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Authors

Collavino, Mónica M
Tripp, H James
Frank, Ildiko E
et al.

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nifH pyrosequencing reveals the potential for location-specific soil chemistry to influence N_2-fixing community dynamics

Mónica M Collavino[1,2], H James Tripp[3], Ildiko E Frank[3], María L Vidoz[1], Priscila A Calderoli[2], Mariano Donato[4], Jonathan P Zehr[3], and O Mario Aguilar[2]

[1]Instituto de Botánica del Nordeste (IBONE), Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste-CONICET, Corrientes, Argentina
[2]Instituto de Biotecnología y Biología Molecular (IBBM), Universidad Nacional de La Plata-CONICET, La Plata, Argentina
[3]Department of Ocean Sciences, University of California, Santa Cruz, CA, USA
[4]Laboratorio de Sistemática y Biología Evolutiva, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, La Plata, Argentina

Corresponding author: O. Mario Aguilar, 49 esq 115 (1900) La Plata, Argentina. Telephone/Fax number 0054-0221-4229777. E-mail: aguilar@biol.unlp.edu.ar.

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Summary

A dataset of 87020 *nifH* reads and 16782 unique *nifH* protein sequences obtained over two years from four locations across a gradient of agricultural soil types in Argentina were analysed to provide a detailed and comprehensive picture of the diversity, abundance, and responses of the N_2-fixing community in relation to differences in soil chemistry and agricultural practices. Phylogenetic analysis revealed an expected high proportion of Alpha-, Beta- and Delta-proteobacteria, mainly relatives to *Bradyrhizobium* and *Methylosinus*/*Methylocystis*, but a surprising paucity of Gamma-proteobacteria. ANOVA and stepwise regression modelling suggested location and treatment-specific influences of soil type on diazotrophic community composition and organic carbon concentrations on *nifH* diversity. *nifH* gene abundance, determined by qPCR, was higher in agricultural soils than in non-agricultural soils, and was influenced by soil chemistry under intensive crop rotation but not under monoculture. At some locations, sustainable increased crop yields might be possible through the management of soil chemistry to improve the abundance and diversity of N_2-fixing bacteria.

Introduction

Biologically available N is often a limiting nutrient in agricultural soil and other environments (Vitousek and Howarth, 1991). To this end, 4.32×10^5 tons of nitrogen fertilizers are used annually in the productive agricultural region of Argentina, which have recently been dedicated to soybean monoculture, due to financial considerations that also lead to minimal

nutrient restoration (FAO, 2012). In an effort to mitigate the negative effects of monoculture on soil quality, principally increased erosion and decreased moisture retention, reduced-till agricultural practices have been widely introduced in Argentina (Viglizzo *et al*., 2011). Studies have shown that while reduced-till practices can improve soil quality by reducing loss of nutrients and increasing moisture retention, it remains to be seen whether the beneficial effects are fully realized under monoculture and low nutrient replenishment (hereafter named poor no-till practices) as compared to intensive crop rotation and nutrient replacement (named good no-till practices) (Abid and Lal, 2009; Souza et al., 2013). Specifically, it is not known if reduced-till practices under monoculture improve the diversity, abundance, and community structure of N_2-fixing microbes in agricultural soil.

Biological N_2 fixation (BNF), which is the biological reduction of molecular N_2 gas to biologically-available ammonium, accounts for approximately 128 million tons nitrogen per year and is considered the main route by which fixed nitrogen enters the biosphere by natural processes (Galloway *et al*., 2004). BNF, catalyzed only by Bacteria and Archaea, requires nitrogenase, an evolutionarily conserved protein in N_2-fixing microorganisms. The nitrogenase enzyme is composed of two multisubunit metallo-proteins. Component I contains the active site for N_2 reduction and is composed of two heterodimers encoded by the *nif*D and *nif*K genes. Component II, also known as dinitrogenase reductase, is composed of two identical subunits encoded by the *nifH* gene. The *nifH* gene sequence is highly conserved across the bacterial and archaeal domains; however, because of codon redundancy for most amino acids, the design of universal *nif*H primers requires a considerable degree of DNA sequence degeneracy. Up to date, several degenerated PCR primers were developed and have been successfully used in culture-independent studies of microbial N_2-fixing communities in terrestrial and aquatic environments for almost 25 years (Zehr and McReynolds, 1989; Zehr *et al*., 1998; Poly *et al*., 2001b; Rosch *et al*., 2002; Steward *et al*., 2004; Bürgmann *et al*., 2005; Izquierdo and Nüsslein, 2006; Farnelid *et al*., 2011; Niederberger *et al*., 2012). As a result, the database for nitrogenase genes (specifically the *nifH* gene) has become one of the largest non-ribosomal gene datasets from uncultivated microorganisms (Zehr *et al*., 2003) This makes *nif*H gene an outstanding reference tool for studying N_2-fixing microbial communities in soil, most of whose members have not yet been cultured. The advent of pyrosequencing now affords the opportunity to study N_2-fixing soil microbial communities in depth, providing tens or hundreds of thousands of *nifH* amplicon sequences per sample.

Like the general survey of soil microbial communities using deep pyrosequencing of 16S rRNA gene (Figuerola *et al*., 2012), this study is part of a larger effort to provide microbial

indicators of the sustainability of no-till practices in four agricultural sites located across a west-east transect in the Argentine Central Pampas (Fig. 1). However, this study is a specific survey of only the N_2-fixing microbial communities in soil, using deep pyrosequencing of the *nifH* gene. The reader is referred to the Introduction and Methods sections of Figuerola *et al.* 2012 for a detailed description of the broader BIOSPAS project and the study sites selected.

The aim of this work was to examine the abundance, diversity and structure of diazotroph communities in a gradient of Argentinean agricultural soils, under good and poor no-till practices, using deep pyrosequencing-based analysis of the *nifH* gene.

Results

Proteobacteria are predominant in the diazotrophic community of Argentinean soils

A total of 87020 *nifH* reads, comprising 16782 unique protein sequences, were obtained from four Argentinean soils subjected to different management. At 98% sequence similarity, the 1558 OTUs for the entire study were imported into an ARB database of *nifH* sequences (Zehr *et al*., 2003) and assigned to 17 subclusters of the four major *nifH* clusters previously defined by Zehr *et al*., (2003) (Table 1). The abundances varied widely among the OTUs; about 1000 OTUs were observed to be represented by no more than 10 sequences, whereas 1375 had less than 50 sequence counts, and only 19 OTUs were represented by more than 1000 sequences (Fig. S1). The proportion of OTU counts and sequence counts was similar in each subcluster (Table 1). Cluster I was the most abundant in the soil samples, particularly the subclusters 1K (40% of the total number of OTUs), 1A (21%) and 1J (20%) which are mainly composed of sequences related to Rhizobiales and Burkholderiales, *Anaeromyxobacter* and Desulfuromonadales, and Rhizobiales and Rhodospirillales, respectively. The next largest group was subcluster 3B (8%), with sequences mainly related to Desulfovibrionales and Verrucomicrobiales (Table 1). No new *nif*H subclusters were identified in this study; however it is noteworthy that new *nif*H sequences within subclusters were observed, i.e. 285 of the 1558 OTUs, most belonging to the subclusters 1A, 3B and 1J, have less than 95% similarity at the amino acid level as compared to the sequences present in the *nif*H database (data not shown).

Nineteen of the most abundant OTUs corresponding to about 48% of our *nifH* database were distributed among the four subclusters 1K (12 OTUs), 1J (4 OTUs), 1A (2 OTUs) and 3B (1 OTU) (Fig. 2). Most of 1K and 1J OTUs were related to Alphaproteobacteria sequences. OTU 3466 of subcluster 1K (Fig. 2) diverged into a separated group, which contains only uncultivated sequences recovered from soil, marine, and plant-associated environments (Zehr

et al., 2003; Hsu and Buckley, 2009; Lovell et al., 2000; Moisander *et al*., 2005). The remaining 1K OTUs clustered with sequences from the Rhizobiales order, which appeared to be highly represented in the database (33% of the total database). These OTUs were more closely related to sequences belonging to *Bradyrhizobium* (7 OTUs) and the *Methylocystis/Methylosinus* methanotrophic group (4 OTUs), which represented 23.6% and 9.4% of the total database, respectively. OTUs of subcluster 1J were related to *Rhodospirillales* sequences; one of them was related to *Azospirillum* while the remaining ones clustered with environmental sequences and were related to sequences from phototrophic purple non-sulfur bacteria belonging to Rhizobiales and Rhodospirillales orders.

Three abundant OTUs were represented by sequences that clustered with Deltaproteobacteria sequences; two of them were classified as 1A and one as 3B. The closest cultivated sequences for the two 1A OTUs were *Anaeromyxobacter* and *Pelobacter/Geobacter,* respectively. The 3B OTU clustered with uncultured soil sequences, which are closely related to sequences of the sulphate-reducing bacteria of the *Desulfobacteraceae* family.

Some major clusters and subclusters were underrepresented in our samples. Major clusters II and IV were represented by only a few OTUs, which is perhaps understandable given that cluster II contains the alternative (FeV and FeFe) nitrogenases and cluster IV is composed of *nifH* paralogs. By contrast, the scarcity of Gammaproteobacteria in subclusters 1O and 1M was surprising, as well as the paucity of Clostridiales, Pseudomonadales and Enterobacteriales representatives from subgroups 3A and 1G (Table 1).

These data revealed that even when soils contain representatives of most of the diversity of *nifH* sequences found in natural environments, sequences belonging to Alpha, Beta and Delta-proteobacteria were predominant.

Geographic location is a major determinant of *nif*H diversity in soil

The diversity of *nifH* in soils was examined using rarefaction curves (Fig. S2) and various estimators of richness and evenness (Table 2). At 2% dissimilarity, none of the rarefaction curves reached saturation. However, the relative coverage was similar among samples, ranging from 59 to 84% and 55 to 77% according to S_{Chao1} and S_{ACE} estimators, respectively. These numbers indicate that although the sequencing effort did not fully cover the potential diversity, a substantial and comparable fraction was assessed in all samples.

As shown in Table 2, the richness and diversity of the diazotrophic community were strongly affected by the geographical location of the sampling site, but not by the soil management practices. One-way ANOVA models confirmed significant differences among the four locations (**$p = 3e^{-6}$ for richness and $p = 4e^{-5}$ for diversity)** but not between years or

142 treatments. Two-way ANOVA did not indicate significant interaction effects. Whereas the
143 sandy soils of Bengolea displayed the lowest number of *nifH* OTUs and the lowest Shannon
144 index (H´) values, Viale clay soils had the highest richness and diversity (Table 2).
145 Stepwise regression models were applied to assess the relationship between diazotroph
146 diversity measured by the Shannon index and soil chemical composition. Only C content was
147 selected as significant predictor ($p = 0.016$, $r^2 = 0.38$). With treatment factor ($p = 0.004$)
148 added to C content ($p = 3e^{-5}$) as predictor, the model improves considerably ($r^2 = 0.60$)
149 indicating that *nif*H diversity is significantly affected by soil organic carbon.
150 **Soil properties affect the composition of diazotrophic community**
151 There were clear differences in the proportion of the eight predominant subclusters with
152 respect to location. The westernmost (Bengolea) and easternmost (Viale) locations showed
153 the largest differences compared to the two central locations (Monte Buey and Pergamino),
154 likely due to contrasting soil types (Fig. 3.A, top row). As noted before, subcluster 1K
155 dominates all locations. The proportion of subcluster 1J sequences decreases from west
156 (Viale) to east (Bengolea). As mentioned above, the lowest diversity among sites was
157 observed at Bengolea, which consists mainly of subclusters 1K and 1J. Correspondence
158 analysis plots show a more comprehensive and detailed view of the similarities between
159 locations as defined by their subcluster proportions (Fig. 3.B). Bengolea samples, which
160 grouped on the left side of the figure, are unique in their high proportions of 1J and 1K, but
161 also 1B, 1E, and 2. Monte Buey and Pergamino samples share similar cluster proportions and,
162 therefore, overlap in the figure. Viale samples separated along the second component, were
163 characterized by high proportions of 1, 1C, 1D, and 3B clusters. Samples do not group
164 according to treatment (data not shown). Chi-sq independence test ($p = 2e\text{-}16$) suggests that
165 locations and *nif*H subclusters are not independent, i.e. subcluster proportions vary
166 significantly across locations.
167 Differences in the proportion of subclusters are subtler across treatments (Fig. 3.A, bottom
168 row). The pie chart shows that the proportion of 1J subcluster is notably high in GAP and
169 PAP samples. The trend in subcluster 1K proportion is NE>GAP>PAP, whereas subcluster
170 3B displays the opposite trend (Fig. 3.A, bottom row).
171 The strong relationship between location and *nifH* cluster proportions was confirmed by
172 linear discriminant analysis. Using the proportion of 13 *nifH* subclusters as predictors, 100%
173 of samples were correctly assigned to location categories (Bengolea, Monte Buey, Pergamino,
174 Viale), while only 88% of samples to treatment categories (NE, GAP, PAP), and 83% of
175 samples to year categories (2010, 2011).

The proportion of the four predominant *nif*H subclusters (1A, 1J, 1K, and 3B) is affected differently by soil chemical properties (Fig. 4). The proportion of subclusters 1A and 3B increased with increasing pH and moisture. Subcluster 1J proportion increased, while 3B decreased with decreasing organic C, N, and P levels. Subcluster 1K was unaffected by soil chemistry, explaining its dominance in all samples. The above findings were confirmed by stepwise regression models (Table S1).

Environment influences abundance of soil N_2-fixing bacteria

The relative abundance of N_2-fixing bacteria was determined by qPCR. In general, the number of *nifH* copies varied significantly across sites (Monte Buey<Bengolea<Pergamino; $r^2 = 0.46$, $p = 4e^{-6}$) and were significantly higher in year 2011 than 2010 ($r^2 = 0.30$, $p = 6e^{-5}$) (Fig. 5). In addition to significant main effects of location and year, their interaction also proved to be significant ($p = 0.002$); this three term ANOVA model describes most of the *nifH* abundance variation (r^2=0.83), revealing a major influence of the environment on abundance of soil diazotrophs. In contrast, the effect of treatment (PAP, GAP and NE), varied across locations and years; hence, when analyzing all samples together, no relationship was found between *nifH* gene abundance and agricultural treatments. However, when abundance was examined separately at each site, year and treatment effects were found to be significant at Bengolea ($r^2 = 0.84$, $p = 0.001$, $p = 0.015$) and Monte Buey ($r^2 = 0.77$, $p = 0.008$, $p = 0.017$). At these two locations, *nifH* abundance was significantly higher under PAP and lower under NE treatment in both years (Fig. 5). This pattern is opposite to the C, P and N levels, i.e. *nifH* abundance is lowest under NE treatment where C, P and N contents are highest.

The relationship between soil chemistry and *nifH* abundances was location- and treatment-specific. This is shown by better fit and prediction of regression models calculated for each location or treatment separately compared to modeling all samples together (r^2=0.57, r^2-pred=0.48). For two of the four locations, Bengolea (r^2=0.97, r^2-pred=0.93) and Pergamino (r^2=0.86, r^2-pred=0.80), as well as for NE (r^2=0.95, r^2-pred=0.88) and GAP (r^2=0.94, r^2-pred=0.85) treatments, soil chemistry parameters showed high r^2 fit and prediction values, suggesting that soil chemistry might influence *nifH* abundance under these conditions. In contrast, low prediction values at Monte Buey (r^2=0.76, r^2-pred=0.58), Viale (r^2=0.84, r^2-pred=0.35) and PAP treatment (r^2=0.54, r^2-pred=0.42) indicate that the models are likely to be overfit to the training data and soil chemistry does not influence *nifH* abundance at these locations and treatment.

The location- and treatment dependence of soil chemistry also manifests in the dissimilar correlation patterns between abundance and soil parameters. For example, under NE and GAP

210 treatment strong significant correlation was found between *nif*H abundance and P (-0.66 and -
211 0.64), while pH (r = -0.74) and moisture (r = -0.56) were the main influencing parameters
212 under PAP treatment. Considering locations, *nif*H abundance was negatively correlated with
213 N (r = -0.79), P (r = -0.88) and moisture (r = -0.84) in Bengolea, whereas in Pergamino the
214 most significant correlation (r = -0.93) was with pH. It is notable that the significant
215 correlations between individual soil chemistry parameters and *nifH* abundance were all
216 negative.

217 **Discussion**

218 This study examined the community structure of N_2-fixing bacteria in soils, and the effects of
219 soil type and management practice on the abundance and structure of the N_2-fixing
220 communities in Argentinean agricultural soils. This database makes a significant contribution
221 to the number of *nifH* sequences coming from soil, which in the recent report by Gaby and
222 Buckley (2011) was estimated to be 3644 unique reads at the Genbank sequence database.
223 The 16782 unique protein sequences and 87020 *nifH* reads we obtained, along with associated
224 metadata, provide the most detailed and comprehensive picture of the diversity, abundance,
225 and environmental responses of the N_2-fixing community in Argentinean soils, which produce
226 much of the world's supply of soybeans. We observed evidence of community members that
227 responded to specific environmental factors, and some evidence of broad community effects
228 related to soil type and soil chemistry.

229 It has been indicated that technical reproducibility and complete removal of sequencing
230 artifacts are important issues in the analysis of sequence data generated by pyrosequencing
231 (Zhou et al., 2011; Knight et al., 2012; Pinto and Raskin, 2012). In this regard, we chose to
232 survey space and time more broadly, without technical replicates. However, to limit the
233 creation of artifacts and to insure their removal, we pooled amplicons from multiple PCR
234 reactions, removed low quality sequences, chimeras and frameshifts, used an equal number of
235 sequences in samples, and removed less frequent OTUs (Schloss et al., 2011). As a result, our
236 dataset is an accurate survey and represent a broad region of agricultural importance for
237 Argentine.

238 We found that Alpha- and Beta-proteobacteria (subclusters 1K and 1J) and Delta-
239 proteobacteria (sublcuster 1A) were well represented in our samples as expected, but not
240 Gamma-proteobacteria (Table 1). The low representation of *nifH* sequences related to
241 Gamma-proteobacteria is striking considering that several genera included in this group
242 (*Pseudomonas*, *Enterobacter* and *Azotobacter*) were found to be common components of soil
243 (Wang *et al*., 2012; Liu *et al*., 2012). As in any other approach based on PCR amplification,

we cannot rule out that underrepresentation of Gamma-proteobacteria sequences should be consequence of primer bias. However, the lack of Gammaproteobacteria has also been reported in the diazotrophic community of some tropical soils examined by both cultivation-dependent and -independent approaches (Izquierdo and Nüsslein, 2006).

The Alpha-proteobacterial sequences related to Rhizobiales order accounted for 33% of the total database. Two of the largest groups of sequences in subcluster 1K, groups 657 and 2991 (Fig. 2), were found in NE and GAP treatments, but poorly represented or not found in PAP practice soils (data not shown), indicating that they prefer either natural conditions or Good no-till Agricultural Practice, such as corn intercropping. Interestingly, groups 657 and 2991 fell into the cluster that groups the photosynthetic *Bradyrhizobium* sp. strains, one of which (clone T1t015) has been reported to be positively affected by the presence of maize residues (Hsu and Buckley, 2009).

Sequences related to the methanotrophic group *Methylosinus/Methylocystis* were well represented in our soil samples (9.4% of the total database). The abundance of these sequences in soil has been attributed to their adaptive advantage in poor-carbon soils and to the positive effect of fermentation processes associated with root exudates or stubble retention (Duc *et al*., 2009; Buckley *et al*., 2008). However, the distribution of methanotrophic sequences found in our soil was uneven and could not be related to any of these factors (data not shown).

The variability in the composition of phylogenetic groups across geographical locations may lend insight into the conditions that affect the different phylogenetic groups. For instance, the distribution of 1B and 1J was mainly associated with the level of soil organic C (Table S1; Fig. 4). Considering that most of the abundant 1J OTUs were related with phototrophic purple non-sulfur bacteria, it could be hypothesized that low carbon content may promote increased phototrophic bacterial populations. Conversely, the proportion of *nifH* sequences from orders Campylobacterales (Cluster 1), Frankia (1D), Desulfovibrionales and Verrucomicrobiales (3B) seems to be associated with high levels of both C and N. Moreover, the proportion of the 3B group, as well as the anaerobes Clostridiales (1C), *Anaeromyxobacter* and Desulfuromonadales (1A), seems to be negatively affected by low levels of soil moisture (Table S1). Consequently, these phylotypes were found underrepresented in sandy soils.

A significant difference in diazotrophic diversity was observed across the four locations but not among management treatments. In a companion study using the same set of soil samples, diversity of the whole bacterial community did not vary by location or management treatment (Figuerola *et al*., 2012), suggesting that diversity variation is a distinctive feature of the N_2-

fixing community. *nifH* diversity and richness levels were consistently low in the sandy soil from Bengolea, whereas the clay soil from Viale displayed the highest diversity (Table 2). Based on regression analysis, the differences of diversity across sites appear to be mainly associated with levels of soil organic carbon. This result is not surprising, given that heterotrophic N_2-fixers often metabolize organic matter both to fix N_2 and to maintain high respiration rates to avoid O_2 inactivation of nitrogenase (Hill, 1992).

Interestingly, *nifH* abundance in agricultural soils was higher than in pristine non-agricultural soils, suggesting that land use or presence of crops somehow promote diazotroph populations. Therefore, our study provided no evidence that no-till production systems either with intense crop rotation or monoculture practices negatively affect the level of the potential N_2 fixation community of soils. It could be that perturbation of soil by cropping promotes increases in abundances unlike the pristine environment which eventually reaches a steady state.

Species abundance, diversity and function are important components of the sustainable agriculture systems. Within this ecological framework, we demonstrated at some locations potential for management of soil chemistry to improve the abundance and diversity of N_2-fixing bacteria. Surprisingly, at locations where good agricultural practices are being followed, it could be beneficial from the standpoint of promoting N_2-fixation not to be overly ambitious with nutrient replacement, as well as with practices that affect soil pH and water content. This conclusion is supported by the negative correlations we sometimes found between *nifH* abundance and nutrient levels, pH, and soil moisture. Considering that higher *nifH* abundances are not always associated with higher N_2-fixation rates or diversity, additional research on *nif* expression and N_2-fixation rates would be useful in order to compare diazotroph community structure with N_2-fixation activity.

Experimental procedures

Sample collection and soil characterization

Soil samples were collected from four geographical locations in the Argentinean Pampas. From west to east, the sampling locations were: Bengolea (33º01′31″S; 63º37′53″W) and Monte Buey in Córdoba Province (32º58′14″S; 62º27′06″W); Pergamino in Buenos Aires Province (33º56′36″S; 60º33′57″W); and Viale in Entre Ríos Province (31º52′59″S; 59º40′07″W) (Fig. 1). The sites comprise three different soil types, a sandy loam (Entic Haplustoll) in Bengolea, a silty loam (Typic Argiudoll) in Pergamino and Monte Buey and a silty clay (Argic Pelludert) in Viale (Table 3).

As previously described by Figuerola *et al*. (2012), three treatments were defined at each of the four sampling geographical locations. Good no-till Agricultural Practices (GAP) treatment is characterized by intensive crop rotation (soybean-maize), nutrient replacement, and low agrochemical use. Poor no-till Agricultural Practices (PAP) treatment is characterized by crop monoculture (soybean), low nutrient replacement, high agrochemical use and lower yields. Grasslands uncultivated for 30 or more years were considered Natural Environment (NE) treatments.

Sampling was performed in February (Southern hemisphere summer) of years 2010 and 2011. Each treatment-site combination was sampled in three replicates from 5 m^2 quadrants separated by at least 50m from each other. Samples consisted of a pool obtained from 16–20 randomly selected subsamples from the top 10 cm of bulk soil. These subsamples were immediately combined and homogenized after field collection, and transported to the laboratory at 4ºC. Within 3 days after collection, samples were sieved through 4-mm mesh to remove roots and plant detritus, and stored at -80ºC until further processing. The replicates of each treatment were independently analyzed for the q-PCR analysis. For pyrosequencing analysis, DNA extraction and amplification were performed independently on each replicate, and amplicons were pooled into a single sample before labelling barcoding, resulting in a total of 24 composite samples (4 sites x 3 treatment x 2 time sampling).

The following soil characteristics were used in the subsequent statistical analyses: soil texture (% silt and clay), pH, gravimetric moisture content (%), total organic carbon measured by dry combustion (C, %), total nitrogen obtained by the Kjeldahl method (N, %), and extractable phosphorous determined by the method of Bray and Kurtz (P, ppm) (Table 3). The measurement and analysis of these physico-chemical characteristics have been previously described (Figuerola *et al*., 2012; Duval *et al*., 2013). Briefly, there was a clear gradient in soil texture from west to east with increasing clay and decreasing sand content from Bengolea to Viale. Regarding chemical parameters, the lowest C and N values were found at Bengolea under all three treatments, whereas Viale C and N levels surpassed the other sites but only for GAP and PAP treatments. P levels were highest in Monte Buey while the lowest levels were observed at Pergamino. With some exceptions, C, N and P were highest in NE treatment and lowest in PAP. These soil properties show no variation between years. By contrast, moisture content in 2011 was only 2/3 of the 2010 level (14% ± 5.9 vs. 22.4% ± 4.7) and pH was also lower in 2011 than 2010 (5.91 ± 0.29 vs. 6.62 ± 0.18).

DNA extraction

DNA extraction and amplification were performed independently for each replicate sample. Soil DNA was extracted from 0.25 g of soil using FastDNA Spin kit for soil (MP Biomedicals), in accordance with the manufacturer's instructions.

Quantification of *nifH*

The relative abundance of the *nifH* gene was quantified via quantitative real-time PCR (qPCR). The qPCR reactions contained 10 ng of soil DNA, 1.2 µl of each primer (5 pM) and 10 µl of 2x SYBR Green iCycler iQ mixture (Bio-Rad) and water for 20 µl final reaction volume. The reaction was carried out on an Applied Biosystem 7500 real-time PCR system (Applied Biosystems) using a program of 95°C for 10 min followed by 40 cycles consisting of 15 s at 95°C, 20 s at 55°C, and 20 s at 72°C. Fluorescence was measured at the end of each cycle. 16S rRNA gene abundances were used to normalize values between the different samples. Relative quantities were calculated using Genorm (http://medgen.ugent.be/~jvdesomp/genorm/). The following specific primers were used: PolF-PolR for *nifH* (Poly *et al*., 2001a) and 338F-518R for bacterial 16S rRNA genes (Park and Crowley, 2005). All qPCR reactions were run in duplicate with DNA extracted from replicates soil samples. For each run, the melting curve was analyzed to ensure specific assessment of *nif*H gene.

Generation of barcoded *nifH* gene libraries

The diversity and composition of diazotrophic communities were assessed by pyrosequencing analysis of the *nifH* gene. PolF-PolR degenerated universal primers were used to PCR amplify an internal fragment (360 bp) of the *nifH* gene (Poly *et al*., 2001a). Primer sequences included the Roche 454 tag sequences A (CACGACGTTGTAAAACGAC) or B (CAGGAAACAGCTATGACC) fused to the 5′ end of the forward and reverse *nif*H-primers, respectively. Amplification was performed using a FastStart High Fidelity PCR system (Roche Applied Science, Mannheim, Germany) with the following parameters: initial denaturation at 95ºC for 5 min, 20 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. The PCR reactions were conducted in triplicate to minimize random PCR bias. Amplicons were purified by using QIAquick Gel extraction (Qiagen, CA, USA) and quantified using a Nanodrop Spectrophotometer. Replicates were pooled in equimolar concentrations in a single treatment library. Each of the 24 libraries was labelled with a unique oligonucleotide barcode and pyrosequenced using 454 GS FLX technology.

Pyrosequencing data analysis

Sequences shorter than the threshold of 300 bp in length or displaying ambiguities were removed from the 24 pyrosequencing-derived datasets. The remaining reads were translated in all six reading frames and compared to a *nifH* reference database (http://pmc.ucsc.edu/~wwwzehr/research/database/) using BLASTx from iNquiry software (Bioteam). A cutoff of $1e^{-10}$ for the *E*-value was set to remove nonspecific blast hits. Putative frame shifts were detected and removed with the FrameBot tool (RDP, Ribosomal Database Project) using a profile hidden Markov model (HMM) (Zehr *et al*., 2003) as reference set. The same HMM was used to align the *nifH* pyrosequences with the hmmalign program (HMMER 2.3.2). The resulting alignment was imported into ARB (Ludwig *et al*., 2004), evaluated by eye and subjected to minor manual corrections. The regions of the primers were trimmed and incomplete sequences were removed. High-quality sequences were subsequently assessed for chimeras using the UCHIME algorithm (Edgar *et al.*, 2011). Following that, the nucleotide sequences were clustered into OTUs (operational taxonomic units) using CD-HIT Suite program (Huang *et al*., 2010) with an OTU threshold value of 98% sequence identity at the DNA level. OTU representative sequences were analyzed with *de novo* mode of UCHIME using its relative abundance data. All putative chimeras detected using UCHIME default settings were eliminated. Finally, the sequences in the database were clustered with OTUs defined at 98% amino acid sequence similarity. The conservative OTU cut off used was defined considering that protein-encoding genes of strains of a given species generally have a high sequence similarity (Konstantinidis and Tiedje, 2005). In an attempt to further reduce potential pyrosequencing errors, OTUs represented by less than three sequences were excluded from the database. The relative abundance of sequences of the remaining OTUs was normalized using the subsampling-based method described in mothur (http://www.mothur.org/wiki/Normalize.shared), prior to comparative analyses.

OTU representative amino acid sequences along with sequences selected from the *nifH* reference database were used to build protein phylogenetic trees. Ambiguously aligned regions were detected by visual inspection and excluded from the analysis. Trees were constructed by performing neighbor-joining analysis (with the Kimura correction) using the ARB program.

The data set was deposited in the NCBI-SRA (Sequence Read Archive) with the submission Accession Number SRP029166.

Statistical Data Analyses

Data were analyzed using R (R Core Team, 2013) and by Minitab Statistical Software, 2010. Richness (Chao1, S_{Chao1} and ACE, S_{ACE}), Shannon diversity (H´), and rarefaction were

calculated with the vegan package in R (Oksanen *et al*., 2013) using the diversity, estimate, rarecurve, and specnumber functions.

Location, treatment, and year effects were explored graphically by parallel boxplots and their significance was assessed by ANOVA models coupled with Tukey's multiple comparison of means in Minitab. One-way and two-way models were calculated to fit richness and diversity parameters as well as relative abundance values.

Relationship between soil chemical parameters and diazotroph community characteristics were investigated calculating correlation and regression models in Minitab. Stepwise regression (both forward and backward) models were calculated to select soil parameters with significant effect on the Shannon diversity and on the relative abundance of diazotrophs.

Variation in community composition across locations and treatments was graphically explored by pie charts created in Minitab. The pie charts were created by adding the corresponding subsets (locations or treatments). Samples were subsetted by location and the sum of six samples (three treatments and two years) was claculated at each location for each cluster. Similarly, samples were subsetted by treatment and at each treatment the sum of eight samples (four locations and two years) was calculated.

Further details about the *nif*H cluster profiles were obtained by correspondence analysis of the contingency table using the ca package in R (Nenadic *et al*., 2007). Location and treatment effects were analyzed by chi-squared test and linear discriminant analysis calculated in R. Abundance of selected *nif*H clusters were projected on principal component biplots calculated from scaled soil parameters in order to examine the effect of soil chemistry on the main diazotroph groups.

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535
536 **Table and figure legends**
537 **Table 1. *nifH* phylotypes obtained from Argentinean soils.**
538 **Table 2. Richness, diversity and relative sample coverage for *nifH* libraries.** The richness
539 estimators (Chao1, S_{Chao1} and ACE, S_{ACE}) and Shannon's diversity index (H´) were calculated
540 for *nif*H libraries with OTUs defined at 98% amino acid sequence similarity.
541 **Table 3. Soil properties according to site and agricultural management at the sampling**
542 **dates.**
543 **Table S1. Soil parameters that explain significant variation in the proportion of *nif*H**
544 **subclusters.**
545 **Fig. 1. Map of the four sampling locations in the Argentinean study area.** Soil samples
546 were collected from four geographical locations: Bengolea and Monte Buey in Córdoba

Province, Pergamino in Buenos Aires Province and Viale in Entre Ríos Province. Template map downloaded from www.google.com/earth/ (30 October 2013).

Fig. 2. Phylogenetic relationships of nineteen largest *nifH* OTUs based on neighbour-joining analysis of partial amino acid sequences. The numbers in shaded boxes identify the largest OTUs observed in this study. The percentage of 500 bootstrap samples that supported each branch is shown. Bootstrap values below 50% are not shown.

Fig. 3. Proportion of the largest subclusters across locations and treatments.

A. Pie charts show the proportion of the eight predominant subclusters with respect to geographical location (top row) and treatment (bottom row). Subclusters 1K, 1J, and 1A dominate at each location and under each treatment (NE = Natural Environment; GAP = Good no-till Agricultural Practices; PAP = Poor no-till Agricultural Practices).

B. Correspondence analysis plot displays rows (samples) and columns (*nifH* subclusters) of a cross-tabulation of sequence counts. Closeness on the plot indicates similarity between samples in terms of subcluster proportions or similarity between subclusters in terms of their distribution across samples. Bengolea and Viale samples are enclosed by ellipses. The first component (horizontal axis) covers 51% and second component (vertical axis) covers additional 26% of variation.

Fig. 4. Proportion of the four largest subclusters related to location, treatment, year, and soil chemistry. Proportion is indicated by circle size at each sample point plotted on the first two principal components of the five soil parameters (represented by arrows). Component 1 (horizontal axis) covers 49% and component 2 covers 30% of the variance in soil chemistry**.** Each sample is labeled by its location (B = Bengolea, M = Monte Buey, P = Pergamino, V = Viale), treatment (NE = Natural Environment, GAP = Good no-till Agricultural Practices, PAP = Poor no-till Agricultural Practices), and year (10 = 2010, 11 = 2011). Circle size is scaled independently for each of the four subclusters.

Fig. 5. Location, year, and treatment effect on *nifH* gene relative abundance

Rows of panels correspond to years (2010, 2011) and columns to locations (Bengolea, Monte Buey, Pergamino, and Viale). Colors indicate soil management treatment: Natural Environment (NE) in white, Good no-till Agricultural Practices (GAP) in gray and Poor no-till Agricultural Practices (PAP) in dark gray. Boxes display the range of three biological and two technical replicates. Levels normalized to 16S rRNA expression are indicated as relative units. A value of 1 was assigned to the lowest detected value (Monte Buey-NE-2010 sample).

Fig. S1. Abundance of 1558 OTUs

580 Abundance is quantified as sequence count in each OTU. The hockey-stick curve indicates
581 that only 19 OTUs are represented by more than 1000 sequences.
582 **Fig. S2. Rarefaction curves grouped by location**
583 Number of sequences (horizontal axis) plotted against number of OTUs (vertical axis). Each
584 curve corresponds to a sample labeled by its location (B = Bengolea, M = Monte Buey, P =
585 Pergamino, V = Viale), treatment (NE = Natural Environment, GAP = Good no-till
586 Agricultural Practices, PAP = Poor no-till Agricultural Practices), and year (10 = 2010, 11 =
587 2011).
588

589 **Tables**

590 **Table 1. *nifH* phylotypes obtained from Argentinean soils.**

591

Cluster	Subcluster	Proportion (%)		Distribution[1] (%)	Group	Orders[2]
		Sequence count	OTU count			
I	1	1.6	1.9	96	Epsilon	Campylobacterales
	1A	18	21.4	100	Delta	Anaeromyxobacter and Desulfuromonadales
	1B	0.8	1.4	87	Cyanobacteria	Nostocales and Chroococcales
	1C	0.2	0.8	62	Firmicutes	Clostridiales
	1D	0.1	0.8	50	Actinobacteria	*Frankia*
	1E	0.5	2.1	96	Firmicutes	*Paenibacillus*
	1G	0.1	0.26	46	Gamma	Pseudomonadales and Enterobacteriales
	1J	20	19.6	100	Alpha	Rhizobiales and Rhodospirillales
	1K	51	40.2	100	Alpha and Beta	Rhizobiales and Burkholderiales
	1M	0.005	0.06	12	Gamma	Methylococcales
	1O	0.01	0.06	4	Gamma	Chromatiales
	1P	2.4	2.7	100	Beta	Rhodocyclales
II	2C	0.01	0.1	21	Alpha	*Rhizobiales* and *Opitutales*
	2	0.02	0.1	42	Firmicutes	*Paenibacillus*
III	3A	0.02	0.1	29	Firmicutes	*Clostridium*
	3B	5	8	100	Delta and Verrumicrobia	Desulfovibrionales and Verrucomicrobiales
IV	4	0.1	0.4	75	Archaea	Methanococcales

592

593 [1] presence across the 24 samples analyzed (e.g. subcluster present in all samples shows a
594 distribution of 100%)

595 [2] orders closest to the predominant sequences observed in the subcluster

596

Table 2. Richness, diversity and relative sample coverage for *nifH* libraries. The richness estimators (Chao1, S_{Chao1} and ACE, S_{ACE}) and Shannon's diversity index (H´) were calculated for *nif*H libraries with OTUs defined at 98% amino acid sequence similarity.

		Bengolea			Monte Buey			Pergamino			Viale		
		NE	**GAP**	**PAP**	**NE**	**GAP**	**PAP**	**NE**	**GAP**	**PAP**	**NE**	**GAP**	**PAP**
2010	SeqN	3142	3193	3763	2460	5870	9067	2632	2950	2756	4752	5278	6330
	$SeqN_n$	1927	1927	1927	1927	1927	1927	1927	1927	1927	1927	1927	1927
	Sobs	252	214	223	391	520	493	237	268	161	501	485	530
	H´	3.46	3.37	3.57	4.77	4.68	4.42	3.81	3.61	3.28	4.59	4.58	4.98
	S_{Chao1}	314	330	319	650	746	786	338	392	191	694	721	742
	S_{ACE}	353	355	319	703	778	774	353	430	209	728	744	767
	RC_{Chao1}	80	65	70	60	70	63	70	68	84	72	67	71
	RC_{ACE}	71	60	70	56	67	64	67	62	77	69	65	69
2011	Seq	2905	2098	4081	2652	2788	2980	1927	2162	2584	3762	4274	2614
	$Seqs_n$	1927	1927	1927	1927	1927	1927	1927	1927	1927	1927	1927	1927
	Sobs	156	183	259	328	285	342	282	297	344	453	427	414
	H´	2.27	3.66	3.55	4.10	4.13	4.26	3.81	4.18	4.48	4.65	4.23	4.68
	S_{Chao1}	233	305	384	503	422	505	428	501	553	661	577	687
	S_{ACE}	237	331	424	540	445	530	471	502	579	710	616	703
	RC_{Chao1}	67	60	68	65	68	68	66	59	62	68	74	60
	RC_{ACE}	66	55	61	61	64	65	60	59	59	64	69	59

Abbreviations: SeqN, number of sequences per sample; $SeqN_n$, normalized number of sequences per sample; Sobs, detected number of operational taxonomic units (OTUs) at 2% distance level; RC, relative coverage calculated as OTU number divided by estimated richness (Chao1 or ACE).

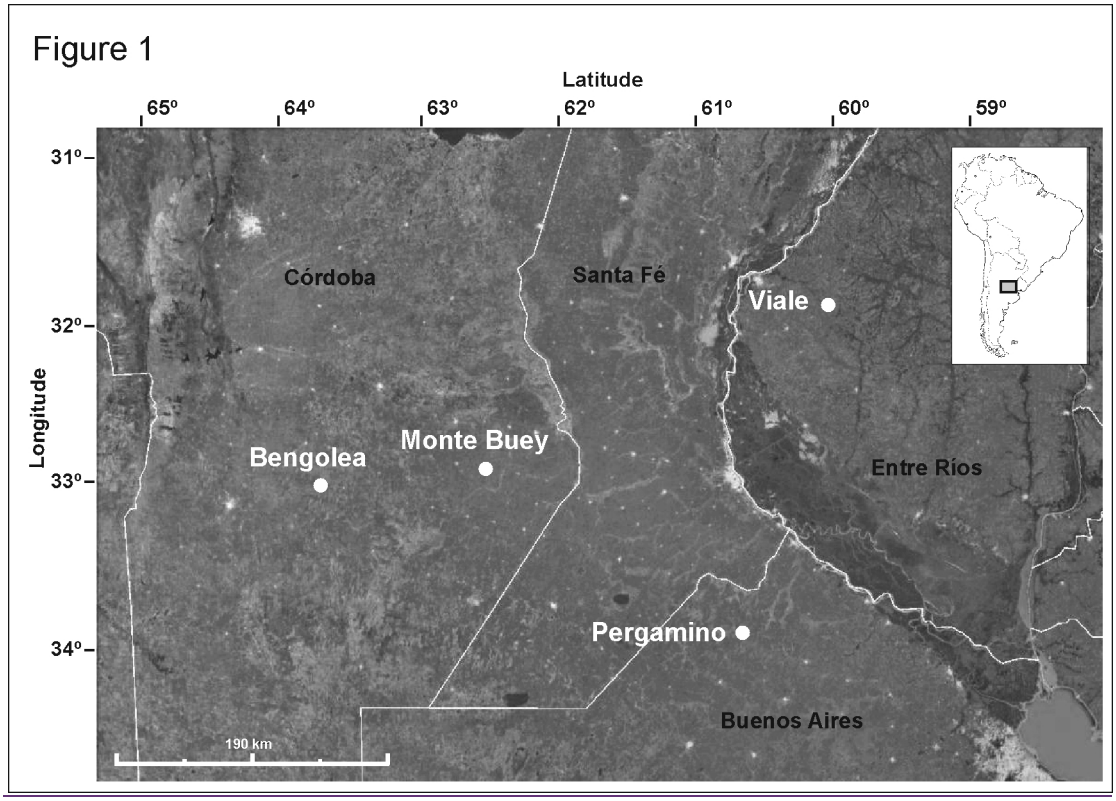
Table 3. Soil properties according to site and agricultural management at the sampling dates.

		Bengolea			Monte Buey			Pergamino			Viale		
		NE	GAP	PAP	NE	GAP	PAP	NE	GAP	PAP	NE	GAP	PAP
	Soil classification	Entic Haplustoll			Typic Argiudoll			Typic Argiudoll			Argic Pelludert		
	Texture	Sandy loam			Silt loam			Silt loam			Silty clay loam		
2010	pH	6.7	6.5	6.6	6.4	6.5	6.6	6.6	6.4	6.6	7	6.9	6.7
	Carbon %	1.69	1.44	1.19	3.74	2.27	1.73	3.21	1.75	1.73	3.52	3.41	2.48
	N %	0.14	0.13	0.11	0.28	0.18	0.13	0.25	0.14	0.14	0.20	0.23	0.16
	P (ppm)	43.10	37.20	26.20	395.10	122.30	17.30	16.60	25.30	22.00	24.30	50.40	43.10
	Moisture %	18.56	15.48	18.00	26.44	25.13	25.32	26.60	22.53	17.41	23.37	28.69	22.08
2011	pH	6.1	5.6	6.1	5.6	5.5	6.2	5.8	5.6	6	6.1	6.5	5.9
	Carbon %	1.71	1.45	1.28	3.83	2.37	1.91	2.99	1.77	1.91	3.57	3.58	2.65
	N %	0.13	0.12	0.10	0.33	0.19	0.14	0.25	0.16	0.14	0.27	0.30	0.20
	P (ppm)	20.93	24.93	8.9	356.13	164.93	8.16	4.76	13.33	2.00	10.23	20.43	36.33
	Moisture %	11.35	16.26	5.63	10.22	15.60	7.95	26.65	19.50	14.98	10.58	19.83	9.92

Table S1. Soil parameters that explain significant variation in the proportion of *nif*H subclusters.

Subcluster	Significant parameters	r^2 fit	r^2 predicted
1	C, N	0.73	0.60
1A	pH, moisture	0.33	0.12
1B	C	0.10	0
1C	moisture	0.12	0
1D	C, N	0.35	0
1E	-		
1G	pH, P	0.52	0.38
1J	C, P	0.44	0.32
1K	moisture	0.17	0
1P	-		
2	moisture, N	0.20	0
3B	moisture, C, N	0.49	0.15
4	-		

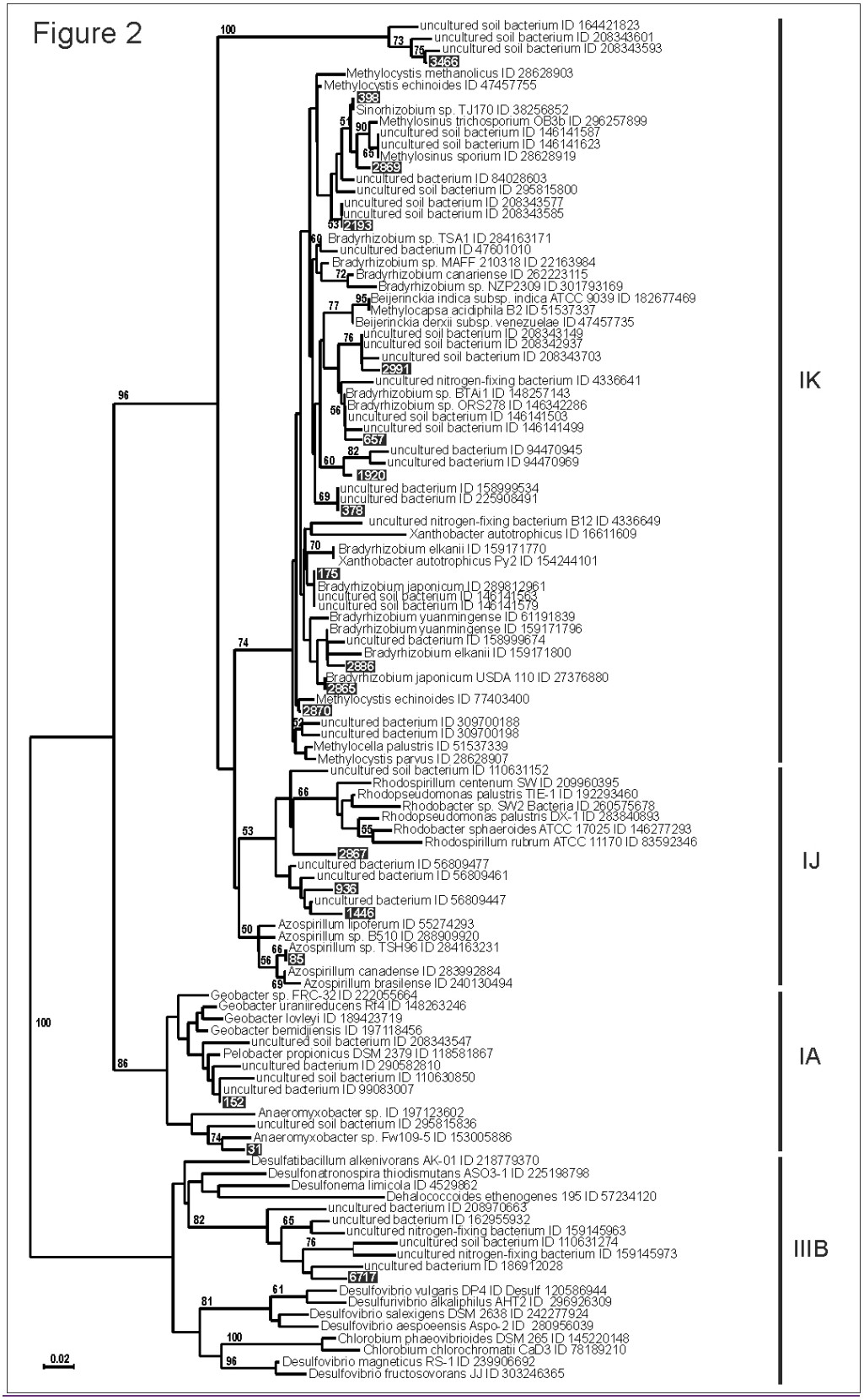
Figure 1



623

624

Figure 2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uncultured soil bacterium ID 208343601
uncultured soil bacterium ID 208343593
3466
Methylocystis methanolicus ID 28628903
Methylocystis echinoides ID 47457755
393
Sinorhizobium sp. TJ170 ID 38256852
Methylosinus trichosporium OB3b ID 296257899
uncultured soil bacterium ID 146141587
uncultured soil bacterium ID 146141623
Methylosinus sporium ID 28628919
2869
uncultured bacterium ID 84028603
uncultured soil bacterium ID 295815800
uncultured soil bacterium ID 208343577
uncultured soil bacterium ID 208343585
2193
Bradyrhizobium sp. TSA1 ID 284163171
uncultured bacterium ID 47601010
Bradyrhizobium sp. MAFF 210318 ID 22163984
Bradyrhizobium canariense ID 262223115
Bradyrhizobium sp. NZP2309 ID 301793169
Beijerinckia indica subsp. indica ATCC 9039 ID 182677469
Methylocapsa acidiphila B2 ID 51537337
Beijerinckia derxii subsp. venezuelae ID 47457735
uncultured soil bacterium ID 208343149
uncultured soil bacterium ID 208342937
uncultured soil bacterium ID 208343703
2991
uncultured nitrogen-fixing bacterium ID 4336641
Bradyrhizobium sp. BTAi1 ID 148257143
Bradyrhizobium sp. ORS278 ID 146342286
uncultured soil bacterium ID 146141503
uncultured soil bacterium ID 146141499
657
uncultured bacterium ID 94470945
uncultured bacterium ID 94470969
1920
uncultured bacterium ID 158999534
uncultured bacterium ID 225908491
378
uncultured nitrogen-fixing bacterium B12 ID 4336649
Xanthobacter autotrophicus ID 16611609
Bradyrhizobium elkanii ID 159171770
Xanthobacter autotrophicus Py2 ID 154244101
175
Bradyrhizobium japonicum ID 289812961
uncultured soil bacterium ID 146141563
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uncultured bacterium ID 158999674
Bradyrhizobium elkanii ID 159171800
2886
Bradyrhizobium japonicum USDA 110 ID 27376880
2365
Methylocystis echinoides ID 77403400
2870
uncultured bacterium ID 309700188
uncultured bacterium ID 309700198
Methylocella palustris ID 51537339
Methylocystis parvus ID 28628907
uncultured soil bacterium ID 110631152
Rhodospirillum centenum SW ID 209960395
Rhodopseudomonas palustris TIE-1 ID 192293460
Rhodobacter sp. SW2 Bacteria ID 260575678
Rhodopseudomonas palustris DX-1 ID 283840893
Rhodobacter sphaeroides ATCC 17025 ID 146277293
Rhodospirillum rubrum ATCC 11170 ID 83592346
2867
uncultured bacterium ID 56809477
uncultured bacterium ID 56809461
936
uncultured bacterium ID 56809447
1446
Azospirillum lipoferum ID 55274293
Azospirillum sp. B510 ID 288909920
Azospirillum sp. TSH96 ID 284163231
35
Azospirillum canadense ID 283992884
Azospirillum brasilense ID 240130494
Geobacter sp. FRC-32 ID 222055664
Geobacter uraniireducens Rf4 ID 148263246
Geobacter lovleyi ID 189423719
Geobacter bemidjiensis ID 197118456
uncultured soil bacterium ID 208343547
Pelobacter propionicus DSM 2379 ID 118581867
uncultured bacterium ID 290582810
uncultured soil bacterium ID 110630850
uncultured bacterium ID 99083007
152
Anaeromyxobacter sp. ID 197123602
uncultured soil bacterium ID 295815836
Anaeromyxobacter sp. Fw109-5 ID 153005886
31
Desulfatibacillum alkenivorans AK-01 ID 218779370
Desulfonatronospira thiodismutans ASO3-1 ID 225198798
Desulfonema limicola ID 4529862
Dehalococcoides ethenogenes 195 ID 57234120
uncultured bacterium ID 208970663
uncultured bacterium ID 162955932
uncultured nitrogen-fixing bacterium ID 159145963
uncultured soil bacterium ID 110631274
uncultured nitrogen-fixing bacterium ID 159145973
uncultured bacterium ID 186912028
6717
Desulfovibrio vulgaris DP4 ID Desulf 120586944
Desulfurivibrio alkaliphilus AHT2 ID 296926309
Desulfovibrio salexigens DSM 2638 ID 242277924
Desulfovibrio aespoeensis Aspo-2 ID 280956039
Chlorobium phaeovibrioides DSM 265 ID 145220148
Chlorobium chlorochromatii CaD3 ID 78189210
Desulfovibrio magneticus RS-1 ID 239906692
Desulfovibrio fructosovorans JJ ID 303246365
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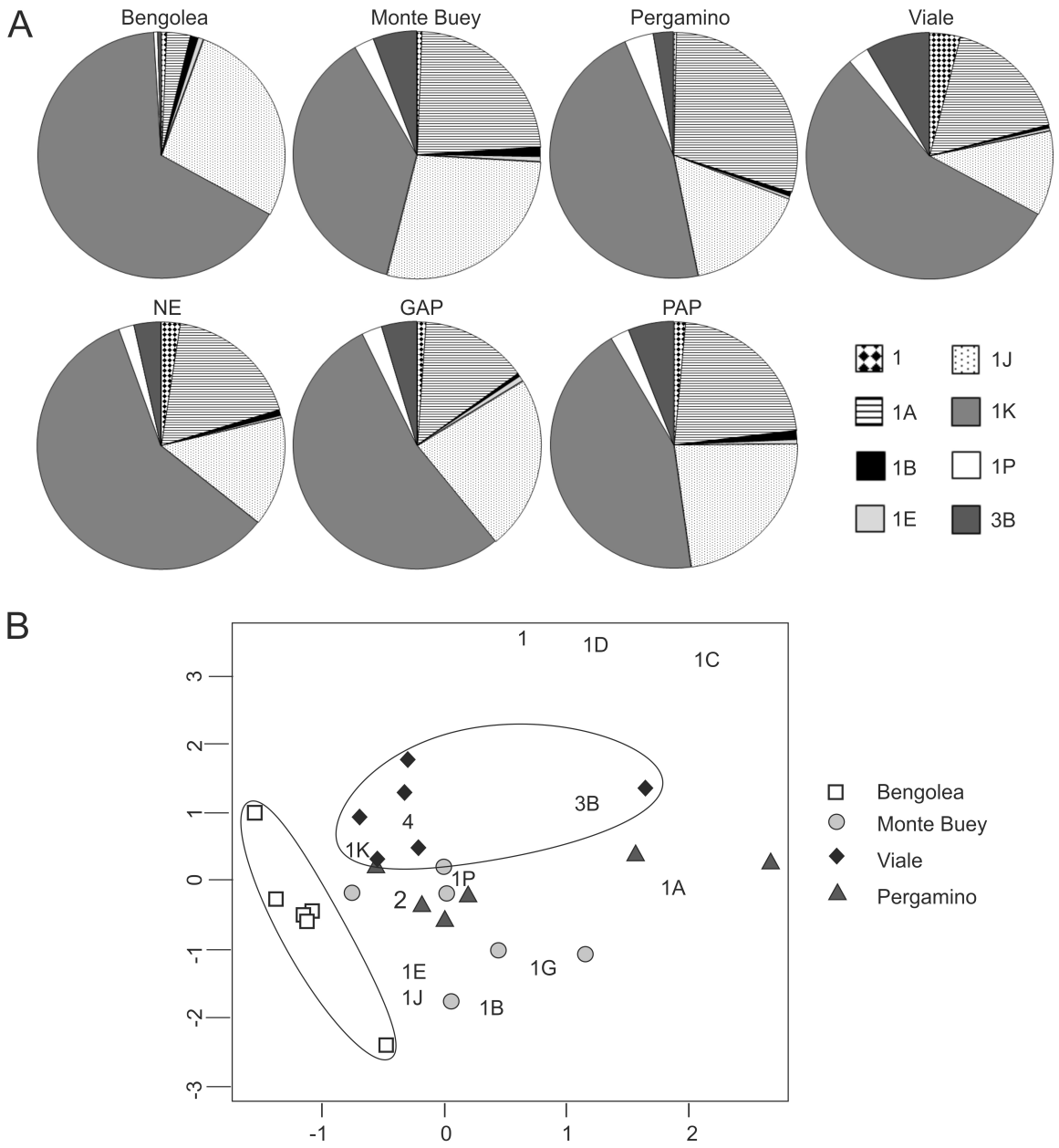
IK
IJ
IA
IIIB

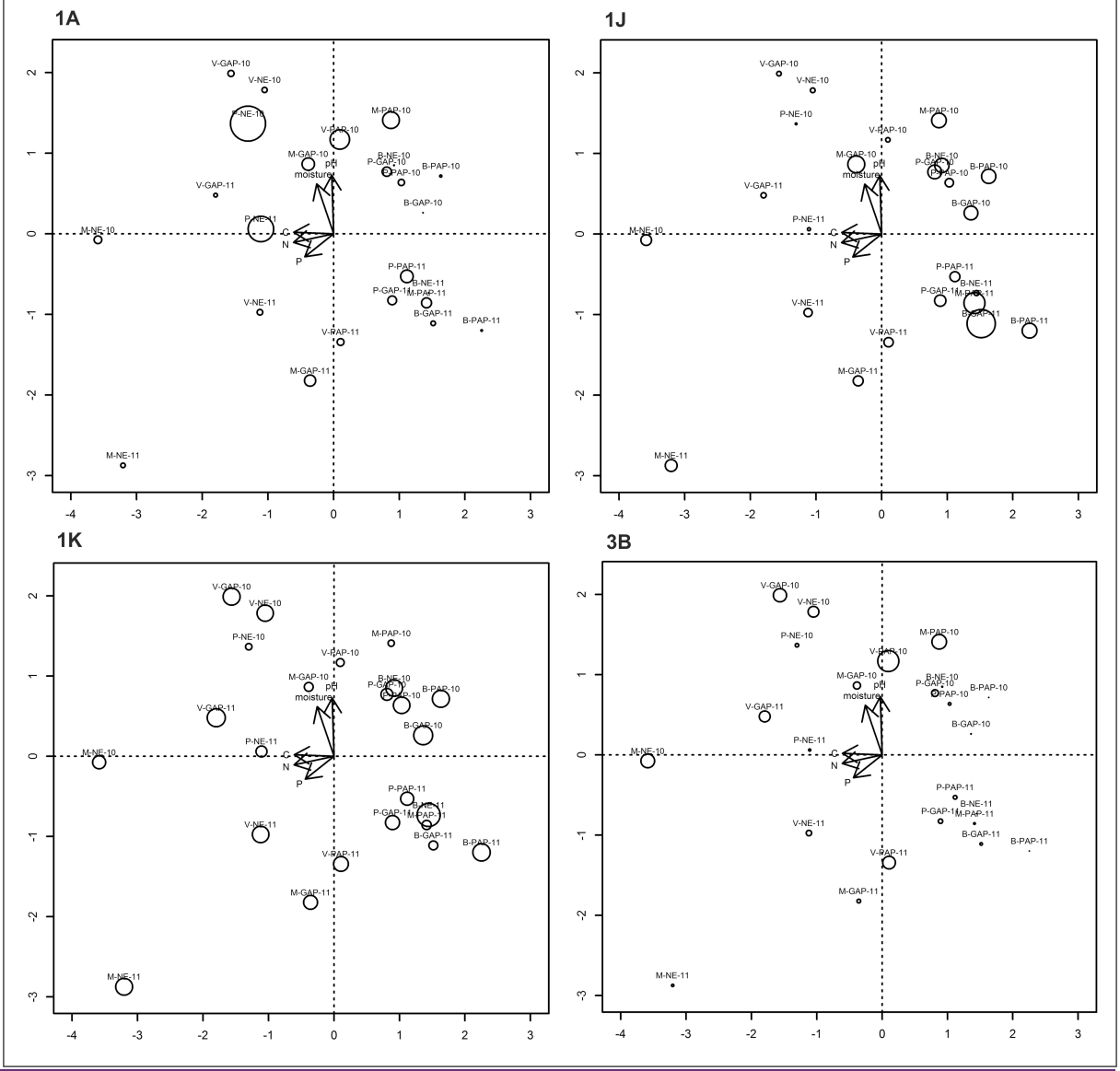
Figure 3



627

628

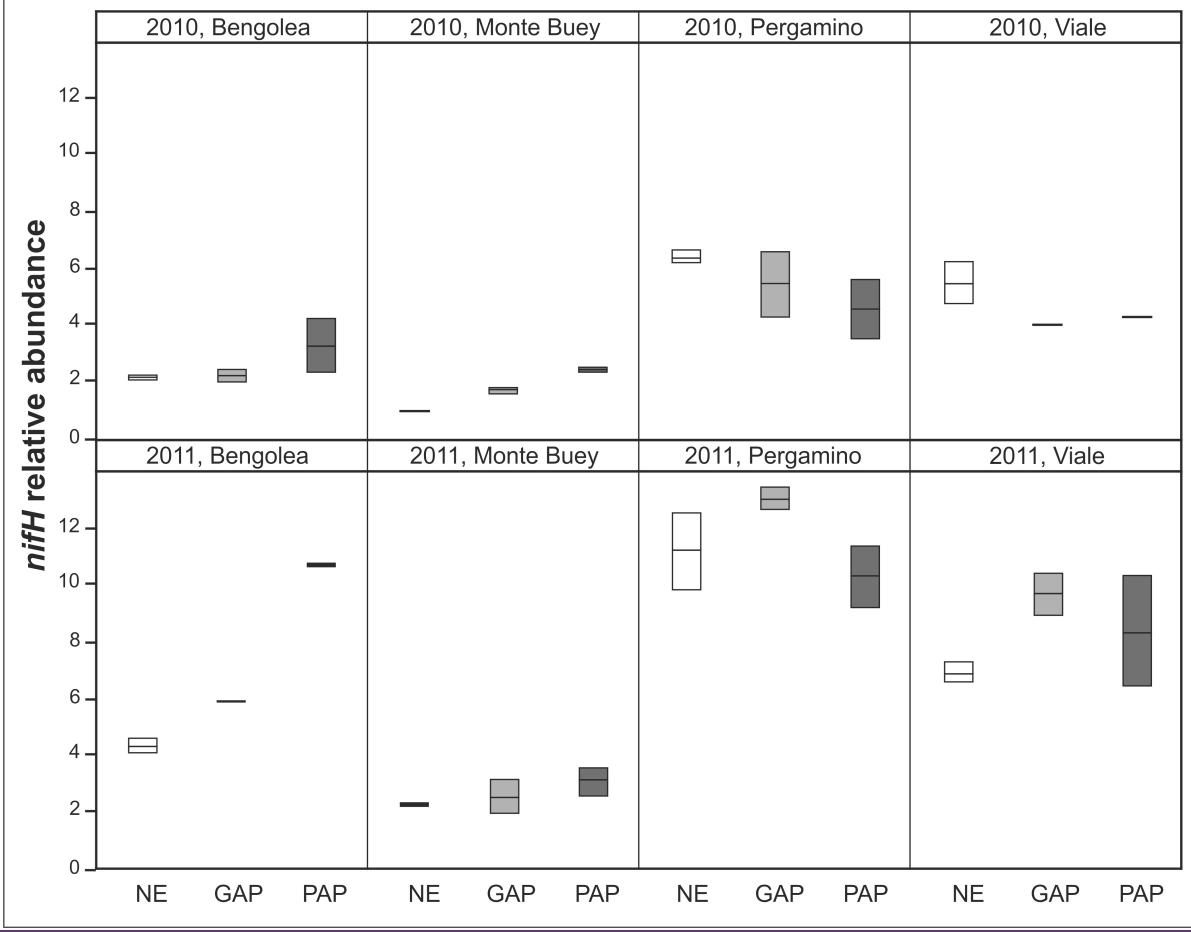
Figure 4



629

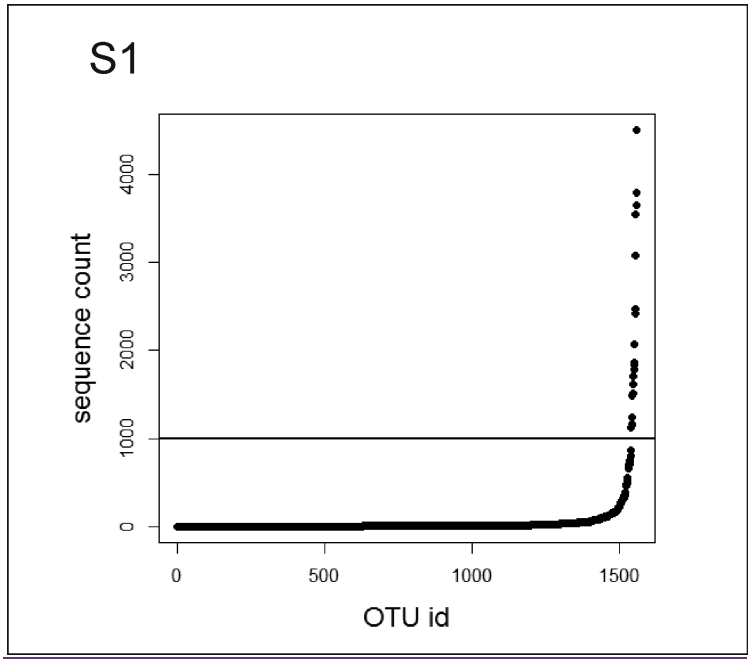
630

Figure 5



631

632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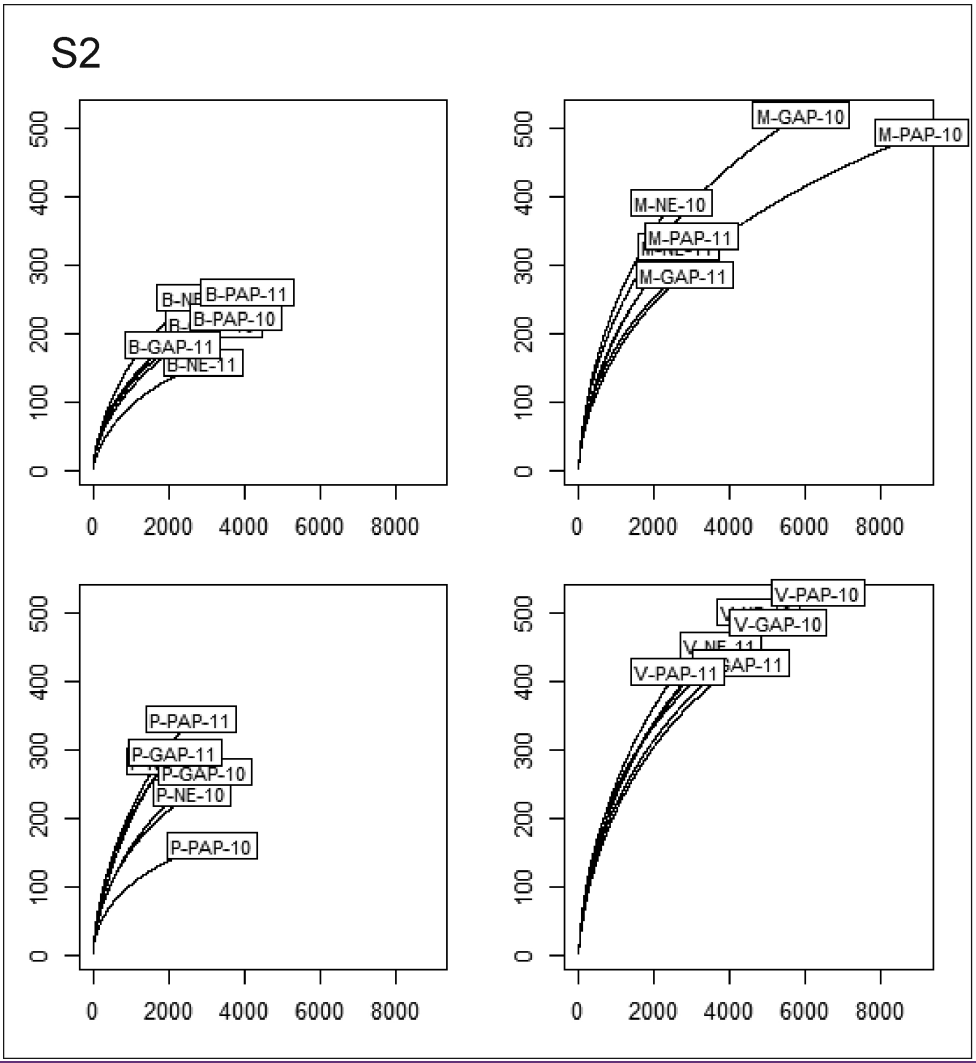
0
500
1000
1500
OTU id

633


S2
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B-PAP-10
B-GAP-11
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M-PAP-10
M-NE-10
M-PAP-11
M-GAP-11
P-PAP-11
P-GAP-11
P-GAP-10
P-NE-10
P-PAP-10
V-PAP-10
V-GAP-10
V-PAP-11
0
100
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300
400
500
0
2000
4000
6000
8000

634