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ARTICLE

Diversity of arbuscular mycorrhizal fungi in soil from the Pampa Ondulada, Argentina, assessed by pyrosequencing and morphological techniques

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Abstract: The aim of this study was to assess the effects of agronomic practices on the arbuscular mycorrhizal (AM) fungal community in soils from the Pampa Ondulada region (Argentina), and to compare conclusions reached when using pyrosequencing or a morphological approach. The AM fungal diversity of 3 agricultural exploitations located in the Pampa Ondulada region (Argentina) was assessed by using 454 amplicon pyrosequencing and morphological (based on spore traits) approaches. Two kinds of soil managements are found in these sites: agronomic and non-agronomic. A total of 188 molecular operational taxonomic units and 29 morphological species of AM fungi were identified. No effect of soil management on AM richness was detected. AM fungal communities were more diverse and equitable in the absence of agronomic management. In contrast, the results on β-diversity varied according to the methodology used. We concluded that agronomic management of soil has a negative effect on AM fungal community biodiversity in the Pampa Ondulada region. We also conclude that both methodologies complement each other in the study of AM fungal ecology. This study greatly improved the knowledge about AM fungi in South America where the molecular diversity of AM fungi was practically unknown.

Key words: glomeromycota, agroecosystem, biodiversity, pyrosequencing technology, morphotyping taxonomy.

Résumé : L'objectif de la présente étude était d'évaluer les retombées des pratiques agronomiques sur la communauté fongique de mycorhizes arbusculaires (MA) dans les sols de la région de Pampa Ondulada (Argentine), et de comparer les conclusions tirées d'approches fondées sur le pyroséquençage ou sur la morphologie. On a évalué la diversité fongique des MA de 3 exploitations agricoles situées dans la région de Pama Ondulada (Argentine) en employant les approches du pyroséquençage d'amplicons par le système 454 et de la morphologie (d'après les traits des spores). Ces sites présentaient 2 sortes de gestion des sols : agronomique et non agronomique. On a identifié un total de 18 unités taxonomiques moléculaires opérationnelles et 29 espèces morphologiques de champignons MA. On n'a remarqué aucune incidence de la gestion du sol sur la richesse des MA. Les communautés fongiques de MA étaient plus diversifiées et équilibrées en l'absence d'une gestion agronomique. En contrepartie, les résultats sur la diversité β ont varié selon la méthodologie employée. Nous avons conclu que la gestion agronomique des sols nuisait à la biodiversité des communautés fongiques de MA dans la région de Pampa Ondulada. Nous concluons également que les deux méthodologies se complètent pour les besoins de l'étude de l'écologie fongiques des MA. La présente étude renseigne grandement au sujet des champignons de MA d'Amérique du Sud, dont on ne savait presque rien sur le plan de la diversité moléculaire. [Traduit par la Rédaction]

Mots-clés : glomeromycota, agroécosystème, biodiversité, technologie de pyroséquençage, taxonomie par morphotypage.

Introduction

Arbuscular mycorrhizal (AM) fungi (phylum Glomeromycota), form mutualistic associations with the roots of the vast majority of terrestrial plants. They are considered one of the most important soil microorganisms because of their abundance, biomass, and key role in ecosystem functioning (Liang et al. 2008; Dumbrell et al. 2011). AM fungi supply phosphorus and others mineral nutrients to host plants through a hyphal network, receiving plant carbohydrates in exchange (Bonfante and Genre 2010). In addition, AM fungi influence host plant growth and reproduction; they also protect their host from pathogens (Wehner et al. 2009).

The diversity of AM fungi in the soil ecosystem is the main bottleneck in mycorrhizal ecology studies (Öpik et al. 2009). When using traditional approaches (based on spores morphology), the difficulty of identifying field-collected spores, the presence of nonsporulating AM fungi, and the failure to distinguish between functional and morphological diversities are some of the challenges to overcome (Douds and Millner 1999). Since there are more than 200 morphological species of AM fungi and the number could even be higher (Öpik et al. 2009), second-generation sequencing technologies have been increasingly considered as useful tools for identifying AM fungi in environmental samples (Dai et al. 2012; Davison et al. 2012; Lekberg et al. 2012).

The ecological factors that affect the composition of AM fungal community are complex. Plant host identity, soil type, habitat fragmentation, and seasonality affect AM fungal community

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structures (Hausmann and Hawkes 2009). Johnson et al. (2003) reported a marked effect of plant community assemblages on the diversity and composition of AM fungal communities in grassland microcosms. In the Pampeana region (Argentina) the natural grasslands have been replaced by productive fields. Areas of soybean monocultures have particularly increased. Monoculture has had consequences for several soil microorganisms, including AM fungi (Friberg 1999).

AM fungal diversity studies in agroecosystems must consider the effect of different factors, such as soil compaction by machinery, chemical fertilization, and biocide use, among others, on the community structure of these beneficial microorganisms. The possible consequences of these practices on AM fungal community structure, and on the symbiosis itself, have been widely investigated (Oehl et al. 2003; Smith and Read 1997). Several studies used conventional methodologies to demonstrate a reduction of inoculum potential of agricultural soils, AM fungal abundance, and species richness (Schalamuk et al. 2007). Recently, studies based on pyrosequencing technologies have provided new approaches for microbial community ecology in a wide range of environments (Unterseher et al. 2011).

Our aim was to assess the effects of agronomic practices on AM fungal community in soils of the Pampa Ondulada region using morphological and metagenomic approaches and to compare the conclusions emerging from the application of each technique. This work also seeks to contribute to the existing theoretical and applied knowledge on the potential of the pyrosequencing method for the study of AM fungi biodiversity.

As traditional methodology would be able to detect only those species that are sporulating, we hypothesize that the use of pyrosequencing could complement and complete the information obtained by morphotyping of spores.

We expected that the structure of the AM fungal community would not vary among agronomically managed soils because of the homogenization of the environment in sites that share a long and equal agricultural history.

Materials and methods

Study site

The study area is located in the Pampa Ondulada region in Buenos Aires province, Argentina, and is considered one of the most productive ecoregions of the world (44 000 km², 33°–39°S) (Morello et al. 2000; Faggi et al. 2006). The studied region was originally grassland (Cabrera 1971). Its soil and climatic conditions allow the development of 2 crops in the same growing season. As a consequence of agricultural expansion, the original plant biodiversity has almost been completely modified (Urricariet and Lavado 1999). Samples were taken in 3 productive establishments: La Estrella (34°42′99″S, 60°4′74″W), Criadero Klein (35°7′29″S, 60°15′71″W), and La Negrita (34°34′77″S, 59°55′13″W), where crop management has been practiced for more than 20 years. The last crop rotation in these establishments was wheat–soybean. Since the 3 selected sites showed similar soil properties, the same rainfall regime (1050 mm), and the same mean annual temperature (16.3 °C), they were considered as replicates (Alvarez and Alvarez 2000).

Sampling was performed under the framework of the SoilGeNe project (www.soilgene.net). Diversity of bacteria and soil fungi were also analyzed, as well as the whole metagenome of each field (Rascovan et al. 2013; Carbonetto et al. 2014).

Soil sampling

Samples were taken in a cultivated field (i.e., with agronomic management) (designated "A" soil) and in its corresponding farmhouse parks, considering them the nearest soil without agronomic management (designated "WA" soil). The sampled parks were not considered as undisturbed soil but places where the original grasses were not removed. Sampling was performed at the end of the soybean cycle (23 June 2010). Twenty soil cores of 3 cm diameter and 20 cm deep were randomly taken in an area of approximately 500 m². Subsamples were homogenized into a unique sample per site to remove the effects of heterogeneity at field scale (Alvarez and Alvarez 2000). They were packed, transported on ice to the laboratory, and stored at 4 °C for subsequent analysis. Each soil sample was divided in 3 parts for its physical and chemical analysis and to characterize AM fungal communities by molecular and morphological techniques.

Plant community composition from WA soils was assessed in each site. Predominant species of plants were sampled for their subsequent classification according Cabrera (1971).

Physical and chemical soil properties were analyzed in the radioisotope laboratory of the Faculty of Agronomy, University of Buenos Aires. The methods used are as follows. The amount of organic carbon was determined through wet digestion with heat (Amato 1983; Nelson and Sommers 1996). Carbon level in carbonates was analyzed by soil acidification and volumetric determination of generated CO_2 (Loeppert and Suarez 1996). Organic matter was estimated by calculating the difference between organic carbon and carbonates values (Nelson and Sommers 1996). Organic nitrogen was determined by the Kjeldahl technique (Bremner 1996). Inorganic nitrogen was quantified by extraction and distillation with Devarda (Mulvaney 1996). Nitrate levels were determined by extraction with KCl (2 mol/L) and phenolsulfonic acid (Bremner 1996). The Bray method was used to determine extractable phosphorus (Kuo 1996). pH was measured by dilution of soil–water (1:2.5) (Thomas 1996). Salinity was estimated as electrical conductivity (Rhoades 1996). Cation exchange capacity was determined by extraction (Sumner and Miller 1996). Total microbial biomass was assessed through quantification of microbial respiration by spraying and the incubation method. Texture (silt + clay) was evaluated with the hydrometer method (Gee and Bauder 1996). To determine soil moisture content (MC), samples of known mass (wet mass) were dried in an oven until a constant mass was achieved, and then MC was calculated using the following equation:

$$MC = [(wm - dm)/wm] \times 100$$

where wm is the wet mass, and dm is the dry mass.

Multivariate analysis of variance (MANOVA) was conducted to compare the values of the assessed parameters in agronomic and non-agronomic soils. Statistical procedures were carried out with the software package SPSS Statistics 17.0 for Windows (SPSS, Inc., Chicago, Illinois, USA). MANOVA did not show significant differences between soil managements, so one-way analysis of variance (ANOVA) was performed to compare soil parameters separately.

Molecular procedures

Soil samples were sieved through 2 mm mesh and preserved at −80 °C until molecular analysis. DNA extractions were performed from 0.25 g of soil with MO BIO PowerSoil DNA Isolation kits, following the manufacturer's instructions (MO BIO Laboratories, Inc., Carlsbad, California, USA).

The amplification primers AMV4.5F and AMDGR were chosen because of their suitable characteristics for the Genome Sequencer FLX 454 (GS FLX 454) (Roche Diagnostics 2009) and their specific amplification of AM fungal DNA (Sato et al. 2005; Lumini et al. 2009; Lin et al. 2012). Oligonucleotides were specifically designed for 454 pyrosequencing with the GS FLX 454 Titanium System. Amplicon fusion primers (see sequences below) contained a directional GS FLX 454 Titanium Primer A or Primer B sequence (in bold letters) and included a 4-base library "key" sequence (underlined) at the 5′ portion of the oligonucleotide and the template-specific sequence at the 3′ end. In the reverse primer, a

Multiplex Identifier (MID) sequence or "barcode" (in parentheses) was added between primer B and the template-specific sequences so that multiple samples could be sequenced in a single run. These MID sequences allowed automated software identification of each sample.

Forward primer (**primer A** – key – template-specific sequence):
5′-**CGTATCGCCTCCCTCGCGCCA**TCAGAAGCTCGTAGTTGAATTTCG-3′.

Reverse primer (**primer B** – key – (MID sequence) – template-specific sequence):
5′-**CTATGCGCCTTGCCAGCCCGCTCAG**(MID 10bp)CCCAACTATCCCTATTAATCAT-3′.

Amplification was done on a FastStart High Fidelity PCR system (Roche Applied Science, Mannheim, Germany) following the instructions of the manufacturer. The PCR conditions were as follows: 95 °C (5 min); 30 cycles of 95 °C (45 s), 57 °C (45 s), and 72 °C (60 s), and a final elongation step at 72 °C (4 min). PCR products were purified using Ampure Beads (Agencourt, Cat. No. A63880), which removed impurities, small spurious products, and primers. Equimolar amounts of each library with different MIDs were pooled to ensure that all were equally represented. Amplification and sequencing on a GS FLX 454 were performed at the Instituto de Agrobiotecnología de Rosario (INDEAR). All steps were performed following the protocols for amplicon sequencing in the Roche Diagostics 454 manuals (available from http://454.com/my454/).

Sequences analyses

The 454 reads were quality-controlled and denoised with the ampliconnoise.py script of the Quantitative Insights Into Microbial Ecology (QIIME) software version 1.5.0 (http://qiime.org/). This script also eliminated chimeras. Sequences were then clustered into molecular operational taxonomic units (MOTUs) using QIIME pipeline (pick_otus.py script with the Uclust method). MOTUs were defined at 97% sequence similarity. This arbitrary value was chosen in accordance with the traditional definition of microbial "species" (Konstantinidis and Tiedje 2007). Singletons were removed to avoid overestimation of species richness (Unterseher et al. 2011).

Representative sequences from each MOTU were compared with 18S rDNA published sequences on the MaarjAM (specific database for AM fungi; http://maarjam.botany.ut.ee/) and the NCBI (National Centre for Biotechnology Information) databases using Nt/nr BLAST (Basic Local Alignment Search Tool) algorithm (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi). Only sequences with query coverage and similarity values higher than 98% (E values equal or close to zero) were considered. After MOTUs identification, non-Glomeromycota sequences (sequences that matched with another eukaryote sequence with E values close to zero) were subsequently removed from the data sets. Taxonomic assignments were done according to the Index Fungorum (http://www.indexfungorum.org).

All analyzed sequences in this study are available as FASTQ at the NCBI Sequence Read Archive database under the accession No. SRX183378.

Glomeromycota sequences were aligned using the ClustalW algorithm (Larkin et al. 2007). Phylogenetic analysis was performed with the MEGA 5.1 program (Tamura et al. 2011) using the neighbor-joining distance method. Bootstrap analysis with 1000 replicates was used to test the confidence of the branches (Sokal and Rohlf 1995). A phylogenetic tree was constructed and edited by Tree Explorer of the MEGA 5.1 program (Supplementary Fig. 1[1]).

Isolation and identification of spores

AM propagules were isolated from 50 g (dry mass) of soil from each sampled site by wet sieving and decanting. Using a dissecting microscope at 40× magnification, we manually selected and removed healthy spores with a micropipette. All propagules were mounted on microscope slides in either polyvinyl alcohol – lactic acid – glycerol (PVLG) or a mixture of PVLG–Melzer reagent. Spore characteristics and subcellular structures were observed under a Nikon light microscope at 100×, 400× and 1000× magnifications (model: Optiphot-2) for morphological characterization. Taxonomic classifications, whenever possible, were made under the supervision of Dr. Gisela Cuenca (Instituto Venezolano de Investigación Científica (IVIC), Caracas, Venezuela), in accordance with the descriptions available at the website of Dr. J. Blaszkowski (http://www.zor.zut.edu.pl/Glomeromycota/index.html) and at the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM) website (http://invam.caf.wvu.edu). Taxonomic assignments were done according to the Index Fungorum.

A trap culture was established in a 1 L pot for each site, to help in the characterization of AM fungal communities. A sterile (100 °C for 1 h, 3 consecutive days) mixture of soil–perlite–vermiculite (1:1:1) was used as growth substrate; sorghum, tomatoes, peas, and clover were used as host plants. AM fungal inoculum consisted of the sampled soil containing spores, mycelia, and mycorrhized roots. Spores isolated from trap cultures were not considered for the abundances analyses.

α-Diversity analyses

A normalization step of the data was applied before diversity analyses. Each data set was subsampled to the smallest reads number obtained for a data set (nWA). This normalization was conducted using QIIME. To asymptotically estimate the AM richness of each site, observed MOTUs (S) and Chao1 index were calculated by rarefaction curves analysis. These analyses were constructed with QIIME pipeline by randomly selecting series of subsets of different sizes from the libraries. Each sampled subset was replicated 10 times by the program.

Simpson index ($\lambda = \sum p_i^2$) and Shannon–Wiener index ($H' = -\sum p_i \cdot \ln p_i$) ($p_i$, relative abundance of the ith species) were calculated to estimate the diversity of AM fungal communities by traditional and metagenomic approaches. As well, community evenness was estimated by Pielou's index ($J' = H'/\ln S$). The number of sequences assigned to each MOTU was considered as an estimator of the abundance of molecular species.

One-way ANOVA was performed to evaluate the effect of agronomic soil management of on the α-diversity of AM fungal communities.

β-Diversity analyses

Bray–Curtis distance matrixes were calculated for the molecular and morphological data sets. Hierarchical cluster analyses were performed with the matrix results to unveil the ecological similarities between sites. With the aim of estimating the relative contribution of soil abiotic factors on AM fungal β-diversity, a distance-based Redundancy Analysis and an analysis of similarity statistics were performed.

α-Diversity and β-diversity analyses were conducted with the software package R statistical language version 2.12.2 using the community analysis-specific package BiodiversityR (R Foundation for Statistical Computing 2011).

Results

Soil analysis

Soil properties are presented in Table 1. Soil management did not influence soil properties, according to MANOVA ($F_{[1,4]} = 28.231$). One-way ANOVAs resulted in significantly ($P < 0.05$) higher values of total phosphorus content ($F_{[1]} = 9.64$), organic

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjm-2014-0364.

Table 1. Physicochemical soil properties of the 6 sampled sites.

Site*	ON (%)	OM (%)	NO_3^{2-} (mg/kg)	Clay (%)	Lime (%)	Sand (%)	P (mg/kg)	pH	Conductivity (dS/m)	Humidity (%)	Microbial biomass (μg C/g soil)	Metabolic rate (μg C/g biomass)
La Estrella												
A	4.3	0.19	40.3	23.3	48	28.7	7.6	5.7	0.16	21.2	393	0.70
WA	6.2	0.35	62.8	19.3	51	29.7	92.3	5.4	0.29	26.1	422	1.37
Criadero Klein												
A	2.9	0.17	39.3	20.3	42	37.7	12.3	5.4	0.16	21.3	652	0.53
WA	3.6	0.23	52.3	14.3	46	39.7	31.8	5.9	0.23	21.8	663	0.65
La Negrita												
A	2.7	0.16	43.0	23.3	51	25.7	11.6	5.6	0.17	23.7	670	0.43
WA	5.8	0.31	62.4	24.3	49	26.7	81.7	6.2	0.17	26.7	588	0.88

Note: ON, organic nitrogen; OM, organic matter; NO_3^{2-}, nitrate; P, phosphorus; C, carbon.

*A, samples were taken from a cultivated field, i.e., incurred agronomic management; WA, samples were taken from a farmhouse park, which was not considered undisturbed but without agronomic management history.

nitrogen ($F_{[1]} = 11.5$), and nitrate ($F_{[1]} = 25.72$) in non-agronomic than agronomic soils.

Flora from the WA areas was composed of the following native and allochthonous plants:

–La Negrita

Cardus sp., *Festuca* sp., *Bowlesia incana*, *Trifolium* sp., *Micropsis spatholata*, *Taraxacum officinale*, *Stenotaphrum secundatum*, *Coronopus didymus*, *Dichondra microcalyx*, *Chaptalia* sp;

–Criadero Klein

Cardus sp., *Festuca* sp., *B. incana*, *Trifolium* sp., *T. officinale*, *D. microcalyx*, *Paspalum dilatatum*, *Ammis majus*;

–La Estrella

Festuca sp., *B. incana*, *Trifolium* sp., *M. spatholata*, *T. officinale*, *D. microcalyx*, *Oxalis* sp., *Plantago tomentosa*, *Carex bonariensis*, *Veronica arvensis*, *Geranium molle*, *Stellaria media*.

α-Diversity analyses: molecular approach

Based on 97% sequence similarity, 489 MOTUs were identified from a total of 57 281 reads (mean length = 268, SD = 16). According to the best BLAST hit, 188 MOTUs belonged to the Glomeromycota phylum. Hits were coincident between the MaarjAM and the NCBI database (Supplementary Table 1[1]). The remaining MOTUs were eliminated, leaving 46 298 sequences in our data set. These AM fungi reads represented 81.38% of total reads, followed by unclassified fungi (4.39%), Basidiomycota fungi (3.3%), Eucaryotes (3.06%), Blastomycota (0.31%), Ascomycota (0.15%), and Zygomycota (0.07%).

At the genus level, *Glomus* showed the highest percentage values (24.9%), followed by *Rhizophagus* (11.29%), *Claroideoglomus* (8.49%), *Gigaspora* (2.85%), *Funneliformis* (2.74%), *Paraglomus* (0.98%), *Scutellospora* (0.70%), *Diversispora* (0.38%), *Ambispora* (0.17%), and *Archaeospora* (0.03%); the remaining 47.32% of AM MOTUs were assigned to the Glomerales order but could not be classified at the genus level (Supplementary Table 1[1]).

Six MOTUs were detected exclusively in soils without agronomic management. They corresponded to 4 fungal taxa: *Septoglomus viscosum*, *Rhizophagus iranicus*, *Rhizophagus proliferum*, and Glomerales.

Fourteen MOTUs were found abundantly in all sampled sites, they were similar to sequences of *Claroideoglomus lamellosum*, *Glomus indicum*, *Glomus sinuosum*, *S. viscosum*, *R. iranicus*, *Rhizophagus intraradices*, *Paraglomus* sp., or unidentified Glomerales.

Rarefaction curves reached the asymptotes in every case, demonstrating that the sequencing effort was enough to cover the entire diversity present in each sample (Fig. 1).

When the different estimators of species richness were compared between agronomic and non-agronomic soils, those from managed soils were always lower, but only the Chao1 index resulted in significant differences between managements ($P = 0.039$, $F_{[1]} = 10.736$) (Fig. 2A).

AM fungal diversity estimated by the Shannon–Wiener index was significantly lower in soils with agronomic management ($P = 0.004$, $F_{[1]} = 36.430$). The same result was obtained when Simpson indices were compared between treatments ($P = 0.031$, $F_{[1]} = 11\,341$) (Fig. 2B). Significant differences were detected among estimated Pielou's indeces of A and WA communities of AM fungi. Evenness was greater in non-agronomic soils (Fig. 2C).

α-Diversity analyses: morphological approach

A total of 487 healthy AM propagules were isolated from soil in this study (Supplementary Table 2[1]). After taxonomic determination, based on morphological characteristics of spores, 10.27% of AM propagules were assigned to the Glomerales order but could not be classified at the genus level. Among the assigned genera, *Glomus* (51.13%) showed the highest percentage values, followed by *Scutellospora* (9.45%), *Funneliformis* (8.01%), *Rhizophagus* (7.6%), *Gigaspora* (6.57%), *Claroideoglomus* (2.67%), *Diversispora* (2.26%), *Acaulospora* (1.64%), and *Entrophospora* (0.41%). The AM fungal morpho-species found in soil were not all found in the trap cultures; moreover other species were observed in those trap cultures but not in the soil samples from each site.

Only *G. sinuosum* occurred in every sampled site, while *Gigaspora margarita* was isolated only from non-agronomic soils (Supplementary Table 2[1]).

AM species richness was higher in soils without agricultural management, but this difference was not statistically significant (Fig. 2D).

Shannon and Simpson indices were compared between agronomic and non-agronomic soils. Both biodiversity estimators resulted in higher values for WA soil. Moreover, only the Simpson index varied significantly between treatments ($P < 0.05$, $F_{[1]} = 35.937$) (Fig. 2E). No significant differences were detected among Pielou's evenness index of AM fungal communities from A and WA soils (Fig. 2F).

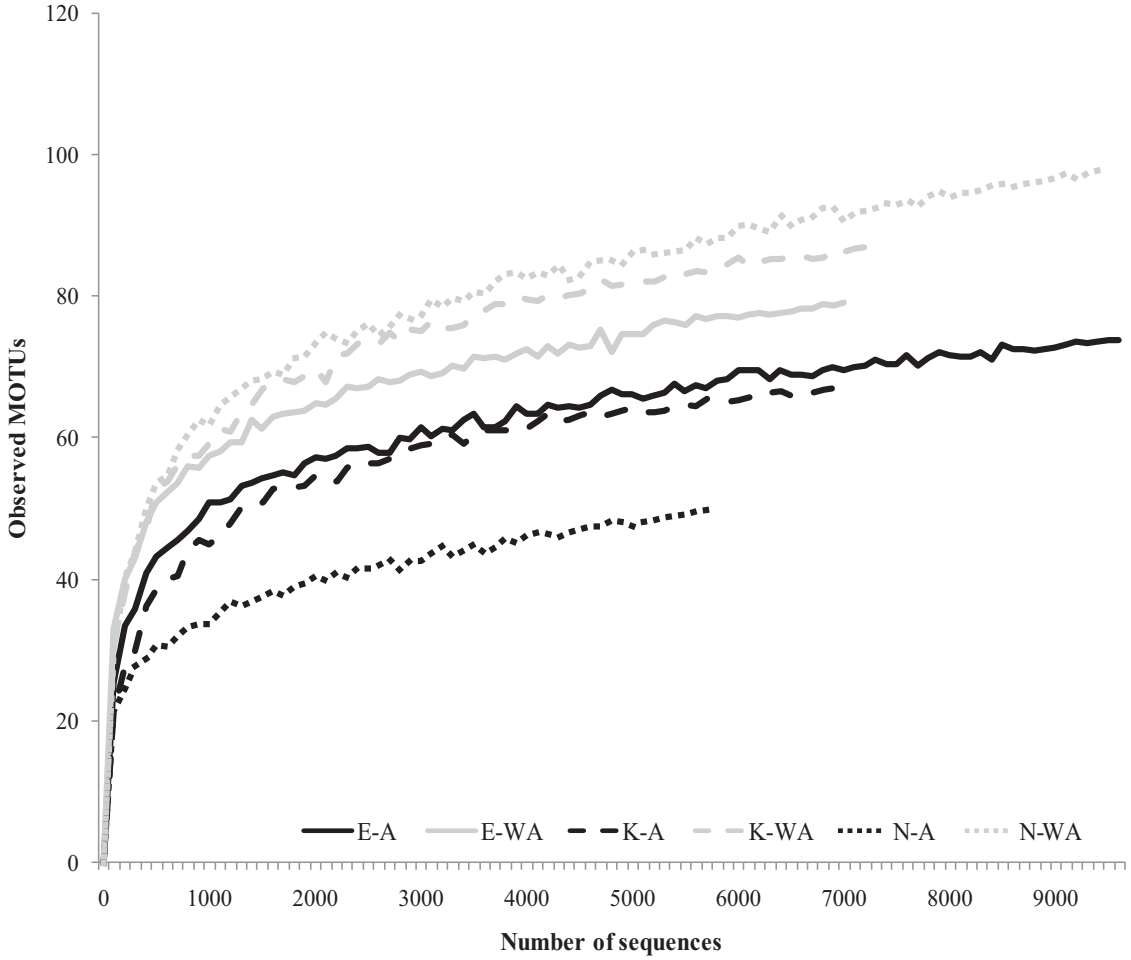
β-Diversity analyses

AM diversity analysis by metagenomic technique showed that 50% of all sequences corresponded to 3–5 or 5–8 MOTUs for A and WA soils respectively. The most frequent MOTUs belonged to order Glomerales and to *Claroideoglomus* sp., *Funneliformis constrictum*, *Funneliformis mosseae*, *G. indicum*, *G. perpusillum*, *R. intraradices*, *R. iranicus*, *Rhizophagus* sp. and *G. margarita*. These MOTUs were abundantly found in every study site.

With regard to the result obtained by morphotyping, 50% of the propagules belonged to 1–2 or 2–3 species in A and WA, respectively. The most frequent morphological species were *Glomus microaggregatum*, *G. sinuosum*, *S. viscosum*, *R. intraradices*, *F. constrictum*, *Claroideoglomus* sp., and *G. margarita*.

Bray–Curtis coefficients were used to compare the similarity between the general structures of AM fungal communities. Soils without agronomic management were clustered together in the metagenomic data set (Fig. 3A).

Fig. 1. Molecular accumulation curves for 6 sites in the Pampa Ondulada region. MOTUs, molecular operational taxonomic units. Codes for the sites are depicted as agricultural site - soil management: E, La Estrella; K, Criadero Klein; N, La Negrita; A, with agronomic management; WA, without agronomic management.



According to cluster analysis of morphotypes, AM fungal communities from A and WA soils from La Negrita were more similar to each other than to other sites. Moreover, according this data set, the AM fungal community of the agronomic soil from La Estrella was most dissimilar (Fig. 3B).

Distance-based redundancy analysis did not reveal a significant relationship between abiotic factors and the β-diversity of AM fungi (data not shown). Analysis of similarity (based on 999 permutations) revealed a significant difference between AM communities from agronomic and nonagronomic soil when molecular data were analyzed ($R = 0.944$, $P = 0.048$). However this difference was not detected with the morphotypes data ($R = 0.444$, $P = 0.25$).

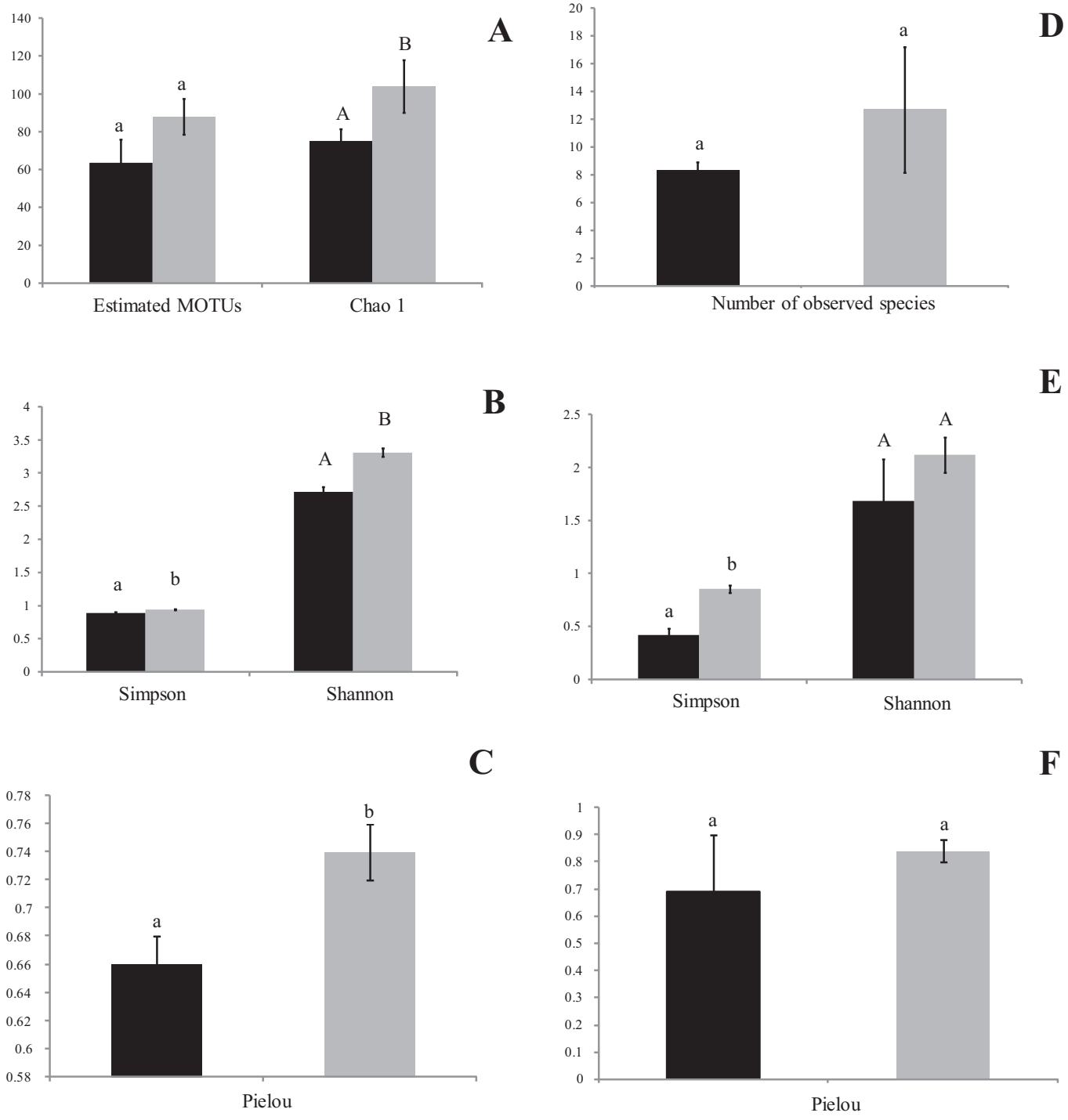
Discussion

Methodological comparison

In our study, AM species richness always showed higher values when estimated by molecular approach. It was observed that representative sequences of different MOTUs were similar to sequences of the same AM fungal morpho-species. The difficulty to correspond the number of morpho-species with the number of MOTUs could be explained from different points of view: the overestimation of fungal diversity, when using a 97% similarity to define MOTUs, or the difficulty for matching environmental sequences with those obtained from taxonomically defined species. Considering that AM fungi are asexual, multinucleated organisms with reported hyphal anastomosis (Giovannetti et al. 1999), a high genetic diversity is observed within a single individual in comparison with other organisms (Fitter 2005). For some AM fungi, Stockinger et al. (2010) detected that intraspecific genetic variability could be higher than interspecific variability. For this reason, a lower resolution among sequences (less than 97% similarity) could represent the same taxonomic unit (Hibbett et al. 2011). Because of this, the molecular definition of "individual" and "species" in the Glomeromycota is still under discussion (Unterseher et al. 2011). As an answer to this challenge, different authors have begun to define virtual taxa (VT) (Davison et al. 2012; Öpik et al. 2013; Öpik et al. 2014). VT are groups of sequences phylogenetically defined that correspond to taxonomic groups. Öpik et al. (2014) postulated that this system would allow a better comparison of the results obtained by morphological and molecular approaches. However, the number of VT defined has already exceeded the number of morphological species.

In this study, spores of *Acaulospora scrobiculata*, *Acaulospora bireticulata*, *Pacispora* sp., and *E. infrequens* were recovered from soil samples and (or) trap cultures, but they were not detected by molecular approach. This is not surprising, as approximately 70% of reported AM morphological species have not yet been sequenced and, consequently, are not represented in GenBank (Öpik et al. 2009). Moreover, the AM species reported in public databases were described using different primers (ITS, SSU, and LSU regions) and their sequences are not comparable. In a recent work, Kohouta et al. (2014) studied the most common primers

Fig. 2. Measurements of arbuscular mychorrizal (AM) fungal diversity in soils with (dark grey bars) or without (grey bars) agronomic management. Results obtained with the metagenomic approach for richness estimators (A), diversity estimators (B), and evenness estimators (C). Results obtained with the morphotyping approach for AM fungal richness (D), diversity estimators (E), and evenness estimators (F). Values are the means with standard deviation bars. Different letters represent significant differences between treatments ($P < 0.05$). MOTUs, molecular operational taxonomic units.

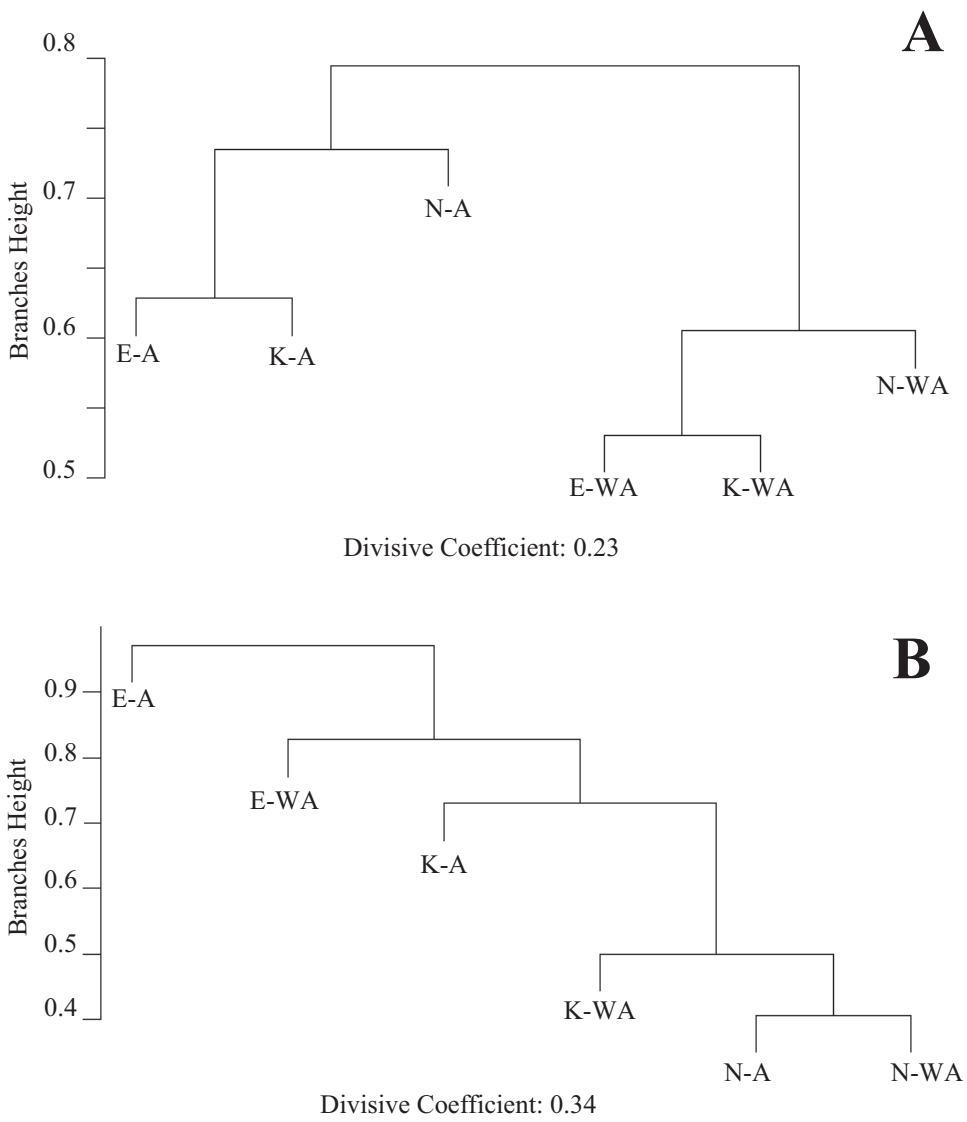


used in AM fungal ecology. The authors reported a strong bias towards the Glomeraceae and Acaulosporaceae in the SSU primer systems. This PCR bias could be a possible explanation for the failure to detect the Acaulosporaceae species listed above.

Another reason for the nondetected sequences could be the low spore density of some particular AM species or the small size of their propagules, which makes it difficult to isolate their DNA or produces a low yield of DNA. An example of the last point is *G. microaggregatum*. Even when it was abundantly detected by morphological technique, this was not always represented in the molecular data set.

Using the Sanger sequencing technique, Hempel et al. (2007) compared the AM community composition of bulk soil, colonized root, and sporulated AM community. The authors reported the

Fig. 3. Bray–Curtis cluster analyses of the sites studied with the metagenomic approach (A) and the morphotyping approach (B). Codes for the sites are depicted as agricultural site - soil management: E, La Estrella; K, Criadero Klein; N, La Negrita; A, with agronomic management; WA, without agronomic management.



dominance of Glomerales species in colonized roots and that Paraglomerales species were exclusively found in soil samples, whereas Diversisporales species were found more frequently in soil (as spores and (or) mycelium) than in roots. These results coincide with those obtained in our work, as Diversisporales species were more frequently observed by using the morphotyping technique than the molecular approach (data obtained from DNA isolated from soil), according to which Glomerales were predominant. Even when sequences belonging to *Paraglomus occultum* were abundantly detected in every site when studying soil DNA, the spores of this genus were observed only in trap cultures. This could be considered evidence that these AM fungi were only present as mycelia at the time of soil sampling. Results obtained exclusively from spores collected in the field are biased to those species sporulated at the time of sampling or that have sporulated and are no longer active.

The species richness of AM fungi estimated by the metagenomic approach is high compared with that estimated by morphotyping, even when considering the possible overestimation of richness due to the use of 97% similarity level, the detection of non-sporulated species, the efficiency of 454 pyrosequencing technique for detecting rare species (Lumini et al. 2009), and the global distribution of most AM taxa (Öpik et al. 2009). For this reason, and in agreement with Öpik et al. (2009), we believe that there would still be a large AM fungal diversity that remains undescribed given that some AM species sporulate only rarely, if at all.

We conclude, in line with Baldrian (2011), that until all morphological taxa are molecularly characterized and a complete AM fungal database is developed, and while the new sequencing methods improve knowledge on the intraspecific variation in Glomeromycota, morphotyping and molecular studies should be combined to best understand AM fungal ecology.

Agronomic management — effects on the AM fungal community structure

In this work we observed a greater richness and evenness in the non-agronomic sites; this was with both methodologies. Both techniques also agreed that non-agronomic soils were significantly more biodiverse than those from agroecosystems.

Our results agree with those of Lumini et al. (2009), who observed that anthropogenic disturbance of soils reduces species richness. The diversity of the AM fungal community is thought to

parallel plant diversity (Fitter 2005). Öpik et al. (2009) supported this hypothesis with molecular data from the comparison of AM fungal and host plant communities from different habitats. Lower plant diversity may be a possible explanation for the lower AM fungal richness observed in the agronomic soils, which were in culture for more than 30 years.

The Glomerales were dominant in the studied sites from the Pampeana region. The dominance of this group has been previously observed in natural and agricultural ecosystems by several authors (Collins Johnson et al. 1991; Schalamuk et al. 2007; Lumini et al. 2009; Dumbrell et al. 2011; Lin et al. 2012; Maherali and Klironomos 2012).

We found that some AM fungal genera and species were present in all soils, while others were observed in a unique site or in soils with a particular kind of management. Oehl et al. (2010) studied these distribution patterns and defined the first ones as "generalist" and the second as "specialist". According to these definitions, generalist MOTUs or morphological species always corresponded to the Glomerales order. An amplicon sequencing study of the bacterial community in the same study sites was already performed (Carbonetto et al. 2014). Some bacterial phyla were abundantly found in non-agronomic soils. However "specialists" phyla from agronomic soils were also reported. In our work, there were not "specialist" AM fungi from agronomic soils but there were in non-agronomic soils.

In this study, we compared results obtained from amplicon pyrosequencing technique with those from the traditional spore-based taxonomy approach and concluded that both methodologies complement each other in the study of AM fungal ecology.

This study reported, for the first time, differences in AM fungal diversity in soils from the Argentinean Pampeana region, with and without agronomic management, using next-generation sequencing technologies. Considering our findings, we concluded that AM fungal biodiversity is negatively affected by agronomic management of soil. Carbonetto et al. (2014) also reported a marked effect of agricultural practices on soil bacterial community in these same study sites.

This DNA-based survey of AM fungal communities is one of very few to have been conducted in South America. Thus, the 188 MOTUs presented here greatly improve the knowledge about AM fungi in South America.

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References

Alvarez, R., and Alvarez, C.R. 2000. Soil organic matter pools and their association with carbon mineralization kinetics. Soil Sci. Soc. Am. J. **64**: 184–189. doi:10.2136/sssaj2000.641184x.

Amato, M. 1983. Determination of carbon ^{12}C and ^{14}C in plant and soil. Soil Biol. Biochem. **15**: 611–612. doi:10.1016/0038-0717(83)90059-7.

Baldrian, P. 2011. Ecology and metagenomics of soil microorganisms. FEMS Microbiol. Ecol. **78**: 1–2. doi:10.1111/j.1574-6941.2011.01184.x. PMID:2209139.

Bonfante, P., and Genre, A. 2010. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. Nat. Commun. **1**: 48. doi:10.1038/ncomms1046. PMID:20975705.

Borrielo, R., Lumini, E., Girlanda, M., Bonfante, P., and Bianciotto, V. 2012. Effects of different management practices on arbuscular mycorrhizal fungal diversity in maize fields by a molecular approach. Biol. Fertil. Soils. doi:10.1007/s00374-012-0683-4.

Bremner, J.M. 1996. Nitrogen-total. *In* Methods of soil analysis. Part 3-Chemical methods. Ed. Soil Science Society of America, Madison, Wis., USA pp. 1085–1121.

Cabrera, A.L. 1971. Fitogeografía de la República Argentina. Boletin de la Sociedad Argentina de Botánica, **14**: 1–42.

Carbonetto, B., Rascovan, N., Alvarez, R., Mentaberry, A., and Vazquez, M. 2014. Structure, composition and metagenomic profile of soil microbiomes associated to agricultural land use and tillage systems in Argentine Pampas. PLoS One, **9**(6): e99949. doi:10.1371/journal.pone.0099949. PMID:24923965.

Collins Johnson, N., Pfleger, F.L., Kent Crookston, R., Simmons, S.R., and Copeland, P.J. 1991. Vesicular-arbuscular mycorrhizas respond to corn and soybean cropping history. New Phytol. **117**: 657–663. doi:10.1111/j.1469-8137.1991.tb00970.x.

Dai, M.L., Hamel, C., Arnaud, M.S., He, Y., Grant, C., Lupwayi, N., et al. 2012. Arbuscular mycorrhizal fungi assemblages in Chernozem great groups revealed by massively parallel pyrosequencing. Can. J. Microbiol. **58**: 81–92. doi:10.1139/w11-111. PMID:22220554.

Davison, J., Opik, M., Zobel, M., Vasar, M., Metsis, M., and Moora, M. 2012. Communities of arbuscular mycorrhizal fungi detecting in forest soil are spatially heterogeneous but do not vary throughout the growing season. PLoS One, **7**: e41938. doi:10.1371/journal.pone.0041938. PMID:22879900.

Douds, D.D., Jr., and Millner, P.D. 1999. Biodiversity of arbuscular mycorrhizal fungi in agroecosystem. Agric. Ecosyst. Environ. **74**: 77–93. doi:10.1016/S0167-8809(99)00031-6.

Dumbrell, A.J., Ashton, P.D., Aziz, N., Feng, G., Nelson, M., Dytham, C., et al. 2011. Distinct seasonal assemblage of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. New Phytol. **190**: 794–804. doi:10.1111/j.1469-8137.2010.03636.x. PMID:21294738.

Faggi, A.M., Krellenberg, K., Castro, R., Arriaga, M., and Endlicher, W. 2006. Biodiversity in the Argentinean rolling Pampa ecoregion: changes caused by agriculture and urbanisation. Erdkunde, **60**: 127–138. doi:10.3112/erdkunde.2006.02.04.

Fitter, A.H. 2005. Darkness visible: reflections on underground ecology. J. Ecol. **93**: 231–243. doi:10.1111/j.0022-0477.2005.00990.x.

Friberg, S. 1999. Distribution and diversity of arbuscular mycorrhizal fungi in traditional agriculture on the Niger inland delta, Mali, West Africa. CBM:s Skriftserie, **3**: 53–80.

Gee, G.W., and Bauder, J.W. 1996. Particle-size analysis. *In* Methods of soil analysis. Part 3. Chemical methods. *Edited by* D.L. Sparks. American Society of Agronomy, Madison, Wis., USA. pp. 383–412.

Giovannetti, M., Azzolini, D., and Citernesi, A.S. 1999. Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. Appl. Environ. Microbiol. **65**(12): 5571–5575. PMID:10584019.

Hausmann, N.T., and Hawkes, C.V. 2009. Plant neighborhood control of arbuscular mycorrhizal community composition. New Phytol. **183**: 1188–1200. doi:10.1111/j.1469-8137.2009.02882.x. PMID:19496954.

Hempel, S., Renker, C., and Buscot, F. 2007. Differences in the species composition of arbuscular mycorrhizal fungi in spore, root and oil communities in grassland ecosystem. Environ. Microbiol. **9**(8): 1930–1938. doi:10.1111/j.1462-2920.2007.01309.x. PMID:17635540.

Hibbett, D.S., Ohman, A., Glotzer, D., Nuhn, M., Kirk, P., and Nilsson, R.H. 2011. Progress in molecular and morphological taxon discovery in fungi and options for formal classification of environmental sequences. Fungal Biol. Rev. **25**: 38–47. doi:10.1016/j.fbr.2011.01.001.

Johnson, D., Vandenkoornhuyse, P.J., Leake, J.R., Gilbert, L., Booth, R.E., Grime, J.P., et al. 2003. Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosms. New Phytol. **161**: 503–515. doi:10.1046/j.1469-8137.2003.00938.x.

Kohouta, P., Sudováa, R., Janouškováa, M., Čtvrtlíkovác, M., Hejdaa, M., Pánkováa, H., et al. 2014. Comparison of commonly used primer sets for evaluating arbuscular mycorrhizal fungal communities: Is there a universal solution? Soil Biol. Biochem. **68**: 482–493. doi:10.1016/j.soilbio.2013.08.027.

Konstantinidis, K.T., and Tiedje, M. 2007. Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. Curr. Opin. Microbiol. **10**: 504–509. doi:10.1016/j.mib.2007.08.006. PMID:17923431.

Kuo, S. 1996. Phosphorus. *In* Methods of soil analysis. Part 3. Chemical methods. *Edited by* D.L. Sparks. American Society of Agronomy, Madison, Wis., USA. pp. 869–920.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., et al. 2007. Clustal W and Clustal X version 2.0. Bioinformatics, **23**: 2947–2948. doi:10.1093/bioinformatics/btm404. PMID:17846036.

Lekberg, Y., Schnoor, T., Kjoller, R., Gibbons, S.M., Hansen, L.H., Al-Soud, W.A., et al. 2012. 454-sequencing reveals stochastic local reassembly and high disturbance tolerance within arbuscular mycorrhizal fungal communities. J. Ecol. **100**: 151–160. doi:10.1111/j.1365-2745.2011.01894.x.

Liang, Z., Drijber, R.A., Lee, D.J., Dwiekat, I.M., Harris, S.D., and Wedin, D.A. 2008. A DGGE-cloning method to characterize arbuscular mycorrhizal community structure in soil. Soil Biol. Biochem. **40**: 956–966. doi:10.1016/j.soilbio.2007.11.016.

Lin, X., Feng, Y., Zhang, H., Chen, R., Wang, J., Zhang, J., and Chu, H. 2012. Long-term balanced fertilization decreases arbuscular mycorrhizal fungal diversity in an arable soil in North China revealed by 454 pyrosequencing. Environ. Sci. Technol. **46**(11): 5764–5771. doi:10.1021/es3001695. PMID:22582875.

Loeppert, R.H., and Suarez, D.L. 1996. Carbonate and gypsum. *In* Methods of soil analysis. Part 3. Chemical methods. *Edited by* D.L. Sparks. American Society of Agronomy, Madison, Wis., USA. pp. 437–474.

Lumini, E., Orgiazzi, A., Borriello, R., Bonfante, P., and Bianciotto, V. 2009. Disclosing arbuscular mycorrhizal fungal biodiversity in soil through a land-

use gradient using a pyrosequencing approach. Environ. Microbiol. **12**: 2165–2179. doi:10.1111/j.1462-2920.2009.02099.x. PMID:21966911.

Maherali, H., and Klironomos, J.N. 2012. Phylogenetic and trait-based assembly of arbuscular mycorrhizal fungal communities. PLoS One, **7**: e36695. doi:10.1371/journal.pone.0036695. PMID:22606282.

Morello, J., Buzai, G.D., Baxendale, C.A., Rodríguez, A.F., Matteucci, S.D., Godagnone, R.E., et al. 2000. Urbanization and the consumption of fertile land and other ecological changes: the case of Buenos Aires. Environ. Urban. **12**: 119–131. doi:10.1177/095624780001200210.

Mulvaney, R.L. 1996. Nitrogen-inorganic forms. *In* Methods of soil analysis. Part 3. Chemical methods. *Edited by* D.L. Sparks. American Society of Agronomy, Madison, Wis., USA. pp. 1123–1184.

Nelson, D.W., and Sommers, L.E. 1996. Total carbon, organic carbon, and organic matter. *In* Methods of soil analysis. Part 3. Chemical methods. *Edited by* D.L. Sparks. American Society of Agronomy, Madison, Wis., USA. pp. 53–90.

Oehl, F., Sieverding, E., Ineichen, K., Mäder, P., Boller, T., and Wiemken, A. 2003. Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of Central Europe. Appl. Environ. Microbiol. **69**: 2816–2824. doi:10.1128/AEM.69.5.2816-2824.2003. PMID:12732553.

Oehl, F., Laczko, E., Bogenrieder, A., Stahr, K., Bösch, R., van der Heijden, M., et al. 2010. Soil type and land use intensity determine the composition of arbuscular mycorrhizal fungal communities. Soil Biol. Biochem. **42**: 724–738. doi:10.1016/j.soilbio.2010.01.006.

Öpik, M., Metsis, M., Daniell, T.J., Zobel, M., and Moora, M. 2009. Large-scale parallel 454 sequencing reveals host ecological group specificity of arbuscular mycorrhizal fungi in a boreonemoral fores. New Phytol. **184**: 424–437. doi:10.1111/j.1469-8137.2009.02920.x. PMID:19558424.

Öpik, M., Zobel, M., Cantero, J.J., Davison, J., Facelli, J.M., Hiiesalu, I., et al. 2013. Global sampling of plant roots expands the described molecular diversity of arbuscular mycorrhizal fungi. Mycorrhiza, **23**: 411–430. doi:10.1007/s00572-013-0482-2. PMID:23422950.

Öpik, M., Davison, J., Moora, M., and Zobel, M. 2014. DNA-based detection and identification of Glomeromycota: the virtual taxonomy of environmental sequences. Botany, **92**(2): 135–147. doi:10.1139/cjb-2013-0110.

Rascovan, N., Carbonetto, B., Revale, S., Reinert, M., Alvarez, R., Godeas, A., et al. 2013. The PAMPA datasets: a metagenomic survey of microbial communities in Argentinean pampean soils. Microbiome, **1**: 21. doi:10.1186/2049-2618-1-21. PMID:24450949.

Rhoades, J.D. 1996. Salinity: electrical conductivity and total dissolved solids. *In* Methods of soil analysis: chemical methods. Part 3. *Edited by* D.L. Sparks. ASA and SSSA, Madison, Wis., USA. pp. 417–435.

Roche Diagnostics. 2009. Using multiplex identifier (MID) adaptors for the GS FLX titanium chemistry — extended MID set. 454 Sequencing Technical Bulletin No. 005-2009. Roche Applied Science, Indianapolis, Ind., USA.

Sato, K., Suyama, Y., Saito, M., and Sugawara, K. 2005. A new primer for discrimination of arbuscular mycorrhizal fungi with polymerase chain reaction – denature gradient gel electrophoresis. Grassl. Sci. **51**: 179–181. doi:10.1111/j.1744-697X.2005.00023.x.

Schalamuk, S., Chidichimo, H., and Cabello, M. 2007. Variación en la composición de especies de Glomeromycota (fungi) en un cultivo de trigo bajo distintos sistemas de labranza. Bol. Soc. Argent. Bot. **42**: 45–53.

Smith, S.E., and Read, D.J. 1997. Mycorrhizal symbiosis. 2nd ed. Academic Press, London, UK.

Sokal. R.R., and Rohlf, F.J. 1995. Biometry. W.H. Freeman and Company, New York, USA.

Stockinger, H., Krüger, M., and Schuβler, A. 2010. DNA barcoding of arbucular mycorrhizal fungi belonging to the phylum *Glomeromycota*. Mycorrhiza, **22**: 247–258.

Sumner, M.E., and Miller, W.P. 1996. Cation exchange capacity and exchange coefficients. *In* Methods of soil analysis. Part 3. Chemical methods. *Edited by* D.L. Sparks. American Society of Agronomy, Madison, Wis., USA. pp. 1201–1229.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony method. Mol. Biol. Evol. **28**: 2731–2739. doi:10.1093/molbev/msr121. PMID:21546353.

Thomas, G.W. 1996. Soil pH and soil acidity. *In* Methods of soil analysis. Part 3. Chemical methods. *Edited by* D.L. Sparks. American Society of Agronomy, Madison, Wis., USA. pp. 475–490.

Unterseher, M., Jumpponen, A., Öpik, M., Tedersoo, L., Moora, M., Dormann, C.F., and Schnittler, M. 2011. Species abundance distributions and richness estimations in fungal metagenomic — lesson learned from community ecology. Mol. Ecol. **20**(2): 275–285. doi:10.1111/j.1365-294X.2010.04948.x. PMID:21155911.

Urricariet, S., and Lavado, R.S. 1999. Indicadores de deterioro en suelos de la pampa ondulada. Cienc. Suelo, **17**(1): 37–44.

Wehner, J., Antunes, P.M., Powell, J.R., Mazukatow, J., and Rillig, M.C. 2009. Plant pathogen protection by arbuscular mycorrhizas: A role for fungal diversity? Pedobiologia, **53**(3): 197–201. doi:10.1016/j.pedobi.2009.10.002.