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ORIGINAL ARTICLE

Preliminary characterization of microbial communities in High Altitude Wetlands of Northwestern Argentina by determining terminal restriction fragment length polymorphisms

Marcela Ferrero,* María E. Farías,* Faustino Siñeriz*,**

ABSTRACT. Laguna de Pozuelos is an extensive wetland in Morthwestern Argentina at 3,600 m above sea level in the Argentinean Andes. The principal lake, placed in the central depression of endorheic basin, is rich in minerals like Cu, As, Fe, etc. It collects water from underground courses and from two main tributaries, namely Santa Catalina River to the north and Cincel River to the south. Following the dry and rainy seasons, the surface of the lake is subject to an annual contraction-expansion cycle, with increasing of salinity during evaporation period. Prokaryotes inhabitants these particular environments have been not described and a few of such places have been surveyed for microbial diversity studies. To systematically explore the underlying communities of *Bacteria* from the water lake of Laguna de Pozuelos wetland and Cincel River, bacterial 16S rRNA genes (rDNAs) were PCR amplified and analyzed by terminal restriction fragment length polymorphism (T-RFLP) analysis. Analysis of the microbial community with T-RFLP identified a minimum of 19 operational taxonomic units (OTU). T-RF patterns derived from multiple-enzyme digestion with *RsaI, HaeIII* and *HhaI* were analyzed in order to provide a preliminary picture of the relative diversity of this complex microbial community. By the combined use of the three restriction endonucleases bacterial populations of this particular place were identified.

Key words: Microbial community–High altitude wetland–T-RFLP-Bacterial biodiversity.

RESUMEN. Laguna de Pozuelos es un extenso humedal en el noroeste de Argentina a 3.600 m.s.n.m. en los Andes argentinos. La principal laguna, ubicada en una depresión central de una cuenca endorreica, es rica en minerales como Cu, As, Fe, etc. El agua es colectada de cursos subterráneos y de dos principales ríos, llamados Río Santa Catalina al norte y Río Cincel al sur. Siguiendo las estaciones seca y lluviosa, la superficie de la laguna es sujeta a ciclos de expansión-contracción, con incremento de salinidad durante el período de evaporación. Los procariotes que habitan estos particulares ambientes no han sido descritos y pocos lugares como éstos han sido examinados para estudiar su biodiversidad microbiana. Para explorar en forma sistemática las comunidades de *Bacteria* del agua de la laguna del humedal de Laguna de Pozuelos y del Río Cincel, genes del RNA 16S ribosomal fueron amplificados por PCR y analizados por análisis de polimorfismos de fragmentos de restricción terminales (T-RFLP). El análisis de la comunidad microbiana con T-RFLP identificó un mínimo de 19 unidades taxonómicas operacionales (UTO). Los patrones de fragmentos derivados de la digestión múltiple con *RsaI, HaeIII* y *HhaI* fueron analizados para proveer un mapa preliminar de la diversidad relativa de esta compleja comunidad microbiana. El uso combinado de las tres endonucleasas de restricción, permitió que fueran identificadas poblaciones bacterianas de este particular lugar.

Palabras clave: Comunidad microbiana-Humedal de altura–T-RFLP- Biodiversidad bacteriana.

INTRODUCTION

The altiplano of the central Andes extends through parts of Argentina, Bolivia, Chile and Peru, between 3,500 and 4,500 m above sea level. It is cold and arid region, exposed to intense solar radiation, strong winds and high daily temperature fluctuations of up to 30 ºC. Numerous endorheic basins form lakes and saltpans and thus constitute patches of aquatic habitat within a desert matrix.

Laguna de Pozuelos is the main water concentration in an extensive internal drainage at 3,600 m above sea level, 66º00´ W and 22º19´S in Northwestern Jujuy province in Argentinean Andes (Fig. 1). It has a total surface of around 70 km^2, shallow waters (maxim depth: 120 cm in its central area) and little vegetation. The Lake, placed in the central depression of endorheic basin, covers an area of approximately 4,000 km^2, rich in minerals like Cu, As, Fe, etc. It collects water from underground courses and from two main tributaries, namely Santa Catalina River to the north and Cincel River to the south. The Lake and its surrounding fringe were declared a National Monument by the National Park ad-

* PROIMI Planta Piloto de Procesos Industriales Microbiológicos Av. Belgrano y Pje. Caseros -(4000) - Tucumán, Argentina CONICET Consejo Nacional de Investigaciones Científicas y Técnicas.
** Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 491, 4000 San Miguel de Tucumán Argentina

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ministration of Argentina in 1980, and a Ramsar site since 1992. The basin is included within the network of Biosphere Reserves of UNESCO 1990. The climate of the basin is cold and semiarid showing an annual temperatures average below 9°C near lake and is markedly seasonal, with a rainy season in summer (between December and March) and a dry season throughout the rest of the year. Average annual rainfall is about 350 mm.

The Lake support a rich diatom flora (estimated density of the diatoms suspended in the water is 4.10^3 cell l^{-1} and 2.10^3 cells gr^{-1} of those in the sediment) consisting mainly *Cocconeis placentula, Nitzschia perspicua, Nitzschia hungarica, Navicula minuta* and *Navicula perminuta*, which are indicators of moderate alkaline (pH = 8.0-8.5) waters.[26] The abundance of *Nitzschia* spp. is evidence of strong salinity, as a result of dry periods.[30] Zooplankton shows an average annual density of 220 individuals l^{-1}. The predominant invertebrate fauna consists of copepods (*Beckella sp, Attheyella sp),* Cladocera *(Daphnia sp)* and rotifers *(Hexarthra sp*).[30] This microfauna supports an extraordinary colony of flamingos that graze by filtering the waters since there are no fish. In summer the colony increases extraordinarily in size.[31] While abundance and richness waterbirds and zooplankton were extensively studied,[26,30] microbial diversity has not been described in this wetland.

Figure 1. *Location of Laguna de Pozuelos and surrounding basin in Jujuy Province, Northwestern Argentina.*

Insights into community structures in environmental samples were achieved by the use of molecular tools such as 16SrRNA genes (rDNAs), which avoid the limitations of culturability.[2,38] Communities of *Bacteria* and *Archaea* have been successfully explored using terminal restriction fragment length polymorphism (T-RFLP) analysis of amplified total community 16S rDNA from different environments.[6,19,22,24,25,29,33,34]

In this paper we carried out a survey on the prokaryotic diversity of water lake from Laguna de Pozuelos and Cincel River by use of 16S rRNA gene amplification and T-RFLP fingerprinting. From a phylogenetic analysis of three endonucleases patterns, we were able to perform a preliminary examination of the diversity of the bacterial communities in Laguna de Pozuelos lake and Cincel River.

MATERIAL AND METHODS

Sampling and fixation. In August 2001 (winter season at South latitudes), 70 lt. of surface water samples were collected in acid-washed and Lake water pre-rinsed polyethylene containers. The sampling was made at 22º 23´39´´ South latitude and 66°01´11´´ West longitude for the Lake sampling. The sampling at Cincel River was made at 22°24´43´´ South latitude and 65°58´43´´ West longitude, as determined by GPS. Water temperature of both places was 15 ºC and pH 6.5.

The sampling location is approximately 700 km of hard access mountain road away from the processing Institute. The samples were stored at 4ºC and further processed within approximately 12 h. The whole water was first pre-filtered through Whatman GF/C filters and 82 µm-pore-size Nucleopore filters. Bacteria from 250 ml were collected in 0.2 µm-pore-size Nucleopore filters and stored at –20ºC

DNA extraction. To extract DNA, the frozen filters were thawed, cut into small strips, and then treated with 15 mg of lysozyme ml^{-1} in 2 ml of buffer (0.15 M NaCl, 0.1 M EDTA; pH8.0) in polypropylene tubes. The suspension was incubated at 37ºC for 30 min. After the addition of proteinase K (100 $\mu g\text{-}ml^{-1}$) and sodium dodecyl sulfate (SDS) (1% wt/vol) samples were incubated 2 h. at 55ºC. The lysate was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), followed by extraction with chloroform-isoamyl alcohol (24:1). The nucleic acids were precipitated at –20ºC overnight with 0.1 volume of 4M sodium acetate (pH 5.4) and 2.5 volume of 100 % (vol/vol) ethanol. After 20 min at –20ºC the sample was centrifuged at 10,000 x *g*

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for 10 min. The pellet was washed with 70% ethanol, dried and resuspended in 200 to 400 µl of distilled water. The samples were visualized in a 0.8% agarose electrophoresis gel.

PCR conditions. PCR amplifications of bacterial 16S rDNAs from total environmental DNA extracts were performed with a total volume of 50 µl in a model 9,700 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn). Bacterial 16S rDNAs from 100 ng of total environmental DNA extract were amplified in reaction mixtures containing 10 pmol of each primer, 200 µM each deoxyribonucleoside triphosphate, 400 ng μl^{-1} of bovine serum albumin (Roche Molecular Biochemicals, Indianapolis, Ind.), 150 mM $MgCl_2$ (Gibco BRL, Gaithersburg, Md.), 0.5 U of *Taq* polymerase (Gibco BRL) and 1/10 volume of a 10X PCR buffer provided with the enzyme. After a denaturation step of 5 min at 95°C, amplification reactions were performed with 30 cycles of denaturation (1 min, 95°C), primer annealing (1 min, 57°C), and primer extension (3 min, 72°C) and a final extension step of 7 min at 72°C. Primers used for amplification of eubacterial 16S rDNAs (8-27F, 5'-AGAGTTTGATCMTGGCTCAG-3', with M for A or C; 1392-1407R, 5'-ACGGGCGGTGTGTACA-3') were described previously[2] and modified by C. L. Moyer (**unpublished results**). Forward primers for amplification of bacterial 16S rDNAs were 5'-end labeled with 5-hexachlorofluorescein (Operon Inc.).

Products of three replicate PCRs each for bacterial 16S rDNA were combined. Aliquots (5 µl) of 16S rDNA PCR products were analyzed by electrophoresis on 0.8% (wt/vol) agarose gels (Gibco BRL), followed by 15 min of staining with Ethidium bromide (0.5 mg $liter^{-1}$). Bands were visualized by UV excitation. 16S rDNA PCR products were loaded onto an analytical gel from which bands were eluted in 35 µl of sterile filtered distilled water using a QIAquick gel extraction kit (Qiagen, Chatsworth, Calif.). The products were again separated on agarose gels to confirm purity and similar concentrations of the purified PCR products.

16S rDNA T-RFLPs. Aliquots (5 µl) were cleaved for 2 h in a water bath at 37°C with 5 U of restriction endonuclease in the manufacturer's recommended reaction buffers. Hydrolysis was performed with three different restriction endonucleases in digestions with a single tetrameric enzyme each (*Hae*III [GG'CC] [where the prime shows the site of cleavage], *Hha*I [GCG'C], and *Rsa*I [GT'CA]; Gibco BRL). To prevent cleavage of the internal standard, the restriction endonucleases were deactivated by heating the reaction mixture to 65°C (80°C for *Hae*III) for 25 min after the reaction was completed. Aliquots (2 µl) of the digest were mixed with 2 µl of deionized formamide, 0.5 µl of loading buffer (Applied Biosystems Instruments [ABI], Foster City, Calif.), and 0.5 µl of a DNA fragment length standard (TAMRA GS 2500; ABI). After denaturing of the DNA at 94°C for 5 min and immediate chilling on ice, aliquots (2.5 µl) were loaded onto a 36-cm-long 6% denaturing polyacrylamide gel of an automated DNA sequencer (373 ABI Stretch). Electrophoresis was run for 14 h with limits of 1,680 V and 40 mA. After electrophoresis, the lengths of fluorescently labeled terminal restriction fragments (T-RFs) were analyzed by comparison with the internal standard using GeneScan 3.1 software (ABI).

Analysis of T-RFLPs. For each sample, peaks over a threshold of 50 units above background fluorescence were analyzed by manually aligning fragments to the size standard. To avoid detection of primers and uncertainties of size determination, terminal fragments smaller than 50 bp and larger than 850 bp were excluded from the analysis. Reproducibility of the patterns was confirmed for repeated T-RFLP analysis of rDNA 16S, using the same DNA extracts from two samples. The number and the height of the peaks characterized communities. The relative abundance of T-RFs was determined by calculating the ratio between the peak height of each peak and the total peak height of all peaks within one sample. Ratios were converted to percentages, and the results are displayed as histograms.

RESULTS

Diversity of 16S rDNAs in Laguna de Pozuelos wetland. T-RFLPs were compared by calculating the diversity and relative abundance of individual T-RFs derived from the three restriction enzymes within two water samples taken from the Lake and from Cincel River in the Laguna de Pozuelos wetlands. Histograms are displayed after cleavage with *HaeIII*, *HhaI* and *RsaI* for bacterial 16S rDNA from each sample. (Fig. 2). Cleavage of amplified bacterial 16S rDNA yielded a total of 43 (*Hae*III), 17 (*Hha*I) and 27 (*Rsa*I) different T-RFs in both samples. *Hae*III yield the highest number of T-RFs and thus represented the highest level of resolution. Only a few T-RFs were found to be shared between these different locations.

After cleavage of whole amplified bacterial 16S rDNAs from the different samples with *Hae*III, 49 fragments were obtained, but only six of the predominant T-RFs (195 bp [3.38 to 3.18%], 213 bp [2.19 to 5.42%], 219 bp [4.26 to 1.35%], 221 bp [2.40 to 2.79%], 226 bp [1.58 to 1.27%] and 291 bp [27.4 to 2.09%]) were common within both samples and had a similar relative low abundance (less than 10%). From the 32 T-RFs derived from cleavage with *Rsa*I, five were found common (111 bp [1.35 to 4.30%],

420 bp [8.89 to 2735%], 424 bp [2.33 to 7.56%], 467 bp [1.08 to 2.67%] and 472 bp [6.95 to 1.82%]). Only two fragments of the 19 T-RFs obtained after cleavage with *Hha*I (65 bp [36.63 to 53.36 %] and 205 bp [4.96 to 2.10%]) were found to be shared between these different locations.

On the other hand, some fragments were unique to one sampling location and, curiously, these are the more abun-

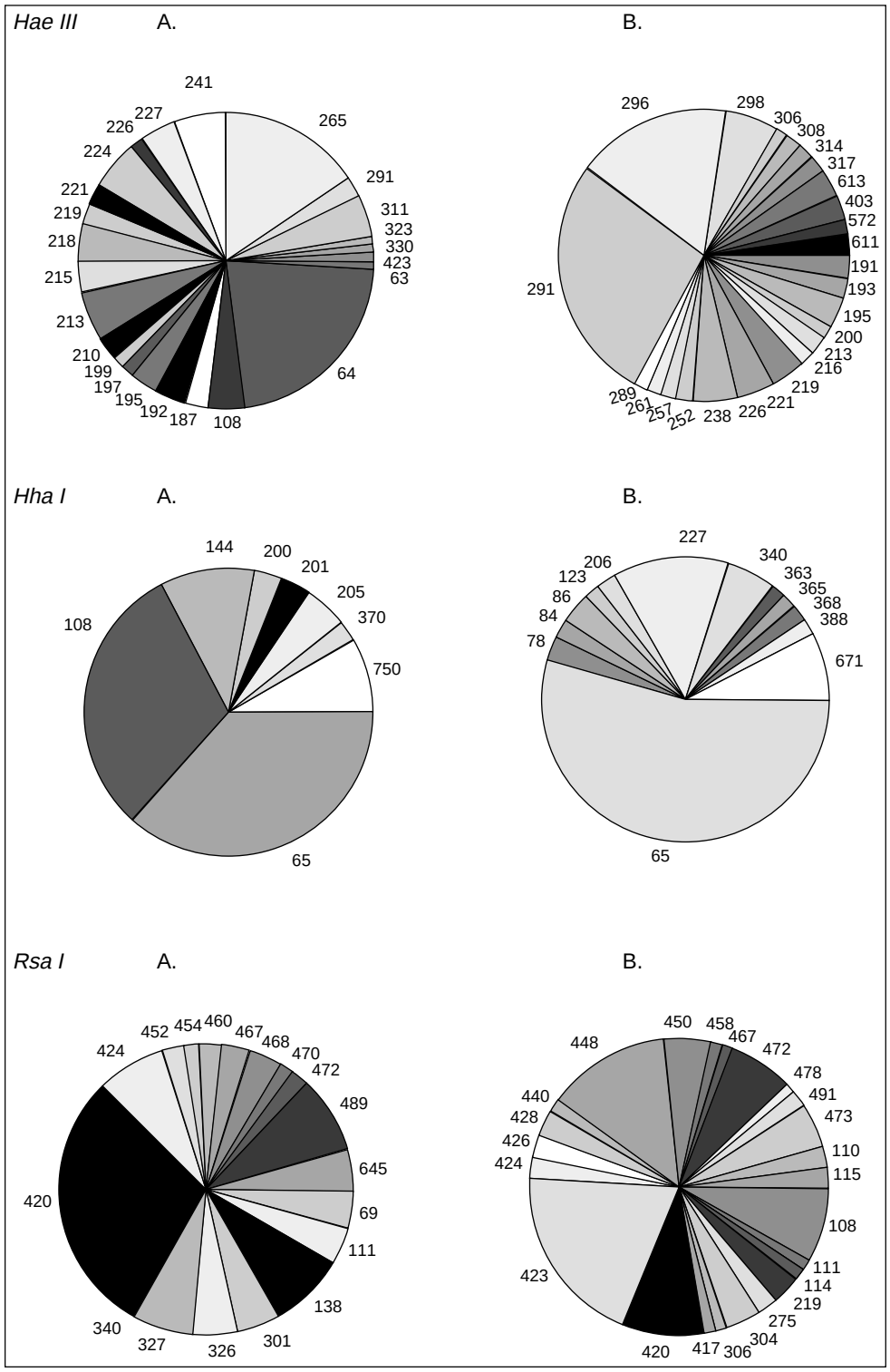

Figure 2. *Relative abundances of T-RF of amplified 16S rDNAs genes of bacteria within Lake (A) and River (B) samples from Laguna de Pozuelos wetland. Diagrams show results after cleavage with* Hae*III,* Hha*I and* Rsa*I. Numbers indicate the lengths of the T-RFs in base pairs.*

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dant in each location. *Hae*III fragments of 64 and 265 bp (22.04 and 15.63%) were restricted to Pozuelos Lake, whereas two fragments of 291 and 296 bp (27.40 and 17.14%) occurred exclusively at the Cincel River sample. Restriction with *Hha*I yield an second and predominant T-RF in lake sample with 108 bp (30.63%), whereas in the river sample, a fragment of 227 bp were the second more abundant T-RF (13.13%) obtained with this restriction enzyme. Unique and with high relative abundance, two fragments with 420 bp (27.35%) in lake sample and with 423 bp (19.74%) in river water sample were obtained by restriction with *Rsa*I.

Fragment lengths and phylogeny. On the basis of T-RFLP analysis 14 different phylotypes were identified from water samples obtained from Laguna de Pozuelos and Cincel River. By combination of the three restriction endonucleases used (*Hae*III, *Hha*I and *Rsa*I) it was possible to assign bacterial genus or specie names or its corresponding EMBL accession number to T-RFs from amplified bacterial 16S rDNAs using the TAP (T-RFLP Analysis Program) tool, a function analysis located at the Ribosomal Database Project web site (http://www.rdp.cme.msu.edu).[28]

Table 1 presents the terminal fragments measured from 5' of three in silico digests (*Hae*III, *Hha*I and *Rsa*I) of the RDP. The fragments were sorted first by the length of the *Hae*III fragments and then by the lengths of the *Hha*I and *Rsa*I fragments. Considering that a difference of ± 2 bp in the sizes of the T-RFs is likely to occur due to the nature of the gel separation, we found phylotypes corresponding to *Proteobacteria* species as well as to the cluster of *Bacillus*/*Clostridium* and *Actinobacteria* groups.

Table 1 lists the phylogenic inference from TAP of Lake and River samples. In the Lake sample (Table 1-A) one

Table 1. Derivation of phylogenic inference from TAP.[1]

A. Fragment length (bp)				
HaeIII	*HhaI*	*Rsa*I	RDP sequence	Phylogenetic group
195	65 (67)	886	*Halorhodospira halochloris*	γ-Proteobacteria
195	65	424	*Methylosinus sp*	α-Proteobacteria
197	204 (205)	879	*Achromatium sp*	γ-Proteobacteria
197	205	472	*Acidovorax sp*	β-Proteobacteria
213	202 (201)	442	*Acidimicrobium ferrooxidans*	Actinobacteria
221	369 (370)	881	*Nitrosomonas sp*	Actinobacteria
227	143 (144)	454	*Rathayibacter toxicus*	Actinobacteria
B. Fragment length (bp)				
*Hae*III	*Hha*I	RsaI	RDP sequence	Phylogenetic group
195	65 (67)	886	*Halorhodospira halochloris*	γ-Proteobacteria
195	65	424	*Methylosinus sp*	α-Proteobacteria
195	207 (206)	467	*Burkholderia sp*	β-Proteobacteria
200	207 (206)	472	*Ralstonia eutropha*	β-Proteobacteria
219	207 (206)	473	*Dechloromonas sp*	β-Proteobacteria
219	207 (206)	428	unidentified bacterium[36]	β-Proteobacteria
219	226 (227)	491	unidentified bacterium [23]	γ-Proteobacteria
219	226 (227)	491	unidentified bacterium [18]	γ-Proteobacteria
219	367 (368)	473	*Iodobacter fluviatilis*	β-Proteobacteria
219	367 (368)	473	*Methylophilus methylotrophus*	β-Proteobacteria
219	367 (368)	473	*Telluria mixta*	β-Proteobacteria
221	368	859	*Mycobacterium marinum*	Actinobacteria
252	366 (365)	875	*Methylophaga thalassica*	γ-Proteobacteria
291	208 (206)	450	unidentified bacterium [40]	B/C
296	227	829	*Anaerobacter polyendosporus*	B/C
306	672 (671)	458	unidentified [18]	Actinobacteria
317	205 (206)	879	*Achromatium sp.*	γ-Proteobacteria

[1]Additional separate enzymatic digestion on the group defined by an *Hae*III terminal fragment size were carried out also with *Hha*I and *Rsa*I from Lake (A) and River (B) samples. Phylogenetic group corresponding to TAP-derived phylotypes was determined by RDP database.

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phylotype corresponded to *Methylosinus sp.* clustered within a- subdivision of *Proteobacteria*. In the extended Actinomycetes group, *Acidimicrobium* ferrooxidans, *Nitrosomonas sp.* and *Ratayibacter toxicus* were tentatively identified. In the γ-subdivision of the Proteobacteria group, *Halorhodospira halochloris* and *Achromatium sp.* were identified.

In total 13 phylotypes were identified in the sample from River (Table 1-B). Within the a-Proteobacteria group, *Methylosinus sp.* was the sole bacterium tentatively identified. *Mycobacterium sp.* clustered within Actinobacteria group. T-RFs corresponded to *Ralstonia eutropha, Dechloromonas* sp, *Burkholderia sp, Iodobacter fluviatilis, Methylophilus methylotrophus,* unidentified bacterium and *Telluria mixta* were clustered within β-Proteobacteria group. T-RFs corresponding to *Halorhodospira halochloris, Methylophaga thalassica, Achromatium sp* and two unidentified bacteria were within the *γ-Proteobacteria*. One RDP sequences corresponded tentatively to unidentified bacterium and other to *Anaerobacter polyendosporus* clustered in the B/C group.

DISCUSSION

The abundance of T-RFs clearly showed low bacterial diversity in Laguna de Pozuelos wetland. Comparing lake and river T-RFs it could be noted that there are not strong differences between both samples. Some fragment produced by *Rsa*I were included in the phylogenetic analysis even though many of them were bigger than 850 bp (maximum size considered) in order to confirm the affiliation of the sequences derived of two restriction enzyme analysis.

One the largest number of RDP sequences were found within the *γ-Proteobaceria*. In general, this phylogenetic group contains organisms that appear to be phylogenetically closely related but are phenotypically very different from each other, so suggestions about possible phenotypes for environmental bacteria that are in the *γ- Proteobacteria* may be considered weak. For example, *Halorhodospira halochloris*, is a phototrophic purple sulfur bacteria generally found in illuminated anoxic zones of lake and other aquatic habitats where H_2S accumulates. The genera *Ectothiorhodospira* and *Halorhodospira* are tipically found in marine environment, saline lakes, soda lake and salterns.[14] *Achromatium oxaliferum* is an uncultivated bacterium that was studied by use of culture-independent approaches.[10] *Achromatium oxaliferum* is a large, morphologically conspicuous, sediment-dwelling bacterium. Nothing is known concerning its phylogeny and it has eluded all attempts at laboratory cultivation.[12] The limited physiological description of *A. oxaliferum* has been based on morphological features of the bacterium such as the presence of intracellular sulphur inclusions.[13] *Halorhodospira* and *Achromatium* were found in both lake and river water samples. *Methylophaga. thalassica*, the another identified γ-proteobacteria, was found only in river sample. *M. marina and M. thalassica*, comprises halophilic methylotrophic bacteria. These organisms utilize C1 compounds through the ribulose monophosphate pathway and are unable to grow on methane. The genus *Methylophaga* was found to be clearly separated from other methylotrophic bacteria and formed a distinct branch within the gamma subclass of *Proteobacteria*.[15] Two unidentified γ-proteobacteria were only found in river sample and their TR-Fs patterns matched with sequences take from cold permanent sediment.[23]

α-Proteobacteria was only represented by *Methylosinus* group. This bacteria was found in lake and river water samples. *Methylosinus*, is a methanotrophic bacterium type II because assimilate C1 intermediates via the serine pathway. Researches found *Methylosinus* as well as lakes, arctic wetlands, contaminated soils, etc.[39,40] Methanotrophs are widespread in aquatic and terrestrial environments, being found wherever stable sources of methane are present. Methane-oxidizing bacteria therefore play an important role in the carbon cycle, converting methane derived from anoxic decomposition back into cell material and CO_2.[39] The alpha-subclass *Proteobacteria* constituted the numerically less abundant bacterial group in Laguna de los Pozuelos wetland. These data agree with previous reports where the mean relative abundance and absolute cell numbers of the alpha-subclass of *Proteobacteria* detected with probe ALF968 were significantly high in marine samples.[8] Previous studies reported by González *et al*.[9] showed that marine alpha *Proteobacteria* decline in abundance with decreasing salinity along estuarine transects and they are not detectable in low-salinity or freshwater samples. We found alpha-*Proteobacteria* by culture independent and dependent methods (data not shown), although the salinity of the lake at the sampling date was low (0.3% salinity). An explanation for the presence of these typical marine bacteria in low salinity water samples may be that this lake suffers dry and rainy seasons with an annual contraction-expansion cycle with concomitant change in the salt concentration. These typical high salinity bacteria could survive during the less saline periods.

β-Proteobacteria constituted the most relative abundant bacterial group detected by T-RFLP analysis in Cincel River. However, there were not representatives of this subclass in the water lake sample. This finding is in agreement with previous reports on the predominance of this widespread group in a variety of other freshwater systems, including fresh water biofilms,[16,27] an olig-

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otrophic high mountain lake,[1,8] activated sludge,[37] and lake snow.[11] *Ralstonia* is an hydrogen-oxidizing bacteria, grow best under microaerobic conditions when growing chemolithrotrophically on H2. Analysis of lake sediment contaminated with high concentrations of copper as a consequence of mine milling disposal, found *Ralstonia* resistant to Cu.[14,20] *Dechloromonas* species, which have been shown to degrade aromatic compounds, including benzene, under nitrate-reducing conditions. It was described a perchlorate-respiring, hydrogen-oxidizing bacterium (*Dechloromonas sp.* strain HZ) that grows with carbon dioxide as sole carbon source and its ability to grow autotrophically with carbon dioxide as the sole carbon source.[41] One unidentified γ-proteobacteria was found in river sample and their TR-Fs patterns matched with sequences take from an activated sludge sample.[36]

Bacteria belong to Actinobacteria group was mainly found in lake water sample. *Nitrosomona*, is a nitrifying bacteria widespread in soil and water, develop in lake, marine, freshwater and soil, especially well in lakes and streams that receive inputs of ammonia.[14] *Acidimicrobium ferrooxidans*, an iron-oxidizing bacterium (also known as *Thiobacillus ferrooxidans*) was found only in the lake sample. This bacterium was isolated from lithotrophic biofilm at an extreme acid mine drainage site,[4] as well as ferric iron-containing minerals.[5] Actinobacteria was found mainly in the lake sample. The unidentified Actinobacteria sequence founded in river sample was described at a bacterial diversity study in permanently cold marine sediments.[18]

In the *Bacillus*/*Clostridium* group two T-RFs were found only in river water sample. One T-RFLP analysis-derived sequence matched with *Anaerobacter plyendosporus*. This bacterium is a member of the Clostridium cluster I, subcluster A and possesses such common phenotypic features as a Gram-positive cell wall structure, anaerobiosis, derivation of energy from carbohydrate fermentation yielding butyric acid among other organic acids and the capacity for endogenous spore-formation. However, the scale of evolutionary change in the 16S rRNA gene between *A. polyendosporus* and phylogenetically related Clostridium species does not correspond to the profound changes in the phenotype of *A. polyendosporus*. Distinctive phenotypic features of the latter are large cell size, polysporogenesis (up to seven spores per cell), alternative modes of development and an unusual membrane ultrastructure.[35]

Although the validity of T-RFs to identify microorganisms is not largely accepted, many reports have assigned a phylogenetic name to the T-RFs peaks,[19] even when a T-RF patterns derived from more than one enzyme digests is provided.[17] Marsh designed a Web-Based as a Research Tool for Microbial Community Analysis that facilitates microbial community analysis using terminal restriction fragment length polymorphism of 16S ribosomal DNA. This program assigns bacterial names or its corresponding EMBL accession number to T-RFs from amplified bacterial 16S rDNAs using the TAP (T-RFLP Analysis Program) tool.[28,29] Avaniss-Aghajani used T-RFLP for rapid identification of *Mycobacteria* in clinical samples.[3]

This molecular approach was used for the ability to rapidly detect and identify microorganisms in the complex community of Laguna de Pozuelos wetland. However, since the 16S rRNA sequences of most microorganisms (cultivated and not yet-cultivated) is yet unknown, it is no possible to determine by this method the composition of the total community. However an approach of the bacterial diversity by a culture independent method was obtained.

In addition, suggestions about the abundance of the phylotypes derived from the TAP analysis of the Laguna de Pozuelos samples and of the five major linages of the *Bacteria* must be made with caution. This type of molecular phylogenetic analysis of environmental T-RFs-derived sequences is reflective of the number of 16S rRNA gene copies present in the sample and not directly of the numbers of different kinds of organisms. The rRNA gene copy number may be 1 to 14 copies per cell and varies from species to species and even from strain to strains.[7] Furthermore, it was demonstrated problems associated with the PCR of 16S rRNA for phylogenetic analysis of microbial communities. Strong biases may be introduced by differential PCR amplification of DNA from heterogeneous templates, product plateau, etc.[7,18,21,32]

We found five major phylogenetic groups represented by the 19 phylotypes (6 in the lake and 13 in the river samples) obtained by the combination of thee endonucleases, but do not conclude that the proportions found in our study are representative of the original samples. Additional steps could be taken to estimate the abundance and proportion of individual species that are detected in a clonal library. For example, fluorescently tagged oligonucleotide probes could be developed and the number of organisms enumerated by this method could be compared to the total number present.[2]

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Correspondence to:

María E. Farías.
Av. Belgrano y Pje. Caseros -(4000) - Tucumán, Argentina
Phone: +54 381 4344888. Fax: +54 381 4344887.
E-mail: mefarias@proimi.edu.ar