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Bacterial community variations in an alfalfa-rice rotation system revealed by 16S rRNA gene 454-pyrosequencing

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Running title: Bacterial diversity in alfalfa-rice crop rotation

Abstract

Crop rotation is a practice harmonized with the sustainable rice production. Nevertheless, the implications of this empirical practice are not well characterized, mainly in relation to the bacterial community composition and structure. In this study, the bacterial communities of two adjacent paddy fields in the 3rd and 4th year of the crop rotation cycle and of a non-seeded sub-plot were characterized before rice seeding and after harvesting, using 454-pyrosequencing of the 16S rRNA gene. Although the phyla *Acidobacteria*, *Proteobacteria*, *Chloroflexi*, *Actinobacteria* and *Bacteroidetes* predominated in all the samples, there were variations in relative abundance of these groups. Samples from the 3rd and 4th years of the crop rotation differed on the higher abundance of groups of presumable aerobic bacteria and of presumable anaerobic and acidobacterial groups, respectively. Members of the phylum *Nitrospira* were more abundant after rice harvest than in the previously sampled period. Rice cropping was positively correlated with the abundance of members of the orders *Acidobacteriales* and "*Solibacterales*" and negatively with lineages such as *Chloroflexi* "Ellin6529". Studies like this contribute to understand variations occurring in the microbial communities in soils under sustainable rice production, based on real-world data.

Keywords: 454-pyrosequencing; bacterial community; crop rotation; diversity; PCoA; rice paddy soil

Introduction

Soil is a privileged habitat for microorganisms and is amongst the most biodiverse environments on Earth (Tamames *et al*., 2010). It is estimated that 1 gram of soil contains about 1 billion of prokaryotic cells (Roesch *et al*., 2007). Although the vast majority of microbial soil species are so far uncultivable, in the last decades, the development and improvement of culture-independent methods (e.g., fluorescent *in situ* hybridization-FISH, phyloarrays, fingerprinting techniques and sequencing of small-insert libraries of environmental DNA) (Newby *et al*., 2009), allowed to get important insights into the phylogenetic diversity of soil microbiomes. Among them, high throughput sequencing technologies gave a new impetus to microbial ecology studies (Mardis, 2008; Mardis, 2011; Suenaga, 2012). In spite of the biases inherent to these technologies (Suenaga, 2012; Zinger *et al*., 2012), the pyrosequencing of phylogenetic marker genes, such as the 16S rRNA gene offers new insights into the composition and structure of environmental microbial communities, allowing inferences about biogeographical or ecological patterns in different habitats (e.g., soil, rhizosphere, sediments) (Gomes *et al*., 2010; Baldrian *et al*., 2012; Zhang *et al*., 2012) or environmental gradients (e.g., depth, pH, N amendments) (Lauber *et al*., 2009; Eilers *et al*., 2012; Ramirez *et al*., 2012).

Due to the increasing demand for productivity, in the beginning of the past century ancient agriculture practices gave way to conventional farming (Matson *et al*., 1997; Ladha & Reddy, 2003). Despite the benefits on productivity, the use of synthetic chemical compounds strongly alters soil microbial communities composition and biogeochemical cycles (Hussain *et al*., 2009; Ramirez *et al*., 2012) and triggers strong negative impacts on sustainable soil fertility and on environmental quality (Matson *et*

al., 1997; Quayle *et al*., 2006; Galloway *et al*., 2008). The expansion of sustainable agriculture practices, which avoid or strongly reduce the use of pesticides and synthetic fertilizers is a priority. Often relying on empirical practices, sustainable agriculture production would benefit from science-based evidences, demonstrating the implications of such management practices.

Crop rotation is an important alternative to conventional farming, because it offers high productivity (Xuan *et al*., 2012) while contributes to environmental health (Rui & Zhang, 2010). The cereal-legume rotation is a worldwide extended crop management, which improves the yield and quality of cereal crops by reducing diseases and weeds (Liebman & Davis, 2000; Fenández-Aparicio *et al*., 2007), fixing atmospheric N_2 (Kelner *et al*., 1997), and contributing to increase the soil organic matter content (Rosen & Allan, 2007). The legumes used in these rotation systems depend on the world region, the water regime and the season where forage is being cropped (Ladha & Reddy, 2003). Alfalfa (*Medicago sativa* L.) is a legume used in different world regions and with different crops, which contributes to maintain soil organic carbon (SOC) and to the accumulation of N in soil (Kelner *et al*., 1997; Pietsch *et al*., 2007). Nevertheless, information about the effect of alfalfa-rice rotation system on the bulk soil bacterial community is scarce. This study was based on the hypothesis that variations on the bacterial community composition and structure (i.e. the groups present and the abundance of each group, respectively) may coincide with i) the presence of rice crop; ii) alterations in the climate conditions and agriculture management (e.g., flooding, rice growth), occurring over the annual rice cycle; iii) alterations in the type of plant litter and nutrients in soil, which are supposed to differ in distinct stages of the crop rotation. To assess such variations, an exploratory comparative study based on the analysis of the 454-pyrosequencing 16S rRNA metagenome of rice paddies, was conducted. Bulk soil

samples of uncropped and cropped rice paddies at different stages of the alfalfa-rice rotation system were collected before rice seeding and after rice harvesting.

Materials and Methods

Site description and soil sampling

The soil samples were collected in an experimental farm of the Portuguese Agriculture Ministry ("Bico da Barca", 40 º 11' N; 08 º 41' W), in the valley of river Mondego, Montemor-o-Velho, Central Portugal. This farm is managed by the technical staff and no special conditions were imposed or controlled for the present study in order to get a perspective of real-world data. Samples were collected in 2010 from two adjacent organically farmed paddy fields, both under a four-year crop rotation system, in which alfalfa (forage crop) rotates with rice (cereal crop). Briefly, alfalfa is continuously cropped for two consecutive years (designated 1st and 2nd year) followed by two consecutive years of rice cropping (designated 3rd and 4th year). In the years of rice cropping, land is under fallow in autumn and winter to avoid the spreading of plant diseases and weeds (Fig. 1). At the beginning of the study, paddies A (total area=3070 m^2) and B (total area=1715 m^2) were in the 3rd and 4th year of the crop rotation cycle, respectively. The field management of both paddies was as follows: field preparation in early April, flooding on 7 April; drainage and mechanical removal of weeds on 19 May; flooding on 20 May; rice seeding on 21 May. A sub-plot (1 m^2 with a periphery margin of about 30 cm) of paddy A was left uncropped and is herein designated as ANS (non-seeded). During rice cropping paddies were drained for a few days and re-flooded at the early stage of rice growth (drainage on the 1st and flooding on the 3rd June) and at active

126 tillering (drainage on the 17th and flooding on the 20th July) to promote the good
127 development of rice plants. Finally, both paddies were drained on the 15th September,
128 and rice was harvested between 15th and 24th September. Since the main objective of the
129 field studies in this experimental farm is the optimization of organic practices, i.e., high
130 productivity without utilization of synthetic fertilizers and pesticides, the regular
131 practice implemented several years ago includes the amendment with a plant residue
132 commercial compost (Fertiormont, 2 t ha^{-1}) and with mineral gafsa (Fertigafsa, 300 kg P
133 ha^{-1}). Therefore, in this study paddy B (4th year) but not paddy A (3rd year), was
134 amended during field preparation in early April before flooding. This was a corrective
135 measure used by the farmers because in previous years rice yields decreased from the
136 3rd to the 4th year of the crop rotation.
137 Soils were sampled on the 6th April 2010 before flooding (samples labelled A_{Apr} I, II, III
138 and B_{Apr} I, II, III, respectively) and after harvesting, on the 29th September (A_{Sep} I, II, III,
139 ANS_{Sep} I, II, III and B_{Sep} I, II, III, respectively) using a soil corer device (2.5-cm
140 diameter by 25-cm depth). In September, samples of paddies A and B were collected
141 between stubble rows. Three different composite samples per plot were obtained at each
142 sampling date. Each composite sample was composed of 20 (5 for ANS) individual soil
143 cores randomly collected over the total area of each plot (paddies A, B and subplot
144 ANS) from the upper 0-25 cm of the soil. To prepare each composite sample, 20 (or 5
145 for ANS) individual soil cores were pooled, homogenized and visible root debris was
146 removed. Each composite sample corresponded to one of the three replicas, named I, II,
147 III, for each plot and sampling date (A_{Apr} I, II, III; As_{ep} I, II, III; $ANSs_{ep}$ I, II, III; B_{Apr} I,
148 II, III; B_{Sep} I, II, III). Soil aliquots of each replica were immediately stored at 4 ºC for
149 soil physicochemical characterization and at -20 ºC for molecular characterization.
150

151 **Soil characteristics**

152

153 The total C and N contents, pH in water and water content of soil were determined
154 following the methods described by Guitián and Carballas (1976). Briefly, total organic
155 C was determined by wet oxidation with potassium dichromate, after treatment with
156 H_2SO_4 to facilitate digestion of the organic matter and to ensure the removal of any
157 inorganic C present. Total N was measured by the Kjeldahl digestion method and the
158 pH in water was determined in a soil:solution mixture (1:2.5 w:v), with a glass
159 electrode. The total available-P was determined after extraction with 0.5 M sodium
160 bicarbonate following the methods described in Trasar-Cepeda *et al*. (1990).

161

162 **16S rRNA gene barcode 454-pyrosequencing**

163

164 A barcode pyrosequencing approach was used for the characterization of soil bacterial
165 communities. Genomic DNA was extracted from 7 aliquots of each soil replica using
166 the Power Soil™ DNA Isolation Kit (MO BIO) as described before (Lopes *et al*., 2011),
167 collecting the seven extracts in a single tube. DNA was further purified (Bacteria
168 genomicPrep Mini Spin Kit, Amersham Biosciences, NJ, USA). The DNA
169 concentration in the final extracts (Qubit® Fluorometer (Invitrogen) with Quant-iT™
170 dsDNA HS assay kit) was approximately 20 μg mL^{-1}. DNA extracts were used as
171 template for the amplification by PCR of the hypervariable V3-V4 region of the 16S
172 rRNA gene. The PCR amplifications, performed in duplicate for each DNA extract,
173 were carried out using barcoded fusion primers containing the Roche-454 A and B
174 Titanium sequencing adapters, an eight-base barcode sequence in adaptor A, and
175 specific sequences for the ribosomal region (V3F 5'-ACTCCTACGGGAGGCAG-3'

and V4R 5'-TACNVRRGTHTCTAATYC-3') (Wang & Qian, 2009). PCR mixtures (25 μL) contained: 0.2 mM dNTPs (Bioron, Ludwigshafen am Rhein, Germany), 0.2 μM of each primer, 5 % DMSO (Roche Diagnostics GmbH, Mannheim, Germany), 1x Advantage 2 Polymerase Mix (Clontech, Mountain View, CA, USA), 1x Advantage 2 PCR Buffer, and 1-3 μL of target DNA (corresponding to 20 ng), and cycling conditions consisted of a first denaturation step at 94 °C for 4 min, followed by 20 cycles at 94 °C (30 s), 44 °C (45 s) and 68 °C (60 s), and a final 2 min extension at 68 °C. The amplicons were quantified by fluorimetry with PicoGreen dsDNA quantitation kit (Invitrogen, Life Technologies, Carlsbad, California, USA) and pooled at equimolar concentrations. Pyrosequencing libraries were obtained using the 454 Genome Sequencer FLX platform according to standard 454 protocols (Roche 454 Life Sciences, Branford, CT, USA) at Biocant (Cantanhede, Portugal). The raw reads have been deposited into the NCBI short-reads archive database (accession number: SAMN01908502 to SAMN01908516).

Post-run analysis

Data generated from pyrosequencing was processed and analysed using QIIME pipeline (Caporaso *et al*., 2010). Briefly, sequences shorter than 280 bp and with quality scores lower than 25 were eliminated. Sequences (280 - 445 bp) were assigned to samples by the 8-bp barcodes, grouped into operational taxonomic units (OTUs) using UCLUST (Edgar, 2010) with a phylotype threshold of ≥ 97 % sequence similarity, and were taxonomically assigned using QIIME defaults. The sequences comprising each OTU were aligned using PyNAST (DeSantis *et al*., 2006) and were classified using Ribosomal Database Project (RDP) classifier (Wang *et al*., 2007). At the 97 % identity

level, the final OTU table consisted of 67 566 sequences (average of 4504 sequences per replica) distributed into 9480 OTUs, of those 4444 were represented by more than one sequence. A phylogenetic tree containing the aligned sequences was produced using FastTree (Price *et al*., 2009).

Both alpha and beta diversity metrics were determined using the QIIME pipeline. Alpha diversity was assessed calculating the richness estimator (Chao 1) (Chao, 1984) and the diversity indices (Simpson, Shannon and phylogenetic diversity-PD) (Simpson, 1949; Shannon & Weaver, 1963; Faith, 1992). Additionally, the sequences data was rarefied (2700 sequences per replica) (Caporaso *et al*., 2010) and beta diversity patterns of rarefied samples were assessed using the UniFrac metric (Lozupone & Knight, 2005).

Statistical analysis

The soil physicochemical properties and alpha diversity metrics among the five soil samples (A_{Apr}, As_{ep}, $ANSs_{ep}$, B_{Apr} and Bs_{ep}) were compared using the two-way analysis of variance (ANOVA) and the post-hoc Tukey test (SPSS Statistics 19, IBM).

Taxon and phylogenetic-based analyses were used to compare 16S rRNA gene sequences among the soil samples and to identify the bacterial groups that significantly changed between i) paddy A and paddy B (crop rotation), ii) April and September (season, i.e., rice crop cycle) and iii) sub-plot ANS_{Sep} and paddies A and B (i.e., that may have been affected by the presence of rice plants). For the taxon-based analyses, the percentage of abundance OTUs were compared using ANOVA combined with post hoc Tukey test. Phylogenetic-based comparisons were done using QIIME pipeline. Jackknifed principal coordinate analysis (PCoA) and dendrograms based on (un)weighted UniFrac distances were obtained. Statistical differences between bacterial

communities from the studied samples plotted on both PCoA were tested using analysis of similarity (ANOSIM). A Mantel test was conducted to evaluate if any of the determined soil physicochemical properties were related to the (un)weighted UniFrac values determined for soil samples and plotted on the PCoA.

Results

Physicochemical properties

The texture of the sampled soil was about 28, 50 and 21 % of sand, silt and clay, respectively (Lopes *et al*., 2011). The analyses of the five soil samples showed similar physicochemical characteristics, although with some significant differences in pH, water and total nitrogen contents, and concentration of total available phosphorus (Table 1). The total carbon content was not significantly different in the analysed samples.

Bacterial diversity

In total, 67 566 high-quality sequences from the 15 analysed metagenomes (triplicate of the samples A_{Apr}, As_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}) were obtained. Approximately 2 % of these sequences were not affiliated to the domain *Bacteria* and were excluded from further analyses. A variable number of high-quality sequences was obtained (ranging from 2754 to 7023). For this reason replicas were normalized by rarefaction to 2700 sequences per replica (total of 8100 sequences per soil sample), as recommended by the QIIME pipeline instructions (Caporaso *et al*., 2010).

Similar values of estimator Chao1 and Simpson index were obtained for all samples, demonstrating similar diversity coverage and an even distribution of sequences in the studied samples, respectively. The number of OTUs per rarefied soil sample varied between 1170 (B_{Sep}) and 1290 ($ANSs_{ep}$). Accordingly, sample ANS_{Sep} showed the highest values of PD and Shannon indices ($P < 0.05$) (Table 2), which indicates that the bacterial community in this sample was more diverse than in those of cropped soils (A_{Apr}, As_{ep} , B_{Apr} and Bs_{ep}).

Taxonomic affiliation of the total rarefied sequences

Each analysed metagenome included sequences that could not be assigned beyond the bacterial domain (average of 2.2 ± 0.3 %). The remaining bacterial sequences were affiliated to 39 phyla, with 19 presenting sequence abundances above 0.1 % (Fig. 2 and Table S1). Among these 19 phyla, sequences classified as *Acidobacteria* (32.4 %), *Proteobacteria* (26.3 %), *Chloroflexi* (8.6 %), *Actinobacteria* (7.5 %), *Bacteroidetes* (7.3 %), and *Gemmatimonadetes* (6.6 %) comprised about 80–90 % of all sequences. Other phyla with average abundance >1 % included *Nitrospira* (2.7 %), *Elusimicrobia* and *Firmicutes* (both averaging 1.0 %). The phyla *Chlorobi*, *Cyanobacteria*, *Verrucomicrobia*, *Planctomycetes* and candidate phyla, such as TM7 and AD3, were less abundant (abundance ranging from 0.1 to 1 %) (Fig. 2).

Acidobacteria, the most abundant phylum, were represented mainly by sequences affiliated to the classes *Acidobacteria* (16.0 %, out of which 99 % belonged to *"Koribacteraceae"*) and "*Solibacteres*" (11.0 %, all included in "*Solibacteraceae*"). Most of the *Proteobacteria* sequences were affiliated to *Alphaproteobacteria* (23.3 %, half of which belonged to the *Sphingomonadaceae*) (Table S2). Thus, these three were

the most abundant families in the analysed metagenomes, representing 26–43 % of the total of sequences (Fig. S1 and Table S4).

Given the small length of the 16S rRNA gene fragments analysed (280 - 445 bp, average of ~360 bp), the low resolution of the 16S rRNA gene sequence analysis for certain bacterial groups (Fox *et al*., 1992; Bennasar *et al*., 1996; Nübel *et al*., 1996; Ibrahim *et al*., 1997; Lebuhn *et al*., 2000; Jaspers & Overmann, 2004), and the eventual existence of organisms not yet affiliated to known taxa, only 33 % of the total sequences could be classified at the genus level. As expected, the most abundant genera belonged to the predominant families, and were "*Candidatus* Solibacter" (10.9 %), "*Kaistobacter*" (9.8 %), and "*Candidatus* Koribacter" (3.7 %) of the families "*Solibacteraceae", Sphingomonadaceae* and "*Koribacteraceae"*, respectively. Other abundant genera included *Rhodoplanes* (1.6 %) and *Nitrospira* (1.3 %) of the families *Hyphomicrobiaceae* and *Nitrospiraceae*, respectively. The abundance of the other genera was below 1 % (Table S5).

OTUs distribution and taxonomic affiliation

Considering the nucleotide sequence similarity threshold value of ≥ 97 %, a total of 6748 OTUs were obtained from rarefied data of the five soil samples analysed. Of these OTUs, 470 were common to all samples, 601 were common to the cropped samples ($A_{Apr,}$ As_{ep}, B_{Apr} and B_{Sep}), and 688 were common to samples A, irrespective of rice plant presence (A_{Apr}, As_{ep} and $ANSs_{ep}$) (Fig. S2a and b).

Among the 6748 OTUs, 371 presented abundance > 0.1 % in the three replicas of at least one sample, and are from this point forward referred to as representative OTUs of that given sample(s). Of these, 276 were common to all samples, 297 were common to

300 samples A_{Apr}, As_{ep}, B_{Apr} and Bs_{ep} and 299 were common to samples A, irrespective of
301 rice plant presence (A_{Apr}, As_{ep} and $ANSs_{ep}$). Thus, about 80 % of the representative
302 OTUs were common to all samples and unique OTUs were, in general, rare (less than
303 0.1 % abundance). Regardless of the threshold of abundance used to create Venn
304 diagrams, sample $ANSs_{ep}$ showed the highest number of unique OTUs (Fig. S2).
305 Among the 276 OTUs common to the five samples, only 82 had abundance > 0.1 % in
306 all the samples, being considered core OTUs. These OTUs were affiliated to the most
307 abundant phyla, except one that was assigned to the candidate phylum AD3 (Table 3).
308 As could be expected, the majority of core OTUs was evenly distributed, presenting
309 abundances between 0.1 and 1 %. Exceptions were two OTUs assigned to the families
310 *Sphingomonadaceae* ("*Kaistobacter*") and "*Koribacteraceae*", which represented, on
311 average, 6.9 and 1.3 % of the total recovered diversity (Table 3 and Fig. S1).
312
313 **Relationship between bacterial communities and edaphic factors**
314
315 To compare the composition and structure of the bacterial communities of the analysed
316 soil samples, the beta diversity was assessed. In this analysis, the three replicas of each
317 of the five samples were compared as 15 independent data sets. The unweighted and
318 weighted UniFrac-based PCoA explained, respectively, 21.6 and 55.9 % of the total
319 variation among the composition and structure of the bacterial communities, and
320 supported the distribution of samples in distinct groups (Fig. 3a and b). ANOSIM
321 confirmed that the groups plotted in both PCoA were significantly different
322 (unweighted UniFrac: R= 0.97, P = 0.001; weighted UniFrac: R= 0.89, P = 0.001).
323 Bacterial lineages affiliated to abundant phyla (Fig. 2) contributed to the variations
324 observed in the weighted UniFrac PCoA (Fig. 3b and c). Correlations between UniFrac

values and edaphic parameters were determined using Mantel tests (Table 4). A mild correlation between unweighted distances and water, total carbon and total available-P contents, and C:P and N:P ratios was observed. When weighted distances were considered only correlations with the water and total carbon contents were observed.

Comparison of rice cropped and uncropped soil

A strong primary clustering of $A_{Apr,}$ As_{ep}, B_{Apr} and B_{Sep} *versus* $ANSs_{ep}$ soil sample, represented in both UniFrac-based PCoA biplots (Fig. 3a and b), evidenced differences between the bacterial communities of cropped and uncropped soil samples. In fact, the average UniFrac distances measured between uncropped ($ANSs_{ep}$) and cropped soil samples (A_{Apr}, $As_{ep,}$ B_{Apr} or Bs_{ep}) were the highest (Fig. S4). Several representative OTUs in cropped samples (including in A_{Sep}), such as those assigned to "*Solibacteraceae*" ("*Candidatus* Solibacter"), "*Koriobacteraceae*" ("*Candidatus* Koribacter" among others), and *Sphingomonadaceae* ("*Kaistobacter*" among others), were absent or rare in the uncropped soil sample. Inversely, several representative OTUs in sample ANS_{Sep}, affiliated to *Chloroflexi* ("Ellin 6529"), "*Acidobacteria*-2", AD3, *Nostocaceae*, among others, were absent or rare in samples A_{Apr}, As_{ep}, B_{Apr} and B_{Sep} (Table S6). The majority of these OTUs were affiliated to bacterial lineages that correlated most with the different groups depicted in the weighted PCoA (Fig. 3b and c). Also the higher abundance of unclassified sequences in ANS_{Sep} than in the other samples contributed to distinguish its bacterial community from the others.

Comparison of soils at different crop rotation stages

The bacterial communities from the 3rd (A_{Apr} and As_{ep}) and 4th year (B_{Apr} and Bs_{ep}) of crop rotation, compared based on UniFrac-based PCoA (Fig. 3a and b), did not cluster (Fig. 3a and b, and S3a and b). Samples of paddies A and B were distributed along axis 2 of the weighted UniFrac-based PCoA biplot (Fig. 3b), with the most divergent being A_{Apr} and B_{Sep}. The highest and lowest average UniFrac distance between each pair of these samples was found for the pairs A_{Apr}/Bs_{ep} and B_{Apr}/$Bs_{ep,}$ respectively (Fig. S4a and b). A higher similarity between the bacterial communities of soil B sampled in April and September than with samples of soil A was evidenced. The bacterial community of sample A_{Sep} was as closer to samples of the 4th year of crop rotation (B_{Apr} and $B_{Sep,}$) as to A_{Apr}.

Sample A_{Apr} contained a higher number of rare and representative unique OTUs than the A_{Sep}, B_{Apr} and B_{Sep} samples (Fig. S2). Representative unique OTUs assigned to *Actinobacteria* (*Cellulomonadaceae*) and *Bacteroidetes* (*Sphingobacteriales*, mainly of the family *Chitinophagaceae*) (Table S7) present in A_{Apr} made this sample the most distinct amongst the cropped soil samples (Fig. 3b and c).

The distinction of the bacterial communities over the crop rotation was supported by some OTUs whose abundance was higher [e.g., assigned to *Bacteroidetes* (*Sphingobacteriales*, *Flavobacteriales)*, and *Alphaproteobacteria* (*Caulobacteraceae*)] or lower [e.g., assigned to *Acidobacteria* (*Acidobacteriales*, "*Solibacterales*"), *Bacteroidetes* (*Bacteroidales*), *Chloroflexi* (*Anaerolineae*) and *Chlorobi* ("SJA-28")] in samples of the 3rd year (A_{Apr} and As_{ep}) than of the 4th year (B_{Apr} and Bs_{ep}) of crop rotation (Table S7).

Comparison of soils before rice seeding and after harvesting

375 The average UniFrac distances obtained between cropped soil samples collected in
376 April and September in the 3rd (A_{Apr} and $As_{ep,}$) or in the 4th year (B_{Apr} and Bs_{ep}) (Fig. S4a
377 and b) indicate variations, although small, of the bacterial communities before rice
378 seeding and after harvesting, mainly in soil A. Unweighted, but not weighted, UniFrac
379 clustering via UPGMA showed some overlapping in the composition of 4th year (B)
380 bacterial communities of April and September (Fig. S3a). It is, thus, suggested that
381 major variations in the bacterial community over this period were mainly due to
382 modifications on the relative abundance of soil bacterial members. OTUs assigned to
383 "*Thermodesulfovibrionaceae*" within the phylum *Nitrospira,* which were more abundant
384 in September than in April in both cropped soils (A and B), may have contributed to the
385 observed variation (Table S8).
386
387 **Discussion**
388
389 **Composition and structure of bulk soil bacterial community**
390
391 The predominance of *Acidobacteria*, *Proteobacteria*, *Chloroflexi*, *Actinobacteria* and
392 *Bacteroidetes* in the paddy soils studied was expected, since these groups have been
393 described as common inhabitants of agriculture soils (İnceoğlu *et al*., 2011; Fierer *et al*.,
394 2012; Kuramae *et al*., 2012), including rice paddy soils (Tanahashi *et al*., 2005;
395 Asakawa & Kimura, 2008; Xuan *et al*., 2012). Half of the 82 core OTUs identified in
396 this study belonged to the classes *Acidobacteria, "Solibacteres"* and
397 *Alphaproteobacteria*. *Alphaproteobacteria* comprise organisms with diverse
398 physiological properties (Rappé & Giovannoni, 2003). Therefore, a clear prediction
399 about the ecology and biochemical role of these members in that habitat seems difficult.

However, it is noted that *Alphaproteobacteria* related with core OTUs enclose chemoorganotrophs, some of which are catalase producers and may be involved in lignin (e.g., *Caulobacter*) (Steinman *et al*., 1997) or aromatic compounds degradation (e.g., *Sphingomonadaceae*) (Kersters *et al*., 2006). Nitrogen fixation is another relevant function found in presumably (photo)organotrophic *Alphaproteobacteria* found in the studied soils (e.g., *Rhodospirillaceae, Hyphomicrobiaceae*) (Heising & Schink, 1998; Boer *et al*., 2005; Kersters *et al*., 2006; DeAngelis *et al*., 2011). In comparison to *Alphaproteobacteria*, *Acidobacteria* and *Gemmatimonadetes* are poorly characterized, since only a small fraction of these bacteria were cultivated (Barns *et al*., 1999; Quaiser *et al*., 2003; Rappé & Giovannoni, 2003; Davis *et al*., 2005; Kielak *et al*., 2008; DeBruyn *et al*., 2011). Nevertheless, *Gemmatimonadetes* represents a phylum with ubiquitous distribution (Rappé & Giovannoni, 2003; DeBruyn *et al*., 2011). *Acidobacteria* mainly from sub-divisions 1, 2, 3, 4 and 6 are common soil inhabitants (Jones *et al*., 2009; Eichorst *et al*., 2011). These organisms are described as chemo-organotrophic, oligotrophic bacteria, with slow growth and K-selected life strategy (Davis *et al*., 2005; Fierer & Jackson, 2006; Pankratov *et al*., 2008; Davis *et al*., 2011; DeAngelis *et al*., 2011; Eichorst *et al*., 2011). The edaphic characteristics of the analysed soils seem to have favoured the abundance of members of this phylum, mainly of the families "*Koriobacteraceae*" and "*Solibacteraceae*", of sub-divisions 1 and 3, respectively. Cultivable members of these sub-divisions, respectively related with "*Candidatus* Koribacter versatilis" and "*Candidatus* Solibacter usitatus", are described as growing optimally at the pH values found in the sampled soils (Eichorst *et al*., 2011). They are capable of decomposing complex substrates (e.g., xylan, hemicellulose, cellulose, pectin), a property that would support an active intervention in the degradation of plant litter in soils (Ward *et al*., 2009; Eichorst *et al*., 2011). In addition,

some members of *Acidobacteria* subdivision 1, in which the family "*Koriobacteraceae*" is included, are capable of feeding on readily oxidizable carbon (Eichorst *et al*., 2011). Based on the characteristics previously described for bacterial groups related with the core bacterial members of the analysed samples, it can be hypothesized that they may have an important role in the cycling of organic carbon derived from plants litter or root exudates (DeAngelis *et al*., 2011; Baldrian *et al*., 2012; Eichorst & Kuske, 2012), a crucial activity in ecosystems, including rice paddies (Asari *et al*., 2007; Rui *et al*., 2009).

Verrucomicrobia have been described as abundant inhabitants of paddy soils (Kikuchi *et al*., 2007; Asakawa & Kimura, 2008; Xuan *et al*., 2012), although presented low abundance in the current study. This observation may be due to the primers used, since *in silico* analyses demonstrated that this primer set may fail the detection of verrucomicrobial sequences (Wang & Qian, 2009), explaining the underestimation of this phylum, even using culture independent methods (Bergmann *et al*., 2011).

Variations in the composition and structure of bulk soil bacterial community

Diversity analyses revealed a richer bacterial community in uncropped soil (ANS_{Sep}) than in rice cropped soil samples (A_{Apr}, As_{ep}, B_{Apr} and Bs_{ep}). This observation may be supported by previous studies which demonstrate that the bacterial communities of the rhizosphere are less diverse than those of bulk soil (Sørensen, 1997; Garbeva *et al*., 2004; Berg & Smalla, 2009; Gomes *et al*., 2010; Uroz *et al*., 2010). Given ANS was a subplot of paddy A, the influence of flooding and climate conditions was identical in samples A_{Sep} and ANS_{Sep}. Hence, a lower diversity in A_{Sep} than in ANS_{Sep} suggests that the presence of rice plants contributed to attenuate eventual bacterial community

variations occurring in sample ANS_{Sep} due to flooding and climate conditions. The dense root mat characteristic of rice cropped paddy soils (Liesack *et al*., 2000), combined with the usual flooding state of paddy fields may promote the distribution of root exudates in flooded soils. Given their presumable nutritional profile, the high relative abundance of "*Solibacteraceae*", "*Koriobacteraceae*" and *Sphingobacteraceae* observed in cropped soils may have been due not only to the presence of rice plants litter, but also of rice exudates. In addition, the proliferation of the organisms of these groups may have contributed to lessen the relative abundance of others, such as unclassified bacteria and members of "*Acidobacteria*-2" and AD3, absent or rare in cropped soil. In contrast, the absence of rice rhizosphere and the consequent scarcity of plant litter and root exudates in the uncropped soil could have favoured the development of other bacterial lineages with slow growth and ability to survive under low substrate availability (e.g., *Chloroflexi* "Ellin6529"), as was described before for members of this phylum (Acosta-Martínez *et al*., 2010). Water content is a parameter affected by several external conditions, including the climate. However, since ANS was a sub-plot of paddy A, it was under the same flood regime. Thus, the presence of rice crop in A_{Sep} may have contributed to the significantly higher water content observed in this sample than in the uncropped soil. Indeed, the ability of plants to increase the retention of water in soil is reported (Chapin & Körner, 1995). Also, the higher abundance of OTUs affiliated to *Nostocaceae* in uncropped than in cropped soil samples may be related with the same abiotic parameter. *Nostoc* members have been referred to as being more prevalent in dry than in wet paddy soils (Roger *et al*., 1993). In addition, the poor plant cover in the uncropped sub-plot may have also favoured the access to sun light, and thus, the development of these photosynthesizing microorganisms.

The stage of the crop rotation affected the bacterial community of cropped bulk soil. Through the weighted UniFrac analysis, a gradient from A_{Apr} to B_{Sep} was observed. The high number of rare and representative unique OTUs (*Bacteroidetes/Sphingobacteriales* and *Actinobacteria/Cellulomonadaceae*) together with the abundance of other presumably aerobic chemoorganotrophs (*Bacteroidetes*/*Flavobacteriales* and *Alphaproteobacteria/Caulobacteraceae)* in sample $A_{Apr,}$ contributed to distinguish this sample from the others. Given that sample A_{Apr} was collected soon after alfalfa harvesting, it can be hypothesized that the root exudates and debris of the forage crop may have favoured the development of these bacterial groups. However, additional studies involving the analysis of samples collected from a subplot where alfalfa was not cultivated are needed to confirm this hypothesis. The abundance of *Alphaproteobacteria (Caulobacteraceae)* and *Bacteroidetes* (*Sphingobacteriales* and *Flavobacteriales)* was higher in paddy A than in paddy B. Inversely, the abundance of *Acidobacteriales* and "*Solibacterales"* was higher in paddy B than in paddy A. These variations may have been due to several alterations in the soil environment. The presence of alfalfa debris in paddy A and of rice root exudates/litter in paddy B may have contributed to the observed differences. However, the influence of soil amendments with mineral P and compost in paddy B cannot be disregarded. Also the abundance of presumable obligate anaerobic chemo-organotrophs (*Chloroflexi/Anaerolineae* and *Bacteroidetes/Bacteroidales)* and phototrophs (*Chlorobi* "SJA-28") was higher in paddy B than in paddy A. Beside the changes in the available nutrients, derived from the substitution of alfalfa for rice root exudates/litter and from the soil amendments, also the higher water content observed in samples B_{Apr} and Bs_{ep} than in A_{Apr} and As_{ep} could have contributed to the increased abundance of these presumable anaerobic bacteria in

paddy B. It is worth mentioning that despite the amendments made, the rice productivity decreased from the 3rd to the 4th year of the crop rotation.

Although this kind of studies in real agriculture fields has several advantages, including the avoidance of microcosms or mesocosms biases, these latter approaches are required to get a reliable assessment of the influence of some variables. Such studies would be useful to assess the contribution of each of the varying parameters, i.e., paddy A/paddy B soil properties, alfalfa/rice litter and/or exudates, P/organic amendments, flooding, soil water content, while maintaining the climate conditions.

According to the results obtained, variations on the bulk soil bacterial communities over time, i.e., variations over the rice annual cycle, were mainly due to alterations in the abundance of some bacterial lineages rather than to presence/absence shifts. Similar findings were reported in previous studies based on community analyses fingerprinting (Kikuchi *et al*., 2007; Lopes *et al*., 2011). Among the representative bacterial lineages occurring in the studied soils, members affiliated to "*Thermodesulfovibrionaceae*", within *Nitrospira*, stood out as having higher abundance in cropped soils after rice harvesting (A_{Sep} and B_{Sep}) than before rice seeding. The increased abundance of this phylum after rice harvesting was reported before (Xuan *et al*., 2012), and may be due to the availability of inorganic compounds resultant from organotrophic metabolism of plant debris and root exudates. Indeed, *Nitrospira* includes chemolithotrophs involved in the N, Fe and S cycles through the activity of nitrite- and ferrous iron-oxidizing and sulfate-reducing bacteria (Henry *et al*., 1994; Ehrich *et al*., 1995; Hippe, 2000). The development of sulfate-reducing bacteria, most probably obligate anaerobes, may have been also favoured by the flooding conditions prevalent during the growth of rice plants. The proliferation of members of these lineages during the late stage of the growth of rice plants, may explain their apparent predominance after rice harvesting.

523 Indeed, the activity of sulfate- and iron-reducers is important in paddy soils (Liesack *et*
524 *al*., 2000), and can be observed in aged rice roots, due to blackening by ferrous sulfide
525 precipitation at the late stage of the growth of rice plants.
526 In summary, the hypothesis supporting this study was confirmed, since bacterial
527 community variations coincided with the occurrence of rice cropping and different
528 periods of the annual rice cycle or crop rotation. The variations observed can be
529 summarized in three major highlights: a) In September, rice cropped soil (A_{Sep})
530 presented a lower diversity and lower relative abundance of rare OTUs (e.g.,
531 *Chloroflexi* "Ellin6529", *Nostocaceae*) than the uncropped (ANS_{Sep}) soil; b) From April
532 to September, a relative increase in the abundance of "*Thermodesulfovibrionaceae*" was
533 observed; c) In the 4th year of the crop rotation, the relative abundance of *Acidobacteria*
534 and of presumably anaerobic bacteria was higher than in the 3rd year; in contrast, the
535 abundance of presumable aerobic bacteria was higher in the 3rd than in the 4th year,
536 mainly in April.
537 In future studies, the interpretation of the observed bacterial community variations in
538 relation to other indicators of nutrients availability and/or microbial activity may bring
539 additional insights into the factors driving the dynamics of bacterial populations in this
540 type of environment.
541
542
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544
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784 **Table 1.** Physicochemical properties of soils A_{Apr}, As_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}. Values are means ± standard deviation (n=3).

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Parameter	A_{Apr}	A_{Sep}	ANS_{Sep}	B_{Apr}	B_{Sep}
pH	5.97 ± 0.03 ^{c}	5.88 ± 0.02 ^{b}	5.87 ± 0.02 ^{b}	5.94 ± 0.06 ^{b,c}	5.70 ± 0.02 ^{a}
Water content (%) (g H_2O /100 g wet soil)	22.4 ± 0.1 ^{b,c}	21.8 ± 0.4 ^{b}	19.2 ± 0.5 ^{a}	27.1 ± 0.8 ^{d}	23.4 ± 0.2 ^{c}
Total C (%)	1.33 ± 0.10	1.33 ± 0.13	1.38 ± 0.27	1.68 ± 0.04	1.60 ± 0.05
Total N (%)	0.14 ± 0.00 ^{a}	0.14 ± 0.00 ^{a}	0.14 ± 0.00 ^{a}	0.15 ± 0.01 ^{a}	0.18 ± 0.00 ^{b}
Total available P (mg Pt kg^{-1}soil)	50.7 ± 0.4 ^{b}	35.7 ± 0.6 ^{a}	36.7 ± 0.6 ^{a}	80.3 ± 2.4 ^{d}	56.4 ± 0.6 ^{c}

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791 The rice productivity was higher in paddy A than in paddy B (productivity ratio B:A = 0.63).

792 $^{a-d}$, Homogeneous subsets among paddies (A_{Apr}, As_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}), as determined by the Tukey test at $P<0.05$.

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Table 2. Diversity of bacterial rRNA gene fragment sequences in soil samples A_{Apr}, As_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}. The diversity measurements (Number of OTUs, Chao1, Shannon and Simpson indices and Phylogenetic diversity) were determined at a rarefaction of 2700 sequences per replica. Values are means ± standard deviation (n =3).

	A_{Apr}	A_{Sep}	ANS_{Sep}	B_{Apr}	B_{Sep}
No. OTUs	1236 ± 12 ^{a,b}	1206 ± 41 ^{a,b}	1290 ± 45 ^{b}	1212 ± 40 ^{a,b}	1170 ± 29 ^{a}
Chao1	3150 ± 114	2967 ± 148	2967 ± 184	3060 ± 163	3010 ± 36
Shannon index	9.30 ± 0.02 ^{b,c}	9.26 ± 0.07 ^{a,b,c}	9.44 ± 0.08 ^{c}	9.25 ± 0.03 ^{a,b}	9.12 ± 0.03 ^{a}
Simpson index	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00
Phylogenetic Diversity (PD)	87.2 ± 1.2 ^{a}	89.7 ± 2.8 ^{a}	99.2 ± 4.1 ^{b}	90.5 ± 2.7 ^{a,b}	88.2 ± 1.7 ^{a}

$^{a-c}$, different letters indicate differences in alpha diversity metrics values among the bacterial communities of the analysed samples (one-way ANOVA followed by Tukey post-hoc test, $P < 0.05$).

800 **Table 3.** Number of core OTUs (abundance > 0.1 % in the three replicates of all the five analysed samples -A_{Apr}, As_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}).

Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs	Genus	No. OTUs
OTUs present in all the samples at abundance > 1 % (core OTUs)											
Bacteria	2	*Acidobacteria*	1	*Acidobacteria*	1	*Acidobacteriales*	1	"*Koribacteraceae*"	1		
		Proteobacteria	1	*Alphaproteobacteria*	1	*Sphingomonadales*	1	*Sphingomonadaceae*	1	"*Kaistobacter*"	1
OTUs present in all the samples with abundances ranging from 0.1 to 1%											
Bacteria	80	*Acidobacteria*	39	*Acidobacteria*	20	*Acidobacteriales*	20	"*Koribacteraceae*"	19	"*Candidatus* Koribacter"	5
								Acidobacteriaceae	1	*Edaphobacter*	1
				"*Acidobacteria* - 2"	3						
				"*Chloroacidobacteria*"	3						
				"*Solibacteres*"	13	"*Solibacterales*"	13	"*Solibacteraceae*"	13	"*Candidatus* Solibacter"	13
		Actinobacteria	3	*Acidimicrobiia*	1	*Acidimicrobiales*	1				
				Actinobacteria	2	*Actinomycetales*	2	*Intrasporangiaceae*	1		
								Micrococcaceae	1		
		AD3	1	"ABS-6"	1						
		Bacteroidetes	3	*Sphingobacteriia*	3	*Sphingobacteriales*	3	*Chitinophagaceae*	2		
		Chloroflexi	1	"Ellin 6529"	1	*Gemmatimonadales*		"Ellin 5301"	1		
		Gemmatimonadetes	10	*Gemmatimonadetes*	6						
				"Gemm-1"	4						
		Nitrospira	2	*Nitrospira*	2	*Nitrospirales*	2	*Nitrospiraceae*	2	*Nitrospira*	2
		Proteobacteria	20	*Alphaproteobacteria*	19	*Rhizobiales*	7	*Bradyrhizobiaceae*	1		
								Hyphomicrobiaceae	4	*Rhodoplanes*	2
										Devosia	1
								Phyllobacteriaceae	1		
						Caulobacterales	1	*Caulobacteraceae*	1	*Phenylbacterium*	1
						Rhodospirillales	3	*Rhodospirillaceae*	3		
						Sphingomonadales	6	*Sphingomonadaceae*	6	"*Kaistobacter*"	6
				Deltaproteobacteria	1	*Myxococcales*	1				

Table 4. Relationship of soil physicochemical properties and (un)weighted unifrac distance measured from soil samples (A_{Apr}, As_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}) (Mantel test)

	Unweighted		Weighted	
Soil physicochemical properties	Mantel *r*	*P* value	Mantel *r*	*P* value
Water content	0.527	0.001**	0.406	0.004*
pH	0.095	0.390	0.055	0.670
Total C	0.230	0.039*	0.271	0.036*
Total N	0.033	0.765	0.138	0.372
Total available - P	0.347	0.003*	0.133	0.290
C:N	0.168	0.100	0.120	0.301
C:P	0.242	0.022*	0.162	0.195
N:P	0.324	0.016*	0.076	0.505

The iterations were set to 999. *$P <$ 0.05 and ** $P \leq$ 0.001 indicate significant correlations.

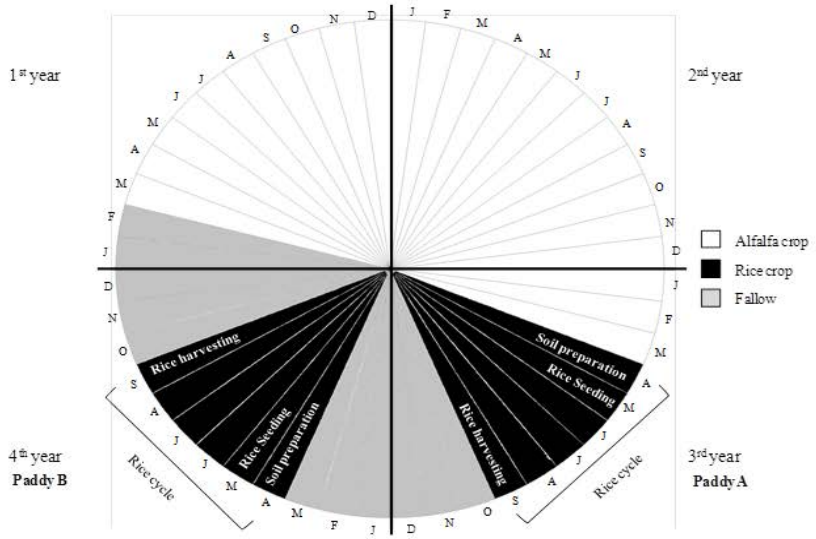

Fig. 1. Scheme of alfalfa-rice crop rotation in the experimental farm "Bico da Barca", Montemor-o-Velho, Portugal.

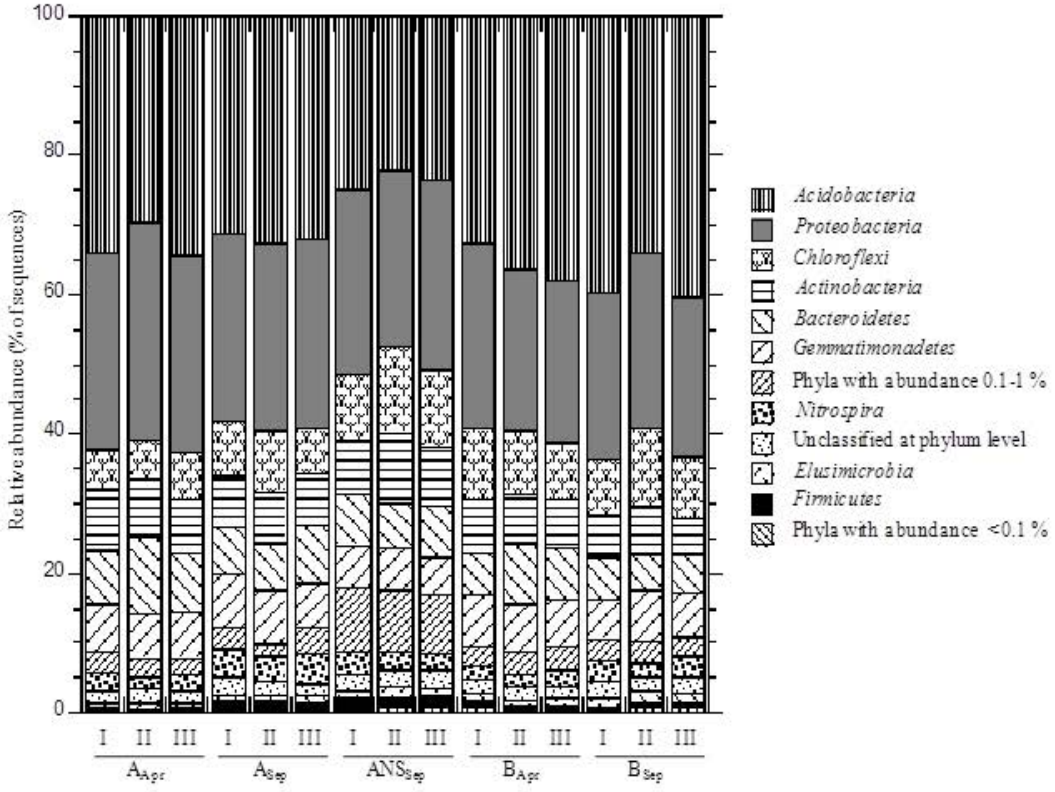

Fig. 2. Relative abundance of different phyla in each replica of samples A_{Apr}, As_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}. The abundance is expressed as the percentage in the total number of rarified bacterial sequences (2700), classified using RDP Classifier at a confidence threshold of 80%. Phyla with abundance ranging from 0.1 to 1% include *Chlorobi*, *Cyanobacteria*, TM7, AD3, OD1, *Armatimonadetes*, *Verrucomicrobia*, *Fibrobacteres*, *Planctomycetes* and OP11. Phyla with abundance < 0.1% are listed in the Table S1.

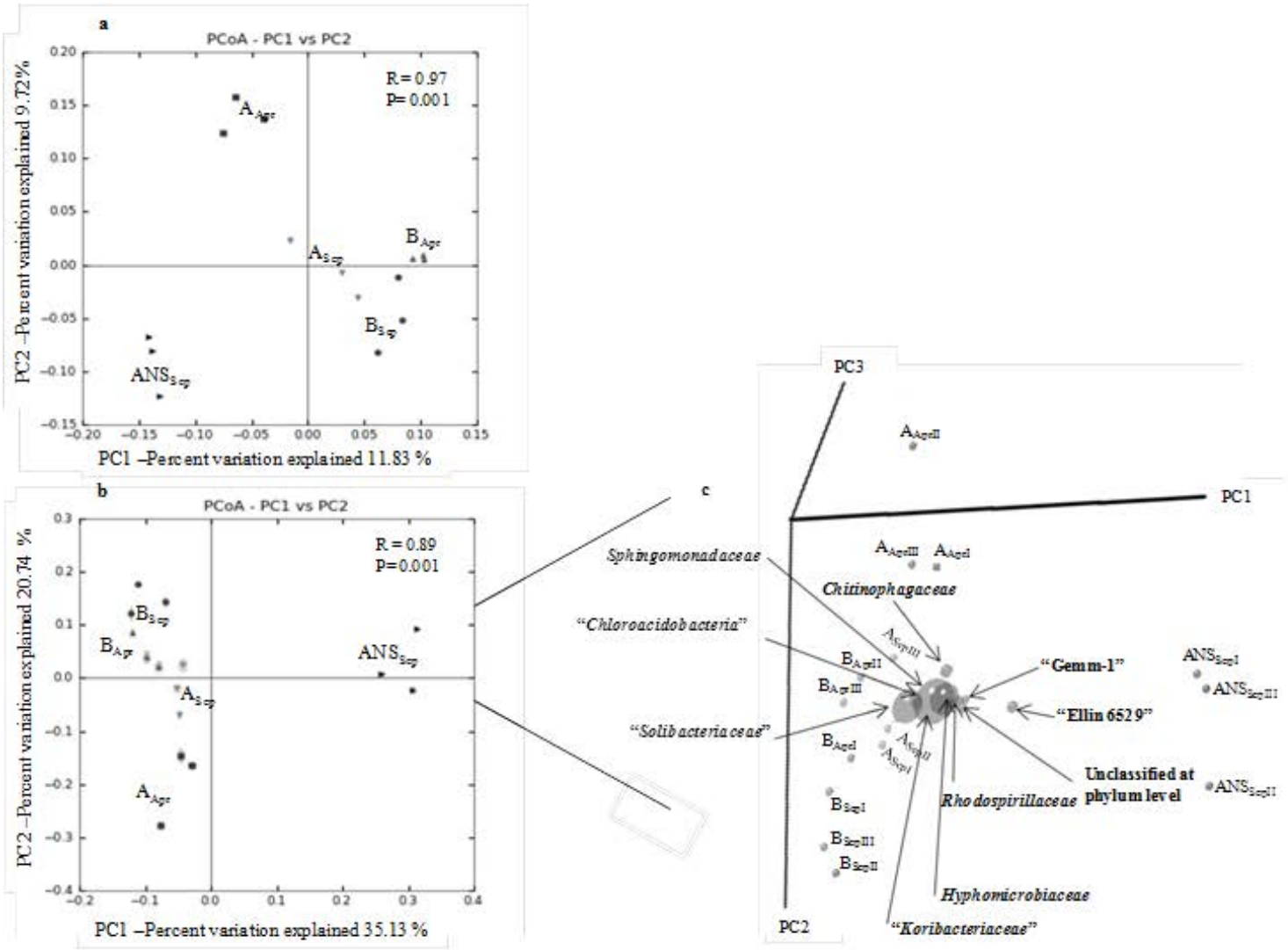

Fig. 3. Jackknifed PCoA plots illustrating distances among bacterial communities of soil samples AApr, Asep, ANSsep, BApr and BSep. (a) Bacterial unweighted UniFrac distances. (b) Bacterial weighted UniFrac distances. (c) Bacterial lineages that varied most among samples.