ARTICLE / INVESTIGACIÓN

Identification and phylogenetic characterization based on DNA sequences from RNA ribosomal genes of thermophilic microorganisms in a high elevation Andean tropical geothermal spring

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Abstract: Several microorganisms can survive in harsh acid environments in geothermal springs at high temperatures across the Equatorial Andes Mountains. However, little is known about their physiological features and phylogenetic composition. Here we identify thermophilic microorganisms (bacteria, fungi, and microalgae) hosted in an almost unexplored geothermal spring (known as “Aguas Hediondas”). The phylogeny of the cultures was determined by analyzing physiological features and DNA sequences of PCR products for 16S rRNA, ITS, and 23S rRNA genes. Twenty pure cultures were isolated from the samples, including 17 for bacteria, one for cyanobacterium, one for eukaryotic microalgae, and one for fungus. Most bacterial strains were gram-positive, spore-forming, and bacilli (*Bacillus*). Cyanobacterium strain belonged to *Chroococcidiopsis* and the eukaryotic microalgae to *Chlorophyta*. The unique fungal strain isolated was closely related to *T. duponti*. Through our study, isolated thermophilic bacteria, microalgae and fungi from the “Aguas Hediondas” geothermal spring were characterized and identified. This study represents one of the first extensive molecular characterizations of extremophile microbes in the Tropical Equatorial Andes.

Key words: Microbial diversity, DNA markers, extremophiles, phylogenetics.

Introduction

There has been a growing interest in studying extreme ecological niches and microorganisms living under extreme environmental conditions in the last decades. The increasing socio-economic and scientific relevance in discovering the biodiversity of extremophile microorganisms is primarily due to the direct and indirect (by-products) use in biotechnology and industries. Historically, a great diversity of microorganisms has been found in hostile conditions of geothermal environments[1–5]. Microorganisms living in geothermal springs have developed several adaptations to survive hostile environments[6]. For instance, these organisms can synthesize enzymes that work under high temperatures, high salt concentrations, high alkalinity or acidity conditions, and under high pressure[7,8]. Their capacity to maintain their metabolic activity under these extreme conditions makes these enzymes (synthesized by thermophilic organisms) incredibly attractive for developing various human applications in diverse fields such as medicine, cosmetics, and the food industry[7,8]. The culture-dependent method has played a crucial role in isolating microbes and preserving them for further research on biotechnological applications and developing new bioproducts[9].

The biodiversity in geothermal springs is an adaptable response to the environmental conditions correlated with the mineral composition, pH, gases, salinity, redox potential, nutrients availability, and temperature variables[10,11]. Additionally, climate change and anthropogenic activities can cause significant changes in the water chemistry of lentic and lotic ecosystems, which may affect the microbial biodiversity of these unique ecosystems. Biodiversity in tropical mountains is wealthy and high, particularly across the Andean mountains around Ecuador[12]. Around 25 % of all terrestrial areas on Earth are mountain regions hosting more than 85% of the world’s species of amphibians, birds, and mammals, many exclusively restricted to mountains[13]. Moreover, it has been well documented that hot spots in the tropical Andean mountains, including water reservoirs, can hold higher diversity than wet lowlands[14]. The biodiversity of the different mountain ecosystems reflects the great importance of the evolutionary and ecological processes in these regions, a history worth understanding, preserving, and protecting.

Our study emphasizes microbial diversity in high-elevation Andean geothermal spring waters within these ecosys-

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tems. The survival of thermophiles depends on temperature regulation processes, activities, and behavior[15–17]. It has been proposed that microorganisms shape ecosystems to make the environment more suitable for life[18–20]. Evidence for symbiotic assemblages has been reported for diverse microbial communities in the microbe mats co-existing in hot springs[21,22]. Main groups of microorganisms found in thermophilic environments, like archaea, bacteria, microalgae, and fungi, have adaptations to adverse conditions[15,23,24].

Only a few studies have addressed the microbial and physicochemical characterization of "Aguas Hediondas" geothermal spring; however, they revealed only the diversity of microalgae communities without considering other important groups of microorganisms[25,26]. Most existing reports have focused exclusively on tourism and geothermal energy production applications[27–30]. It is believed by the local people that water from the geothermal spring has medicinal and healing properties[31,32]. We find these proposed properties interesting to investigate, therefore relevant to characterizing the microbial communities in "Aguas Hediondas."

This study aimed to identify thermophilic microorganisms in a little-explored hot spring in the Equatorial Andes using culture-dependent methods and classification based on DNA sequences from RNA ribosomal genes.

Materials and methods

Site characteristics and sample collection

Sediment and water samples were collected from the "Aguas Hediondas" geothermal spring located in Carchi province in northern Ecuador (00º 48.587'N & 77º 54.362'W) at 3428 m.a.s.l. The samples were taken from three different places in the same geothermal spring in April 2016. Three replicates were taken at each sampling point. Each replicate consisted of 50 ml of water and a portion of sediment from the bottom of the spring. The samples were maintained at 50 ºC during transportation and storage until the culture using a portable incubator (BIOBASE). The average temperature recorded in the three geothermal water points was 54.67±1.63 ºC. Environmental water and sediment samples were collected under permit # MAE-DNB-CM-2017-0071 granted by the Ministry of Environment of Ecuador. Water and sediment samples were sent for physicochemical analysis to a commercial service provider.

Media and culture conditions

Samples were cultured within six hours after collection. Sub-samples of 100 µl were spread on specific media for each group of microorganisms. The pour plate culturing method was used to get pure isolations. Bacteria were cultured on agar M9 (Difco) between 51 and 56 ºC for 72 hours[33]. Cultivation of fungi was performed on PDA (Difco) supplemented with 50 mg l^{-1} of chloramphenicol (Sigma) at 55 ºC for 2 weeks[34]. Microalgae were cultured on BG11 (Sigma) solidified with 1.5% Difco Bacto agar at room temperature (25 ºC) with illumination between 1000-2000 lux with 12 hours light and 12 hours dark photoperiod[35]. Pure microalgae cultures were obtained by transferring part of each algal colony in 3 ml of liquid BG11 (Sigma). All the culture media were adjusted to the pH of the geothermal spring, around 5. Individual bacterial and fungal colonies were isolated by transferring to a fresh plate and incubated as indicated before prior to phenotypic characterization. Phenotypic features were examined according to colony pigmentation, texture, appearance, shape, and edge. After identification, bacterial and microalgal strains were cryopreserved in broth-glycerol (8:2) at -20ºC. Fungal isolates were preserved by culturing on PDA (Difco) dishes and then coated with mineral oil and stored at 4ºC.

Optical microscopy

All isolates were examined using a 100X magnification with a CX21 Olympus® microscope. Each isolate was characterized based on colony pigmentation, texture, appearance, shape, and edge (Supplemental Material). Mycelium structure was also observed in fungal isolates[36]. Bacterial isolates were Gram-stained for easier identification.

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA from bacteria, fungi, and microalgae was extracted by different protocols established by Moore *et al.*[37], Weising *et al.*[38], and Cai & Wolk[39], respectively. NanoDrop 8000 UV-Vis quantified DNA purity and concentration. Fragments of 16S rRNA, ITS, and 23S rRNA genes were amplified with specific primers (Table 1) through PCR. A 25 µl PCR reaction was carried out with the kit GoTaq® Green Master Mix from Promega. The PCR reaction mix was composed of 7 µl of ultrapure water, 12.5 µl of GoTaq® Green Master Mix (2X), 1.5 µl of each primer (10 µM) and 2.5 µl of DNA template. The protocol used for PCR amplification was 94°C for 5 min, 35 cycles of denaturation at 94°C for 30-sec primer annealing at 50°C for 1 min and extension at 72°C for 7 min and 30 sec with a final extension at 4°C for 10 min. The PCR products were visualized on 1 % agarose gels using GelStar™ dye. Amplified DNA fragments were sent for sequencing to a commercial sequencing service provider (Macrogen, South Korea).

Gen	Sequence (5'-3')	Target	Size (pb)	Reference
16S rRNA	27F: AGATTTGATYMTGGCTCAG	Bacteria	~1500	37
	1492R: ACGGYTACCTTGTTACGACTT			
23S rRNA	p23srV_f1: GGACAGAAA-GACCCTATGAA	Microal-gae	~410	39
	p23SrV_r1: TCAGCCTGTTATCCCTA-GAG			
ITS	ITS1F-Bt1: CTTGGTCATTTAGAG-GAAGTAA	Fungi	~650	38
	ITS4Rbt: TCCTCCGCTTATTGATATGC			

Table 1. PCR primer sequences used in this study.

Blast, sequence alignment, and phylogenetic analyses

DNA sequences used for this analysis are available under GenBank accession numbers MT765288-MT757927, MT757926, and MT764950-MT764966. To search for the similarity of the DNA sequences, the GenBank database (NCBI, National Centre for Biotechnology Information) was applied using the Basic Local Alignment Search Tool (BLAST) based on the most similar matches (>99% similarity). The obtained sequences were aligned using Geneious Prime® 2020.2.2 software through MUSCLE and ClustalW alignment methods and the online tool (https://mafft.cbrc.jp/alignment/server/index.html) for the MAFFT 7 alignment method. Phylogenetic reconstruction was performed with the Markov Chain Monte Carlo (MCMC) Bayesian approach implemented in BEAST version 1.10.4. Phylogenetic analysis was carried out with the GTR+Gamma as the best substitution model suggested by the software jModelTest for DNA sequences alignments[40]. A non-parametric Bayesian Skyline (Piecewise-constant) coalescent model was used with a Strict molecular clock method. MCMC was developed with 20 million generations, subsampled every 1000 generations by applying a Random as the starting tree. Moreover, a Marginal Likelihood Estimation (MLE) using path sampling (PS) / stepping-stone sampling (SS), which performs an additional analysis after the standard MCMC chain has finished, was implemented. The MCMC/MLE analysis output was summarized using TreeAnnotator software included in the Beast package (.log files are provided in the supplemental material as a support of the analysis). The maximum Clade Credibility tree was produced after discarding 10 % of burn-in. The final tree was visualized through FigTree version 1.4.4.

Results

The mean water pH measured was 4.8, while the sediment means pH was 5.8. Water and sediment samples showed distinct compounds and concentrations. Water contained 0.054 mg l^{-1} of arsenic, 83.1 mg l^{-1} of chlorides, 0.53 mg l^{-1} of iron, 2.5 mg l^{-1} of manganese 225 mg l^{-1} of sodium, 45.4 mg l^{-1} of potassium, 168.44 mg l^{-1} of magnesium, 72 mg l^{-1} of calcium, and 744.8 mg l^{-1} of sulphates. Water samples composition included volatile and non-volatile solids with 120 and 1 290 mg l^{-1} respectively. The sediment portion had the following compounds 1.3 mg kg^{-1} of cadmium, 29.3 mg kg^{-1} of copper, 312 mg kg^{-1} of potassium, 12 500 mg kg^{-1} of iron, 79 mg kg^{-1} manganese, and 1 860 mg kg^{-1} of magnesium. Sediments also contained organic matter, which represents approximately 60.59 % mean for all the samples. Electrical conductivity (EC), an indicator of water quality and inorganic constituents' presence, was 0.1725 Sm^{-1} in water and 0.0164 Sm^{-1} in sediments. Higher EC levels denote higher total solids dissolved concentrations (TSD). EC is an indicator of dissolved minerals.

A total of 20 pure cultures were isolated, including 17 bacterial isolates, one cyanobacterium, one eukaryotic microalga, and one fungus. Most bacterial isolates were gram-positive spore-forming bacilli. The unique fungal strain isolated produced a grey dusty mycelium. Microalgae colonies were light green (eukaryotic) and bluish-green (cyanobacterium) (Figure 1).

Table 2 shows the relationships of isolated geothermal strains with the most similar sequences from the Genbank database.

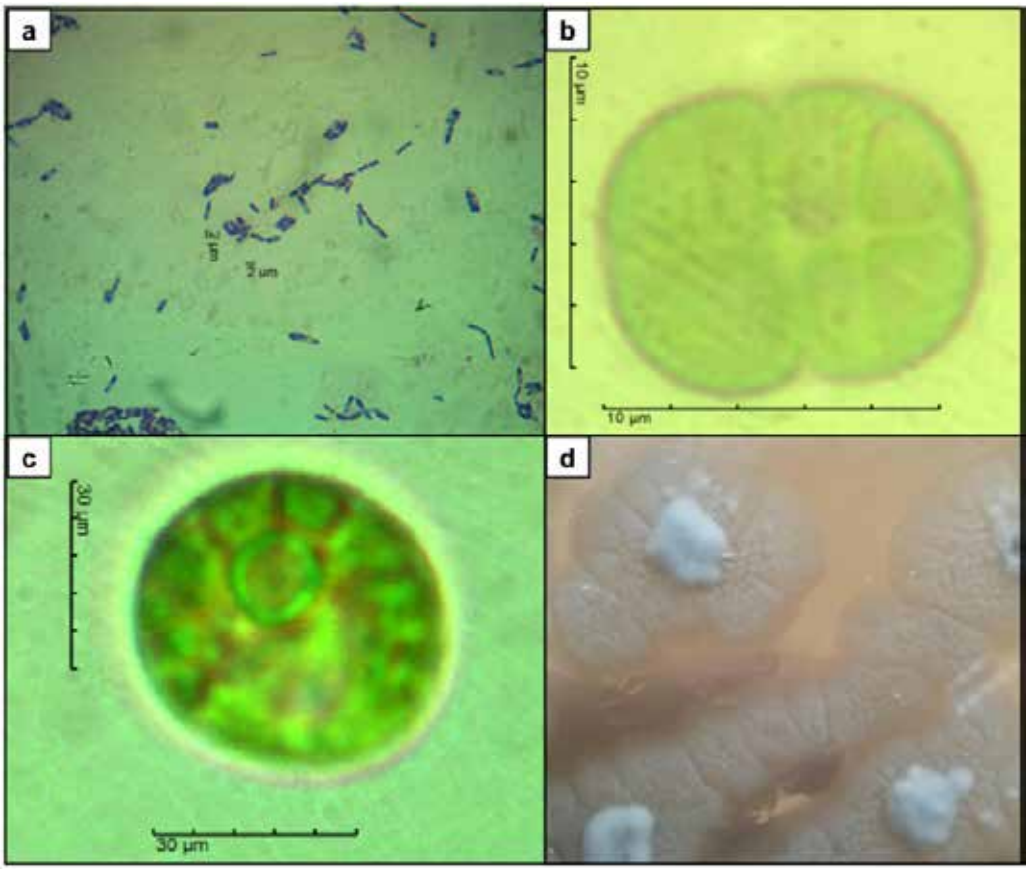

Figure 1. Microbial strains isolated. (a) Bacteria (*B. licheniformis*). (b) Cyanobacterium (*Chroococcidiopsis*). (c) Eukaryotic microalgae (*C. vulgaris*). (d) Fungi (*T. thermophilus*).

strain	BLAST	Accession no.	Coverage (%)	Identity (%)
1R	*Bacillus licheniformis*	NR_118996	100	97.3
2R	*Bacillus licheniformis*	NR_118996	98	96.1
3R	*Bacillus licheniformis*	NR_118996	97	94.7
6R	*Bacillus licheniformis*	NR_118996	100	97.2
7R	*Bacillus licheniformis*	NR_165685	100	99.1
10R	*Bacillus licheniformis*	NR_165685	100	99.0
18R	*Bacillus licheniformis*	NR_118996	100	96.1
20R	*Bacillus licheniformis*	NR_118996	99.93	99.4
21R	*Bacillus licheniformis*	NR_118996	100	98.1
24R	*Bacillus licheniformis*	NR_165685	81.29	97.7
25R	*Bacillus licheniformis*	NR_118996	100	98.6
30R	*Bacillus licheniformis*	NR_118996	100	95.3
32R	*Bacillus thermoamylovorans*	NR_117028	99.93	99.5
34R	*Bacillus licheniformis*	NR_118996	100	99.5
35R	*Bacillus licheniformis*	NR_165685	100	98.7
36R	*Bacillus thermoamylovorans*	NR_144578	99	98.47
37R	*Bacillus licheniformis*	NR_118996	99	97.4
R1A	*Chroococcidiopsis thermalis*	NR_102537.1	100	95.1
R2A	*Chlorella mirabilis*	KM462865.1	99	94.1
RR	*Thermomyces duponti*	NR_163517.1	99	99.8

Table 2. Sequences from GenBank producing significant alignments.

Figure 2 presents the phylogenetic tree of 17 isolated bacterial DNA strains. Two clades are marked, where the distance was compared by measures based on rRNA sequence divergence. All the bacteria (17) were contained within the genus *Bacillus* in the phylum *Firmicutes*. The nucleotide similarity in the ribosomal RNA gene sequences reveals that the bacterial strains were separated into two main groups: a branch closely related to *Bacillus licheniformis* (Figure 2a), and a group closely related to strains of *Bacillus thermoamylovorans* (Figure 2b).

On the other hand, the BLASTn analysis exhibits that the only fungal isolate recovered from the hot spring was classified as closely related to *Thermomyces duponti* (Table 2).

Finally, the prokaryote microalgal isolate was a cyanobacterium classified as closely related to *Chroococcidiopsis thermalis*, and the eukaryote microalgal isolate was categorized as closely related to *Chlorella mirabilis* within *Chlorophyta* (Table 2).

Discussion

The predominant presence of bacteria compared to fungi and microalgae may be due to the intrinsic geochemical properties of hot springs[41]. However, the culture process must also be considered a restrictive issue for the determination of abundance. It is well established that approximately 1% of microorganisms can be readily cultivated in vitro[42,43]. Because most microorganisms remain unculturable, the diversity in our study is inevitably underestimated using culture-dependent methods. For instance, M9, PDA, and BG11 are media that might not be the best for the recovery of 'unculturable' microorganisms from environmental samples[44]. Methods, including the use of dilute nutrient media, could be a great approach to the recovery of 'unculturable' microorganisms adapted to oligotrophic conditions[45].

Seventeen bacterial strains isolated in our study belong to the genus *Bacillus*, gram-positive and spore-forming. Similar findings were reported by Darland and collaborators[46], who observed that the genus *Bacillus* predominates in geothermal springs at Yellowstone National Park, especially those gram-positive with a pronounced tendency to form endospores. Bacterial strains isolated were classified as closely related to two species: *B. licheniformis*, and *B. thermoamylovorans* (Fig. 2). There are several reports of same species growing at similar conditions of temperature and pH in other geothermal springs around the world[47–54]. *B. licheniformis* has been found in different geothermal springs in Ecuador, including Papallacta, Chachimbiro, Guapán, and Baños de Agua Santa[55–57].

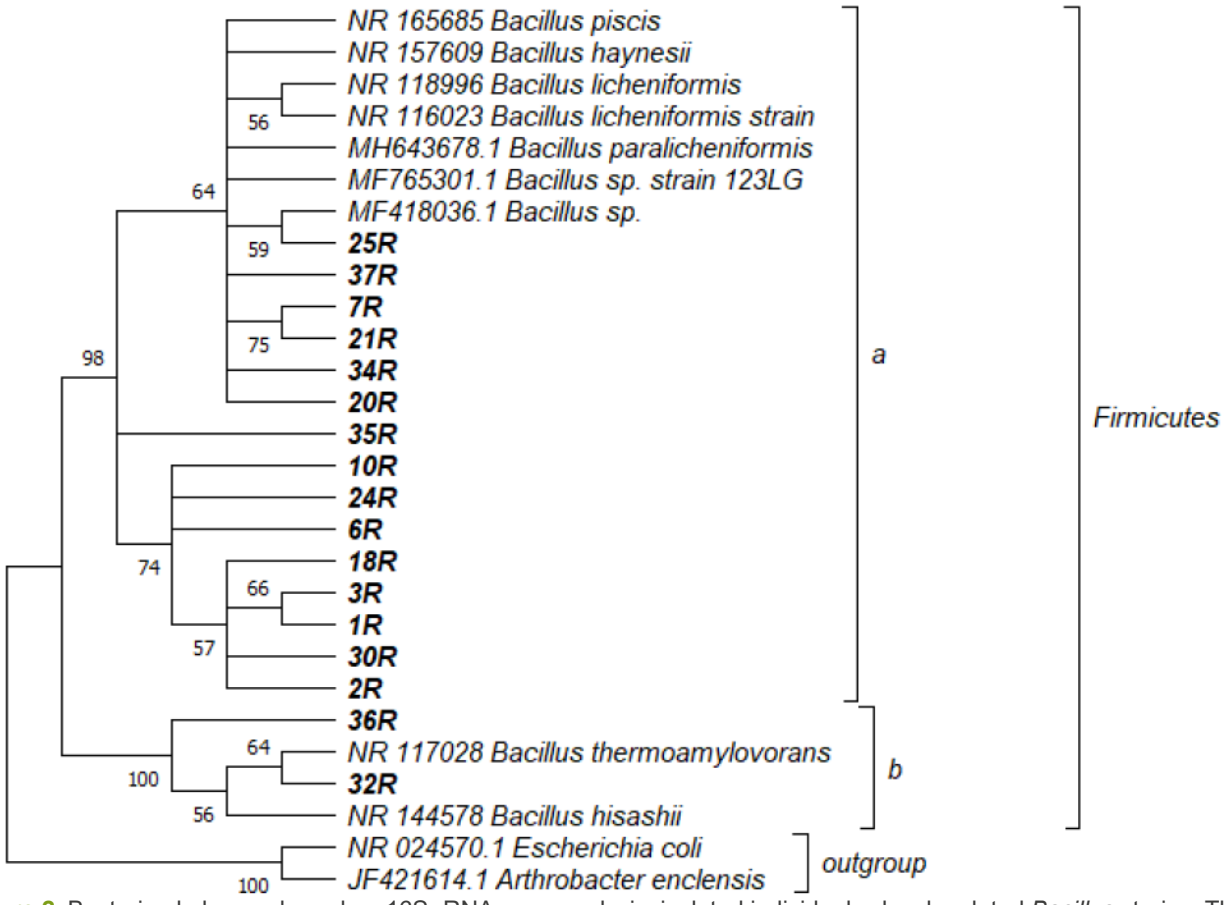

Figure 2. Bacteria phylogeny based on 16S rRNA gene analysis: isolated individuals closely related *Bacillus* strains. The phylogenetic tree was constructed using 1456 bp and maximum likelihood analyses under a GTR+Gamma Sites model. Numbers at nodes are support values generated from 1000 bootstrap replicates. GenBank accession no. MT764950-MT764966.

In the phylogenetic tree, *E. coli* and *A. enclensis* are located outside of the branch close to the genus *Bacillus*, as it should be expected, because these organisms belong to different phyla: *Proteobacteria*, *Actinobacteria*, and *Firmicutes* respectively[58–62]. Wei Wang[63], also confirmed the position of *B. thermoamylovorans* concerning *B. licheniformis*. Furthermore, Yang Liu *et al.*[64] and Christopher Dunlap *et al.*[65] described that *B. licheniformis* and *B. paralicheniformis* are sister taxa sharing a common ancestor.

The Fungal ITS sequence analysis exhibited an association between *T. duponti* and the isolated strain (Table 2). *T. duponti* has been previously isolated from sediment samples in China's Tengchong Rehai National Park hot springs[66]. Optimal growth conditions are similar with these found in “Aguas Hediondas” spring. Pan *et al.*[66] established an optimal growth temperature for this fungus of 45-50°C and tolerable pH ranges of 4-12. *Thermocyces dupontii* belong to the order *Eurotiales* (Phylum *Ascomycota*)[67,68].

The examined samples in “Aguas Hediondas” geothermal spring contained prokaryotic and eukaryotic microalgaes. According to our analysis, isolated algae strains were classified as closely related to two species: *Chroococcidiopsis thermalis*, and *C. mirabilis* (Table 2). *C. thermalis* are part of the phylum *Cyanobacteria*[69]. Moreover, *C. mirabilis* is an eukaryotic algae[69]. Both species have been previously isolated from a similar environment. The genus *Chroococcidiopsis* has mainly been isolated from the interior and exterior of rocks in hot and cold deserts of the planet[66,70]. It appears to be highly tolerant to desiccation[66,70]. Other studies have identified strains of the genus *Chroococcidiopsis* in geothermal springs in northern Thailand with an optimal growth temperature of 50°C and pH 8[71]. The eukaryotic microalgae *C. mirabilis*, which belongs to *Chlorophyta*, has been established as a phylum with an important role in algal communities from geothermal springs of Bulgaria[72]. The data showed that 75 of the 200 species surveyed correspond to the phylum *Chlorophyta*, which can grow at temperatures in between 30 to 101ºC, and pH between 1-10[72]. Our study is significantly different from local previously algal characterizations. Morales *et al.*[25] reported the predominant presence of *Closterium sp.*, *Maougeotia sp.*, *Navícula sp.*, *Dictyosphaerium sp.*, and *Ulothrix sp.* in the Aguas Hediondas geothermal spring.

Ribosomal RNA barcodes were used for the identification and phylogenetic analysis of isolates because these kinds of markers are universal and composed, at the same time, of highly conserved as well as variable domains[73]. Even if some researchers consider that ribosomal RNA is the best target for studying phylogenetic relationships, choosing the most suitable barcode could contribute to the resolution enhancement of the analysis presented in this study[74]. Due to the increasing amount of information, 16S and 18S rRNA barcodes could improve the characterization of prokaryotic and eukaryotic microalgal isolates.

Because of their potential to produce thermostable extracellular enzymes with essential biotechnological uses, thermophilic and thermotolerant microorganisms are of significant economic value[75–77]. The benefits of using ther-

mostable enzymes for biotechnological processes at elevated temperatures include reduced risk of contamination by mesophilic microorganisms[78], decreased reaction-medium viscosity, increased bioavailability and solubility of organic compounds, and increased substrate and product diffusion coefficients, resulting in faster reaction rates[79]. The species of microorganisms isolated in this study have been previously reported as producers of thermostable lipases[51], cellulases[80], xylanases[81], and pectinases[82]. There are several reports of the use of these kinds of enzymes in biotechnological processes such as biobleaching of paper pulp[83], animal feed production[83], fermentation of sugars to obtain biofuel from cellulosic wastes[84,85], fruit juice extraction and clarification[84,85], refinement of vegetable fibers[86], degumming of natural fibers[86], curing of coffee[86], cocoa[86], and tobacco[86], and waste-water treatment[86]. Further research is needed to elucidate the biotechnological applications of the isolated microorganisms in this study.

Conclusions

Through our study, isolated thermophilic bacteria, microalgae, and fungi from the “Aguas Hediondas” geothermal spring were characterized and identified. These results are confirmed by previous studies in the phylogeny and characterization of other geothermal waters. Our results represent an initial contribution to the study of thermophilic microorganisms in the geothermal spring “Aguas Hediondas”.

Author Contributions

Conceptualization, R.R.-P. and A.I.; methodology, R.R.-P., A.A.-N. and A.I.; software, R.R.-P. and A.A-N.; validation, R.R.-P.; formal analysis, R.R.-P., A.A.-N.; investigation, R.R.-P.; resources, A.I.; data curation, R.R.-P.; writing—original draft preparation, R.R.-P, K.S., D. B.-G.; writing—review and editing, R.R.-P, K.S., D. B.-G., A.A.-N., A.I.; visualization, R.R.-P.; supervision, A.I.; project administration, A.I.; funding acquisition, A.I. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

DNA sequences used for this analysis are available under GenBank accession numbers MT765288-MT757927, MT757926, and MT764950-MT764966.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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