Environmental Microbiology (2006) **8**(12), 2136–2149 doi:10.1111/j.1462-2920.2006.01096.x

Cultivation-independent analysis of *Pseudomonas* species in soil and in the rhizosphere of field-grown *Verticillium dahliae* host plants

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Summary

Despite their importance for rhizosphere functioning, rhizobacterial *Pseudomonas* spp. have been mainly studied in a cultivation-based manner. In this study a cultivation-independent method was used to determine to what extent the factors plant species, sampling site and year-to-year variation influence *Pseudomonas* community structure in bulk soil and in the rhizosphere of two *Verticillium dahliae* host plants, oilseed rape and strawberry. Community DNA was extracted from bulk and rhizosphere soil samples of flowering plants collected at three different sites in Germany in two consecutive years. *Pseudomonas* community structure and diversity were assessed using a polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) system to fingerprint *Pseudomonas*-specific 16S rRNA gene fragments amplified from community DNA. Dominant and differentiating DGGE bands were excised from the gels, cloned and sequenced. The factors sampling site, plant species and year-to-year variation were shown to significantly influence the community structure of *Pseudomonas* in rhizosphere soils. The composition of *Pseudomonas* 16S rRNA gene fragments in the rhizosphere differed from that in the adjacent bulk soil and the rhizosphere effect tended to be plant-specific. The clone sequences of most dominant bands analysed belonged to the *Pseudomonas fluorescens* lineage and showed closest similarity to culturable *Pseudomonas* known for displaying antifungal properties. This report provides a better understanding of how different factors drive *Pseudomonas* community structure and diversity in bulk and rhizosphere soils.

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Introduction

Verticillium dahliae causes wilt of a broad range of crop plants and significant annual yield losses worldwide (Tjamos *et al*., 2000). Control of *V. dahliae* in soil had been largely dependent on the application of methyl bromide in the field. As this ozone-depleting soil fumigant has been recently phased-out, the use of alternative, ecologically friendly practices to combat *V. dahliae* is a subject of increasing interest (Martin, 2003). Proper rhizosphere management is of utmost importance when implementing such strategies. Recently, efforts have been made to better understand the structure and diversity of microbial communities in the rhizosphere of different *V. dahliae* host plants. Smalla and colleagues (2001) have shown major shifts in bacterial community structure in response to plant species and plant development in the rhizosphere of potato, strawberry and oilseed rape grown at one field site, with the most pronounced changes observed at the time of flowering. Costa and colleagues (2006) have investigated the selective force exerted by plant roots in shaping community structure of different microbial guilds in the rhizosphere of strawberry and oilseed rape at three different geographic locations. The so-called 'rhizosphere effect' on indigenous bulk soil microbial communities was found to be characterized by a reduced diversity in the rhizosphere compared with the bulk soil for all taxa studied. Culture-dependent studies conducted by Berg and colleagues (2002; 2006) have shown that *Pseudomonas* spp. were the most abundant group of culturable rhizobacteria displaying *in vitro* antagonistic activity towards *V. dahliae.* Considering such an impressive potential of *Pseudomonas* for the biocontrol of *V. dahliae* in the field, a better comprehension of the geographical distribution, community structure and diversity of the members of this genus in the rhizosphere of *V. dahliae* host plants is of fundamental need.

As *Pseudomonas* spp. are supposed to be easy to cultivate and amenable to genetic manipulation (Haas and Keel, 2003), their study in soil environments has been almost exclusively performed by means of culture-dependent approaches. Primers designed to specifically targeting *Pseudomonas* (*sensu stricto*) 16S rRNA gene fragments have first been developed by Widmer and colleagues (1998). Since then culture-independent studies performed to assess the diversity of *Pseudomonas* 16S rRNA genes polymerase chain reaction (PCR)-amplified from soil community DNA have been scarcely reported (Gyamfi *et al*., 2002; Garbeva *et al*., 2004; Milling *et al*., 2004; Pesaro and Widmer, 2006). DNA fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) overcome limitations related to preferential culturing and allow a fast analysis of multiple environmental samples. They are, thus, advantageous for a broader and less biased evaluation of the impact of major forces (e.g. agricultural management, environmental triggers, pollutants, plant species) in driving microbial community structure in the rhizosphere. No such studies on the distribution, abundance and diversity of *Pseudomonas* spp. in the rhizosphere of field-grown *V. dahliae* host plants have been conducted so far.

We report on a culture-independent study of *Pseudomonas* spp. occurring in bulk soils and in the rhizosphere of flowering, field-grown strawberry and oilseed rape, two *V. dahliae* host plants, over two consecutive years. We have used a PCR-DGGE system to specifically fingerprint *Pseudomonas* 16S rRNA gene fragments amplified from a large set of soil DNA samples. Factors affecting the distribution, abundance and diversity of *Pseudomonas* spp. in soil were surveyed, such as rhizosphere selection of soil populations, plant specificity and the effects of sampling site and year-to-year variation on these two attributes. To determine to what extent such factors influence *Pseudomonas* structure and diversity in the rhizosphere, DGGE fingerprints were analysed using different statistical tools. To identify ribotypes behind dominant or differentiating *Pseudomonas* populations in bulk and rhizosphere soils, DGGE bands were reamplified, cloned and sequenced. Results are discussed in the context of biological control.

Results

The impact of geographic location, plant species and year-to-year variation on Pseudomonas *community structure in rhizosphere and bulk soils*

Pseudomonas-specific DGGE profiles obtained for bulk soils collected at Braunschweig, Berlin and Rostock markedly differed from each other (Fig. 1a and c), with fingerprints from each sampling site forming well-defined groups after cluster analysis (data not shown). On the other hand, such a clear differentiation was not observed when rhizosphere samples collected at these same locations were compared. Especially in 2003 (Fig. 1c and d), rhizosphere DGGE profiles from different sampling sites shared higher similarity indices than did their corresponding bulk soil samples (data not shown). In order to test for the significance of this trend, pairwise permutation tests were performed using matrices of similarity from two different gels (for instance, gels 1a and 1b or 1c and 1d). Results are shown in Table 1. For samples collected in 2003, rhizosphere DGGE fingerprints of all sampling sites were considered to be more similar to each other than their corresponding bulk soil profiles ($P < 0.05$) (Table 1). Ordination techniques were used to find out to what extent factors such as geographic location, plant species and year-to-year variation contributed to the differences in ribotype composition and abundance in bulk and rhizosphere soils of flowering plants as revealed by DGGE fingerprinting. Figure 2a is an ordination biplot of samples fingerprinted by DGGE and environmental variables as determined by redundancy analysis (RDA). Each symbol represents the DGGE profiles of four replicates treated as blocks. Nominal (qualitative) environmental variables were plotted according to their centroid position in the ordination diagram. The factors sampling site, plant species and year, in this order, significantly influenced the variation in the relative abundance ($P < 0.05$) of DGGE bands. The distribution of samples in the ordination diagram followed

Table 1. Pairwise comparisons between similarity measures obtained within rhizosphere and bulk soil replicates collected at different sampling sites.

	Oilseed rape			Strawberry		
Year	BS vs. B	BS vs. R	B vs. R	BS vs. B	BS vs. R	B vs. R
2002	**0.25623**	0.02837	**0.85413**	**0.20079**	0.02837	**0.45675**
2003	0.02825	0.02837	0.0284	0.02825	0.02837	0.0284

Values of *P* indicate whether measures of similarity among rhizosphere samples from different sites are higher than the values of the corresponding soil samples ($P < 0.05$). Values in bold indicate no significant differences.
BS, Braunschweig; B, Berlin; R, Rostock.

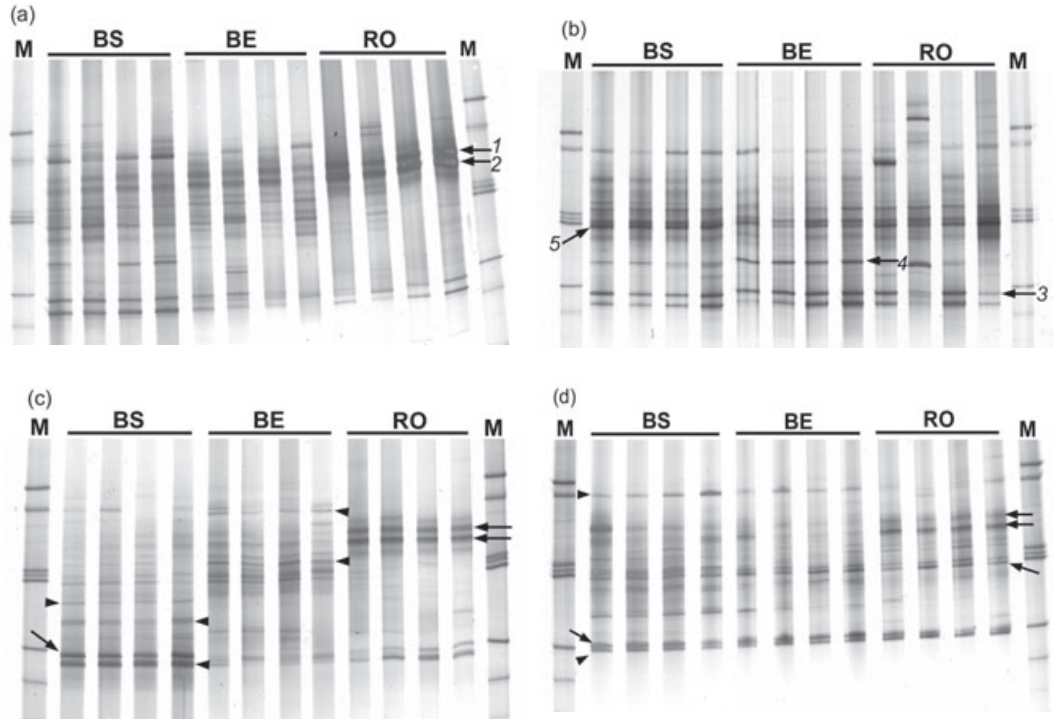

Fig. 1. Sampling-site comparisons among DGGE fingerprints of *Pseudomonas*-specific 16S rRNA gene fragments PCR-amplified from whole-community environmental DNA. Four replicates per field treatment are shown.
a and c. Bulk soil fingerprints obtained in 2002 and 2003 respectively.
b and d. Strawberry rhizosphere fingerprints, years 2002 and 2003 respectively. Numbered arrows in (a) and (b) indicate dominant or differentiating DGGE ribotypes which were extracted from these gels and submitted to cloning and sequencing (see Table 2). Short arrows in (c) and (d) show differentiating ribotypes. Long arrows in (c) and (d) resemble the ribotypes shown in (a) and (b). BS, Braunschweig; BE, Berlin; RO, Rostock; M, marker.

mainly a gradient determined by the factors plant species and year along the first axis and sampling site along the second axis, accounting for 35.6% and 26.0% of the variation in DGGE ribotype/environment correlations respectively. To assess the impact of field treatments on *Pseudomonas* community structure in the rhizosphere, we have run RDA taking exclusively the rhizosphere fingerprints into account, without considering bulk soil fingerprints. Sampling site was again the major factor affecting the relative abundance and distribution of PCR-DGGE ribotypes, but the factor year-to-year variation was considered to have a slightly greater impact than plant specificity on ribotype composition in this case. When plotted in the ordination space, rhizosphere samples followed a similar pattern as displayed in Fig. 2a (data not shown). Figure 2b represents a general perspective on the richness (i.e. quantity of bands) of *Pseudomonas* DGGE fingerprints analysed in this study. The graphic shows the distribution of all bulk and rhizosphere soil samples in the ordination space as determined by RDA. The higher the richness of a given profile, the higher the area of the symbols plotted in the ordination space. No obvious trend was found in the differences of 16S rRNA gene richness between rhizosphere and bulk soils as fingerprinted by DGGE. While rhizosphere fingerprints of samples from Rostock displayed a higher richness than some of their corresponding bulk soil samples in both years, this striking observation was not made for the other sampling sites and eventually soil samples displayed higher richness than rhizosphere samples at sampling sites Berlin and Braunschweig (Fig. 2b).

The rhizosphere effect on Pseudomonas *diversity at each sampling site*

We have performed a more detailed evaluation of the rhizosphere effect on *Pseudomonas* community structure and diversity by performing RDA separately for each sampling site and estimating Shannon's diversity indices for each DGGE profile. Figure 3 shows DGGE fingerprints obtained for samples collected in 2002 (Fig. 3a, c and e) and RDA ordination plots of rhizosphere and bulk soil samples collected at each sampling site in both years (Fig. 3b, d and f). Distribution of samples in the ordination diagram was strongly influenced by both factors, the plant species and the growing season (2002 or 2003). For all

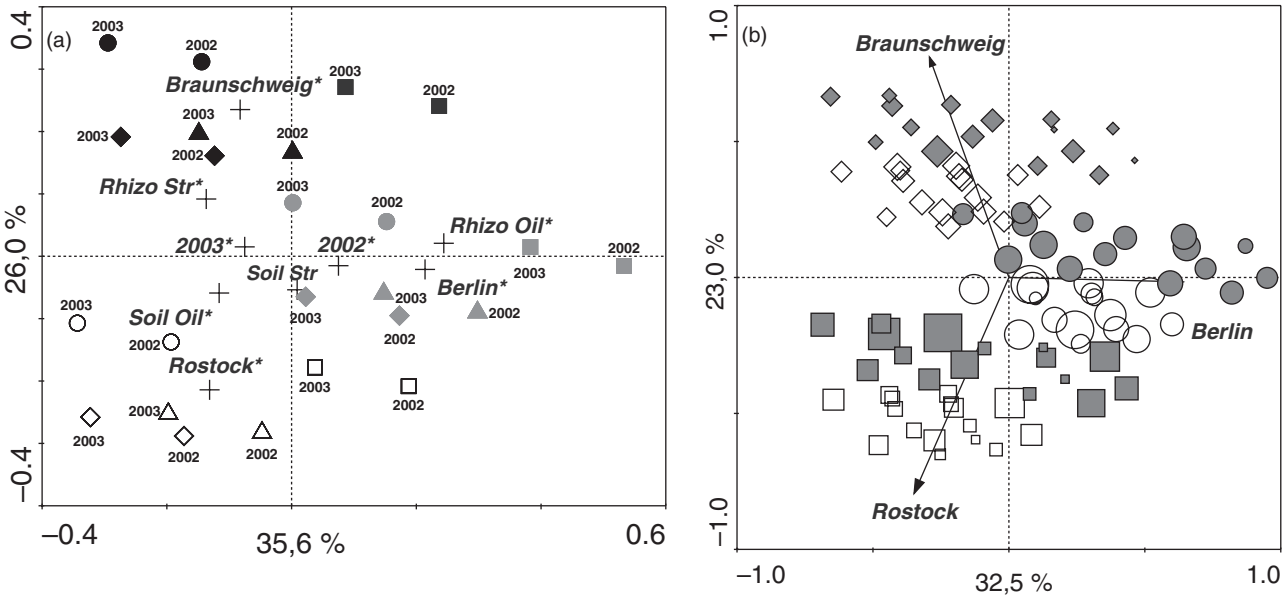

Fig. 2. Ordination biplots generated by redundancy analysis (RDA) using DGGE fingerprints (i.e. band positions and relative intensities) of *Pseudomonas* 16S rRNA gene fragments as predictors of environmental samples.
a. Redundancy analysis ordination of bulk and rhizosphere soil samples ($n = 96$). Each symbol represents the profiles of four replicates which were used in the statistics as blocks. Crosses represent the centroid positions of environmental (nominal) variables. Asterisks indicate those environmental variables found to have a significant ($P < 0.05$) influence on abundance variation of DGGE ribotypes. Labels displayed on diagrams' axes refer to the percentage variation of PCR-DGGE ribotypes–environment correlation accounted for the respective axis. Black symbols: samples from the Braunschweig site. Grey and white symbols: samples collected in Berlin and Rostock respectively. Squares and circles: Oilseed rape and strawberry rhizosphere samples respectively. Diamonds and triangles: Bulk soil samples from oilseed rape and strawberry fields respectively. Year in which samples were collected (2002 or 2003) is shown.
b. Overall comparison between bulk and rhizosphere soil ribotype richness as revealed by DGGE. Samples ($n = 96$) are plotted individually in the ordination space as determined by RDA. The higher the richness of a given DGGE profile, the higher the area of the symbols. Only the 'sampling site' variables, represented by arrows to facilitate visual interpretation, are shown. Solid symbols: rhizosphere samples; empty symbols: soil samples. Diamonds: samples collected in Braunschweig; circles: Berlin; squares: Rostock.

sampling sites, rhizosphere fingerprints were grouped separately from the corresponding bulk soil fingerprints. Overall, RDA data supported the results obtained by cluster analysis and pairwise permutation tests (data not shown). Although it was clear that the rhizosphere imposed a selective force on soil-borne *Pseudomonas* populations, the rhizosphere effect was rather different at different sampling sites and in the rhizosphere of the two plant species. For instance, diversity measures estimated for oilseed rape rhizosphere and bulk soil samples from Braunschweig in 2002 followed the typical description normally associated to the rhizosphere effect: fewer and more abundant populations (PCR-DGGE ribotypes) in the rhizosphere in comparison to bulk soil profiles, where the presence of a higher number of fainter bands leads to higher evenness and less dominance, enhancing diversity (Fig. 3a and b). On the other hand, DGGE fingerprints of samples collected in Berlin in 2002 revealed different community structures in both rhizospheres compared with bulk soil without a significant change in diversity (Fig. 3c and d). After repeated cultivation in 2003, DGGE profiles of bulk soils showed a higher diversity ($P < 0.05$) than their corresponding rhizosphere samples at this site (Fig. 3d). The factor plant specificity seemed to be of particular importance at this sampling site: Strawberry and oilseed rape *Pseudomonas* rhizosphere communities differed markedly from each other (Fig. 3c and d). In addition, plant-specific DGGE ribotypes were found in Berlin: bands 4 and 6 were found to be positively correlated to the strawberry and oilseed rape rhizospheres respectively (Fig. 4). In contrast, at the sampling site Rostock, rhizosphere fingerprints of both strawberry and oilseed rape were characterized by higher diversity than bulk soil profiles (Fig. 3e and f) in both years. Here, bulk soil fingerprints were strongly dominated by DGGE bands 1 and 2 (Fig. 1a) and the presence of a higher number of bands in rhizosphere samples was evident mainly in 2002. Although clearly different from those obtained for bulk soil samples, *Pseudomonas* rhizosphere DGGE fingerprints could not be regarded as plant-specific at the Rostock site (Fig. 3f). To avoid superposition of rhizosphere samples in the ordination diagram, Fig. 3f shows results obtained only for samples collected in 2002. The higher diversity in the rhizosphere of both plant species was also observed in 2003 and can be easily depicted for the strawberry rhizosphere by comparing Fig. 1c and d. A rather striking

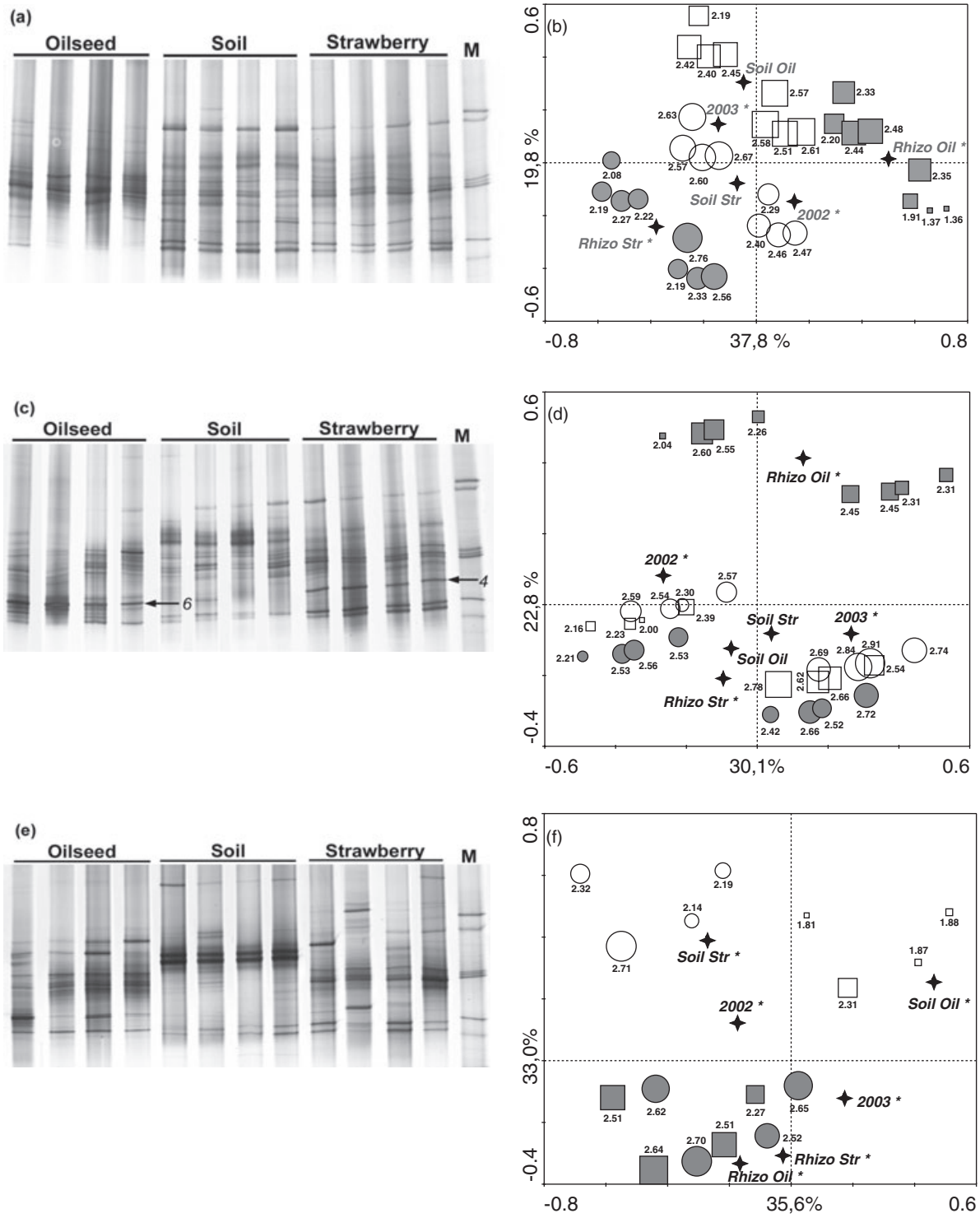

Fig. 3. Analysis of diversity, plant specificity and seasonal variation according to RDA ordination of bulk and rhizosphere soil samples fingerprinted by *Pseudomonas* PCR-DGGE. Denaturing gradient gel electrophoresis profiles obtained for samples collected in 2002 at the sites Braunschweig (a), Berlin (c) and Rostock (e) are shown. Their respective RDA ordination diagrams, including also the samples fingerprinted in 2003, are displayed (b, d and f). Symbols' areas correspond to Shannon's indices of diversity estimated for each DGGE profile. Percentage variances of PCR-DGGE ribotypes–environment correlations accounted for each ordination axis are shown. Solid symbols: rhizosphere samples; empty symbols: bulk soil samples; squares: oilseed rape; circles: strawberry. Stars represent the centroid position of nominal environmental variables; asterisks indicate variables that contributed significantly to variation in ribotype data ($P < 0.05$). Arrows in (c) indicate bands which were sequenced and cloned. To avoid superposition of samples and allow visual interpretation, only samples collected in 2002 are shown in the ordination diagram of the Rostock site (f).

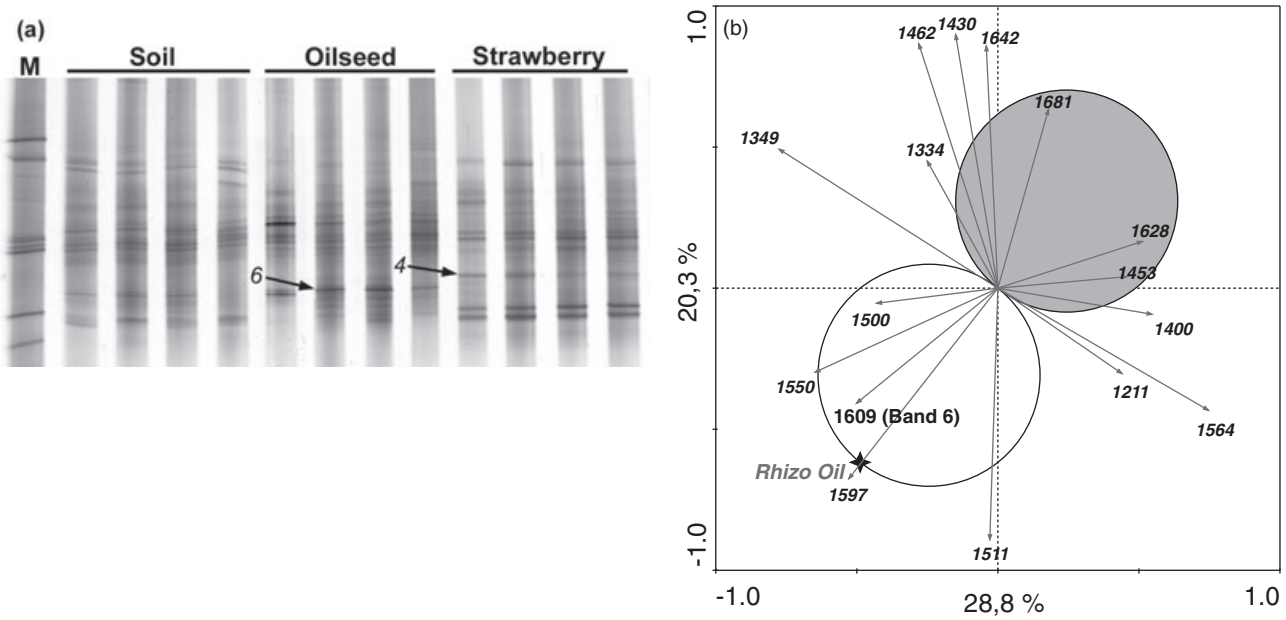

Fig. 4. a. *Pseudomonas*-specific DGGE fingerprints of 16S rRNA gene fragments PCR-amplified from bulk and rhizosphere DNA samples collected at sampling site Berlin, year 2003. Arrows indicate the cloned and sequenced DGGE bands 4 and 6.
b. Redundancy analysis *t*-value biplot of DGGE ribotypes and environmental variables showing a significant correlation between band 6 (position '1609' on DGGE profiles; *Pseudomonas aeruginosa*-like sequences) and the environmental factor 'oilseed rape rhizosphere' (Rhizo Oil). Other environmental variables were suppressed from the ordination space to facilitate visual interpretation. Modified RDA settings were used to search for DGGE bands, represented by arrows (numbers at the arrow tips refer to band positions on DG-gels), found to display large variance in relative abundance in response to environmental factors. Percentage variances of PCR-DGGE ribotypes–environment correlations accounted for each ordination axis are shown. Circles drawn in the ordination plots (Van Dobben circles) delimitate the area within which a given PCR-DGGE ribotype is considered to have significant positive (*t*-value larger than 2.0, empty circle) or negative (*t*-value less than −2.0, dashed circle) correlation with the factor 'oilseed rape rhizosphere'. Bands 4 and 6 (a) were found to have a positive correlation with the strawberry and oilseed rape rhizospheres, respectively, according to the *t*-value biplot technique (see also Fig. 3c).

level of variability within replicates was detected in the strawberry rhizosphere profiles from Rostock in 2002 (Fig. 1b), while patterns were more homogeneous in the subsequent year (Fig. 1d).

Sequencing analysis of DGGE bands

Bands 1 and 2 (Fig. 1a) were present in bulk soil profiles of all sampling sites and were particularly dominant in Rostock. They were also observed in the rhizosphere fingerprints of all three sites (Fig. 1b and d), but their relative abundance was less pronounced. Bands 3 and 5 were found to represent typical rhizosphere-associated ribotypes (Fig. 1b and d). Band 5 occurred in strawberry and oilseed rape rhizospheres at all sites and in both years. This band could also be detected and observed in bulk soils from Braunschweig and Berlin (Fig. 1a and c). The relative abundance of ribotypes corresponding to band 5 was negligible in Rostock bulk soils but strongly increased in both rhizospheres (Fig. 3e). Bands 4 and 6 had a positive correlation with the strawberry and oilseed rape rhizospheres, respectively, especially in the profiles of the Berlin site (Figs 3c and 4). The above-mentioned DGGE bands were chosen for cloning and subsequent sequence analysis. At least two clones per band excised were submitted to sequencing. Clones derived from band 3 did not display the original band's electrophoretic mobility after reamplification and cloning and were thus discarded from further analysis. Bands 1, 2 and 5 were excised from profiles of more than one sampling site in order to determine whether PCR-DGGE ribotypes with the same electrophoretic mobility detected at different locations resemble similar 16S rRNA gene sequences. All sequences analysed in this study were considered to belong to the genus *Pseudomonas* with 95% confidence according to the Naive Bayesian rRNA Classifier [Version 1.0 – Ribosomal Database Project (RDP)]. Table 2 presents a summary of the bands excised, their origin, number of clones sequenced and their respective closest phylogenetic relatives. Multiple closest hits, representing both *Pseudomonas* strains and clones, were found in the database for many of our sequence queries (data not shown) and Table 2 shows at least two representatives of the closest relatives for such cases. Exceptions to multiple matching were the sequences retrieved from band 6B, which had a unique closest hit in the database each, as

Table 2. Closest phylogenetic relatives of partial 16S rRNA gene sequences (regions V6 to V8) derived from dominant or differentiating *Pseudomonas* denaturing gradient gel electrophoresis bands.

					Closest phylogenetic relative	
Band[a]	DGGE profile[b]	*n* [c]	Clone[d]	Accession no.	Identity (accession number)	%
1BS	Bulk soil Braunschweig	3	1BS-06 (3)	DQ376187	*Pseudomonas* sp. NZ065 (AY014815.1) *Pseudomonas fluorescens* strain PCL1751 (AY900171.1)	100
1R	Bulk soil Rostock	5	1R-15 (3)	DQ376188	*Pseudomonas* sp. NZ009 (AY014802.1) *Pseudomonas fluorescens* strain F (DQ146946.1)	99.8
			1R-20 (2)	DQ376189	*Pseudomonas* sp. NZ009 (AY014802.1) *Pseudomonas fluorescens* strain F (DQ146946.1)	100
2BS	Bulk soil Braunschweig	3	2BS-12 (3)	DQ376190	*Pseudomonas* sp. 'ARDRA PS1' (AY364085.1) *Pseudomonas* sp. TB3-6-I (AY599721.1)	100
2B	Bulk soil Berlin	4	2B-31 (3)	DQ376193	*Pseudomonas* sp. NZ009 (AY014802.1) *Pseudomonas fluorescens* strain F (DQ146946.1)	99.8
			2B-22 (1)	DQ376191	Uncultured bacterium clone BG.d4 DQ228373 *Pseudomonas* sp. NZ009 (AY014802.1) *Pseudomonas fluorescens* strain F (DQ146946.1)	100 99.8
2R	Bulk soil Rostock	5	2R-27 (1)	DQ376192	*Pseudomonas* sp. NZ009 (AY014802.1) *Pseudomonas fluorescens* strain F (DQ146946.1)	100
4B	Strawberry Berlin	2	4B-02 (2)	DQ376195	Uncultured *Pseudomonas* sp. clone BBS8w15 (AY682184.1) *Pseudomonas fluorescens* strain F113 (AJ278814.1)	98.5
5BS	Strawberry Braunschweig	3	5BS-05 (2)	DQ376196	*Pseudomonas fluorescens* strain PCL1753 (AY900172.1) *Pseudomonas fluorescens* VKM B-2168 (AY271793.1)	100
			5BS-10 (1)	DQ376197	Uncultured gamma bacterium clone BBS8w2 (AY682190.1) *Pseudomonas fluorescens* strain F113 (AJ278814.1)	100
5R	Strawberry Rostock	3	5R-04 (2)	DQ376198	Uncultured *Pseudomonas* sp. clone BBS8w6 (AY682190.1) *Pseudomonas fluorescens* strain F113 (AJ278814.1)	99.5
			5R-10 (1)	DQ376199	Uncultured gammaproteobacterium clone BBS8w2 (AY682193.1) *Pseudomonas fluorescens* strain PCL1753 (AY900172.1) *Pseudomonas fluorescens* VKM B-2168 (AY271793.1)	100 99.7
6B	Oilseed rape Berlin	3	6B-01 (1)	DQ376200	*Pseudomonas aeruginosa* ATCC BAA-1006 (AY631058.1)	98.6
			6B-03 (1)	DQ376201	*Pseudomonas aeruginosa* ATCC BAA-1006 (AY631058.1)	99.0
			6B-05 (1)	DQ376202	*Pseudomonas aeruginosa* ATCC BAA-1006 (AY631058.1)	98.5

a. Numbers refer to the DGGE ribotypes marked in Figs 1a and b and 3d.
b. All bands were extracted from DGGE profiles of year 2002.
c. Number of clones with the same DGGE mobility of the original excised band which were submitted to sequencing.
d. In brackets: number of clones sharing identical 16S rRNA gene sequences. Only non-identical sequences were submitted to GenBank.
BS, Braunschweig; B, Berlin; R, Rostock.

well as the clones 2B-22 and 5R-10 (Table 2). Although microheterogeneities within sequences retrieved from bands with the same electrophoretic mobility were observed, they accounted only for one to four different base identities in the *c.* 400-bp-long sequences and, if not equal, very similar phylogenetic affiliations were obtained for clones retrieved from the same DGGE band (Table 2). Phylogenetic trees were constructed to relate our partial 16S rRNA gene sequences to full-aligned sequences of *Pseudomonas* type strains present in the ARB database. Sequences derived from bands 1 and 2 belonged to a subgroup of the *Pseudomonas fluorescens* lineage (Moore *et al*., 1996) with closest affiliation to species such as *Pseudomonas rhodesiae* and *Pseudomonas gessardii* (see Fig. S1). The clones 4B-02 and 5R-04 were affiliated to a second subgroup of this same lineage, composed by type-strains such as *Pseudomonas taetrolens*, *Pseudomonas brassicacearum* and *Pseudomonas thivervalensis* (see Fig. S1). Alignment of clones obtained from DGGE bands 5BS (Braunschweig) and 5R (Rostock) presented a certain level of microheterogeneity (up to three differences in base identities). These clones appear scattered throughout different subclusters (see Fig. S1). Despite of this, they were all found to share the highest similarity to *P. fluorescens* strains known to act as biocontrol agents, such as strains F113 (Ramette *et al*., 2001) and PCL1753 (Kamilova *et al*., 2005) (Table 2). The highly similar clones 6B-01, 6B-03 and 6B-05, originated from the oilseed rape-specific band 6, resembled *Pseudomonas aeruginosa* sequences (Table 2, see Fig. S1).

Discussion

In this study, field experiments were performed to assess 16S rRNA gene diversity of naturally occurring *Pseudomonas* spp. in bulk and rhizosphere soils of strawberry and oilseed rape, two *V. dahliae* host plants, in two consecutive years and at three different geographical locations. For the first time the relative strengths of the rhizosphere effect, plant specificity, sampling site and

year-to-year variation in shaping *Pseudomonas* community structure in bulk and rhizosphere soils were simultaneously assessed in field trials with cultivation-independent methods. Recently, considerable knowledge on the functional and structural microbial diversity in the rhizosphere of both plant species has been achieved (Berg *et al*., 2006; Costa *et al*., 2006). Berg and colleagues (2006) screened more than 6000 bacterial isolates retrieved from the soil and rhizosphere samples fingerprinted in this study for *in vitro* antagonism towards *V. dahliae*. Seventy-seven percent of the antagonists obtained were considered to belong to the genus *Pseudomonas*. Such an impressive antagonistic potential is of obvious importance for the implementation of alternative strategies for the control of *V. dahliae*. Curiously, none of the cultivation-independent studies on the *Pseudomonas* community structure performed so far (Gyamfi *et al*., 2002; Reiter *et al*., 2003; Garbeva *et al*., 2004; Milling *et al*., 2004; Pesaro and Widmer, 2006) has attempted to assess differences between rhizosphere and bulk soil patterns. As stated by Lugtenberg and Bloemberg (2004), plant root exudates and rhizodeposits are supposed to deeply influence the composition of *Pseudomonas* species in the rhizosphere. We showed that, at all three sites studied, *Pseudomonas* rhizosphere communities differed significantly from those in bulk soil. Moreover, based on the RDA of the DGGE fingerprints, the influence of the sampling site, each with its particular soil characteristics and history of land use, on *Pseudomonas* community structure in soils was considered to be decisive. Previous molecular studies on bacterial (Crecchio *et al*., 2004; Kennedy *et al*., 2005), *Burkholderia* (Salles *et al*., 2004; 2006) and *Pseudomonas* (Garbeva *et al*., 2004) communities have shown that different land use history and management affected indigenous soil bacteria to a large extent. Along with intrinsic soil properties and local climatic conditions, previous land use management might have contributed substantially to the observed differences among bulk soil *Pseudomonas* communities at the three sites. Moreover, indications for plant specificity in the rhizosphere were evident: *Pseudomonas* communities in the vicinities of the roots of strawberry and oilseed rape plants grown at the same sampling site were affected in a particular manner (Fig. 3). Nevertheless, the effects exerted by both rhizospheres on soil-borne *Pseudomonas* spp. varied at different geographic locations and also in different years at the same location. Using the same pool of samples evaluated in this study, Costa and colleagues (2006) reported on 16S rRNA gene fingerprinting of *Bacteria*, *Actinobacteria*, *Alpha-* and *Betaproteobacteria*. At all sampling sites, the bulk soil patterns consisted of a large number of faint bands representing equally abundant ribotypes, whereas the relative intensities of few particular bands were enhanced in the rhizosphere. Similar observations describing the rhizosphere effect have been reported elsewhere (Gomes *et al*., 2001; Smalla *et al*., 2001). Such a trend fits well to the hypothesis of microspatial habitat heterogeneity leading to higher community diversity in soil (Stotzky, 1997; Nicol *et al*., 2003). Coexistence of diverse microorganisms is believed to be supported in soil because of its complexity and niche heterogeneity. In comparison to soil not affected by roots, the rhizosphere represents a habitat with higher resource availability. Specific populations fitter in assimilating and allocating such resources for growth would be selected in the rhizosphere, resulting in dominance of a few members within the community and leading to reduced evenness and diversity. Our data revealed, however, that this trend was not as clear as we originally hypothesized for *Pseudomonas* communities. We did observe a reduced diversity in the oilseed rape rhizosphere profiles from Braunschweig in 2002 (Fig. 3a and b). Nevertheless, for samples collected in Berlin, we found evidences for differences in community structure without shifts in diversity indices (Fig. 3c and d). The most striking contrast to the hypothesis of reduced diversity in the rhizosphere in comparison to bulk soils was indeed the consistent patterns observed in sampling site Rostock. Here, diversity indices estimated for rhizosphere DGGE fingerprints were significantly higher than those obtained for the corresponding soil samples (Fig. 3e and f). Possible explanations for this outcome might be due to particular physiological characteristics of *Pseudomonas* spp. Despite the higher diversity observed in rhizosphere fingerprints of Rostock, we cannot exclude that this could have been caused by pseudomonads carrying multiple 16S rRNA gene operons with sequence heterogeneities. Alternative hypotheses of habitat heterogeneity might explain the patterns observed in Rostock. Recent studies allowed a better understanding of the complexity and dynamics in the rhizosphere, an environment where the distribution of resources indeed varies substantially in both space and time (Yang and Crowley, 2000; Marschner *et al*., 2004). Habitat changing with time, for instance, can lead to recurrent substitution of dominant populations in the rhizosphere so that, at any given time point, the coexistence of diverse populations with different densities might be observed. As *Pseudomonas* spp. have the ability of assimilating a broad range of organic compounds and of quickly responding to environmental changes, they comprise a bacterial group for which such responses to root exudation might be particularly pronounced. Most likely, a complex interaction of many biotic and abiotic factors (Costa *et al*., 2006) is the explanation for the different rhizosphere effects on the *Pseudomonas* populations at different sites. It is not the first time that higher 16S rRNA gene diversity was detected in the rhizosphere in compar-

ison to bulk soils. Using taxon-specific nested PCR-DGGE systems, Stafford and colleagues (2005) reported on higher diversity of *Actinobacteria* and the streptomycetes in the rhizosphere of Proteaceae species in comparison to bulk soils in the South African Cape Floral Kingdom. Taken together, our results led to the conclusion that the rhizosphere effect on the structure of indigenous soil *Pseudomonas* spp. does consistently occur. Nevertheless, its strength and, more importantly, its kind of impact, especially considering the diversity of this group in the rhizosphere, may vary in a case-by-case manner. This outcome highlights the importance of targeting particular microbial guilds in the rhizosphere and indicates that more efforts are needed to better comprehend factors that drive *Pseudomonas* community diversity in this environment.

Despite the variations in *Pseudomonas* spp. community patterns at different sites, dominant PCR-DGGE bands occurring in the bulk soils of all sites (e.g. bands 1 and 2), bands considered to be rhizosphere-associated (e.g. bands 3 and 5) and plant-specific bands (e.g. bands 4 and 6) could be consistently detected. Such PCR-DGGE ribotypes might correspond to *Pseudomonas* populations able in persisting and surviving in the bulk and rhizosphere soil environment. This is an essential requirement for successful performance of any biocontrol agent. Thus, we were interested in the identity of these ribotypes so that an indication of their potential role in the rhizosphere could be depicted. In comparison to the other PCR-DGGE systems so far developed to fingerprint *Pseudomonas* 16S rRNA gene fragments, the system employed in this study (Milling *et al*., 2004) allows the retrieving of the longest 16S rRNA sequences (*c*. 400 bp, primers excluded). Nevertheless, one should bear in mind that the phylogenetic information provided by 400-bp-long 16S rRNA gene sequences is rather limited, especially considering the genomic complexity of *Pseudomonas* (Palleroni, 2003). Not surprisingly, multiple closest hits, representing both *Pseudomonas* strains and clones, were found for the majority of our sequences (Table 2). A slight degree of microheterogeneity within 16S rRNA gene sequences obtained from bands sharing the same melting behaviour on DGGE was detected. This suggests that dissimilarity between *Pseudomonas* 16S rRNA gene fingerprints as assessed by DGGE might be higher in case microdiversity within populations is taken into account. Maximum parsimony phylogenetic analysis (see Fig. S1) helped finding out the lineages within the genus *Pseudomonas* to which our sequences most probably belong. The vast majority of our sequences were affiliated to the *P. fluorescens* lineage (Moore *et al*., 1996; Anzai *et al*., 2000). Members of this group have been reported to act as biocontrol agents (Keel *et al*., 1992; Leeman *et al*., 1995) and to display a range of antifungal properties (Walsh *et al*., 2001). Almost all partial 16S rRNA gene clone sequences derived from bands 1 and 2 (Table 2), were shown to be most similar to two *Pseudomonas* strains, NZ009 and NZ065, which have antifungal activity towards *Agaricus bisporus*, causing the blotch disease of this fungus (Godfrey *et al*., 2001). The sequences of clones retrieved from band 5 showed the highest hits to the 16S rRNA gene sequences of the biocontrol agents *P. fluorescens* F113 (Ramette *et al*., 2001) and PCL1753 (Kamilova *et al*., 2005) (Table 2). *Pseudomonas fluorescens* F113 is capable to produce 2,4 diacetylphloroglucinol (2,4-DAPG), an antibiotic that has already been unequivocally associated to biological control efficiency (Keel *et al*., 1992; Raaijmakers and Weller, 1998). Strain PCL1753 is a superior root-tip colonizer that reduced tomato foot and root rot caused by *Fusarium oxysporum* (Kamilova *et al*., 2005). We propose that such dominant *Pseudomonas* PCR-DGGE ribotypes may represent rhizosphere-competent populations that harbour plant-beneficial characteristics and might have the capability to act as *V. dahliae* biocontrol agents in the rhizosphere. Further research indicates that ribotypes 1, 2 and 5, identified in this study, indeed correspond to oilseed rape and strawberry rhizosphere-associated *Pseudomonas* populations that carry multiple antifungal properties (R. Costa, unpublished results), indicating their potential use as biocontrol agents (BCAs).

In this study, we evaluated the extent to which different factors affected *Pseudomonas* community structure and diversity in the rhizosphere of *V. dahliae* host plants. The processing of large sample sets is essential for such purposes. Our analyses provided an advanced understanding of the *Pseudomonas* dynamics in the rhizosphere, a particular microenvironment where multiple microbial interactions take place. Although many of the partial 16S rRNA gene sequences reported here could be assigned to previously described sequences of culturable *Pseudomonas*, it has been argued that there may still be pseudomonads lacking the ability to grow on culture media tested so far (Sørensen and Nybroe, 2004). Indeed, Pesaro and Widmer (2006) have recently reported on a new lineage of soil-borne bacteria, named *Cellvibrio*-related *Pseudomonadaceae*, based on molecular studies conducted to specifically target *Pseudomonas* in agricultural soils. As *Pseudomonas* spp. can act in the rhizosphere as plant pathogens (Bais *et al*., 2004; 2005), plant growth-promoters (Dell'Amico *et al*., 2005; Gray and Smith, 2005), biocontrol agents (Haas and Keel, 2003; Guo *et al*., 2004) and biodegraders (Lorenzo, 2000), unravelling their diversity in soil by means of nucleic acid-based studies not only permits a less biased understanding of their community structure but also may help revealing so far unknown genetic resources of potential use in applied biotechnology.

Experimental procedures

Field design and sampling

Strawberries [*Fragaria ananassa* (Duchense) Decaisne and Naudin cv. Elsanta] were planted and oilseed rape (*Brassica napus* L. cv. Licosmos) was sown in field plots using a randomized block design consisting of four replicate plots per plant. For each plot, one composite bulk soil sample and one composite rhizosphere sample were taken at the flowering development stage of the plants as described in detail by Costa and colleagues (2006). Thus, four composite samples per treatment (strawberry rhizosphere, oilseed rape rhizosphere, soil from strawberry field and soil from oilseed rape field) were collected at each sampling event and immediately transported to the laboratory. Samplings took place in two consecutive years (2002 and 2003) at three locations in Germany: Braunschweig (52°16′N, 10°31′E), Berlin (52°31′N, 13°24′E) and Rostock (54°05′N, 12°07′E). Soil texture was classified as sand in Berlin and weakly loamy sand in Braunschweig and Rostock. Physical-chemical parameters of soils were determined by Berg and colleagues (2005). All sampling sites differ from each other in respect to land use. At the Braunschweig site rotation of maize, potatoes, legumes and *Phacelia tanacetifolia* had been carried out for several years. Soils at the Berlin site had been planted with Summerflowers in rotation with garden pansy (*Viola tricolor hortensis*) and barley during 10 years before the implementation of our field trials. The Rostock site has been used for agricultural purposes already for 100 years. Oilseed rape is normally planted at this site in rotation with a variety of cereals. Conventional practices of soil fertilization and crop protection have been applied at all sites, such as N amendment (50–75 kg N/ha) and moderate application of herbicides. Thus, due to the intricacies of a field experiment, where many variables are not to be controlled, a range of environmental and biotic factors such as crop use history, soil management and physical structure, local climatic conditions, nutrient availability and others may simultaneously contribute to comprise what is referred to as 'sampling site factor' in this study. The term 'year-to-year variation', in its turn, refers to the variation in DGGE profiles obtained for bulk and rhizosphere soils collected at the time of flowering in two different growing seasons (2002 and 2003).

Total community DNA extraction

An indirect DNA extraction method was applied to isolate DNA from soil matrices. Microbial cells were dislodged from soil matrices and pellets were obtained for each sample by employing successive blending/centrifugation treatments to the samples as explained by Costa and colleagues (2006). The BIO-101 DNA extraction kit (Q Biogene, Carlsbad, CA, USA) was used to extract total community DNA from cell pellets according to the manufacturer's recommendations. DNA samples were repurified with the GENECLEAN Spin kit (Q Biogene) following the supplier's instructions. Genomic DNA yields were checked under UV light after electrophoresis in 0.8% agarose gels and ethidium bromide staining. DNA concentrations were estimated visually by applying the 1-kb gene-ruler™ DNA ladder (Fermentas, St. Leon-Rot, Germany) on the agarose gels as a mass ruler. Genomic DNA samples were diluted differentially to obtain *c.* 1–5 ng DNA to be used as PCR templates.

Polymerase chain reaction amplification of Pseudomonas *16S rRNA gene fragments for DGGE analysis*

A nested PCR approach was used to amplify *Pseudomonas* 16S rRNA gene fragments for DGGE fingerprinting of total community DNA. A first, taxon-specific, PCR amplification was carried out using 1 μl of soil DNA template (*c.* 5 ng) and 25 thermal cycles. A reaction mixture (25 μl) was prepared containing 1 μl of template DNA (1–5 ng), 1× Stoffel buffer (10 mM KCl, 10 mM Tris-HCl, pH 8.3), 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 5% DMSO, 0.2 μM of both primers F311Ps (5′-CTG GTC TGA GAG GAT GAT CAG T-3′) and R1459Ps (5′-AAT CAC TCC GTG GTA ACC GT-3′) (Milling *et al*., 2004) and 1 U *Taq* DNA polymerase (Stoffel fragment, Applied Biosystems). After initial denaturation at 94°C for 7 min, 30 thermal cycles of 1 min 94°C, 1 min 63°C and 2 min 72°C were carried out. A final extension step of 10 min at 72°C was performed to finish the reaction. One to 20 diluted PCR products were used as templates (1 μl) for the amplification of 16S rRNA gene fragments using the primers F984GC and R1378 (Heuer and Smalla, 1997) and PCR conditions as described by Costa and colleagues (2006) prior to DGGE analysis. This primer pair amplifies a fragment ranging from the V6 to V8 hyper-variable regions (*c.* 430 bp, primers included) of the 16S rRNA gene. Polymerase chain reaction products were checked after electrophoresis on agarose gels under UV transillumination (254 nm). All PCR amplifications were performed with a Tgradient thermal cycler (Biometra, Göttingen, Germany).

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis analysis was performed with the Dcode System apparatus (Bio-Rad, Hercules, CA, USA). A double gradient consisting of 26–58% denaturants (100% denaturants defined as 7 M urea and 40% formamide) and 6–9% acrylamide was prepared (Costa *et al*., 2004). Aliquots of PCR products (*c.* 3 μl) were loaded on the gel and electrophoresis was carried out with 1× Tris-acetate-EDTA buffer at 58°C and constant voltage of 220 V for 6 h. Polymerase chain reaction products amplified from four replicates per treatment (each representing one composite sample) were loaded side by side on the gels. A mixture of the DGGE-PCR products from 11 bacterial species was applied at the extremities of the gels as a marker to check the electrophoresis run and to compare fragment migration between gels (Smalla *et al*., 2001). Gels were silver-stained according to Heuer and colleagues (2001) and air-dried.

Denaturing gradient gel electrophoresis image conversion

Gels were scanned transmissively (Epson 1680 Pro, Seiko-Epson, Japan) with high resolution settings (720 dpi). The GelCompar 4.0 program (Applied Maths, Ghent, Belgium) was used to convert and normalize gel image as proposed

by Rademaker and colleagues (1999) with the modifications of settings listed by Smalla and colleagues (2001). The external marker applied at the extremities of the gels was used as reference to align DGGE tracks and standardize internal distortions. Background subtraction was carried out using the rolling disk mechanism. Banding patterns were analysed by a suite of statistical techniques.

Cluster analysis and pairwise permutation tests of significance

Clustering of DGGE fingerprints was performed only for samples loaded on the same gel. Analyses were performed using the GelCompar 4.0 software. Pearson's correlation coefficients (*r*) were calculated for each pair of lanes within a gel as a measure of similarity between the community fingerprints. Samples were grouped by applying the unweighted pair group method with average linkages (UPGMA) to the matrix of similarities obtained. Pairwise similarity measures obtained with the Pearson's correlation coefficient were submitted to 100 000 unrestricted permutations to test for significant differences between the treatments characterized by DGGE using the permtest software developed by Kropf and colleagues (2004). As applied here, pairwise similarity values within a given matrix were randomly permuted in order to determine whether similarity measures calculated within groups (among replicates of the same treatment) were significantly higher than those ones obtained between groups (replicates from different treatments). Moreover, comparisons of different matrices (i.e. DGGE gels) were performed to verify whether soil samples from different sampling sites, loaded on one DGGE gel, differed more from each other than their corresponding rhizosphere samples, loaded on another gel (Fig. 1). The gels are considered as two different blocks in a statistical sense, and comparisons between similarity values obtained for both gels can be carried out (Kropf *et al*., 2004). As similarity matrices were calculated separately for each gel and used further for the permutation test, this kind of comparison avoids problems related to gel-to-gel variation.

Multivariate analysis

To assess correlations between DGGE bands (i.e. PCR-DGGE *Pseudomonas* ribotypes) and environmental variables (i.e. field treatments), multivariate analysis of data was required. To perform it, the standardization of six DGGE gels containing all 96 samples fingerprinted in this study was needed. Images were processed in GelCompar 4.0 with the highest resolution provided by the software (2000 points). Positioning and quantification of bands present in each lane was carried out by setting tolerance and optimization at 12 points, i.e. 0.6%. Thus, bands from different gels whose positions were ranging within the limit determined by the tolerance setting were considered to be the same. Standardization of bands automatically performed by GelCompar was carefully checked manually further and correction was done when needed. Single strands and staining artefacts were removed from the analysis. A table containing band positions and their corresponding relative intensities (peak areas) was constructed and data (i.e. presence/absence and relative abundance of DGGE bands) were used as predictors to characterize different treatments according to their *Pseudomonas* PCR-DGGE ribotype composition. The software package Canoco 4.5 (Microcomputer Power, Ithaca, NY, USA) was used for the ordination of samples fingerprinted by DGGE and environmental variables. Firstly, detrending correspondence analysis was applied to the data set to check the length of gradient in PCR-DGGE ribotype variation. This analysis serves as a reliable tool for choosing the ordination techniques that better fit to a given data set. As a linear model of distribution was the model that better fitted to the variation of our data, RDA was chosen to estimate the extent to which the variation in the relative abundance of *Pseudomonas* DGGE bands can be explained by the environmental variables (i.e. field treatments) taken into account in this study. Monte Carlo permutation tests, based on 499 unrestricted permutations, were performed to test for the significance of PCR-DGGE ribotype and environment correlations, assuming the null hypothesis that DGGE ribotype data are not related to environmental data.

Estimation of community diversity from DGGE profiles

The Shannon index of diversity (H′), determined as $H' = -\sum pi \cdot \log pi$, where pi represents the abundance of the i^{th} category within the sample, was employed to estimate the diversity of *Pseudomonas* 16S rRNA gene fragments of bulk and rhizosphere soil DNA samples fingerprinted by DGGE. Band positions and intensities were used as parameters to indicate categories (PCR-DGGE ribotypes) and relative abundance (peak area) of each category within one sample respectively. Indices were calculated for each DGGE track analysed in this study using the software package Canoco 4.5 for windows. Values obtained for replicates were averaged and diversity indices of each treatment were tested for significant differences by applying the two-sample *t*-test of Student as described by Brower and colleagues (1997).

Extraction, cloning and sequencing of DGGE bands

Bands representing ribotypes of interest were excised with a scalpel from the gels, put into sterile microcentrifuge tubes containing 50 μl of 10 mM Tris-HCl (pH 8.0) and stored at 4°C overnight. Two microlitres of the resulting elution suspension were used as templates for a DGGE-PCR with the same conditions mentioned above to reamplify 16S rRNA gene fragments from the excised band. After reamplification, the electrophoretic mobility and purity of the excised bands were checked by DGGE. Only reamplified bands that confirmed their original positions on DGGE were used for cloning. Appropriate, reamplified PCR products (without GC-clamp) were ligated into pGEM-T vectors (Promega, Madison, USA) and transformed into competent cells (*Escherichia coli* JM109; Promega) following the supplier's instructions. The 16S rRNA gene fragments amplified from clones and from the original community DNA profiles were loaded on DGGE to carefully check whether the cloned 16S rRNA gene fragments comigrated with the band of the corresponding community pattern. Clones containing inserts which shared the electrophoretic mobility of the original band were selected for

sequence analysis. Prior to sequencing, amplified clone inserts were purified with the GENECLEAN Spin kit (Q Biogene). Two to five clones per band were submitted to sequencing.

Sequence analysis and phylogeny

Clone sequences from DGGE bands were firstly classified at the genus level with the Naive Bayesian rRNA Classifier (Version 1.0) of the Ribosomal Database Project II (RDP – http://rdp.cme.msu.edu/) with 95% limit of confidence. The nucleotide-nucleotide BLAST search tool (BLASTN) of the National Center for Biological Information (NCBI, USA) was used to find sequences in the relevant databases that better match to our queries. Phylogenetic trees were constructed using the ARB software and database (http://www.arb-home.de) as follows. Clone sequences were automatically aligned with full 16S rRNA gene *Pseudomonas* sequences by using the ARB sequence editor with the fast-aligner tool. The resulting alignment was manually proof-read and corrected when necessary. For each clone sequence, the similarity to the best database hits was calculated by using a distance matrix (Jukes-Cantor) with the particular sequence itself as a filter. An initial tree was first calculated using the neighbour-joining method and a large number of full 16S rRNA gene sequences from the database (*c.* 50 000 sequences). Partial sequences derived from clones were then accommodated into the built 16S rRNA phylogenetic tree using maximum parsimony. This tool, available in the ARB software, enables the construction of phylogenetic trees containing sequences of different lengths. The resulting tree was optimized and set to contain full sequences of *Pseudomonas* type strains and the partial sequences retrieved in this study. The full 16S rRNA gene sequence of *Cellvibrio elegans* was used as outgroup. Representative (i.e. non-identical) partial 16S rRNA gene sequences obtained in this study were deposited in the GenBank database under Accession numbers DQ376187–DQ376202.

Acknowledgements

We thank Nicole Mrotzek for her valuable technical assistance and Arno Buchner for his support on phylogenetics. This study was funded by the Deutsche Forschungsgemeinschaft (DFG SM59-2, DFG BE). R. Costa was granted a Deutscher Akademischer Austauschdienst (DAAD) scholarship to perform this work.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Maximum parsimony phylogenetic tree constructed with nearly full 16S rRNA gene sequences of *Pseudomonas* (*sensu stricto*) type strains and partial sequences of clones retrieved from DGGE bands in this study. Tree was generated using the ARB project database and software. Reference bar at the bottom of the tree indicates substitutions per site.

This material is available as part of the online article from http://www.blackwell-synergy.com

