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ORIGINAL ARTICLE

Culture-independent analysis of *Pseudomonas* community structures in fertilized and unfertilized agricultural soils

James Tabi Tambong · Renlin Xu

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Abstract *Pseudomonas* community structures were investigated by analyzing 16S rRNA clone libraries derived from fertilized and unfertilized soil plots under corn–alfalfa rotation in a long-term experiment. Amplified 16S rRNA fragments derived by polymerase chain reaction (PCR) were cloned and sequenced. A total of 729 clone sequences were analyzed, of which 51 were possible chimeras and discarded. The remaining clone sequences (678) belonged to γ-proteobacteria with 61.8 % (419) classified to the genus *Pseudomonas*. Unclassified Gammaproteobacteria accounted for 23.4 % of total clones sequences. Rarefaction analyses showed a more diverse community structure of both Gammaproteobacteria and *Pseudomonas* in unfertilized than fertilized field soils irrespective of plant types under cultivation. Bacterial or *Pseudomonas* community structures differed significantly between fertilized and unfertilized soil plots. Clone sequences that are affiliated to *Pseudomonas putida* and *P. oryzihabitans* were more prominent in libraries from fertilized plots, while those that clustered with *Pseudomonas fredericksbergensis* were more often retrieved from unfertilized soil plots. A strong influence of fertilizer applications on community structure was supported by principal component analysis. We conclude that long-term use of mineral fertilizers could influence *Pseudomonas* community structure.

Keywords Culture-independent · *Pseudomonas* · 16S rRNA · Diversity · Rarefaction

J. T. Tambong (✉) · R. Xu
Environmental Health Program (Biodiversity),
Eastern Cereal and Oilseed research Center,
Agriculture and Agri-Food Canada in Ottawa,
960 Carling avenue,
Ottawa, ON K1A 0C6, Canada
e-mail: tambongj@agr.gc.ca

Introduction

Mineral and organic fertilizer applications are used primarily to enhance nutrient availability to plants, but they can also affect soil microbial community structure and diversity. Interest in environmentally sound agricultural practices has resulted in an increase in studies that assess how different cropping regimes affect microbial diversity in soil (Garbeva et al. 2004; Lemanceau et al. 1995; O'Donnell et al. 2001). However, due to the complexity of agricultural soil ecosystems, and the vast diversity and the enormity of the population inhabiting the environment, it is a challenging task (McCaig et al. 1999; O'Donnell and Gorres 1999; Smit et al. 2001; Torsvik et al. 1990). Perturbation of bacterial community equilibrium populations by changes in environmental conditions and soil management practices due to seasonal and temporal variations have been extensively reported (Peacock et al. 2001; Smit et al. 2001; van Elsas et al. 2002), but data interpretation is not always conclusive and reliable (Amann et al. 1995; Smit et al. 2001). This could be predicted, since variations of microbial community structure and diversity due to seasonal and temporal changes in nutrients and physical factors are slow and gradual (Sun et al. 2004). Long-term (>10 years) studies on bacterial community structure shifts in various land use might provide a more reliable dataset required to identify sustainable agricultural management practices (Cruz-Martinez et al. 2009; Sun et al. 2004). We had a unique opportunity to examine soil bacterial communities following a long-term monoculture corn–alfalfa rotation experiment with regular mineral fertilizer applications for over 40 years. The long-term experiment was started in 1959 in southwestern Ontario, Canada, to study the effects of fertilization, crop rotation, and climatic factors on yields (Drury and Tan 1995).

One of the most important bacterial communities in soil, which have recently been considered potential agricultural

soil health bio-indicatorss, belongs to the genus *Pseudomonas* (Garbeva et al. 2004). Members of this genus belong to several distinct functional groups of environmental interest, such as plant pathogens (Endert and Ritchie 1984; Ferrante and Scortichini 2010; Sarkar and Guttman 2004), plant growth promoters (Patten and Glick 2002; Raaijmakers and Weller 2001), and xenobiotic degraders (Clausen et al. 2002; Park et al. 2004). Moreover, *Pseudomonas* species can also play important roles as biological control agents against soil-borne plant pathogens (Bolwerk et al. 2003; Mazurier et al. 2009; Tambong and Höfte 2001).

Various culture-independent molecular methodologies have become the preferred approach to study population dynamics and genetic diversity of soil microbial communities (Torsvik et al. 1996). Analysis of the PCR-based cloned16S rRNA gene libraries is routinely used to assess microbial diversity in complex systems, as well as estimate effects of disturbance on diversity (Duineveld et al. 2001; Dunn and Stabb 2005; Lin et al. 2010; Sun et al. 2004). Also, PCR-denaturing gradient gel electrophoresis (PCR-DGGE) of 16S rRNA fragments is a highly sensitive culture-independent tool for investigating changes in the bacterial community structure (Ibekwe et al. 2001; Muyzer et al. 1993; Torsvik et al. 1996). In preliminary studies, we used PCR-DGGE to assess changes in the gross bacterial community structure. PCR-DGGE band patterns from soils of different treatments were consistent and indicated that bacterial community structure could have been influenced by long-term mineral fertilizer applications in samples collected in 2007 and 2008. Sequencing and BLAST search of some unique PCR-DGGE bands showed high similarity to members of the genus *Pseudomonas*. Based on these PCR-DGGE results, it was hypothesized that long-term mineral fertilizations could influence *Pseudomonas* community structure and diversity.

The objective of this study was to analyze the community structures of *Pseudomonas* in soils under long-term (>40 years) applications of inorganic fertilizers (NPK) in a corn–alfalfa rotation in comparison to control plots by cloning and sequencing PCR-amplified 16S rRNA gene fragments using soil microbial community DNA as the template. The 16S rRNA clone sequences obtained were used to perform rarefaction, phylogenetic, and principal component analyses to assess the effects of fertilizer applications on *Pseudomonas* community composition.

Materials and methods

Experimental site description and management

The land at the Eugene Whelan Experimental Farm site was cleared of deciduous trees in the late 1800s or early 1900s and the long-term study started in 1959. Detailed descriptions of the experimental site and soils have been reported (Drury and Tan 1995; Drury et al. 2004). Briefly, the soil at the experimental site, located at Woodslee, Ontario (42° 13′N, 82°44′W), was a Brookston clay loam (Humic Gleysol) with mean particle distribution of 28.0 % sand, 35 % silt, and 37 % clay (Drury and Tan 1995). Based on climate data over the period 2006–2008, the average annual temperature of this site was 10.6 °C; an average maximum temperature during sampling (September 2007) of 24.9 °C and an average minimum temperature of 14.4 °C were recorded with average monthly precipitation of 139.6 mm (Environment Canada 2008: http://climate.weatheroffice.ec.gc.ca/climateData/monthlydata_e.hml). In 1959, 12 large unreplicated plots (76.2 m long by 12.2 m wide) were established and consisted of different cropping treatments with or without fertilizer applications. Fertilized plots received annual applications of 16.8 kg Nha^{-1}, 29.3 kg P ha^{-1}, and 27.9 kg Kha^{-1}, applied to all crops. Fertilized maize received an additional application of 112 kg Nha^{-1} per year of side-dressed incorporated ammonium nitrate. A permanent grass treatment (S11) was mowed to a height of 13–15 cm four to five times each year, when the grass reached a height of 25 cm, and the residues were left on the plots after mowing. Planting densities and management including herbicides to control weeds have been reported (Drury and Tan 1995).

Two fertilized plots (S2 and S5) and two unfertilized plots (S8 and S7) were sampled for the study. Five replicate cores of soil samples (0–10 cm depth) were collected in September 2007 from fertilized and unfertilized plots under corn or alfalfa cultivation. The field-moist soil samples were passed through a 2-mm sieve and stored at 4°C.

PCR, cloning and sequencing of partial 16S rRNA gene sequence

Total soil DNA was extracted from 10-g samples using the PowerMax™ Soil DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA, USA). The quality of the DNA was verified by agarose gel electrophoresis and the concentration determined using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologist, Wilmington, DE, USA. Aliquots of the DNA were stored at −80°C.

Total soil community DNA was used as template for PCR amplification of 16S rRNA gene using F311-Ps (CTGGTCTGAGAGGATGATCAGT; Widmer et al. 1998) and R1459Ps (AATCACTCCGTGGTAACCGT; Milling et al. 2004). PCR mixture and conditions were as previously reported (Tambong et al. 2006). PCR amplifications (40 cycles) were performed in a reaction mixture that included 1× buffer, 200 μmol l^{-1} dNTP, 400 nmol l^{-1} of each primer and 0.75 U Titanium *Taq* DNA polymerase (Clontech, Palo Alto, CA, USA). PCR primers were synthesized by Invitrogen (Invitrogen, Carlsbad, CA, USA). PCR amplicons were

cloned using TA PCR2.1-TOPO plasmid kit (Invitrogen). A total of 800 clones were screened by PCR for the correct inserts (approximately 1.1 kb) using M13 primers and the resulting PCR amplicons sequenced with T7 and SP6 primer pair (Invitrogen) following the manufacturer's instructions.

Sequencing was done as previously described (Tambong et al. 2006) using ABI BigDye Terminator chemistry v.3.0 (Applied Biosystems, Foster City, CA, USA) and run on an ABI 3100-Avant automated sequencer (Applied Biosystems/Hitachi). DNA sequences were edited in SeqMan software (DNASTAR, Lasergene 8.1.5, Madison, USA) and sequences were checked for chimeras using Mallard computer program (Ashelford et al. 2006) with a cut-off of 99.9 % deviation from expectation statistics using 16S rRNA of *Escherichia coli* (GenBank entry U00096) as reference.

Taxonomic, rarefaction and phylogenetic analyses

16S rRNA sequences were assigned to taxonomic classes using the Classifier, Ribosomal Database Project 10 (RDP Classifier; Wang et al. 2007), a naïve Bayesian tool based upon taxonomic classifications in Bergey's *Taxonomic Outline of the Prokaryotes* (Garrity et al. 2004). The sequences were assigned to operational taxonomic units (OTUs) and used to perform rarefaction analysis as implemented in Distance-Based OTU and Richness Determination (DOTUR) software with the furthest-neighbor clustering algorithm (Schloss and Handelsman 2005). Sequences with more than 97 % homology were considered to belong to the same OTU. Pair-wise collector's curves were established to compare the relative diversity and coverage of each library by plotting the number of OTUs against the number of clones retrieved.

Initial phylogenetic analysis was performed using clone sequences classified to the genus *Pseudomonas* and 107 type strains reported by Mulet et al. (2010). Type strains of *Pseudomonas* species that did not cluster with any clone sequence from our study were excluded. Maximum likelihood was implemented in MEGA 5.0 (Tamura et al. 2011) using 1,000 bootstrap replicates to generate the final phylogenetic trees for pair-wise comparison of clustering patterns. The best-fit model (General Time Reversible) was selected using the jMODELTEST 0.1.1(Posada 2009).

Statistical analyses

Bacterial communities were statistically compared using analysis of similarity (ANOSIM) (Clarke 1993) as implemented in mothur (Schloss et al. 2009). The ANOSIM procedure generates a test statistic, R, used to assess the congruence among individuals grouped according to their respective populations. Under the null hypothesis, the test statistic R changes little when labels identifying populations are rearranged randomly, indicating no differentiation. The values of R range between 1 (maximum) and 0 (no separation). Also, Cramer-von Mises test statistic (Libshuff method; Singleton et al. 2001) was used to test whether the different *Pseudomonas* communities have the same structure. The significance of the test statistic indicates the probability that the communities have the same structure by chance. ∫-Libshuff analysis was implemented in mothur (Schloss et al. 2009) using the exact and integral form (Schloss et al. 2004) of the Cramer-von Mises statistic. Finally, principal coordinate analysis was performed to visualize the effects of fertilizer applications on *Pseudomonas* community structures based on the 16S rRNA sequences retrieved from each soil plot. This analysis was implemented using Unique Fraction metric (UniFrac; Lozupone and Knight 2005) software, a web-based program for comparing microbial communities.

Nucleotide sequence accession numbers

A total of 419 *Pseudomonas* 16S rRNA gene sequences obtained in this study are available in the National Center for Biotechnology Information database under the accession numbers HM011621–HM012039.

Results

Our goal was to evaluate *Pseudomonas* community structure and diversity in a long-term fertilizer application study using PCR-based cloning, sequencing, and analysis of amplified 16S rRNA gene fragments.

Taxonomic and rarefaction analyses of 16S rRNA clone libraries

Five 16S rRNA gene sequence libraries were constructed from unfertilized and fertilized plots under corn or alfalfa and from a fallow plot with permanent grass. A total of 729 sequences were generated, of which 51 identified as chimeric and discarded. The proportion of chimeric sequences in each treatment was not statistically different. All the remaining sequences (678) were assigned to taxonomical hierarchy within Gammaproteobacteria (domain bacteria).

Figure 1 shows the distribution of the retrieved taxa within each clone library. All libraries had sequences that are affiliated to the genus *Pseudomonas*, unclassified Gammaproteobacteria, and the genus *Lysobacter*; and these 3 distinct groups constituted 96.2 % of all the clones retrieved. Clones belonging to the genus *Pseudomonas* made up 61.8 % of all the clones retrieved. Clone library from plot 8 (unfertilized corn) showed a significantly ($p<0.01$) higher number of clones that belonged to *Lysobacter* and unclassified Gammaproteobacteria and a significantly low number

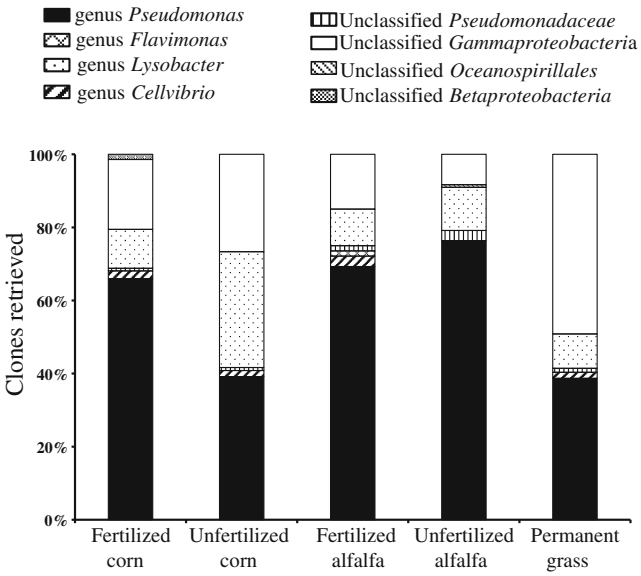


Fig. 1 Distribution of 678 16S rRNA sequences to hierarchical taxa using the Ribosomal Database Project 10 Classifier (Wang et al. 2007), a naïve Bayesian tool based upon taxonomic classifications in Bergey's *Taxonomic Outline of the Prokaryotes* (Garrity et al. 2004). Sequences were obtained in 2007 after subcloning of PCR-amplified products of total community DNA extracted from fertilized and unfertilized soils under long-term rotation plots

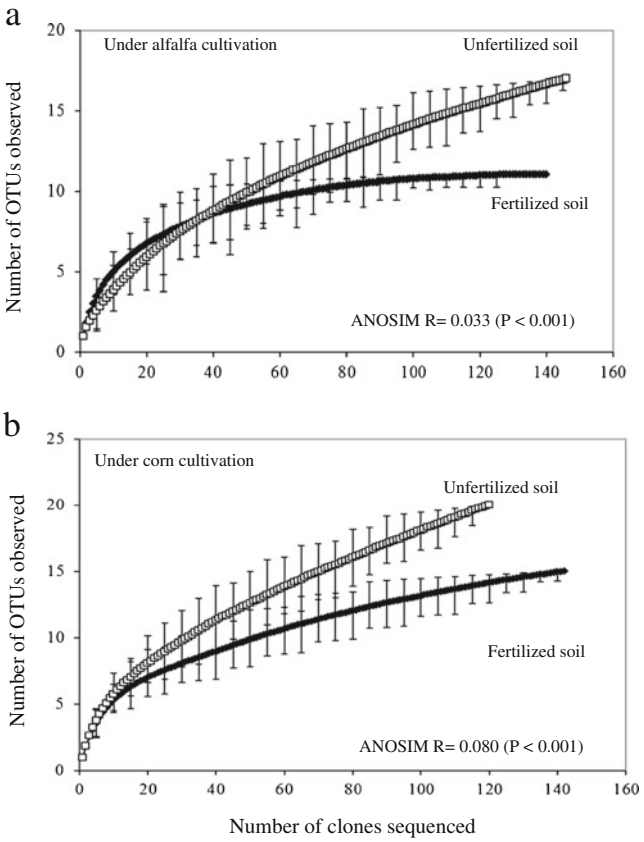


Fig. 2 Rarefaction curves (3 % distance level) generated from 16S rRNA sequences of *Gammaproteobacteria* community in the bacterial clones retrieved. Numbers of operational taxonomic units were significantly lower in fertilized compared to unfertilized soil plots under alfalfa (S5 vs. S7) (**a**) or corn (S2 vs. S8) (**b**). Analysis was performed with DOTUR (Schloss and Handelsman 2005) using furthest neighbor assignment algorithm with 16S rRNA library of the different treatments. *Error bars* 95 % confidence intervals. The provided Analysis of Similiarity (ANOSIM) R statistic values show highly significant population structure differences between fertilized soils and their corresponding unfertilized field plots

of clone sequences that could be affiliated with *Pseudomonas* (Fig. 1). Plot 11 (fallow, under grass, and unfertilized) had significantly ($p<0.01$) higher number of clone sequences affiliated to unclassified Gammaproteobacteria. Other minor bacterial groups retrieved could be affiliated to the genera *Cellvibrio* and *Flavimonas* or unclassified Betaproteobacteria class or unclassified Oceanospirillales order.

Rarefaction analysis performed by plotting the numbers of OTUs at 3 % distance level against the number of clones sequenced (Fig. 2) showed that the bacterial sequence populations from unfertilized soil treatments were more diverse statistically compared with corresponding groups from fertilized soil plots. The calculated rarefaction curves did not reach a clear saturation for unfertilized soil plots under either corn or alfalfa cultivation (Fig. 2). Soil samples from plots under long-term mineral fertilizer applications showed apparent saturation especially in plots under alfalfa cultivation (S5). Comparison of rarefaction curves between unfertilized plots (S7 and S8) and permanent grass (S11) did not reveal significant differences in diversity (data not shown). Also, in pair-wise comparisons, ANOSIM showed a clear separation among corresponding populations derived from fertilized and unfertilized plots, with global R statistics of 0.033 ($P<0.001$) or 0.080 ($P<0.001$) under alfalfa or corn cultivation, respectively. Pair-wise ∫-Libshuff analysis supported observations of ANOSIM with ΔCxyscores of 0.00145 ($P<0.0001$) or 0.00072 ($P<0.0001$) between corresponding populations derived from fertilized and unfertilized soil plots under alfalfa or corn cultivation respectively.

Analyses of *Pseudomonas* community structures

From the 5 libraries, a total of 419 clones were classified to the genus *Pseudomonas.* We analyzed the different resulting *Pseudomonas* populations to determine whether fertilizer applications affected the community structure associated with each soil plot. The Cramer-von Mises statistic of pairwise comparisons of the different populations revealed highly significant differences with $P<0.0001$ except for unfertilized soil plot pair that showed a P value of 0.017 (Table 1).

Phylogenetically, the 419 clone sequences clustered with 19 type strains. Representative ML trees are shown in

Table 1 ∫-Libshuff pairwise comparison of *Pseudomonas* community structures in fertilized and unfertilized soil plots using Cramer-von Mises test statistic[a]

Soil treatment	ΔCXYScore	*P*
Fertilized soil under corn (S2) versus fertilized soil under alfalfa (S5)	0.00043446	<0.0001
Fertilized soil under corn (S2) versus unfertilized soil under alfalfa (S7)	0.00198467	<0.0001
Fertilized soil under corn (S2) versus unfertilized soil under corn (S8)	0.000963	<0.0001
Fertilized soil under alfalfa (S5) versus unfertilized soil under alfalfa (S8)	0.00121828	<0.0001
Fertilized soil under alfalfa (S5) versus unfertilized soil under corn (S8)	0.00106402	<0.0001
Unfertilized soil under alfalfa (S7) versus unfertilized soil under corn (S8)	0.00020923	0.0172

Highly significant differences in *Pseudomonas* community structures except for unfertilized soil plot pair (S7 vs. S8); *P* probability

[a] Based on Libshuff method as implemented in mothur (Schloss et al. 2009)

Fig. 3a, b. The majority of the sequences within the different populations did not cluster with any of the type strains and were considered as uncultivated potential new *Pseudomonas* genotypes (group I) with low similarity to *P. amygdali* or *P. avellanae*. The percentage number of uncultivated genotypes was similar between fertilized soil plots and their corresponding unfertilized plots. Under corn cultivation, 42.0 or 42.6 % of clones retrieved did not group with any described type strains, while a 65.0 % or 70.0 % rate was observed for plots under alfalfa cultivation. The main clusters of sequences retrieved from fertilized soil plots under corn cultivation (S2) could be associated to *P. oryzihabitans* (Fig. 3a, cluster A) and *P. putida* (Fig. 3a, cluster B), while the clones from the corresponding unfertilized soil plot (S8) were strongly associated with *P. frederiksbergensis* (Fig. 3b, cluster C). Similar clustering patterns were observed between *Pseudomonas* populations retrieved from fertilized soil plot under alfalfa cultivation and the corresponding unfertilized soil plot (data not shown).

A principal coordinate analysis supported the hypothesis that long-term mineral fertilizer applications to soil plots could influence *Pseudomonas* community structure. *Pseudomonas* clone libraries from fertilized field plots, irrespective of the crop type under cultivation, clustered closely (PC1 and PC2) while a similar trend was observed with clone libraries from unfertilized plots (Fig. 4). The analyses also revealed a potential but less pronounced effect of crop species (PC1 vs. PC3; Fig. 4). The unfertilized permanent grass plot (S11) showed a loose relationship (P3 vs. P2) with the other unfertilized plots (S7 and S8) (data not shown).

Discussion

This study demonstrated that the community structure of *Pseudomonas* is influenced by long-term (>40 years) mineral fertilizer amendments. Analysis of 16S rRNA clone libraries using a suite of tools supports this hypothesis. The use of a culture-independent method for studying 16S rRNA bacterial diversity has proved to be a powerful tool for evaluation of shifts in community structures (Amann et al. 1995; Baati et al. 2010; Sun et al. 2004). This approach was adopted because of the limitations of traditional techniques using pure cultures, which are reported to represent only a small proportion (0.1–10 %) of the total soil bacteria (Amann et al. 1995; Colwell 2009; van Elsas et al. 2002). This is corroborated to an extent in our study since a high proportion (>40 %) of retrieved clone sequences did not cluster with cultured described type strains. The ability of 16S rRNA cloning to sample the phylogenetic diversity in natural communities more comprehensively than cultivation is characteristic of the method (Dunbar et al. 1999; Ludwig et al. 1997).

The primer set (F311 Ps/R1459 Ps) reported to be *Pseudomonas*-specific (Costa et al. 2007; Milling et al. 2004) consistently amplified 16S rRNA fragments of the genus *Lysobacter* (Xanthomonadaceae Family) and genus *Cellvibrio* (Pseudomonadaceae family). A few of the retrieved clones were classified as members of the genus *Flavimonas*, an indication of 16S rRNA sequence similarity to the primer set used in this study. This is not surprising given that the genera *Flavimonas* and *Chryseomonas* are junior subjective synonyms of *Pseudomonas* (Anzai et al. 1997).

Our study supports the general view that soil management practices could influence shifts in bacterial community structure (Buckley and Schmidt 2003; Peacock et al. 2001; Sun et al. 2004). However, long-term soil management practices would show a more significant impact than short-term land use (Buckley and Schmidt 2003; Sun et al. 2004). We analyzed soil plots that have been treated or not with mineral fertilizer for more than 40 years, and obtained substantive data on its influence on *Pseudomonas* community structure. For example, based on the rarefaction analysis, all fertilized plots consistently and significantly showed less diverse gammaproteobacteria populations than unfertilized plots, suggesting that recognizable gammaproteobacteria community structures have established in relation to long-term (>40 years) mineral fertilizer amendments. These

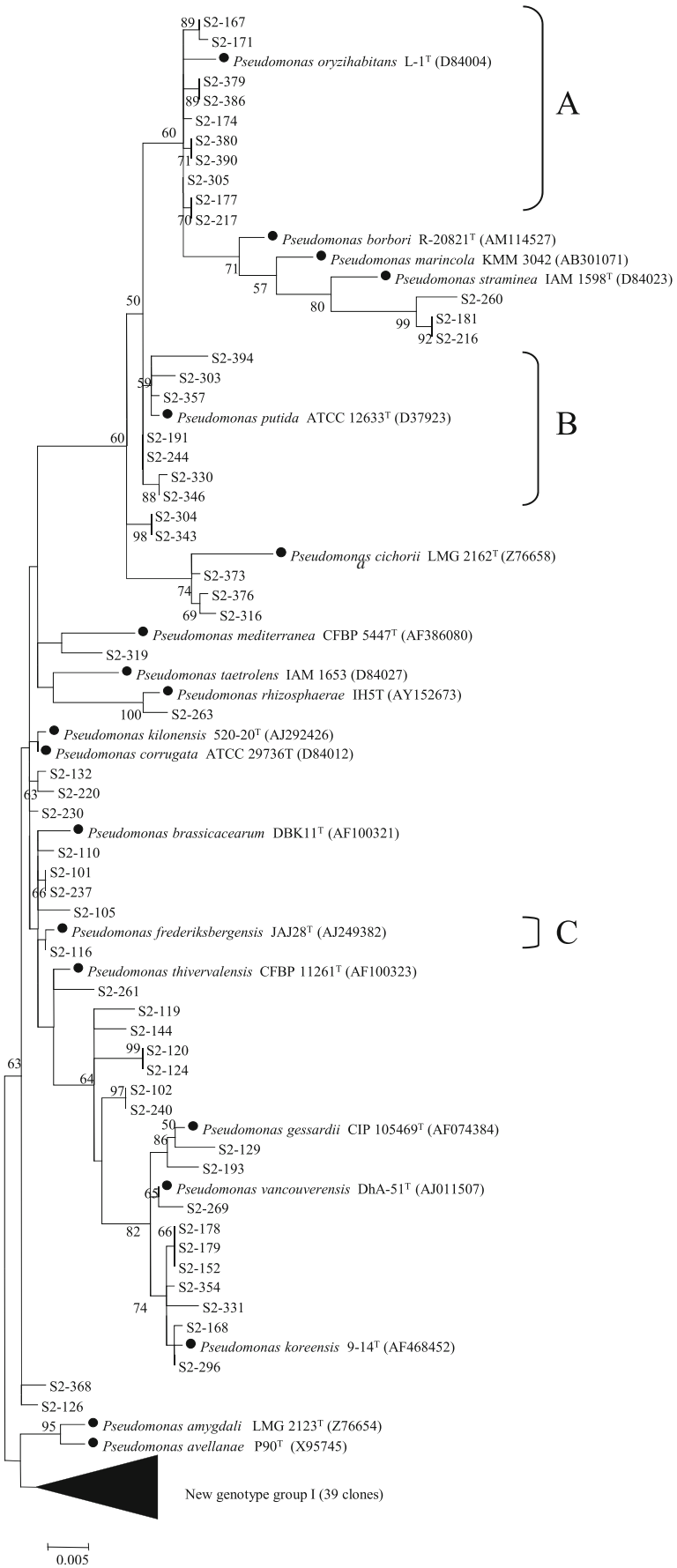
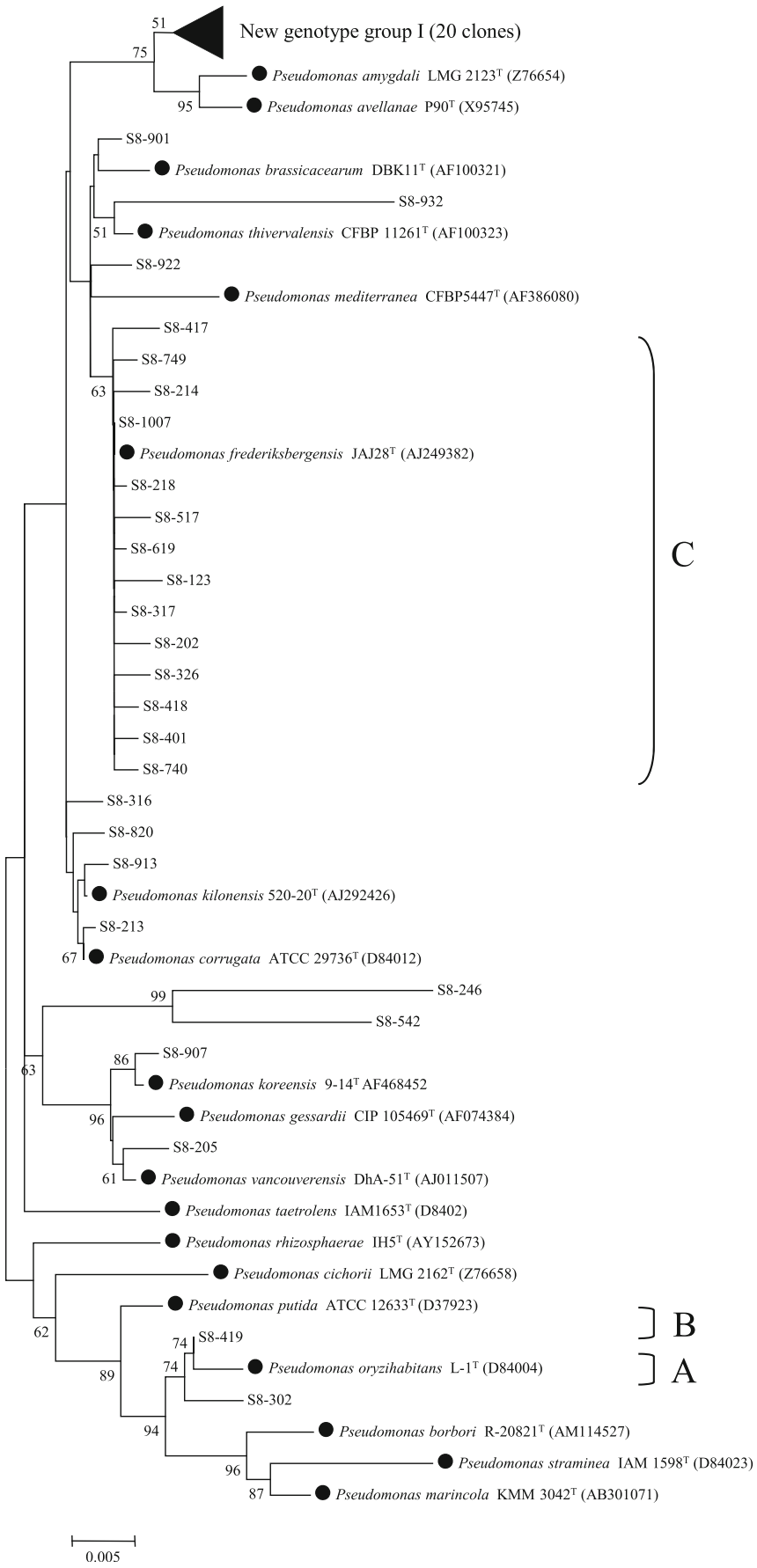
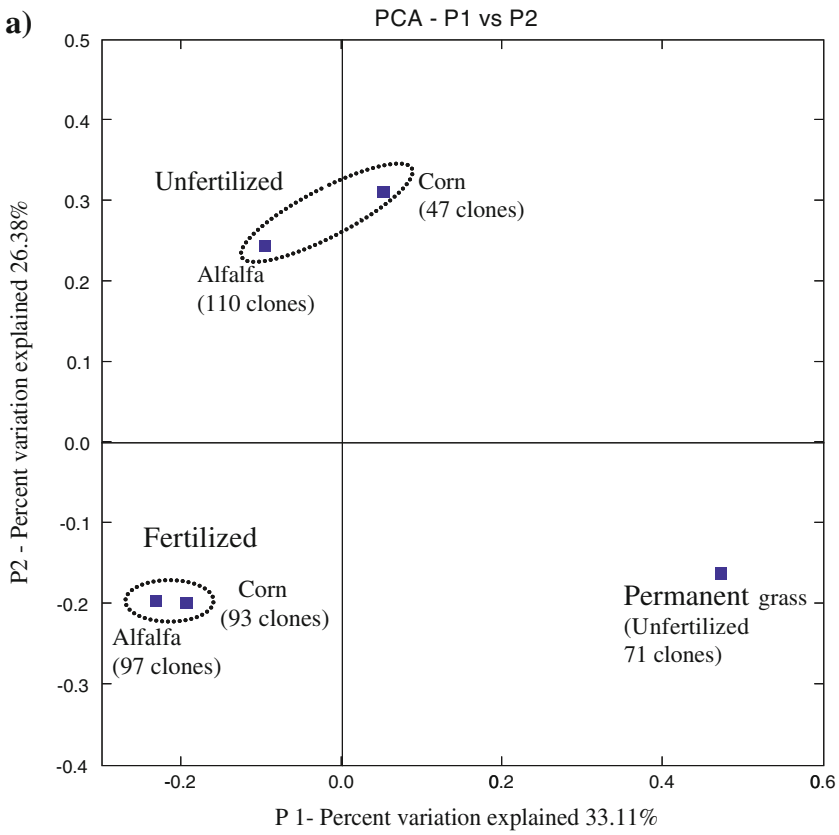


Fig. 3 Maximum likelihood (ML) phylogenetic tree showing *Pseudomonas* community clones recovered from fertilized plot S2 (**a**) and corresponding unfertilized soil plot S8 (**b**) under corn cultivation. ML was implemented in MEGA5 (Tamura et al. 2011) using the general time reversible (GTR) substitution model. Bootstrap values below 50 % are not shown. *Solid circle* indicates the type strain

Fig. 3 (continued)





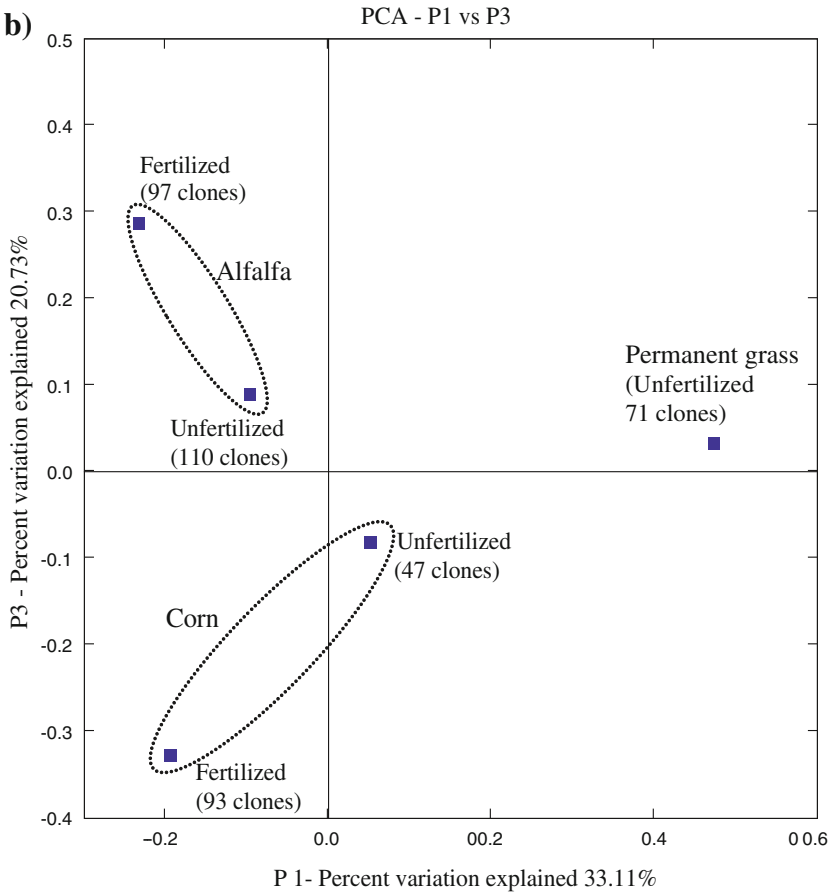


Fig. 4 First three principal coordinates from principal component analysis (PCA) based on 419 rRNA *Pseudomonas* sequences of unfertilized and fertilized soil plots under long-term corn-alfalfa rotation (cf. "Materials and methods" for details). PCA was implemented using UniFrac software package (Lozupone and Knight 2005) to measure the relative contributions of different factors (fertilizer treatment and plant type) to similarities between samples. The percentages in the axis labels represent the percentages of variation explained by the principal coordinates

results are in agreement with the conclusions of Buckley and Schmidt (2003) and Sun et al. (2004). A similar trend in *Pseudomonas* community structure was observed in fertilized and unfertilized plots under alfalfa cultivation.

Unfertilized plot (S8) under corn cultivation showed a low number of clones that belonged to the genus *Pseudomonas* compared to the other plots. This low number of sequences of *Pseudomonas*-like clones retrieved could be attributed to the unexpectedly high *Lysobacter*-like clones in the same soil plot. The proportion of *Lysobacter*-like clones retrieved from the unfertilized soil plot (S8) under corn cultivation was at least three times higher than in each of the other field plots. It is not clear why this unfertilized plot exhibited a high abundance of *Lysobacter*-like clones. It is possible that the order of crop rotation, a year of oat/alfalfa followed by a 2-year continuous alfalfa cultivation on this plot, might have enhanced the population of *Lysobacter*. The ecology of *Lysobacter* is not clear, but soil and plant types and seasonal factors are reported to influence their population (Hayward et al. 2010). Cultivation of clover seems to stimulate *Lysobacter* populations (Postma et al. 2008). However, its corresponding fertilized soil plot (S2) under the same rotation sequence did not show a similar abundance level of *Lysobacter*, which could be attributed partly to potential effects of long-term mineral fertilizer applications. Also, the unfertilized fallow (grass) plot (S11) had at least twice the clone abundance of unclassified Gammaproteobacteria (>50 % of retrieved clones), an indication that this plot harbors potential new genotypes of this predominant class of bacteria. Based on BLAST results, some of the clone sequences from this soil plot (11) were highly similar to *Pseudomonas syringae*, an indication that this plot could be a reservoir for this potential plant-pathogenic bacterium.

Phylogenetic analysis of the 16S rRNA clones indicated high association of *P. putida* and *P. oryzihabitans* with plots that received long-term mineral fertilizer applications. This could be partly due to the versatility of members of these two species in soils. *Pseudomonas putida* is a well-studied versatile Gram-negative bacterium that possesses most genes of any known species involved in bioremediation of several organic environmental pollutants. Aerobic denitrification by some *P. putida* strains has been reported (Lin 1999; Kim et al. 2008), while Carter et al. (1995) reported the expression of a periplasmic nitrate reductase in *Pseudomonas putida* 2.9 (Carter et al. 1995). Unlike *P. putida*, *P. oryzihabitans* is not well studied, but most of its strains reduce nitrate to nitrite, without denitrification (Palleroni 2005). The recovery of clone sequences that affiliated with *P. putida* or *P. oryzihabitans* mainly from soil plots that received long-term fertilizer applications species is, probably, the first report in which these 2 species are associated strongly with long-term mineral fertilizer applications.

A strong and consistent effect of fertilizer applications was evident in this study. Principal component analysis allowed for visualization of the influence of fertilizer application. While fertilizer effects were strong, only a relatively weak influence of crop type on bacterial community structure and diversity was observed. This is contrary to the conclusions of Buckley and Schmidt (2003) and Sun et al. (2004) on continuous winter wheat (*Triticum aestivum* L.). This discrepancy could be attributed to the fact that our study was in a crop rotation experiment in which the plant type is changed more frequently. An alfalfa crop effect was more pronounced than that of corn, probably because of the implemented sequence of rotation which favored the former. For example, in a 4-year rotation period, alfalfa was cropped three times either as monoculture or intercropped with oat. This duration of alfalfa cropping could have a significant influence on bacterial community structure and diversity. This sequence of crop rotation was adopted since corn disproportionately depletes soil nutrients, while alfalfa plants, because they have root nodules containing nitrogen-fixing bacteria, could improve soil fertility.

In conclusion, despite limitations inherent to culture-independent approaches, such as DNA extraction efficiency, competitive PCR amplifications of non-target DNA, and DNA amplification of non-viable cells, the use of this approach to investigate *Pseudomonas* community structure supports the accumulating evidence that mineral fertilizer application could influence shifts in microbial populations. The strength of our data in corroborating this general view could be due to the fact that we had the unique advantage of sampling long-term field plots that would have allowed for preferential accumulation of specific bacterial genotypes. The sustainability of agricultural systems is dependent on adapting management practices that conserve and maintain soil microbial biodiversity.

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