	AF234130	Chlorobi
35	Uncultured Acidobacteria bacterium	94.6	AY571790	Acidobacteria
36	Uncultured soil bacterium	95.6	DQ412816	Acidobacteria
39	Uncultured soil bacterium	96.7	AJ871258	Acidobacteria
41	Uncultured bacterium	97.6	AY921754	Gemmatimonadetes
42	Uncultured eukaryote chloroplast	94.3	AY153455	Chlorop.[a]
44, 45	*Microcoleus vaginatus*	93.5–99.8	AF355357	Cyanobacteria
47	Uncultured Acidobacteria bacterium	95.7	AY921835	Acidobacteria
52	Uncultured cyanobacterium	88.1	AY858013	Cyanobacteria
54	*Taxeobacter* sp.	95.6	AY167829	Bacteroidetes

[a] Closely related to eukaryotic chloroplast sequences.

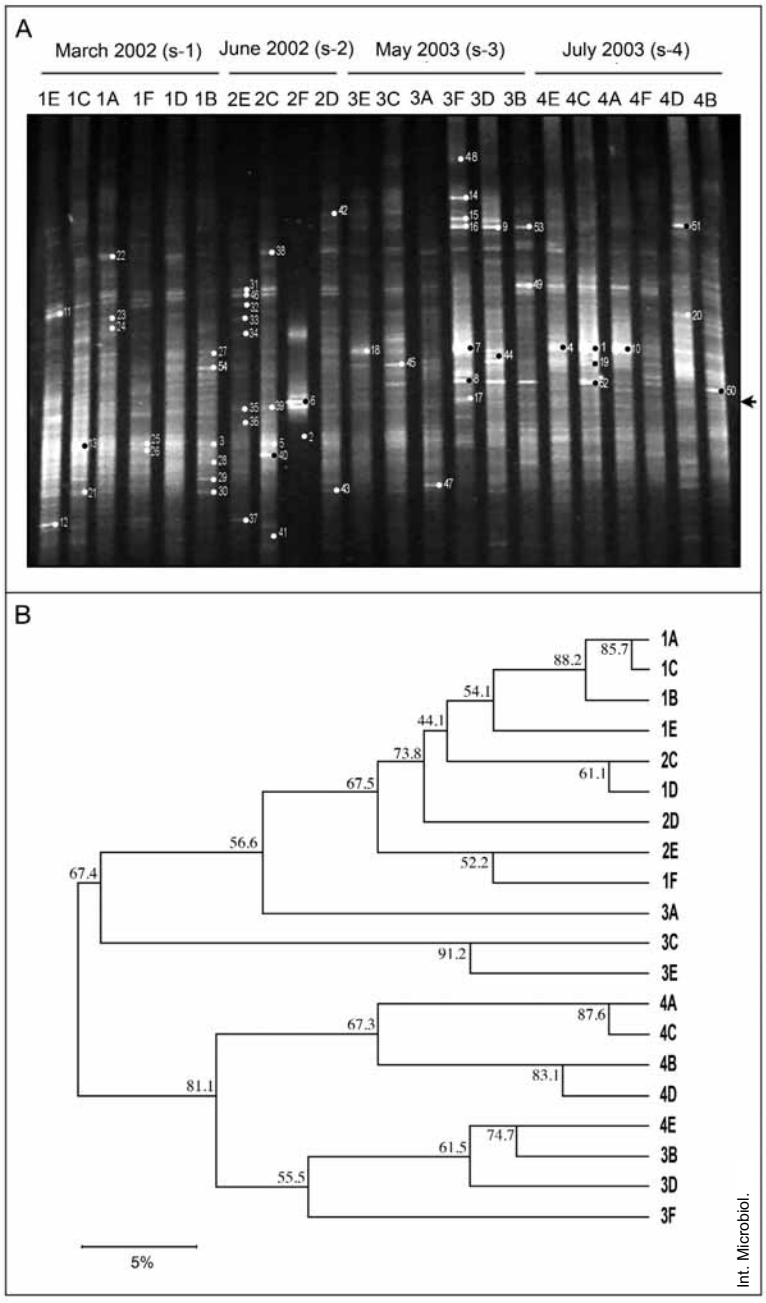


Fig. 2. (**A**) DGGE banding patterns of 16S ribosomal DNA obtained using universal primers for members of the domain *Bacteria*. Lane 1, 1E; lane 2, 1C; lane 3, 1A; lane 4, 1F; lane 5, 1D; lane 6, 1B; lane 7, 2E; lane 8, 2C; lane 9, 2F; lane 10, 2D; lane 11, 3E; lane 12, 3C; lane 13, 3A; lane 14, 3F; lane 15, 3D; lane 16, 3B; lane 17, 4E; lane 18, 4C; lane 19, 4A; lane 20, 4F; lane 21, 4D and lane 22, 4B. Each lane designation includes a number indicating the sampling time (1, March 2002; 2, June 2002; 3, May 2003 and 4, July 2003) and a letter that refers to the plot sampled (A and B, *Conference* cultivar; C and D, *Blanquilla* cultivar; E and F, *Williams* cultivar). Plant chloroplast position is indicated by the arrow. (**B)** UPGMA cluster analysis from DNA band fingerprints obtained by PCR-DGGE with specific primers for the domain *Bacteria*. The bar indicates 5% divergence. Each sample is defined by a code that includes a number indicating the sampling time (as above) and a letter that refers to the plot sampled (as above). Bootstrap values are given at nodes (1000 resamplings).

that of S4 (81% similarity). Differences were also evident for a given cultivar (A-B, C-D, E-F) between sampling times for the years 2002 (e.g., 1E compared to 2E) and 2003 (e.g., 3A compared to 4A and 3E compared to 4E).

Principal component analysis. The PCA is shown in Fig. 3. The first two principal components accounted for 65.81% of the total variance and effectively captured the main patterns of variation in the original variables. Samples separated into distinct groups in the PCA ordination revealed the connection of environmental variables and bacterial fingerprints. The most important variations were temperature-dependent. The first axis (44.82%) was positively correlated with temperature and tree phenological development; the second (20.99%) was positively correlated with cultivar. Rainfall was negatively correlated with both components. Bacterial community composition shifts, evaluated using DGGE fingerprints, were positively correlated with component 2 (PC2) and negatively correlated with component 1 (PC1). PC1 reflected seasonality, since it contained variables subject to cyclic annual variations, while PC2 had a strong biotic component, including ribotype richness and cultivars.

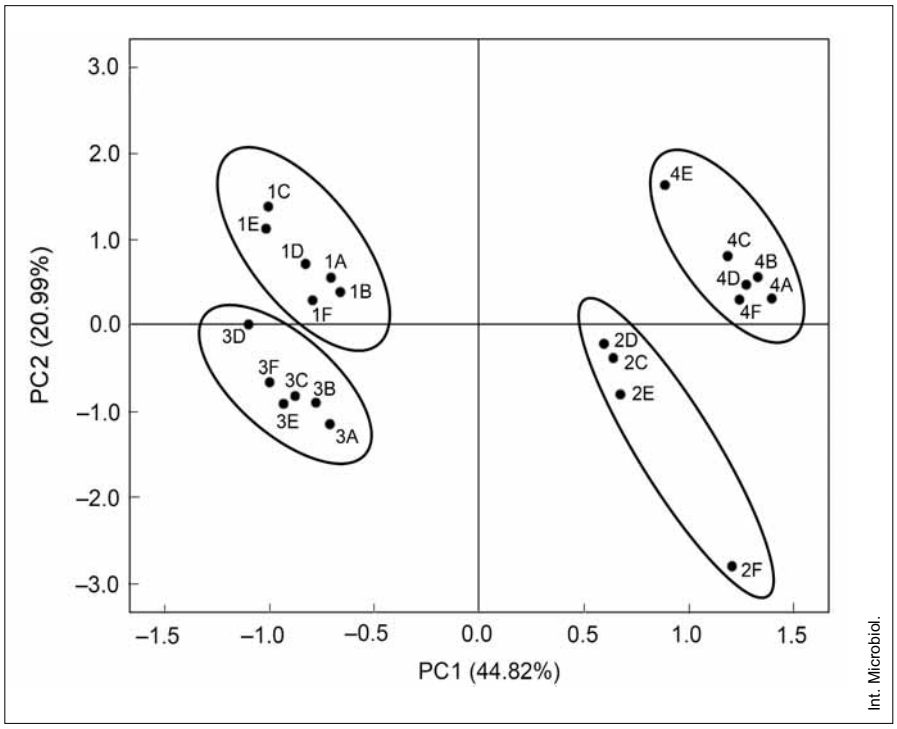


Fig. 3. Two-dimensional plot of the principal component analysis (PCA) performed for the whole dataset, including abiotic and biotic data. Each sample represented in the figure as a dot is defined by a code that includes a number indicating the sampling time (as in Fig. 2) and a letter that refers to the plot sampled (as in Fig. 2).

Bacterial identities. Fifty-five prominent bands were excised from the gel and sequenced to obtain further information about the dominant bacterial populations. The positions of the sequenced bands are shown in Fig. 2A. Most of the excised bands produced legible DNA sequences; only one band was discarded due to its ambiguous chimerical nature. Table 2 shows the sequenced bands, their similarity values compared to the most closely related GenBank sequences, and their phylogenetic affiliations. Sequence similarity values compared to previously reported sequences were 85% in all cases. Overall, 46 sequences fell into seven phyla of the domain Bacteria (Fig. 4A–D). On average, Bacteroidetes (30.4%) and Cyanobacteria (26.1%) were the most abundant phylogenetic groups, followed by Acidobacteria (15.2%), Nitrospirae (10.9 %), Proteobacteria (8.7%), Gemmatimonadetes (6.5%), and Chlorobi (2.2%). In addition, seven bands (13.0% of the total bands sequenced) were closely related to the chloroplast sequences of diatoms, which are common inhabitants of soils. In the same gel position, sequences sometimes resulted in the same phylogenetic affiliation (e.g., bands 1 and 31 compared with 4 and 49, respectively), but others were affiliated with different sequences (e.g., bands 1 and 45 compared with 27 and 54, respectively). However, bands located at the same gel site tended to have the same phylogenetic affiliation (Fig. 2A and Table 2).

Finally, on the basis of the bands sequenced, there was no clear correlation between phyla and pear cultivars. A comparison of the relative abundance of specific bacterial groups from the three pear cultivars showed no significant differences ($P > 0.05$). However, for the *Blanquilla* cultivar, phylum Cyanobacteria was more abundant than Bacteroidetes; whereas the opposite was observed for the *Conference* cultivar (Fig. 5). In addition, with respect to the phyla present in low proportions, the most relevant differences corresponded to the phyla Gemmatimonadetes and Chlorobi, since the former was only detected in *Williams* and *Blanquilla* cultivars and the latter only in *Conference*.

Discussion

Shifts of the bacterial community in surface soil fruit tree orchards. The enormous diversity in the microbial community composition of soils may be driven by factors such as crop types, physical and chemical soil properties, environmental variables, and other environmental factors (e.g., heavy-metal contamination, fertilization, and tillage) [25,28,42,46]. The first few centimeters of a soil horizon are subject to relatively extreme environmental conditions and undergo much more rapid changes, with regard to temperature, water status, and solar irradiation, than occur in deeper zones. These particular characteristics strongly affect water infiltration rates, run-off, and soil detachment [3]; thus, their impact on the biota present in the surface is much greater than on the biota from deeper soil zones.

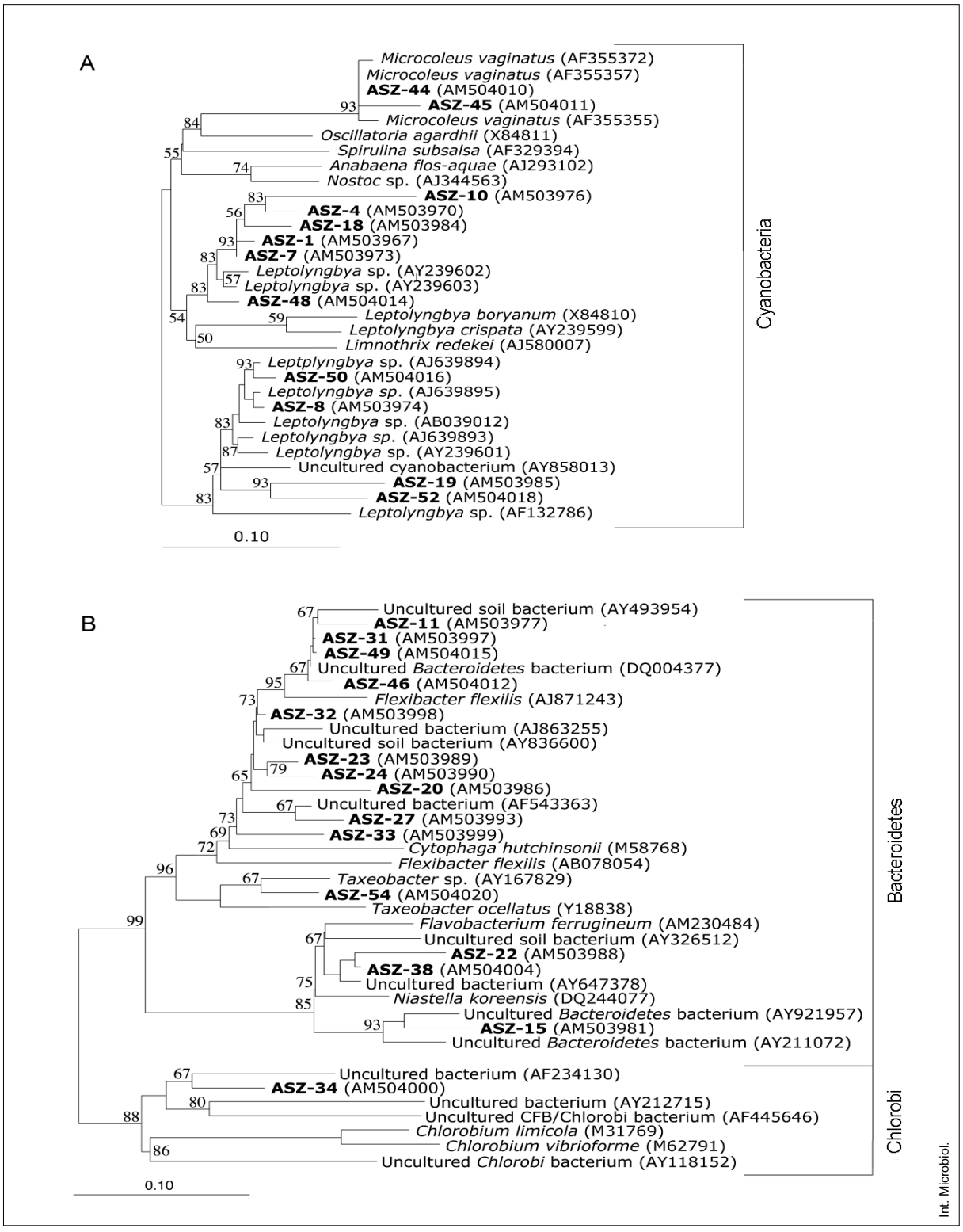
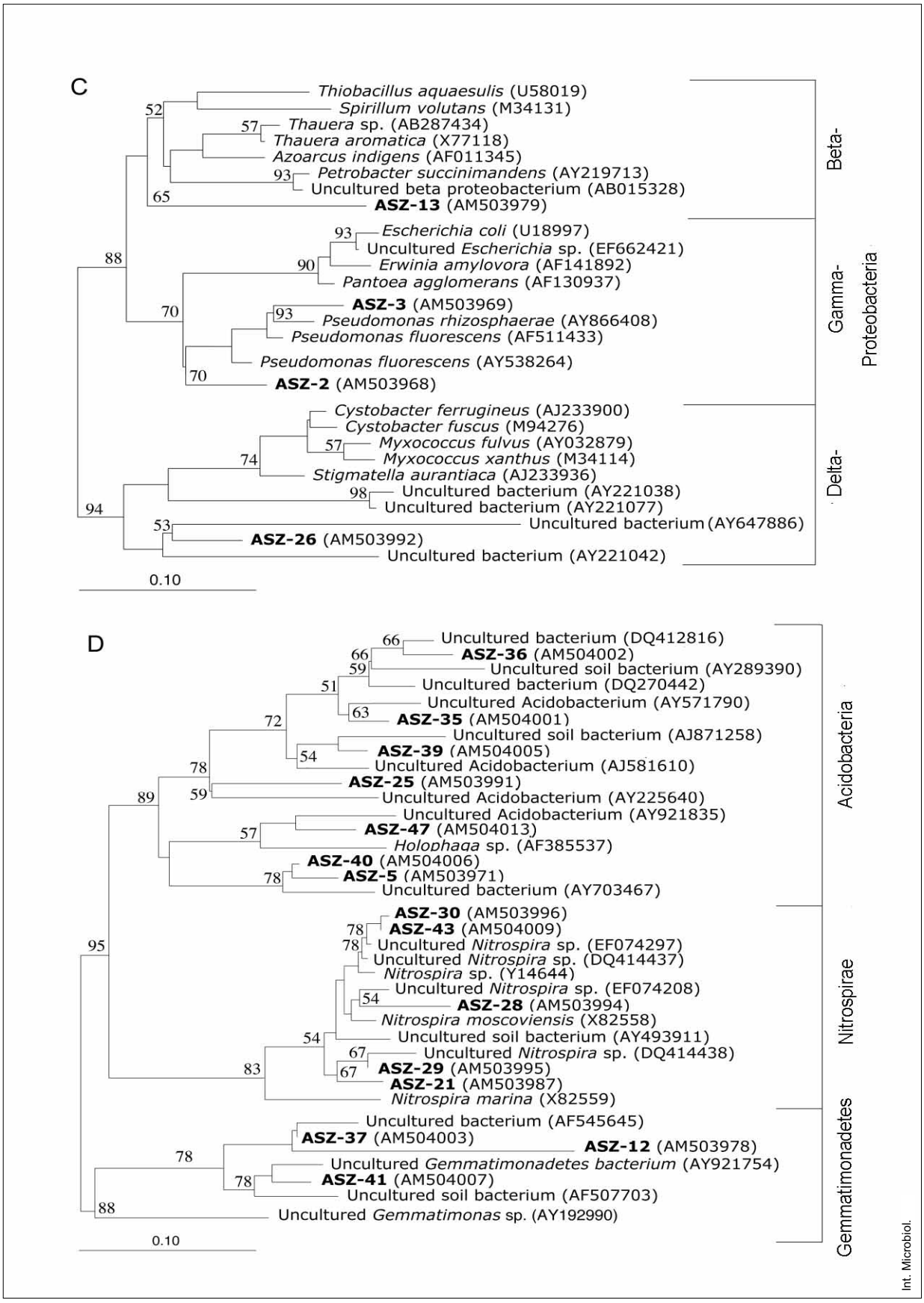


Fig. 4. Phylogenetic affiliation of 16S ribosomal DNA sequences retrieved from the agroecosystem studied, related to (**A**) Cyanobacteria, (**B**) Bacteroidetes and Chlorobi, (**C**) Proteobacteria and (**D**) Acidobacteria, Nitrospira, and Gemmatimonadetes. Trees were constructed with the neighbor-joining method using the ARB software package, including virtually complete sequences available publicly and selected according to initial BLAST similarity to our novel (partial) sequences. Partial sequences were then inserted into the corresponding tree using maximum parsimony without changing the overall tree topology. Scale bar represents 10% sequence divergence. Bootstrap values (>50%) are indicated at nodes (1000 replications).


C
Thiobacillus aquaesulis (U58019)
Spirillum volutans (M34131)
Thauera sp. (AB287434)
Thauera aromatica (X77118)
Azoarcus indigens (AF011345)
Petrobacter succinimandens (AY219713)
Uncultured beta proteobacterium (AB015328)
ASZ-13 (AM503979)
Escherichia coli (U18997)
Uncultured Escherichia sp. (EF662421)
Erwinia amylovora (AF141892)
Pantoea agglomerans (AF130937)
ASZ-3 (AM503969)
Pseudomonas rhizosphaerae (AY866408)
Pseudomonas fluorescens (AF511433)
Pseudomonas fluorescens (AY538264)
ASZ-2 (AM503968)
Cystobacter ferrugineus (AJ233900)
Cystobacter fuscus (M94276)
Myxococcus fulvus (AY032879)
Myxococcus xanthus (M34114)
Stigmatella aurantiaca (AJ233936)
Uncultured bacterium (AY221038)
Uncultured bacterium (AY221077)
Uncultured bacterium (AY647886)
ASZ-26 (AM503992)
Uncultured bacterium (AY221042)
0.10
Beta-Proteobacteria
Gamma-Proteobacteria
Delta-
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Uncultured bacterium (DQ412816)
ASZ-36 (AM504002)
Uncultured soil bacterium (AY289390)
Uncultured bacterium (DQ270442)
Uncultured Acidobacterium (AY571790)
ASZ-35 (AM504001)
Uncultured soil bacterium (AJ871258)
ASZ-39 (AM504005)
Uncultured Acidobacterium (AJ581610)
ASZ-25 (AM503991)
Uncultured Acidobacterium (AY225640)
Uncultured Acidobacterium (AY921835)
ASZ-47 (AM504013)
Holophaga sp. (AF385537)
ASZ-40 (AM504006)
ASZ-5 (AM503971)
Uncultured bacterium (AY703467)
ASZ-30 (AM503996)
ASZ-43 (AM504009)
Uncultured Nitrospira sp. (EF074297)
Uncultured Nitrospira sp. (DQ414437)
Nitrospira sp. (Y14644)
Uncultured Nitrospira sp. (EF074208)
ASZ-28 (AM503994)
Nitrospira moscoviensis (X82558)
Uncultured soil bacterium (AY493911)
Uncultured Nitrospira sp. (DQ414438)
ASZ-29 (AM503995)
ASZ-21 (AM503987)
Nitrospira marina (X82559)
Uncultured bacterium (AF545645)
ASZ-37 (AM504003)
ASZ-12 (AM503978)
Uncultured Gemmatimonadetes bacterium (AY921754)
ASZ-41 (AM504007)
Uncultured soil bacterium (AF507703)
Uncultured Gemmatimonas sp. (AY192990)
0.10
Acidobacteria
Nitrospirae
Gemmatimonadetes
Int. Microbiol.

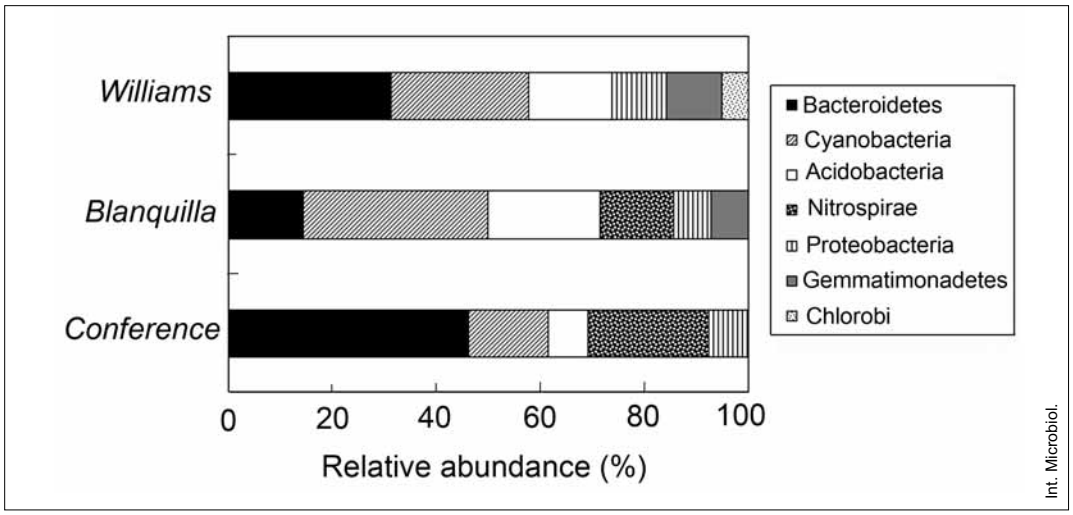

Fig. 5. Relative phylum abundance from the three pear cultivars.

In this study, PCA revealed that seasonal changes were responsible for the main variations during the growing season in the structure of the surface soil bacterial community, with the close relationship between temperature and the first principal component ($r > 0.9$) being particularly relevant. The fact that the behavior of rainfall was different from that of the temperature regime was due to seasonal differences. However, the loadings in both components grouped samples as a function of seasonality. Temperature and moisture gradients influence soil biogeochemical processes and play an important role in determining differences in the composition of released nutrients. Therefore, nutrient shifts may profoundly affect both the microbial community and its activity; in turn, the composition of soil populations is a key factor in the dynamics of organic-matter decomposition and turnover rates.

Another source of variation in the bacterial populations was related to the pear trees. Cultivars were closely related ($r > 0.7$) to the second principal component and also contributed to explaining the variations in the soil samples analyzed but to a lesser degree (Fig. 3). Distinct cultivars differ with respect to the chemical composition of their leaves and fruits [1,13], which may in part reflect differences in nutrient supply to soil microorganisms upon leaf fall. Organic matter degradation is the primary function of soil microorganisms, and plant residues are usually considered the largest contributors to organic matter in soil. While there is a lack of information about leaf-litter decomposition rates in orchard soils, investigations carried out to date have provided evidence that these values are highly heterogeneous and may range from 0.03 to 0.76 per year depending on many factors, especially climate and vegetation type [40,45]. The results obtained in this study suggested that differences in the leaf compositions of the pear cultivars *Conference*, *Blanquilla*, and *Williams* are not sufficient to strongly alter the microbial composition of the soil surface after leaf fall and leaf degradation. However, it cannot be ruled out that both climate and organic matter from leaf litter undergo similar temporal variations, or even that the effect of leaf-litter input is diluted in the presence of very high decomposition rates.

The surface-soil bacterial community. The largest proportion of bands recovered and sequenced from the surface soil of the pear orchard in this study belonged to Bacteroidetes (ca. 30%) and Cyanobacteria (ca. 26%). The former group has a high diversity of phenotypes and is one of the major bacterial groups detected in agricultural soils [5]. Members of this phylum have been implicated in the degradation of lignocellulosic litter due to their ability to degrade complex biopolymers. The latter group comprises the major components of the phototrophic bacterial assemblage. Cyanobacteria are typically found in soil crusts and laminated ecosystems. In these extreme environments, which are characterized by sharp fluctuations of environmental factors, they are considered to play a fundamental role, together with diatoms, in soil stability and nutrient cycling [41]. The particular characteristics of the surface soil could favor the more competitive members of Cyanobacteria due to their physiological flexibility [23,32,36].

Acidobacteria and Proteobacteria, the third and fourth most dominant phyla based on sequence analysis of excised DGGE bands, are two of the most abundant phyla in agricultural soils [5,6,10,11,21,26]. Several works have suggested that the ratio between the ribotype numbers of Acidobacteria

and Proteobacteria increase with trophic status, ranging from 0.16 in an oligotrophic soil [10] to 0.87 in a high-input agricultural soil [30]. The ratio in the pear orchard in this study was 0.57, which agrees with the above-mentioned values.

Members of the phyla Nitrospirae, Gemmatimonadetes, and Chlorobi were detected as minor components in the pear-orchard soil. Nitrospirae is ubiquitous in terrestrial environments and is thought to play a major role in the biological nitrogen cycle. At least four subgroups can be clearly delineated within the phylum Gemmatimonadetes, one of which is restricted to soil and other terrestrial ecosystems [34], but their functional significance in such ecosystems is not well understood. Chlorobi comprises a group of anoxygenic phototrophic bacteria with close affinity to Bacteroidetes [19] and includes a significant group of unclassified members closely related to heterotrophic degraders, some of them forming symbiotic consortia [43] (Fig. 4B).

Gram-positive bacteria, which are usually detected in soils by culture-based methods, were not detected in this work. These bacteria likely formed only a minor fraction of the surface soil analyzed. Several works have indicated that gram-positive bacteria account for >10% of the soil-retrieved clones from different geographical locations [9,11,24,26], whereas others have not detected representatives of this bacterial group [20,31]. The relative abundances of gram-positive bacteria tend to increase with depth, whereas the highest gram-negative bacteria abundances are found at the soil surface [4,16]; these shifts have been attributed to changes in soil resource availability. This observation is consistent with the results obtained in the present study, which was based on surface soil samples, where microbial populations, such as gram-negative bacteria, that are more dependent on inputs of fresh organic material could be enhanced.

The fairly stable DGGE-band patterns observed indicated that a certain number of bacteria found in the agronomic crop system represented by a pear orchard are well adapted to survive under changing environmental conditions. Reports of spatial homogeneity in soil over distances ranging from several hundred meters to soils from different continents [5,6,14,30] suggest that certain characteristics of soil environments lead to overall similarities, permitting an almost universal presence of the most successful bacterial groups. Furthermore, habitat specificity is not prohibitive. The immense heterogeneity and complexity within the soil environment together result in the formation of many discrete microhabitats suitable for populations that are well adapted to these microenvironments. Such populations may be evident in DGGE profiles as a series of discrete bands of different intensities, sometimes difficult to analyze, but responsible for the changes observed.

Recently, Fierer et al. [15], based upon their own results as well as meta-analyses, introduced two useful terms, “copiotrophs” and “oligotrophs,” to classify abundant bacterial phyla in soil ecosystems. These terms correspond to the classic *r*- and *K*-strategies used to describe ecological traits of plants and animals. In their work, the authors found a negative correlation of Acidobacteria abundance and carbon availability, which corroborates that this phylum has oligotrophic attributes, while for Betaproteobacteria and Bacteroidetes, both copiotrophs, a positive correlation between carbon availability and bacterial abundance was established. According to Fierer et al. [15], Proteobacteria are more abundant in rhizosphere soils than in bulk soils, while Acidobacteria are less abundant in rhizosphere soils than in bulk soils. Bacteroidetes are equally abundant in both ecosystems, but their abundances are highly variable. Furthermore, Alphaproteobacteria and gram-positive bacteria do not respond in any predictable manner to changes in carbon availability and both show a high degree of variability between experimental replicates. These observations could further our understanding of the structure and function of soil bacterial communities in general, and our own data in particular.

Overall, similarity analysis of band profiles revealed that temperature was responsible, to a great extent, for the changes observed in the bacterial community structure in the pear-orchard agroecosystem of this study. Differences in the organic matter composition of fallen leaves from distinct cultivars were far less important. The functional roles of most of the phyla detected in the pear orchard’s surface soil ecosystem remain unknown, due to the lack of cultivated representatives. Nonetheless, the widespread and possibly ubiquitous distribution of many of these phyla suggests that they play major roles in plant-soil-microbes interactions.

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