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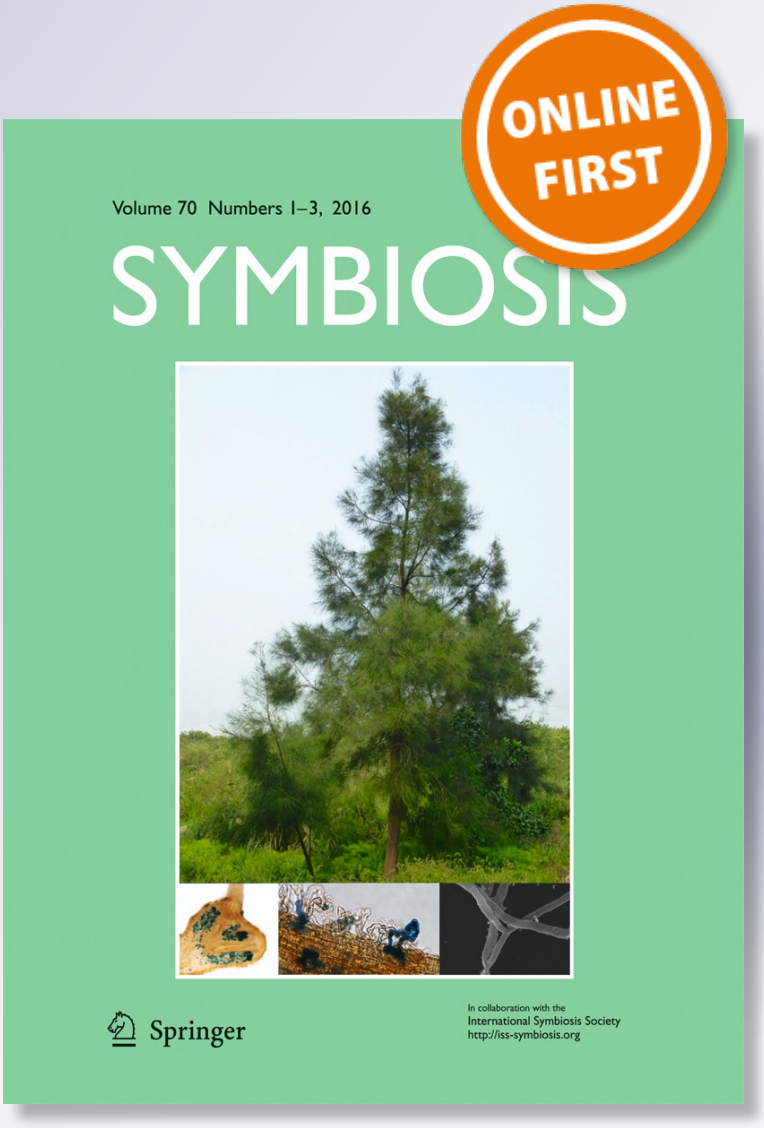

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Arbuscular mycorrhizal fungal diversity in high-altitude hypersaline Andean wetlands studied by 454-sequencing and morphological approaches

Vanesa Analía Silvani[1] · **Roxana Paula Colombo**[1] · **María Victoria Scorza**[1] · **Laura Fernández Bidondo**[1] · **Carolina Paola Rothen**[1] · **Adalgisa Scotti**[2] · **Sebastián Fracchia**[3] · **Alicia Godeas**[1]

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Abstract The *Laguna Brava* Nature Reserve is a stressful habitat in the Andean Mountains (Argentina) dominated by extreme abiotic factors: high altitude and UV radiance, hypersalinity, alkalinity, and high concentrations of toxic elements in the soil. The sparse native vegetation that inhabits *Laguna Brava* and *Mulas Muertas* wetlands is frequently colonized by arbuscular mycorrhizal (AM) fungi. It is, however, unknown which AM species can survive in such a harsh environment and how those environmental conditions influence the AM communities. To answer these questions, 454-amplicon pyrosequencing and morphological (based on spore traits) approaches were used to assess fungal diversity. A total of 23 molecular operational taxonomic units and 14 distinct morphospecies of AM fungi were identified. The morphological characterization of AM fungal communities in *Laguna Brava* and *Mulas Muertas,* supported by the molecular data, revealed that *Glomeraceae* and *Claroideoglomeraceae* were the dominant families, confirming the predominance of generalist and ruderal AM fungal taxa but with stress-tolerant life history traits. Our results showed that the presence of AM fungi is strongly associated with local environmental variations in *Laguna Brava* (hypersalinity and high Na^+, Sr, As and U contents in soils). The AM fungal communities in *Laguna Brava* and *Mulas Muertas* wetlands were similar according to the Simpson diversity index and the ecological distance estimated by Bray Curtis index. These results were also supported by the environmental parameters measured, as they did not vary between the studied sites. This study represents the first characterization of AM fungal community in a high-altitude Andean wetland in Argentina, improving our knowledge about these fungi from extreme environments.

Keywords Arbuscular mycorrhizal fungi · High-altitude wetlands · Extreme environment · Salinity · Heavy metal · Pyrosequencing

Vanesa Analía Silvani and Roxana Paula Colombo contributed equally to this work.

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1 Introduction

Vegetation exposed to extreme environments has developed different evolutionary strategies to overcome the prevailing stressful conditions, such as the establishment of mutualistic symbiotic associations in its roots (Rodriguez et al. 2004). Arbuscular mycorrhizae (AM) are the most widespread symbiosis on Earth and involve soil fungi (phylum: *Glomeromycota*) and the majority of plant families (Smith and Read 2008). It has been widely demonstrated that AM fungi influence plant fitness through the improvement of plant

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growth and tolerance under stressful conditions (Evelin et al. 2009; Bompadre et al. 2014). They also play a key role in plant and soil microbial community assemblages and participate in important soil ecosystem processes (Liu et al. 2011).

Numerous studies have been performed to assess AM fungal diversity in a variety of hospitable environments using many methods. In the last decade, new techniques have made it possible to carry out metagenomic studies on different ecosystems and to detect a great number of non-cultivable species (Lentendu et al. 2011; Dai et al. 2012; Öpik et al. 2013). However, it has also been shown that the traditional approach (based on morphological description of AM spore traits) is needed for a complete description of an AM fungal community (Colombo et al. 2014; Wetzel et al. 2014). Many studies on the diversity and distribution of AM fungi in natural extreme environments have been completed, e.g. at geothermal sites (Appoloni et al. 2008; Lekberg et al. 2011), on serpentine and gypsum soils (Schechter and Bruns 2012), from hypersaline soils (Wang et al. 2004), or in extremely cold environments (Lentendu et al. 2011; Liu et al. 2011). However, in South America, studies on AM fungal biodiversity occurring in extreme natural environments are scarce (Becerra et al. 2014).

The high Andean wetlands, in the cold and arid central Andes, are one of the rarest, most pristine and vulnerable ecosystems on Earth, located more than 4300 m above sea level (m.a.s.l.), an elevation close to the limit for plant growth. The *Laguna Brava* Nature Reserve is a protected area in the northwest of the Andean Mountains in Argentina, and is recognized as being of significant value on the List of Wetlands of International Importance (the Ramsar List) (Carrizo et al. 1997). The hypersaline, high-altitude wetland gathers several environmental conditions considered extreme for living organisms: high incidence of UV radiation, low oxygen pressure, high salinity and alkalinity, toxic levels of heavy metals, cold temperatures, and oligotrophy (especially low phosphorous content) (Silvani et al. 2013). This natural ecosystem has been preserved over time due to its isolation; however, in recent years, it has become exposed to anthropogenic disturbance (e.g., mining, tourism) and climate change. Investigating the natural occurrence of AM fungal associations under such harsh environmental conditions where they have evolved is an important contribution to the knowledge of AM fungal diversity.

In a previous study, AM fungal colonization was observed in some individuals of *Puccinellia frigida*, a dominant plant species growing near the margins of *Laguna Brava* and *Mulas Muertas* (Silvani et al. 2013). However, the *Laguna Brava* Nature Reserve has never been studied in terms of AM fungal diversity. Furthermore, the *Laguna Brava* Nature Reserve is likely a reservoir of AM fungal ecotypes that are polyextremophiles, capable of tolerating physical (low temperatures, UV radiation, or low oxygen pressure) and geochemical extremes (alkalinity, salinity, or high heavy metal content in soils).

AM fungal species can be classified into functional groups based on their life history strategies: competitors, stress tolerators and ruderals (Chagnon et al. 2013). Members of *Glomeraceae* family are associated with a "ruderal" strategy, because they cope with disturbance (i.e. faunal grazing) by having a short life cycle, a high growth rate and early production of abundant asexual spores. By contrast, the stress tolerators, such as *Acaulosporaceae* species, produce less biomass and have low growth rates which reduce their exposure to abiotic stressors. Finally, the competitive life history strategy is associated with *Gigasporaceae* members. They mainly produce high extraradical hyphal densities and show a delay in sporulation when stress or disturbance conditions are low. Thus, biodiversity patterns in natural communities may be predicted. As a result of the high levels of stress and disturbance in these high-Andean wetlands, we expected to find stress tolerators and ruderals as AM fungal taxa.

In the present study, we set out to describe and characterize the AM fungal communities in *Laguna Brava* Nature Reserve using both 454-pyrosequencing and traditional (based on spore traits) approaches. We also wished to discover which AM functional groups could thrive under these harsh environmental conditions and whether soil and vegetation influence the AM fungal communities.

2 Material and methods

2.1 Description of sampling sites

The study area was located in the Central Andes Mountains of La Rioja Province (northwest of Argentina). The weather is cold and dry during the year, and the annual mean air temperature is 0.8 °C (Minimum temperature: −25 °C; Maximum temperature = 15 °C). The annual total precipitation does not exceed 20 mm, falling mainly as snow or hail accompanied by strong western winds (maximum wind speed of 120 km/h) and is predominantly during the fall and winter (March to September). The UV-B irradiance for a nearby Andean wetland (*Laguna Azul*, Catamarca Province, 4500 m.a.s.l.) had a maximal value of 10.8 Wm^{-2} for the 300 to 325 nm range at noon in the austral summer (Dib et al. 2009). Soils are classified as entisols, suborder torriorthents. For all sampling sites, the soil texture is gravelly sand. The *Laguna Brava* wetland is a network of hypersaline shallow lakes associated with small communities of wet-marshy meadows. *Laguna Brava* (28°16′ S, 68°50′ W, 4300 m.a.s.l.) is the most important hypersaline lake with an area of approx. 5100 ha followed to the north by *Mulas Muertas* (28°17′ S, 68°44′ W, 4100 m.a.s.l.) with an area of approx. 210 ha (Fig. 1). The plant community is mainly composed of a few species of *Poaceae, Cyperaceae* and *Juncaceae* families, such as *Puccinellia frigida*, *Deyeuxia*

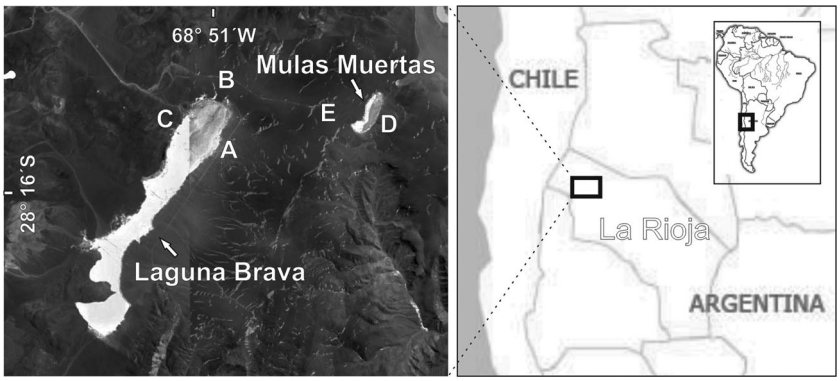

Fig. 1 Geographical location (C: *Laguna Brava*, D: *Mulas Muertas* and E: area between wetlands)

curvula and *Zameioscirpus atacamensis*. Plant communities are consistent across seasons (Silvani et al. 2013).

Three sampling sites were studied (A, B and C) in *Laguna Brava*, one site (D) in *Mulas muertas* and a site (E) in a nearby area between both lakes on a high mountain slope (site E; 28° 26′ S; 68° 50′ W, 3500 m.a.s.l.). The sites A, B, C, and D were selected on the basis of access, plant cover and environmental properties (Silvani et al. 2013), and the site E was chosen because of its location between the wetlands.

2.2 Soil and plant root sampling

Samples of rhizospheric soil and roots were harvested at each sampling site in September 2011. Each sample of rhizospheric soil and roots was taken at 15 cm depth and was a composite from three individual of plants. Five composite samples were collected at each sampling site, sealed in polyethylene bags and stored at 4 °C until processed.

The mycorrhizal status of the plant community was assessed at each site and individual plants were collected for the taxonomic identification. A root sample from each plant was processed according to the method of Barrow (2003) for dual staining (sudan IV and trypan blue). With this method, AM fungi, septate fungi and Chytridiomycetes could be recognized in roots; chitin stained dark blue with trypan blue and fungal and plant lipids stained bright red with sudan IV (Silvani et al. 2013). Thirty randomly selected root fragments (5 mm) were mounted on microscope slides in groups of ten and examined using a Nikon Optiphot-2 microscope at 200× magnification. The frequency of mycorrhizal colonization was calculated as the percentage of root fragments containing hyphae, arbuscules or vesicles (Ai-Rong and Kai-Yun 2007). Five subsamples of soil and roots were taken at each sampling site and bulked into a composite sample of about 1 kg. This was then divided into three equal portions to determine: (a) soil physicochemical parameters, (b) establish trap cultures, (c) molecular characterization of AM fungal communities (the soil and roots were stored at −80 °C until processed). For economic reasons, we used one composite sample for molecular characterization, so no statistical comparisons could be performed.

2.3 Soil analyses

Soil moisture was determined by drying 10 g of soil overnight at 105 °C and computing the difference between dry and wet sample weight (in triplicate). Electric conductivity (EC), soil cation exchange capacity (CEC) and pH was measured in 1:2.5 suspension of soil:water. Total dissolved salts (g/L) values were estimated by Rhoades et al. (1999) methodology. Total C (C_t) was measured following the Walkley and Black procedure (1934); total available P (P_t) according to the Bray and Kurtz method (1945), and total N (N_t) by Kjeldahl digestion (Bremner 1996). NH_4^+ was extracted with KCl 1 M and NO_3^- by Morgan's extracting solution (0.01 M $CuSO_4$). The S (SO_4^{-2}) content was measured by inductively coupled plasma spectroscopy after extraction solution Ac.NH_4 (pH 5). Soluble ions (Ca^{+2}, Mg^{+2}, Na^+, K^+) were quantified by Atomic Absorption Spectrophotometry (AAS) using a PerkinElmer Analyst 200 Spectrophotometer after digestion with Ac.NH_4 (pH 5). The heavy metal concentrations in soils (Zn, Mn, Sr, Cd, Ni, Cu and Pb) were also analyzed with the same spectrophotometer, and As content with a Varian AA1475 Spectrophotometer. Determination of U was measured by Laser-induced Fluorescence Spectroscopy with a Scintrex UA-3 Fluorometer. These analyses were performed by the Geochemistry Laboratories Division of Atomic Energy National Commission (*Regional Cuyo*, Argentina).

2.4 Morphological characterization of AM fungal spores

AM fungal spores from 50 g dry mass of soil from each field sample (five composite replicates per site) were extracted by wet sieving and decanting. Soil samples were passed through 850-, 450-, 125-, and 29-μm sieves. The contents of each sieve were transferred to a counting dish (Doncaster 1962) and checked for AM fungal spores, clusters of spores and sporocarps. This counting dish enables a complete sample to be counted in a more orderly way and facilitated the separation of spores from soil particles. We manually removed all AM fungal propagules with a micropipette by using a dissecting microscope at 40× magnification. Collected spores were

mounted in polyvinyl-alcohol glycerol (PVLG) and a mixture of PVLG and Melzer reagent (1:1, *v*/v) to observe their morphological characters with a Nikon Optiphot-2 microscope at 100×, 400× and 1000× magnifications. Identification to species level, whenever possible, was based on taxonomic criteria (spore size, color, surface ornamentation and wall structure) following species descriptions (Blaszkowski 2012) and the online guide provided by INVAM (http://invam.caf.wvu.edu). Taxonomic assignments were done according to the Index Fungorum. Permanent slides were deposited as voucher material at the *Banco de Glomeromycota* in Vitro (www.bgiv.com.ar, Buenos Aires, Argentina).

To facilitate the identification of AM fungal species from the field samples, trap cultures were established for each site. Trap cultures consisted of pots (10 L) filled with soil samples mixed with an autoclaved perlite and vermiculite (3:1:1 by volume). Pre-germinated seeds of mycotrophic host plant species (*Pisum sativum* and *Allium porrum*) were planted in each pot. Individuals of *Puccinellia frigida* also grew naturally in the trap cultures. Plants were kept in a greenhouse (450 μE. m^{-2} s^{-2}, 400–700 nm; 16/8 light-darkness; 23/15 °C day/night; 60–70 % relative humidity) for 10 months and watered approximately once per week to maintain the soil moisture level close to field capacity. Spores isolated from trap cultures were not considered for abundance and diversity analysis (both morphological and molecular approaches).

The spore density (= spore abundance) and species richness (number of species detected) were assessed by measuring the total number found in the field soil samples.

2.5 DNA extraction, PCR amplification and sequencing

The molecular procedures were followed according to the protocol in Colombo et al. (2014). Field soil samples (approx. 50 g) were sieved through 2 mm mesh and collected field root samples were crushed with liquid nitrogen. Genomic DNA extractions were carried out from 0.25 g of soil and roots for each site with the MO BIO PowerSoil DNA isolation kit (MO BIO Laboratories, INC., Carlsbad, CA, USA). The partial SSU rRNA gene was amplified using the AMV4.5F and AMDGR primers, specifically designed for the Genome Sequencer (GS) FLX 454 Titanium System (Roche applied Science, Mannheim, Germany) (Lumini et al. 2009; Lin et al. 2012). PCR reactions were run as follows: initial denaturation at 95 °C (5 min.); 30 cycles at 95 °C (45 s.), 57 °C (45 s.) and 72 °C (60 s.) and a final elongation at 72 °C (4 min.). Amplification was done on a FastStart High Fidelity PCR system (Roche applied Science, Mannheim, Germany). The PCR products were purified and then sequenced on a GS-FLX 454 Titanium pyrosequencer following instructions of the manufacturer. Amplification and pyrosequencing was carried out at the *Instituto de Agrobiotecnología de Rosario* (INDEAR) in Argentina.

2.6 Analysis of pyrosequencing data

Sequences were quality-controlled and edited using Quantitative Insights Into Microbial Ecology (QIIME) software v1.5.0 (http://qiime.org/). Chimeras were also eliminated with the same script. Sequences were clustered into molecular operational taxonomic units (MOTUs) using a 97 % similarity threshold (Konstantinidis and Tiedje 2007) and QIIME pipeline (pick_otus.py script with the Uclust method). Singletons were removed to avoid overestimation of species richness (Unterseher et al. 2011). Comparisons between representative sequences from each MOTU with closest sequences published on the MaarjAM (http://maarjam.botany.ut.ee/) and National Centre for Biotechnology Information (NCBI) databases were performed with the BLAST algorithm. Only sequences with query coverage and similarity values higher to 98 % similarity (E values equal or close to zero) were considered. Taxonomic names were assigned to MOTUs according to the Index Fungorum (http://www.indexfungorum.org). The non-*glomeromycotan* and unqualified sequences (sequences that matched with another eukaryote sequence with E values close to zero) were eliminated from the dataset.

The representative sequences have been deposited at the NCBI Sequence Read Archive (SRA) database with the accession number SRAO58463. Phylogenetic analyses were conducted with MEGA 6.0 program (Tamura et al. 2013). All *glomeromycotan* sequences were aligned using ClustalW algorithm. A Maximum Likelihood consensus tree was constructed with the Tamura-Nei model (Tamura and Nei 1993) and 1000 replicates of non-parametric bootstrapping. Phylogenetic trees were viewed and edited by Tree Explorer and CorelDraw Graphic Suites X5.

2.7 Statistical analysis and diversity

Asymptotic AM richness estimators (rarefaction index and Chao1 index) of each site were calculated by rarefaction analysis. These rarefaction curves were obtained with QIIME pipeline by random selection of a series of different-sized subsets from the libraries. This procedure was repeated 10 times for each sampled subset.

The diversity of AM fungal communities by the morphological approach was estimated with the Simpson and Shannon-Wiener indexes. Differences between spore density, species richness and diversity parameters across the sampled sites (five replicates from each site) were tested using Fisher's least significant difference (alpha =0.05) after a one-way ANOVA. All above statistical analyses were performed using the SPSS 13.0 (SPSS Inc., IL).

Bray–Curtis distance matrixes were calculated for the molecular and morphological data sets. Principal component analyses (PCA) were performed with the matrix results to unveil the ecological similarities between sites. With the aim

Table 1 Physicochemical properties of sampled soils and mycorrhizal status of plants occurring in each study site of *Laguna Brava* Reserve

Study sites	*Laguna Brava*			*Mulas Muertas*	Area between wetlands
Sampling sites	A	B	C	D	E
Plant species	*Puccinellia frigida* (−)	*P. frigida* (−)	*P. frigida* (12.5 ± 1.3 %); *Deyeuxia curvula* (7.5 ± 0.2 %); *Zameioscirpus atacamensis* (−); *Nastanthus caespitosus* (−); *Patosia clandestina* (−); *Ranunculus exilis* (−); *Calandrinia compacta* (−)	*P. frigida* (5.5 ± 0.7 %); *D. curvula* (3.2 ± 1.0 %); *Z. atacamensis* (−)	*P. frigida* (15 ± 2.5 %); *D. curvula* (2.5 ± 0.3 %); *Stipa* sp. (8.5 ± 1.0 %); *Z. atacamensis* (−)
pH	9.5	8.1	9	8.7	8.3
Soil moisture (%)	26.1	25.3	36	30.9	12.3
CEC (cmol/kg)	9.5	6.8	7.3	21.9	13
EC (dS/m)	12.4	21.3	2.5	2.1	3.1
Total dissolved salts (g/l)	7.9	13.6	1.6	1.3	1.9
Ct (g/kg)	0.4	0.5	2.5	1.0	BDL
Available Pt (mg/kg)	BDL	0.7	16	BDL	3.5
Nt (g/kg)	0.7	0.3	0.6	1.1	0.4
NH_4^+(mg/kg)	7.2	BDL	10	8.8	8.4
NO_3^-(mg/kg)	2.8	43.6	61.4	39.4	15.3
$S(SO_4^{-2})$ (cmol/kg)	260	316	100	34.3	295
Ca^{+2} (cmol/kg)	23.8	34.9	8.8	29	17.9
Mg^{+2} (cmol/kg)	2.7	5.2	2.6	7	2.7
Na^+ (cmol/kg)	26.1	24.1	2.9	4.9	3.2
K^+ (cmol/kg)	2.1	1.4	1.3	2.9	1

BDL Below the detection limit. The mean frequency (%) of AM fungal colonization is shown between parentheses (means ± SD). (−) No AM fungal structures

of estimating the relative contribution of soil abiotic factors and plant data on AM fungal communities, a distance-based Redundancy Analyses (dbRDA) were performed. These analyses were conducted with the software package R, statistical language version 3.3.1 (32-bit) using the community analysis-specific package BiodiversityR (R Foundation for Statistical Computing 2011).

3 Results

3.1 Environmental soil factors

A further description of physical and chemical rhizosphere soil properties for all sampled sites is shown in Table 1. All soil samples were alkaline with pH values ranging from 8.1 to

Table 2 Total heavy metal concentration of the sampled soils in each study site of *Laguna Brava* Reserve

Total heavy metal concentration (μg/g)					
Study sites	*Laguna Brava*			*Mulas Muertas*	Area between wetlands
Sampling sites	A	B	C	D	E
Mn^{+2}	410	440	440	330	460
Zn^{+2}	50	60	130	50	50
Sr	1400	770	640	490	230
U	5	8	2.8	2.2	2
As	30	25	2	1.7	12
Cd	10	10	<5	<5	<5
Ni	20	20	20	20	20
Cu	15	10	20	15	15
Pb	20	20	<10	<10	<10

9.5. The CEC (6.8 to 21.9 cmol kg^{-1}) and water content (12.3 % to 36 %) varied greatly among samples. The EC values and total dissolved salts were higher in sites A and B of *Laguna Brava*, mostly found as Na^+ ions (26.1 and 24.1 cmol kg^{-1}, respectively). The lowest concentrations of Ca^{2+} and Mg^{2+} ions were recorded at site C in *Laguna Brava*. Soil carbon and available Pt were very low in most sites, whereas the highest values of both compounds were registered from site C (Ct: 2.45 g kg^{-1}; Pt: 16 mg kg^{-1}). In relation to soil heavy metal contents (Table 2), As, Cd, U, Sr and Pb concentrations at sites A and B were higher than at the other study sites; in contrast, elevated concentrations of Zn were found at site C.

3.2 Mycorrhizal status of plants

At the highest salt levels in soils (sites A and B), intraradical structures of AM fungi were not observed in *Puccinellia frigida*, but dark septate endophytes and chytrids occurred in these roots. However, AM fungi were detected in roots of this plant species at site C, D, and E at low percentage values of colonization (mean ± SD, 12.5 ± 1.3 %, 5.5 ± 0.7 % and 15 ± 2.5 %, respectively) (Table 1). The percentage of AM colonization of *Deyeuxia curvula* found at sites C, D, and E was 7.5 %, 3.2 % and 2.5 %, respectively. Low AM colonization in roots of *Stipa* sp. was observed at the E site (8.5 ± 1.0 %). Arbuscules, coils and intercellular hyphae were observed in field roots of mycorrhizal plants at C, D and E sites. Vesicles were more frequently found in root samples of *P. frigida* from site D than those from sites C and E. Fine endophytes were often detected in *P. frigida* roots at site C and E, but no spores or sequences of *Glomus tenue* were obtained at the present study.

No AM fungal spores were found in field soil samples and trap cultures from sites A and B. By contrast, the spore density varied significantly among sampling sites C, D, and E, ranging from 335.7 ± 50.4 (D) to 1115.0 ± 70.4 (E) spores per 50 g soil (mean ± SD) (Table 3).

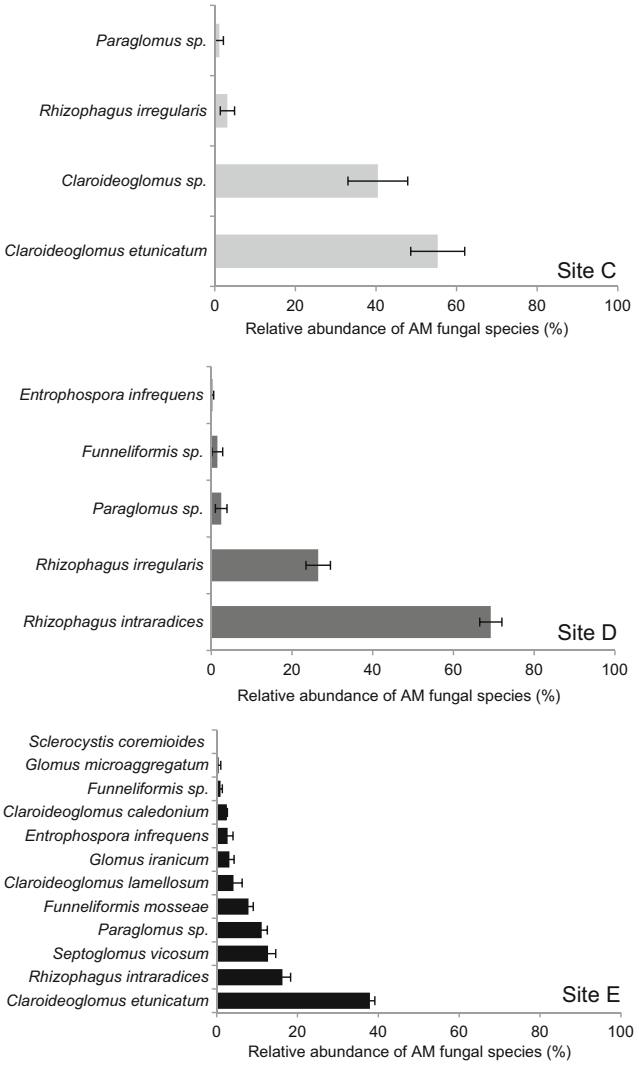

Fig. 2 Relative spore abundance and composition of AM fungal species in *Laguna Brava* (site C), *Mulas Muertas* (D) and the area between wetlands (E) by morphological analysis (mean ± SD; $n = 5$)

3.3 Morphological characterization of AM fungal community

At least 14 AM fungal species belonging to eight genera were found on the *Laguna Brava* Reserve according to

Table 3 Spore density and measurements of AM fungal diversity in *Laguna Brava* (C), *Mulas Muertas* (D) and area between wetlands (E)

Study sites	*Laguna Brava* C	*Mulas Muertas* D	Area between wetlands E
Spore density (spores per 50 g soil)	871.0 ± 11.8a	335.7 ± 50.4b	1115.0 ± 70.4c
Species richness	3.7 ± 0.6a	4.3 ± 0.6a	11.0 ± 1b
Shannon index	0.84 ± 0.02a	0.74 ± 0.1b	1.86 ± 0.1c
Simpson index	0.52 ± 0.02a	0.45 ± 0.03a	0.79 ± 0.02b

Data are reported as mean and standard deviations of five composite replicates per site. Significant differences among study sites are shown by different letters, determined using Fisher's Least Significant Difference (LSD) at the alpha = 0.05 level following one-way ANOVA

morphological analysis of spores from field samples (Fig. 2; Supplemental Fig. 1). *Claroideoglomus* species were dominant at site C (*Claroideoglomus etunicatum* and *Claroideoglomus* sp.), where as spores of *Rhizophagus irregularis* were less abundant in soil samples from this site. Conversely, the most predominant AM fungal species present at site D was *Rhizophagus intraradices*, followed by *R. irregularis*, whilst the less abundant AM fungal species were *Funneliformis sp.* and *Entrophospora infrequens*. Spores of *C. etunicatum* and *R. intraradices*, followed by *Septoglomus viscosum* were the most numerous at site E. *Sclerocystis coremioides*, *Glomus microaggregatum*, *Claroideoglomus caledonium*, *Glomus iranicum and Claroideoglomus lamellosum* were only present in soil samples from site E. *Paraglomus* sp. was found at sites C, D and E in low proportions.

3.4 Molecular characterization of AM fungal community

Samples from sites A and B were not included in the molecular analysis because we found no evidence of mycorrhizal fungal spores or root colonization in field samples and trap cultures. Subsequently, only field samples from sites C, D and E were processed. A total of 4673 sequences were obtained (average length of 268 ± 16 bp) corresponding to 118 MOTUS based on 97 % similarity. At the phylum level, the majority of fungal sequences belonged to the *Glomeromycota* (81.1 %), followed by *Chytridiomycota* (15.2 %), *Basidiomycota* (2.2 %) and *Zygomycota* (0.8 %) (Supplemental Table 1). The remaining of sequences (0.7 %) matched other fungal lineages and unclassified fungi. A total of 23 MOTUS from 3467 sequences belonging to *Glomeromycota* were detected according to the best BLAST results using the MaarjAM and the NCBI database (Supplemental Table 2).

The molecular phylogenetic analysis by Maximum Likelihood method showed that MOTUs were grouped into three families of *Glomeromycota* (*Glomeraceae*, *Claroideoglomeraceae,* and *Paraglomeraceae*) (Fig. 3). The *Glomeraceae* family represented by the '*Rhizophagus intraradices* clade' (*Glomus* Group Ab), and a second subclade represented by the '*Funneliformis mosseae* clade' (*Glomus* Group Aa) clustered together with *Septoglomus viscosum* sequences. The '*Claroideoglomus claroideum* clade' (*Glomus* Group B) and *Paraglomeraceae* sequences clustered apart from the *Glomeraceae* group.

The number of *Glomeromycota* sequences of each MOTU varied among the study sites (Fig. 3). *Rhizophagus* sequences close to *Rhizophagus* aff. *intraradices-irregularis,* C. *etunicatum,* and *C. lamellosum* were found on site C, whereas sequences identified as *Claroideoglomus* sp. were the most abundant taxa. *Rhizophagus* MOTUs genetically similar to

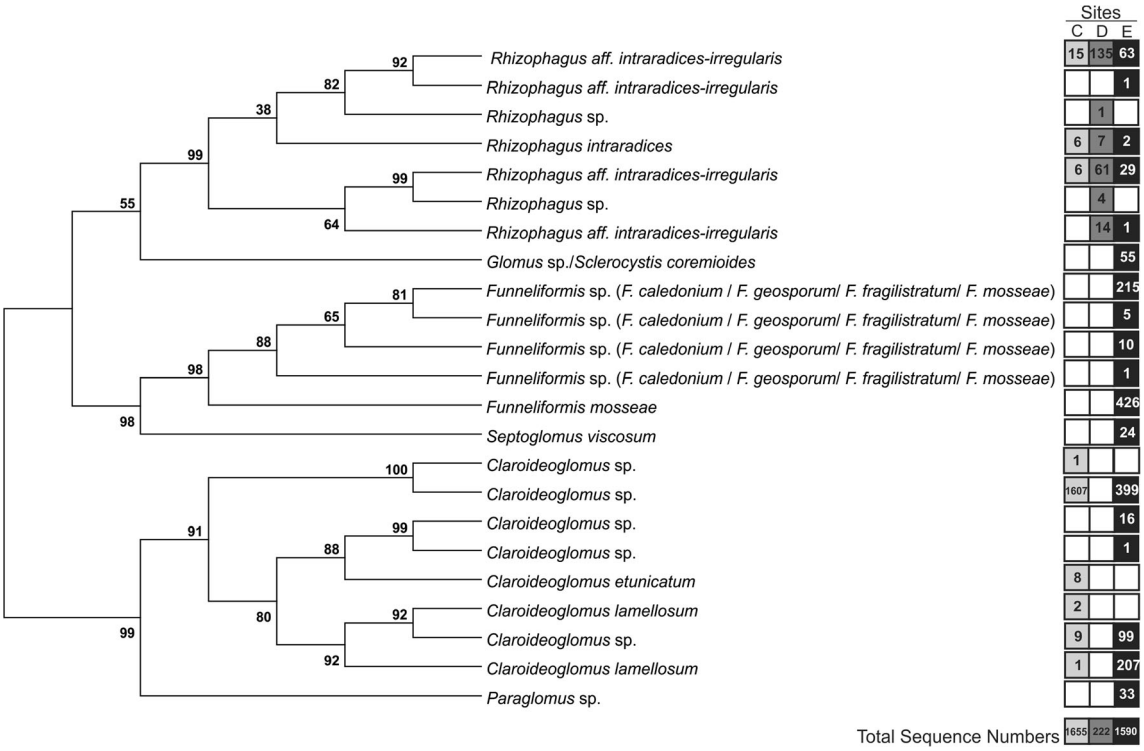

Fig. 3 Maximum Likelihood tree showing the phylogenetic positions of AM fungi inferred from partial SSU sequences obtained by pyrosequencing. Values above the branches are bootstrap values (1000 replicates). Numbers of sequences from each MOTU are provided for each site. *Laguna Brava* (C) is represented by light grey, *Mulas Muertas* (D) by grey and the area between wetlands (E) by black

Rhizophagus intraradices and *R. irregularis* were the only AM fungal species registered in D. Two MOTUs identified as *Rhizophagus* sp. were exclusively found in this area. *Claroideoglomus* sp., *Funneliformis mosseae*, *Funneliformis* sp., and *Rhizophagus* aff. *intraradices-irregularis* were the dominant taxa on site E. Many sequences belonged to *Funneliformis* sp. and *F. mosseae* were exclusively detected in the area E; sequences identified as *Sclerocystis coremioides*, *Septoglomus viscosum* and *Paraglomus* sp. were also detected in a low proportion in this study area.

The rarefaction curves reached the asymptotes in each library, suggesting that the sequencing effort covered the most AM fungal diversity in each sample (Supplemental Fig. 2). The number of obtained MOTUs was found to be close to the number of estimated MOTUs for all sites (index CHAO1). The richness estimator was higher on E site, followed by sites C and D (Supplemental Fig. 2).

3.5 Structure community of AM fungi

The diversity analysis of AM fungal community based on morphological results showed that site E had a significantly higher species richness (mean ± SD: 11 ± 1) than sites C and D (3.7 ± 0.6 and 4.3 ± 0.6, respectively) (Table 3). Shannon indices were statistically different among sites. The highest AM fungal diversity was registered at site E and the lowest at site D. The sites C and D reached similar Simpson index values, which differed significantly from those of site E (Table 3).

Bray–Curtis coefficients were used to compare the similarity between the AM fungal community composition. The AM fungal communities from site C (*Laguna Brava*) and site D (*Mulas Muertas*) were more similar to each other in both data sets, morphological (Bray-Curtis coefficient: 0.987) and molecular one (Bray-Curtis coefficient: 0.971), than with site E. The highest dissimilarities were recorded between the AM fungal communities belonging to sites C and E when assessing morphological (Bray-Curtis coefficient: 0.537) and molecular (Bray-Curtis coefficient: 0.734) techniques. According to PCA analysis, the first and second principal component explained 72.27 % and 27.72 % of similarity between sites, respectively, when studying AM fungal community by morphological technique (Fig. 4a). In addition, the same statistical analysis for molecular data showed that the first and second principal component explained the 68.49 % and 31.51 % of the ecological distance among communities, respectively (Fig. 4 b).

DbRDA did not reveal a significant relationship between environmental physicochemical parameters, plant species richness and the diversity of AM fungal communities (data not shown).

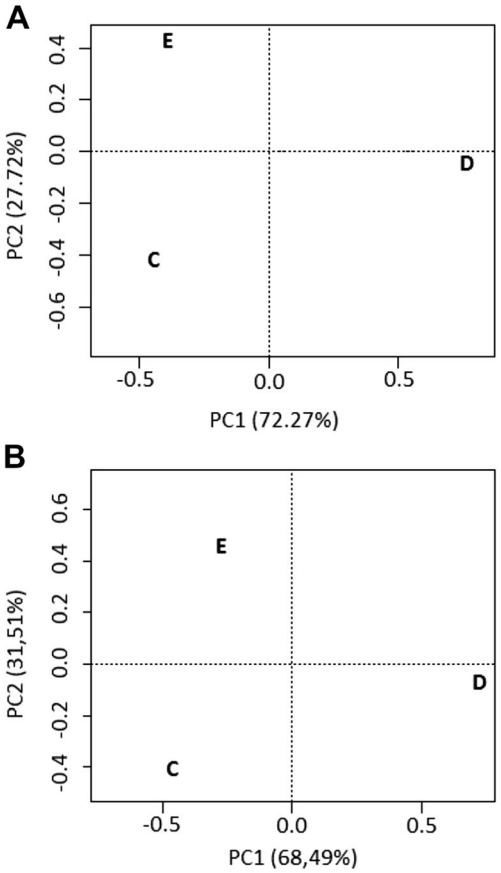

Fig. 4 Principal component analyses (PCA) performed with the Bray–Curtis distance matrixes with the morphological (**a**) and molecular (**b**) data sets. C: *Laguna Brava*; D: *Mulas Muertas*; E: area between wetlands

4 Discussion

In this work we describe for the first time, the AM fungal communities on the *Laguna Brava* Nature Reserve, an ecosystem with extreme environmental conditions. Our survey showed that AM fungal diversity is less diverse than in other natural extreme ecosystems (Wang et al. 2004; Appoloni et al. 2008; Liu et al. 2011; Oehl et al. 2011). One possible explanation is that the low plant species richness along with extreme abiotic conditions restricted the AM fungal diversity and spore production of these fungi in the *Laguna Brava* wetlands. We detected local environmental and biological variations within *Laguna Brava* wetland. For example, the establishment of AM fungi in plant roots and soils appeared to be negatively affected by high Na^+ levels and high heavy metals (Sr, U and As) contents in the soil at two sampling sites (sites A and B), and possibly by the low plant species richness and coverage (one plant species distributed in small patches). Other researchers have demonstrated that AM fungal abundance decreases with increasing soil salinity as this affects AM fungal spore germination (Campagnac and Khasa 2014) and mycorrhizal colonization (Silvani et al. 2013; Guo and

Gong 2014). It is also known that the heavy metal content of soils can strongly affect the development of external AM fungal structures and limit the production of arbuscules (del Val et al. 1999; Mendoza et al. 2015).

The morphological characterization of AM fungal communities in *Laguna Brava* and *Mulas Muertas,* supported by the molecular data, indicated the dominance of *Glomeraceae* and *Claroideoglomeraceae*, and at a lower taxonomic level, of *R. intraradices*, *R. irregularis,* and *Claroideoglomus* species. These results accord with previous studies on extreme natural environments (Appoloni et al. 2008; Liu et al. 2011; Becerra et al. 2014; Guo and Gong 2014). Although *Glomus* is a dominant genus in most cases, we highlight the high abundance of *Claroideoglomus* at the two sites on the *Laguna Brava* Reserve. Moreover, neither sequences nor spores belonging to *Diversisporales* and *Archaesporales* order were detected in *Laguna Brava*. Lugo et al. (2008), in a study set on *Puna* grassland (Argentina), discovered that *Acaulosporaceae* and *Gigasporaceae* were not present above 3520 m.a.s.l. We consider that our molecular results provide a reliable characterization of the AM fungal community of *Laguna Brava* wetlands. When comparing these results with those obtained through morphological approach, it is clear that some AM fungal species were not detected (e.g., *G. microaggregatum* and *E. infrequens*) by the molecular study. This difficulty in detecting some AM species may have been due to the low yield obtained during DNA isolation, as suggested in Colombo et al. (2014) and likely caused by the small-size of spores and their low abundance in the field soils.

The dominance of *Glomerales* in our study could be explained by their life history strategies (Klironomos and Hart 2002; Hart and Reader 2005; Silvani et al. 2014). In particular, members of the genera *Rhizophagus* and *Claroideoglomus* are widely considered to have both a generalist and ruderal lifestyle. Chagnon et al. (2013) proposed that a short life cycle leading to an early and constitutive investment in asexual spores could be a strategy by which ruderal AM fungi cope with disturbed habitats. In contrast to expectations, the ruderal fungal isolates from *Laguna Brava* produced both low extraradical propagules and internal root structures in concordance with stress-tolerant strategies. These functional traits may be a way to reduce exposure to abiotic stress factors and deal with the limited amount of carbon supplied by the plants for AM fungal growth.

In the present study, the Shannon index indicated that AM fungal diversity varied significantly among study sites. However, no differences between *Laguna Brava* and *Mulas Muertas* wetlands were found in species richness and Simpson index values. These results were consistent with the Bray–Curtis analyses that showed similarity between the AM fungal communities from *Laguna Brava* and *Mulas Muertas* wetlands, and with a nearby area (site E). This suggests that environmental factors could explain the AM community assemblages in the margins of these two Andean wetlands, and that found at sampling site E which was in close proximity to *Mulas Muertas*. Moreover the DbRDA analysis showed no significant interaction between the physicochemical soil parameters and plant data with the three AM fungal communities.

In conclusion, more data are clearly needed, and exploring new sampling sites and collecting data on other environmental factors (eg. temperature, hypoxia, faunal grazing) would further elucidate the diversity and structure of AM fungi in this unique ecosystem. Further research should also be conducted to explore the roles of that endemic AM fungal ecotypes play under stress conditions which could increase our scope to manipulate the AM symbiosis and enhance their use in conservation and remediation schemes.

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