High diversity and suggested endemicity of culturable Actinobacteria in an extremely oligotrophic desert oasis

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The phylum Actinobacteria constitutes one of the largest and anciently divergent phyla within the Bacteria domain. Actinobacterial diversity has been thoroughly researched in various environments due to its unique biotechnological potential. Such studies have focused mostly on soil communities, but more recently marine and extreme environments have also been explored, finding rare taxa and demonstrating dispersal limitation and biogeographic patterns for Streptomyces. To test the distribution of Actinobacteria populations on a small scale, we chose the extremely oligotrophic and biodiverse Cuatro Cienegas Basin (CCB), an endangered oasis in the Chihuahuan desert to assess the diversity and uniqueness of Actinobacteria in the Churince System with a culture-dependent approach over a period of three years, using nine selective media. The 16S rDNA of putative Actinobacteria were sequenced using both bacteria universal and phylum-specific primer pairs. Phylogenetic reconstructions were performed to analyze OTUs clustering and taxonomic identification of the isolates in an evolutionary context, using validated type species of *Streptomyces* from previously phylogenies as a reference. Rarefaction analysis for total Actinobacteria and for Streptomyces isolates were performed to estimate species´ richness in the intermediate lagoon (IL) in the oligotrophic Churince system. A total of 350 morphologically and nutritionally diverse isolates were successfully cultured and characterized as members of the Phylum Actinobacteria. 105 from the total isolates were successfully subcultured, processed for DNA extraction and 16S-rDNA sequenced. All strains belong to the order Actinomycetales, encompassing 11 genera of Actinobacteria; the genus *Streptomyces* was found to be the most abundant taxa in all the media tested throughout the 3-year sampling period. Phylogenetic analysis of our isolates and another 667 reference strains of the family Streptomycetaceae shows that our isolation effort produced 38 unique OTUs in six new monophyletic clades. This high

biodiversity and uniqueness of Actinobacteria in an extreme oligotrophic environment, which has previously been reported for its diversity and endemicity, is a suggestive sign of microbial biogeography of Actinobacteria and it also represents an invaluable source of biological material for future ecological and bioprospecting studies.

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Abstract. (332 words)

The phylum Actinobacteria constitutes one of the largest and anciently divergent phyla within the Bacteria domain. Actinobacterial diversity has been thoroughly researched in various environments due to its unique biotechnological potential. Such studies have focused mostly on soil communities, but more recently marine and extreme environments have also been explored, finding rare taxa and demonstrating dispersal limitation and biogeographic patterns for *Streptomyces*. To test the distribution of Actinobacteria populations on a small scale, we chose the extremely oligotrophic and biodiverse Cuatro Cienegas Basin (CCB), an endangered oasis in the Chihuahuan desert to assess the diversity and uniqueness of Actinobacteria in the Churince System with a culture-dependent approach over a period of three years, using nine selective media. The 16S rDNA of putative Actinobacteria were sequenced using both bacteria universal and phylum-specific primer pairs. Phylogenetic reconstructions were performed to analyze OTUs clustering and taxonomic identification of the isolates in an evolutionary context, using validated type species of *Streptomyces* from previously phylogenies as a reference. Rarefaction analysis for total Actinobacteria and for *Streptomyces* isolates were performed to estimate species´ richness in the intermediate lagoon (IL) in the oligotrophic Churince system. A total of 350 morphologically and nutritionally diverse isolates were successfully cultured and characterized as members of the Phylum Actinobacteria. 105 from the total isolates were successfully subcultured, processed for DNA extraction and 16S-rDNA sequenced. All strains belong to the order Actinomycetales, encompassing 11 genera of Actinobacteria; the genus *Streptomyces* was found to be the most abundant taxa in all the media tested throughout the 3-year sampling period. Phylogenetic analysis of our isolates and another 667 reference strains of the family Streptomycetaceae shows that our isolation effort produced 38 unique OTUs in six

new monophyletic clades. This high biodiversity and uniqueness of Actinobacteria in an extreme oligotrophic environment, which has previously been reported for its diversity and endemicity, is a suggestive sign of microbial biogeography of Actinobacteria and it also represents an invaluable source of biological material for future ecological and bioprospecting studies.

Introduction

The phylum Actinobacteria are gram-positive bacteria with a high G+C content, and it constitutes one of the largest phyla within the Bacteria domain (Parte, Whitman, Goodfellow 2012). Actinobacteria diversity and community structure have been thoroughly researched in various environments. However, such studies had focused mostly in soil communities (Coombs & Franco 2003; Gremion, Chatzinotas & Harms 2003; Mohammadipanah & Wink 2015; Zhao, Guo, Li 2016); but more recently, marine environments have also been explored (Ward & Bora 2006; Maldonado, Fragoso-Yanez, Perez-Garcia 2009; Claverias, Undabarrena, Gonzalez 2015; Duran, Bielen, Paradzik 2015; Chen, Zhang, Guo 2016; Mahmoud & Kalendar 2016; Undabarrena, Beltrametti, Claverias 2016).

As an indicator of their ecological importance, Actinomycetes, filamentous members of the phylum Actinobacteria account for about 10% of bacteria colonizing marine aggregates (Grossart, Schlingloff, Bernhard 2004). Initially, marine Actinomycetes were poorly characterized (Goodfellow & Williams 1983), but more recently, culture independent studies have shown that marine Actinomycetes are diverse and abundant (Ward & Bora 2006). Rare marine Actinomycetes taxa have been isolated from a range of depths, sediments and other microbial communities such as stromatolites (Allen, Goh, Burns 2009). Actinomycetes also comprise about 10% of the microbiome of extreme habitats, showing extensive taxonomic diversity (Kuhn, Ichimura, Peng 2014; Mohammadipanah & Wink 2015; Liu, Salam, Jiao 2016; Qin, Li, Dastager 2016). However, careful population studies must still be done to determine if Actinomycetes are cosmopolitan, or if they do have local ecotypes, i.e., some degree of biogeography. Endemism would be the clearest demonstration of microbial biogeography, as it is for other organisms such as *Salinispora*[obj](Jensen, Dwight & Fenical 1991; Johnson 2005; Jensen & Mafnas 2006; Winsborough, Theriot & Czarnecki 2009; Coghill, Hulsey, Chaves-Campos 2013; Prieto-

Davo, Villarreal-Gomez, Forschner-Dancause 2013). Nevertheless, to unambiguously accept the idea of unlimited dispersal of microorganisms, we need data from studies employing good sampling. Such is the case, for example, of *Escherichia coli,* human-related strains of which travel with their host all around the world, or the case of *Bacillus subtilis* that can form endospores and travel with the air (Souza, Eguiarte, Travisano 2012). Even in such cosmopolitan bacteria, there are local ecotypes that are unrelated to any other known strains (Gonzalez-Gonzalez, Sanchez-Reyes, Delgado Sapien 2013; Avitia, Escalante, Rebollar 2014; Valdivia-Anistro, Eguiarte-Fruns, Delgado-Sapien 2015). *Streptomyces*, a filament and spore producer, and the most extensively studied genera of Actinomycetes, has been studied and it had shown environmental gradients and regional endemism in some localities (Davelos, Xiao, Samac 2004; Antony-Babu, Stach & Goodfellow 2008; Kinkel, Schlatter, Xiao 2014; Andam, Doroghazi, Campbell 2016).

Actinobacterial diversity and community structure have been thoroughly investigated, not only for their ecological importance, but also by virtue of their unique biotechnological potential due to their robust secondary metabolism and incomparable ability to produce a plethora of bioactive molecules with extensive medical, industrial and agricultural applications. Actinomycetes, are the source of most clinically relevant antibiotics in use today (Barka, Vatsa, Sanchez 2016). Nevertheless, the growing emergence of antibiotic multirresistant pathogenic strains, challenges the scientific community to overcome the problem of rediscovery of known compounds. Recent studies have concluded that discovery of unkown bioactive molecules will be facilitated by focusing heavily on “gifted” (secondary-metabolites-rich), readily culturable microbes that have been isolated from untapped environments, such as marine ecosystems, which enhance the isolation of large-genome (>8 Mb), thus, rare culturable bacteria (Tiwari & Gupta 2012; Zotchev 2012; Subramani & Aalbersberg 2013; Tiwari & Gupta 2013; Baltz 2016;

Katz & Baltz 2016; Smanski, Schlatter & Kinkel 2016).

Correspondingly, efforts towards describing the extent of the diversity of culturable actinomycetes on different conditions and extreme environments have been done, as evidenced by recent reports of bioprospecting and diversity studies of actinobacteria on deserts, marine sediments and vents, coral reefs, glaciers, as well as in symbiotic relationships (Maldonado et al., 2009; Rateb, Houssen, Harrison 2011; Lee, Zainal, Azman 2014; Duncan, Haltli, Gill 2015; Duran et al., 2015; Jami, Ghanbari, Kneifel 2015; Kuang, Li, Zhang 2015; Mohammadipanah & Wink 2015; Trujillo, Riesco, Benito 2015; Yang, Li, Huang 2015; Andam et al., 2016; Chen et al., 2016; Liu et al., 2016; Mahmoud & Kalendar 2016; Undabarrena et al., 2016).

To assess the extent of morphological and metabolic diversity and the distribution of culturable actinobacteria populations on a local scale, we chose the extremely oligotrophic and biodiverse Cuatro Cienegas Basin (CCB), an endangered oasis in the Chihuahuan desert (Souza, Siefert, Escalante 2012). This is a site where endemic *Bacillus* (Alcaraz, Olmedo, Bonilla 2008; Cerritos, Eguiarte, Avitia 2011), *Pseudomonas* (Escalante, Caballero-Mellado, Martinez-Aguilar 2009) and *Exiguobacterium* (Rebollar, Avitia, Eguiarte 2012) have been described. Particularly, within the CCB, the Churince System has been studied with more intensity by a large team of scientists since it is the most endangered hydrological system due to its relatively high altitude within the valley (730 m above sea level, compared to, ca. 700 m above sea level which is the average of most of the CCB), and because the San Marcos Sierra near this site of the basin is too step to efficiently recharge the aquifer locally. Hence, the system depends mostly on deep ancient water with a magmatic influence (Wolaver, Crossey, Karlstrom 2012). This, together with the calcium sulfate soil matrix, and extreme oligotrophy in terms of phosphorus-limitation (Elser, Schampel, Garcia-Pichel 2005), makes Churince the most unusual site within the CCB

(Minckley & Cole 1968). This analysis is relevant not only for understanding the extensive biodiversity of this bacteria in such a peculiar environment, but also, for allowing us the biological material for the elucidation of biochemical strategies for survival in conditions of scarcity, future experimentation of bioactive molecules, as well as studies of ecological interactions, including cooperation and competition analyses to understand the processes that are relevant to structure these complex bacterial communities. In contrast to what is commonly expected in an extremely oligotrophic site, we found high morphological and unique taxonomic diversity of culturable Actinobacteria, and we were able to isolate enriched abundance of the genus *Streptomyces*. When compared to available databases, we observed six novel monophyletic clades and seven single-member clusters, containing a total of 31 OTUs of the genus *Streptomyces* that are presumably different from other species previously described, and thus, good candidates for consideration as endemic to the CCB. These unique groups of *Streptomyces* strains represent key clades in evolutionary history of an anciently divergent Phylum of the Bacteria domain.

MATERIALS AND METHODS

Study Site and Sampling

The Churince hydrological system (Figure 1) is located in the western part of the CCB, at 740 m above sea level, surrounded by large and mostly pure gypsum dunes. This system consists of three main zones connected by small water causeways: a spring, an Intermediate Lagoon (IL), and a desiccation lagoon (Lopez-Lozano, Heidelberg, Nelson 2013). The Intermediate Lagoon (IL), where sampling took place, has low seasonal variations such as: salinity ranging ~1.5–7.1 ppt, pH 7.6 to 8, and water temperature fluctuation from 14-20 °C in winter and 20 to 30 °C in summer (data of this study).

147 Sampling took place during 2013-2016 at the following times: February 2013, March
148 2013, October 2013, October 2014, January 2015, February 2015, July 2015, April 2016.
149 Samples were obtained from water and upper layer sediment from six locations along the shore
150 in the Intermediate Lagoon in the Churince system (Figure 1) in Cuatro Cienegas, Coahuila with
151 the permission of Federal authorities to collect in the Natural Protected Area (SEMARNAT
152 scientific sampling permit No. SGPA/DGVS/03121/15): Location A: 26°50′53.79″N,
153 102°08′30.29″W; location B: 26°50′53.53″N, 102°08′31.81″W; location C: 26°50′54.37″N,
154 102°08′32.96″W; location D: 26°50′55.30″N, 102°08′33.63″W; location E: 26°50′55.63″N,
155 102°08′35.28″W; location F: 26°50′56.57″N, 102°08′36.03″W. At each site, water and surface
156 sediments (0.2-1 cm) were transferred to sterile conical tubes (50 mL). Samples were transported
157 to a nearby laboratory in the town of Cuatro Cienegas at room temperature (≤ 1.5 h) and were
158 used for streaking out primary plates immediately.

159

160 **Selective isolation of culturable Actinobacteria**

161 Nine selective Actinobacterial Isolation Media (AIM) were designed for this work to enhance the
162 isolation of actinobacteria of aquatic and sediment environment. **AIM1** ([per liter]: 21g yeast
163 extract agar, 10g Malt extract, 4g Dextrose, 25g Reef salt mix); **AIM2** ([per liter]: 20g mannitol,
164 20g soy flour, 20g Agar, 25g Reef salt mix); **AIM3** ([per liter]: 50g chitin, 16g agar, 25g Reef
165 salt mix); **AIM4** ([per liter]: 10g starch, 1g Casein, 15g agar, 25g Reef salt mix); **AIM5** ([per
166 liter]: 20g Oat meal, 0.001g $Fe_2(SO_4)_3$, 0.001g $MgCl_2$, 0.001g $ZnSO_4$, 18g agar, 25g Reef salt
167 mix); **AIM6** [per liter]: 10g starch, 1g K_2HPO_4, 1g $H_{14}MgO_{11}S$, 2g $H_8N_2O_4S$, 1g NaCl, 2g
168 $CaCO_3$, 0.001g $FeH_{14}O_{11}S$, 0.001g $MgCl_2$, 0.001g Z_nSO_4, 20g agar, 25g Reef salt mix); **AIM7**
169 ([per liter]: 40g Soy Tripticasein agar, 25g Reef salt mix); **AIM8** ([per liter]: 10g Bactopeptone,

5g Yeast extract, 16g agar, 25g Reef salt mix); **AIM9** ([per liter]: 100µl humic acid, 0.02 g CaCO3, 0.5 g Na2HPO4, 0.5 g MgSO4, 1.7 g KCl, 0.01 g FeSO4, 0.5 mg Vitamin B12, 18g agar, 25g Reef salt mix).

All isolation media were autoclave-sterilized and supplemented with 0.20 µm pore size filtered Nystatin (100 µg/ml) to inhibit fungal growth, nalidixic acid (50µg/ml) to inhibit gram-negative bacteria growth and to favor the growth of slow-growing Actinobacteria.

Prepared media were used for primary selective isolation of Actinobacteria by plating 150 µl directly from fresh samples, and using sterile 3mm glass beads. Inoculated plates were incubated at 27 C for 1-6 weeks. Isolates were selected based on colony morphology and Gram stain, picked and re-streaked several times to obtain pure cultures. Isolates were maintained on AIM1 and AIM6 agar plates for short-term storage, and long-term strain collections were set up in 50% glycerol and preserved at −20°C (sporulated) and −80°C (non-sporulated).

Nucleic acid extraction

To confirm Actinobacteria identity and further phylogenetic analysis of isolates, after testing several techniques, genomic DNA was prepared using a modified phenol/ chloroform method that yielded the best quality DNA for our isolates: colonies of putative Actinobacteria were carefully scraped from agar plates and placed in centrifuge tubes; cell pellets were washed 2× 10mL of 10% (w/v) with sucrose and resuspended in 400µl of lysis solution (4% Triton x-100, 20% SDS, 5M NaCl, 2M Tris-HCl pH 8, 500mM EDTA pH 8). After resuspension, 400 µl of Phenol/Chloroform and 0.1mm glass beads were added to lysis mix and this was mechanically disrupted for 2 minutes. The lysates were centrifuged (12,000 x rpm, 15 min) and DNA in aqueous phase was precipitated with 2 volumes of ethanol and 1/10 volume of 3M sodium

192 acetate, pH 5.2; after overnight incubation at -20 °C, DNA was centrifuged (12,000 x rpm, 10
193 min at 4°C), washed with 70% ethanol and eluted in TE with RNase.

194 **Molecular Identification and Phylogenetic Analysis**

195 Genomic DNA from putative Actinobacteria was sent to Macrogen, Inc., USA, to perform 16S
196 rDNA gene amplification by PCR and sequencing using the universal primers 27F (5′-
197 GAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), as
198 well as phylum-specific primers: S-C-Act-235-a-S-20 (5′CGCGGCCTATCAGCTTGTTG-3′)
199 (Stach, Maldonado, Ward 2003) and 23SR (5′-AGGCATCCACCGTGCGCCCT3′) (Yoon, Lee,
200 Kim 1997).

201 The 16S rDNA gene sequences were edited and assembled using CodonCode Aligner 5.1
202 software (CodonCode Corporation, Dedham, MA); assembled contigs were compared to 16S
203 rDNA gene sequences in the NCBI database (http: //www.ncbi.nlm.nih.gov/) using the Basic
204 Local Alignment Search Tool (BLAST) to determine genus-level affiliations and are deposited in
205 GenBank, which is associated with this document and are also available as Supplementary
206 Material.

207 Our 16S rDNA gene sequences sharing a phylogenetic affiliation with Actinobacteria and
208 reference sequences were aligned with ClustalW (Higgins 1994) using Molecular Evolutionary
209 Genetics Analysis MEGA Version 7 (Kumar, Stecher & Tamura 2016).

210 Phylogenetic reconstructions were performed to analyze CCB OTUs clustering and
211 taxonomic identification of the isolates in an evolutionary context. The phylogenetic tree of total
212 Actinobacterial isolates was constructed by Maximum Likelihood (ML) algorithm using MEGA
213 software v. 7 (Kumar, Stecher & Tamura 2016) and Tamura–Nei I+G (Tamura 1992) parameter

as an evolutionary model with 1,000 replicates. For a more comprehensive interpretation of results, 16S sequences of previously characterized species of Actinobacteria with closest affiliations to our isolates, were obtained from GenBank databases and added to reconstructions of this Phylum. Criteria for selection of reference sequences was based on similarity and length of nucleotide sequences, but also, the selection of 16S sequences from study model organisms (such as *S. coelicolor)* and also microorganisms originally isolated from water and sediments from aquatic environments. Other reference strains were added to provide biological interpretation, and were selected from previous work reporting isolation of *Streptomyces* from deserts (Okoro, Brown, Jones 2009; Rateb et al., 2011). Model selection was performed using statistical and evolutionary analysis of multiple sequence alignments TOPALi v2 (Milne, Lindner, Bayer 2009).

Abundance and diversity were clearly remarkable for *Streptomyces.* From these early observations, we decided to compare distances between our *Streptomyces* isolates, to available information from previous studies, so we included a dataset of 667 16S-rDNA sequences of validated species of *Streptomyces;* most of them were selected for a wide phylogenetic analysis within the family (Labeda, Goodfellow, Brown 2012; Labeda, Dunlap, Rong 2017). We first performed a phylogenetic reconstruction using parameters and conditions reported by Labeda, et. al., 2012. Obtaining a preliminary Neighbour Joining (NJ) tree and leading us to the identification of relevant information regarding evolutionary relationships as well as the extent of the isolated diversity. It also provided criteria for selection of ideal reference strains for a later, more stringent analysis.

To reconstruct a second phylogenetic tree of the members of family Streptomycetaceae, we used the Maximum-likelihood (ML) method using MEGA software v. 7 and the Tamura–Nei

I + G parameter as an evolutionary model. The reliability of nodes was estimated by ML bootstrap percentages (Felsenstein 1985) obtained after 1,000 replications. A total of 41 16S sequences obtained in this study were included, and 73 reference strains belonging to the genera *Streptomyces,* 6 of *Kitasatospora* and 3 *Streptoacidophilus,* which were the most closely related to our isolates, were selected (trimmed to 1074 bp).

To provide support to ML tree, we conducted a Bayesian analysis employing MrBayes v3.2.5 (Ronquist, Teslenko, van der Mark 2012) with 10,000,000 Markov chain Monte Carlo generations and the GTR+ G model of evolution with a nucmodel= 4by4, nruns = 2, nchains = 4, and sampled freq = 100. The average standard deviation of split frequences was below 0.001. The nodes that had posterior probabilities greater than 95 % (Bayesian), were considered well-supported and were shown in the resulting tree.

Estimation of diversity of Actinobacteria in CCB

To estimate species richness in the IL in the Churince system, we performed a rarefaction analysis for total Actinobacteria isolates, and another for only *Streptomyces* isolates. The definition of operational taxonomic units (OTUs) was conducted with MEGA software v. 7 at 97% cutoff according to their pairwise distances. Then we conducted the rarefaction curve using the EstimateS 9.1.0 software package (Colwell & Elsensohn 2014) at the 95% confidence level.

RESULTS

Diversity of culturable Actinobacteria within the Churince system in CCB

A total of 350 morphologically and nutritionally diverse isolates were successfully cultured and characterized as members of the Phylum Actinobacteria throughout the three-year period. AIM2 and AIM4 were the best nutrient conditions for culturing Actinomycetes (Figure 2). Soy flour and mannitol-based medium allowed an isolation of 5 different genera of Actinobacteria and the greatest number of total isolates. The genus *Streptomyces* was found to be the most abundant taxa, accounting for over 50% of total sequenced isolates.

Diversity of cultured Actinobacteria varied in relation to sampling sites within the Churince. Among all sampling sites, C was the location where we found the highest diversity and abundance of *Streptomyces* strains. Only *Streptomyces* was ubiquitous in Churince IL and through the seasons, while isolation of the other 10 genera showed fluctuations.

From the entire isolated collection, 105 strains were successfully subcultured, processed for DNA extraction and 16S-rDNA sequenced (Supplementary Table 1). These strains belong to the order Actinomycetales, and to suborders Corynebacterineae, Pseudonocardineae, Streptosporangineae, Frankineae, Streptomycineae, Micromonosporineae, Glycomycineae, and Micrococcineae, encompassing 11 genera of Actinobacteria. For phylogenetic analysis, a radial tree is presented in supplementary material (Supp. Fig.1) showing the extent of macrodiversity of the genera of Actinobacteria retrieved from CCB.

Two rarefaction curves showed that the potentially yet-to-be-cultured diversity at both taxonomic levels (Actinobacteria phylum and *Streptomyces* genus) is large (Figure 3) in fact, far higher than the 30 and 12 OTUs for Actinobacteria and *Streptomyces* respectively, defined with a 97% cutoff according to their pairwise distances of the 16S-rDNA sequences, as seen by the curves, which are far from reaching the asymptote.

High diversity and phylogenetic clustering of *Streptomyces* from Cuatro Cienegas.

Primary isolation plates were enriched with *Streptomyces*-like colonies in every sampling culture, with characteristic morphologies and geosmin-like odor. *Streptomyces* isolates account for 54% of the total sequenced isolates and since this genus was the most abundant in all media, sampling site and season, we first characterized these isolates based on their morphology to avoid picking clonal individuals for later DNA sequencing. Morphologies and other culture-related phenotypes varied among all selected individuals throughout the process of subculturing, such as colony morphology, pigment production, colony sporulation, optimal growth temperature and growth rate. Some of the different colony morphologies in *Streptomyces* are shown in Figure 4.

A preliminary phylogenetic reconstruction of the family Streptomycetaceae was performed using isolates from this study and a dataset of 667 16S-rDNA sequences from *Streptomyces* previously used for a broad phylogenetic analysis within the family Streptomycetaceae (Labeda et al., 2012) (Supplementary Material Fig. 2). The analysis shows that numerous CCB isolates are closer to each other and separated along the tree topology from most reference organisms. To construct a summarized and well-supported phylogenetic analyses, two different methods were used (Bayesian and ML), including 95 close reference strains, as well as sequences from isolates from the Atacama Desert and other ecologically similar isolates (Figure 5). In this summarized analysis, we can unambiguously identify six novel monophyletic clades with 31 new OTUs and 7 single-member clusters, all of them isolated in the present study.

DISCUSSION

Actinobacteria from oligotrophic CCB are diverse and abundant.

Several different culture media were defined and applied for maximum recovery of culturable Actinobacteria in this study over a 3-year period, including different seasons. From this effort, 350 morphologically diverse isolates of Actinobacteria within the Churince system, were successfully cultured making a large, valuable, indigenous collection of different cultivated morphologies within one particular site. Nevertheless, due to well-known difficulties in genotyping this phylum (Yoon et al., 1997; Stach et al., 2003; Farris & Olson 2007; Kumar, Aiemsum-Ang, Ward 2007), we were able to extract DNA and sequence 16S-rDNA of only 105 of them. In light of our observations of the abundance and uniqueness of the 16S sequence of the *Streptomyces* from the CCB and the reported biases from other studies in Actinobacteria (Hansen, Tolker-Nielsen, Givskov 1998; Farris & Olson 2007; Krogius-Kurikka, Kassinen, Paulin 2009; Rajendhran & Gunasekaran 2011), it is not difficult to speculate that this group of microorganisms would require a different approach for a detailed characterization, such as whole-genome analysis of culturable strains. Ongoing work in our research group is applying this strategy for the most peculiar strains of our collection.

Although gram-positive bacteria are more commonly observed in organic rich habitats (Fenical 1993), isolated strains from the extremely oligotrophic Churince IL encompass 11 genera of Actinobacteria (Figure 2), which is comparable to the culturable diversity found in rich marine environments (Duncan et al., 2015; Duran et al., 2015; Kuang et al., 2015; Chen et al., 2016; Undabarrena et al., 2016). Interestingly, *Streptomyces* was the most abundant taxa, representing over 50% of the total sequenced isolates varying in relation to sampling point within the Churince system (figure 2). This result is comparable to the *Streptomyces*-enriched isolation in extreme environments such as the Atacama Desert (Okoro et al., 2009), nonetheless CCB culturable diversity within the Phylum Actinobacteria is greater.

328 CCB culturable *Streptomyces* diversity is still far from being exhaustively explored as
329 shown by rarefaction analysis (figure 3), suggesting a complex community structure, both in
330 sediment and in the water column.
331 Morphological and genetic diversity of this phylum in the Churince does not come totally
332 as a surprise since in concurrent studies using Illumina16S rRNA tags (Souza et al., in review) it
333 was observed that Actinobacteria are the most successful lineage in CCB water, with notable
334 presence of genera *Streptomyces, Yaniella, Arthrobacter, Trueperella*, as well as several putative
335 Actinobacteria from non-culturable marine lineages, in particular a strain closely related to the
336 marine PeM15, which is very sensitive to nutrient enrichment (Lee et al., submitted) and other
337 clades unique to soil and sediment. These analyses are consistent with our isolation efforts,
338 which yielded abundant and diverse *Streptomyces* and abundant *Arthrobacter* isolates. It is
339 possible to speculate that those several putative non-culturable Actinobacteria lineages detected
340 by Illumina in concurrent projects, relate to our great numbers of cultured isolates which were
341 not able to be detected by universal and phylum-specific primers.
342 Many interesting morphotypes could not be identified using 16S rDNA sequences, and in
343 addition, many were lost as the purification of a single colony proceeded. Success at bringing the
344 environment into the laboratory culture is not sufficient for successful cultivability of bacteria.
345 Subsequent culturing of Actinomycetes to obtain axenic (pure) cultures from the Churince,
346 dramatically reduced the total number of unique pure isolates, suggesting obligate mutualism and
347 cross-feeding (Tanaka, Hanada, Manome 2004; Kim, Kim, Masui 2011; Seth & Taga 2014).
348 It is quite interesting to observe that previous bacterial isolation efforts in the IL of the
349 Churince in the CCB, using a culture-dependent approach initially based on thermo-resistant
350 aquatic strains, did not lead to the isolation of *Streptomyces* individuals among the numerous

isolated Actinobacteria (Cerritos et al., 2011). Many variables can play a role in this marked difference, most probably the different culture methods of Cerritos et al. (2011) through which thermoresistant bacteria in Marine Agar media were selected, thus enriching the isolation of Micrococcineae members. In contrast, our study applied several media with different carbon and nitrogen sources to maximize the possibility of culturing a wider diversity. Even so, the rarefaction curve shows that the potentially yet-to-be-cultured diversity is large (Figure 3), as commonly occurs in highly diverse communities (Colwell, Mao & Chang 2004; Colwell & Elsensohn 2014).

Another possible factor that could explain differences between our study and Cerritos et al. (2011) is the years which passed between sampling periods, including possible temporal variation in the community structure. Notably in the CCB, after the time of the initial isolations described in Cerritos et al. (2011), a decline of the Churince aquifer occurred. As shown in experiments with UV and temperature increase in mesocosms (Pajares, Eguiarte, Bonilla-Rosso 2013; Pajares, Souza & Eguiarte 2015), endemic CCB Actinobacteria are particularly susceptible to perturbation. Hence, it is possible that enrichment of *Streptomyces* after 2010 is a succession response to the shrinkage and concomitant changes in the Churince aquifer system.

Endemicity of* Streptomyces *in CCB

As expected from previous studies finding endemic microorganisms at CCB (Alcaraz et al., 2008; Rebollar et al., 2012), we found 38 unique operational taxonomic units (OTU´s) for *Streptomyces*. Moreover, these 38 novel OTUs are in six new monophyletic clades in a deeply represented and well-supported phylogeny of the family Streptomycetaceae, which is a sign of endemicity. What makes this result unprecedented in a relatively very well-known cosmopolitan

374 genus, *Streptomyces* (Barka et al., 2016), is the discovery of this degree of diversity and
375 endemism in such an oligotrophic extreme environment.
376 Even though these data do not represent evidence of dispersal limitation *per se*, the phylogenetic
377 clustering of OTUs of the CCB among themselves, and the genetic distance between OTUs from
378 667 reported species of Streptomycetaceae family from other sites around the world (Fig. 5 and
379 Supplementary Fig. 2), could be explained by migration limitation to and out of the CCB.
380

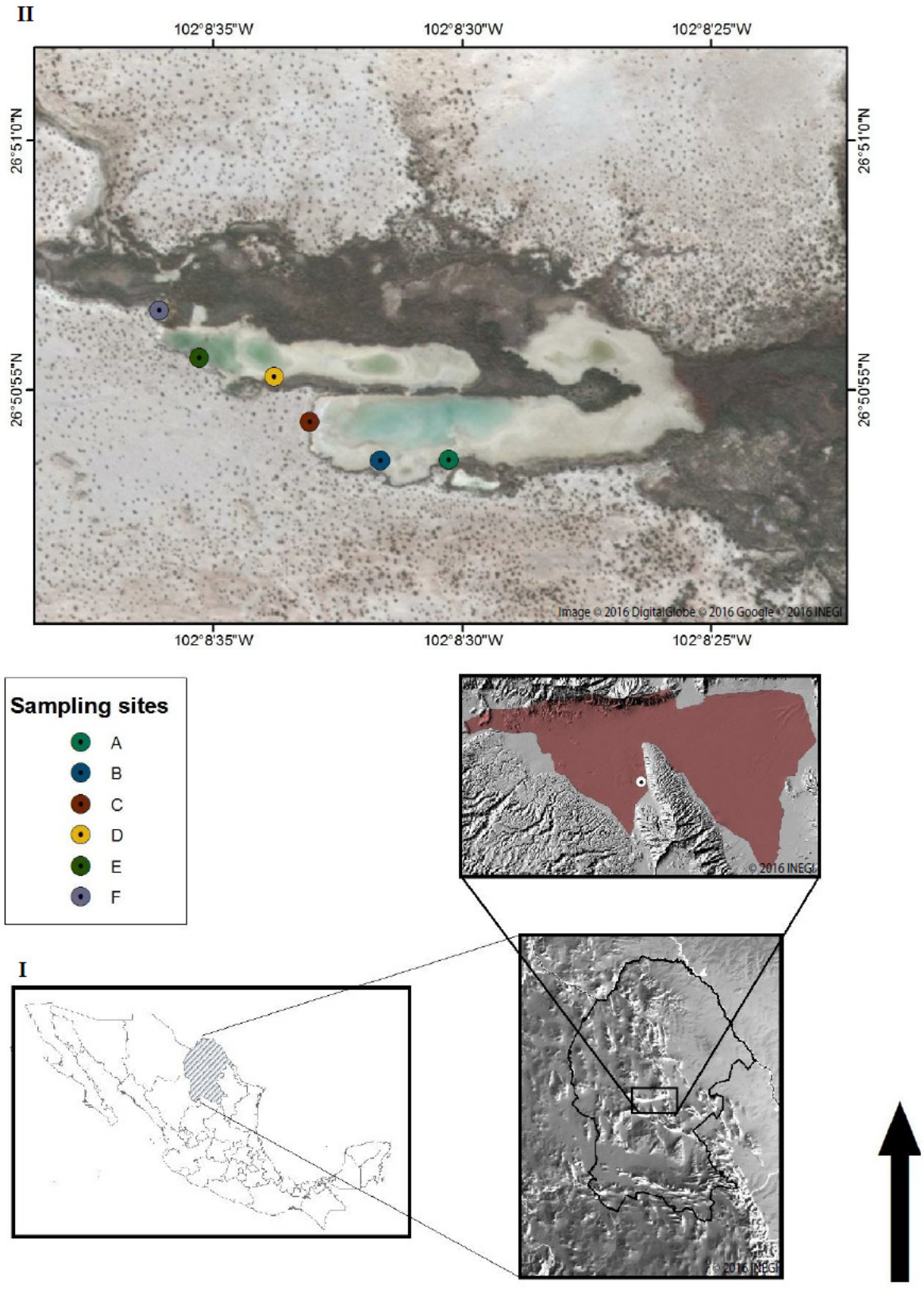
381 ***Relevance of culturing new Actinobacteria strains and lineages***
382 Only a tiny fraction of the universal bacterial diversity has been pure cultured (Pace 2009), and
383 with this, the description of the biological diversity of the prokaryotic branch of the tree of life
384 remains limited. Moreover, as culturable Actinobacteria diversity available for the study and
385 characterization has been still insufficient when searching for bioactive compounds, there has
386 been an increasing urge to culture untapped diversity within under-explored habitats (Katz &
387 Baltz 2016).
388 While genome mining represents a major paradigm shift for exploration of rare taxa
389 (Cano-Prieto, Garcia-Salcedo, Sanchez-Hidalgo 2015; Tang, Liu, Peng 2015; Iftime, Kulik,
390 Hartner 2016; Smanski, Schlatter & Kinkel 2016), recent studies from genome mining for
391 secondary metabolites gene clusters of unculturable Actinobacteria support the culturable
392 approach for natural product discovery targeting “gifted microbes”, obtaining samples from
393 unexplored habitats. In particular, untapped marine sediments are recommended when searching
394 for cultivable potentially bioactive natural products from Actinobacteria (Baltz 2016).
395 Although clades and clusters of CCB-isolates along the phylogeny might suggest that
396 OTUs within the same groups are very close to each other, figure 4 shows distinctive

morphologies that clearly reflect the uniqueness of each isolate. Hence, this collection of Actinobacteria from Cuatro Cienegas represents an invaluable source of great diversity for microbial ecology and biotechnology studies considering that: *i)* phylogenies constructed with the sequenced portion of our collection indicate six novel clades of *Streptomyces,* but they only represent a third of the successfully cultured collection; *ii)* this collection has been isolated from an environment of a diversity and endemicity, that has previously been considered comparable to that in the Galápagos Island (Souza et al., 2012), and as revealed by our six clades cointaining only CCB isolates (Fig. 5), it is quite likely that we have cultured several unique species yet to be described; *iii)* the great diversity shown here has been calculated using the conserved 16S rDNA marker, but it is well known that single-gene phylogenies might not always reflect the evolutionary history of a species due to the high degree of horizontal gene transfer (Marri, Hao & Golding 2006), a phenomenon particularly common in *Streptomyces* (Huguet-Tapia, Lefebure, Badger 2016; Tian, Zhang, Yang 2016).

In conclusion, we can mention that our findings suggest a very high, albeit still uncalculated richness in microbial diversity in CCB, as well as suggested endemism. Our main result show that the CCB is not only a special place to study community structure where Actinobacteria diversity plays a major ecological role in such an oligotrophic environment, but it also represents a promising area for bioprospecting studies that will require concerted long-term efforts to search for genuine and substantial contributions to the discovery of natural products.

420

421 **FIGURES**


II
102°8'35"W
102°8'30"W
102°8'25"W
26°51'0"N
26°50'55"N
Image © 2016 DigitalGlobe © 2016 Google © 2016 INEGI
Sampling sites
A
B
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© 2016 INEGI
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Figure 1. The Churince hydrological system. (I) Map of Mexico displaying the State of Coahuila and the location of the Cuatro Cienegas Basin (CCB) and the Churince hydrological system (circle) © 2016 INEGI. (II) Aerial view of the intermediate lagoon (IL) in the Churince hydrological system. The circular forms point out the sampling sites. Image © 2016 DigitalGlobe © 2016 Google © 2016 INEGI.

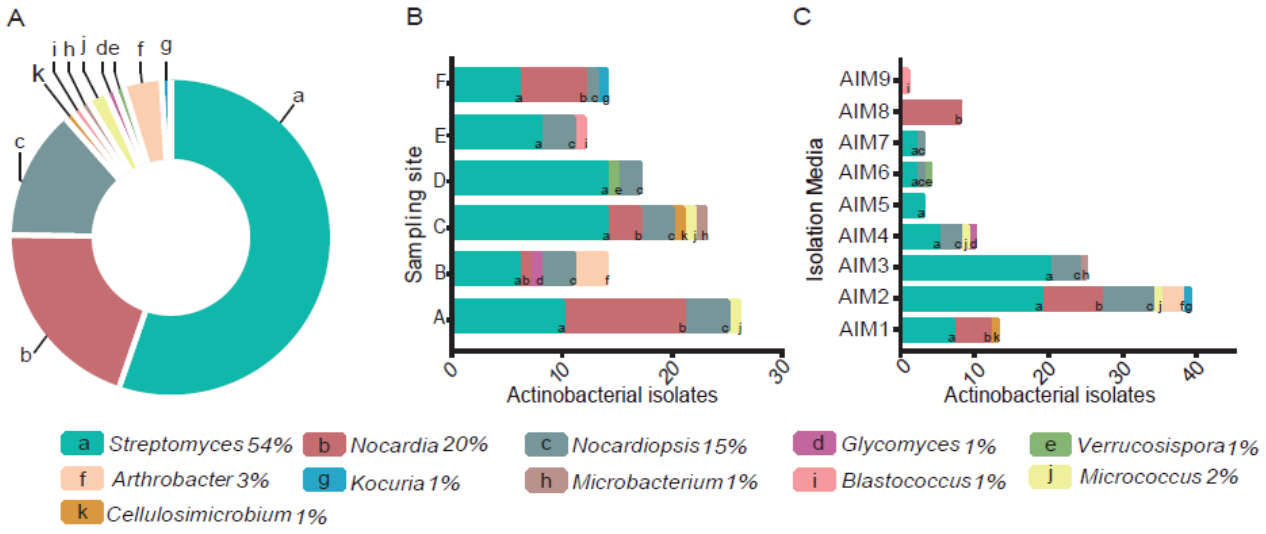

Figure 2. (A) Pie chart of the percentage of Actinobacteria genera isolated from the intermediate lagoon in Churince system. (B) Number of Actinobacteria isolated according to the sampling sites. (C) Number of Actinobacterial isolated according to the culture media used.

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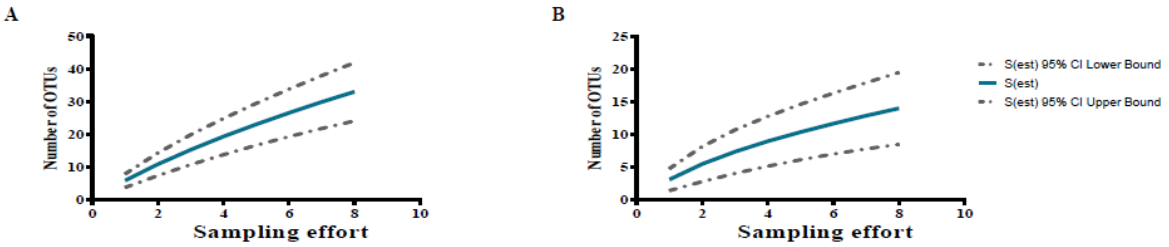

443

444 Figure 3. Rarefaction curves show sampling effort on the estimation of the numbers of OTUs at
445 97% sequence identity from cultured Actinobacteria (A), and total isolated *Streptomyces* (B)
446 from CCB.

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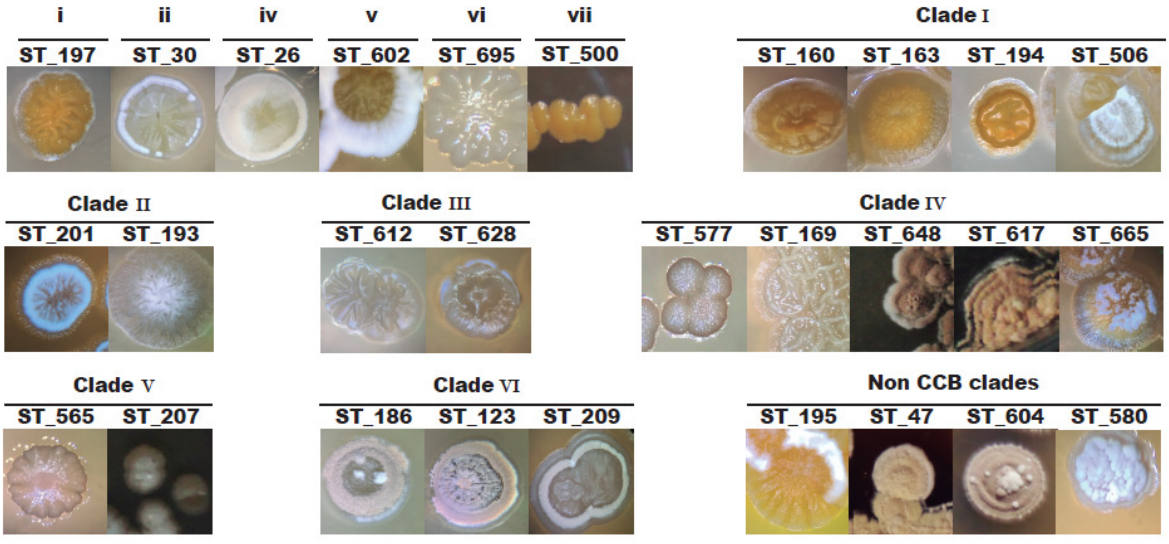

452

453 Figure 4. Colony morphological diversity of *Streptomyces* isolated from CCB within clades.

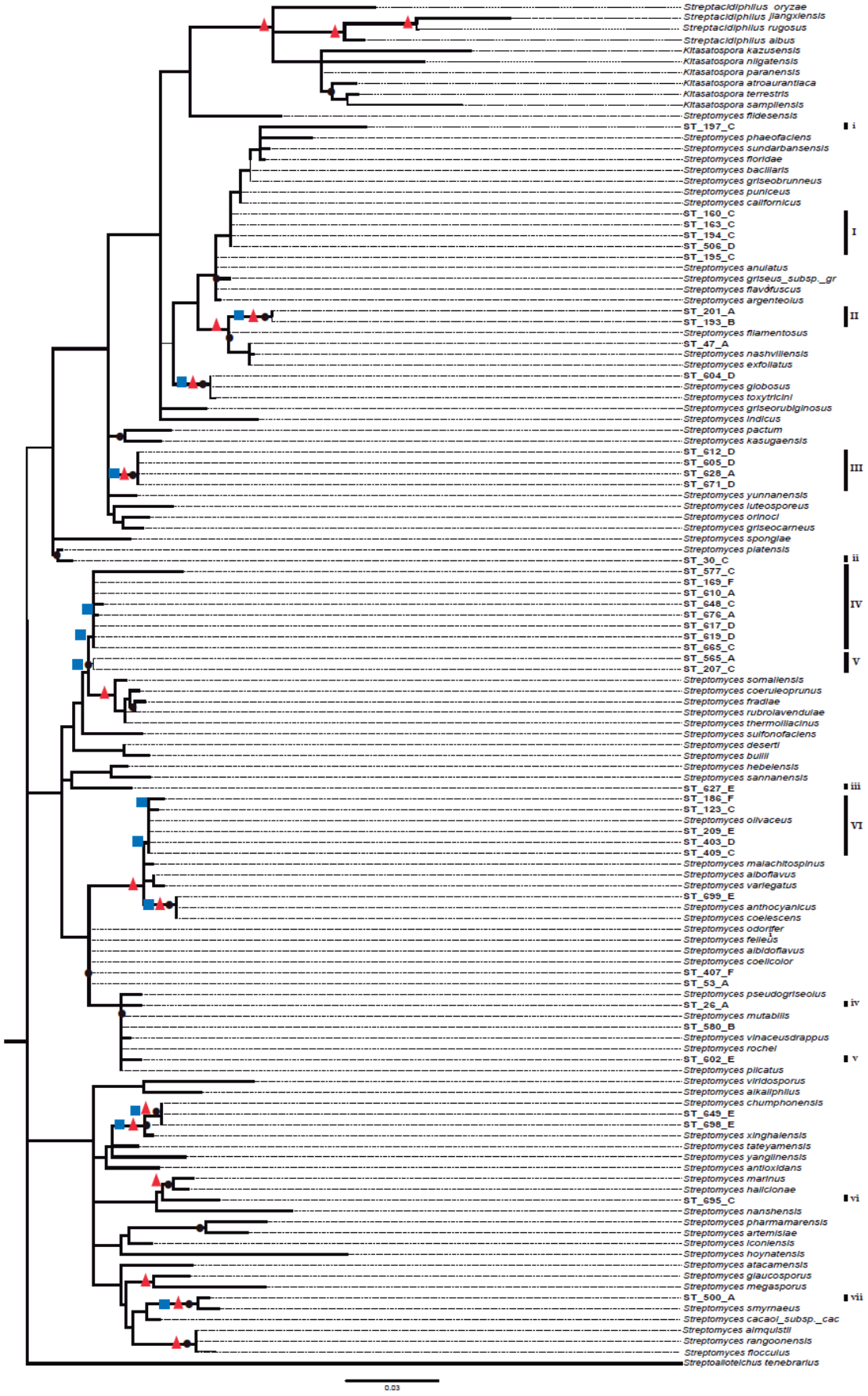
Streptacidiphilus oryzae
Streptacidiphilus jiangxiensis
Streptacidiphilus rugosus
Streptacidiphilus albus
Kitasatospora kazusensis
Kitasatospora niigatensis
Kitasatospora paranensis
Kitasatospora atroaurantiaca
Kitasatospora terrestris
Kitasatospora samplensis
Streptomyces flidesensis
ST_197_C
i
Streptomyces phaeofaciens
Streptomyces sundarbansensis
Streptomyces floridae
Streptomyces bacillaris
Streptomyces griseobrunneus
Streptomyces puniceus
Streptomyces californicus
ST_160_C
ST_163_C
ST_194_C
ST_506_D
ST_195_C
I
Streptomyces anulatus
Streptomyces griseus_subsp._gr
Streptomyces flavofuscus
Streptomyces argenteolus
ST_201_A
ST_193_B
II
Streptomyces filamentosus
ST_47_A
Streptomyces nashvillensis
Streptomyces exfoliatus
ST_604_D
Streptomyces globosus
Streptomyces toxytricini
Streptomyces griseorubiginosus
Streptomyces indicus
Streptomyces pactum
Streptomyces kasugaensis
ST_612_D
ST_605_D
ST_628_A
ST_671_D
III
Streptomyces yunnanensis
Streptomyces luteosporeus
Streptomyces orinoci
Streptomyces griseocarneus
Streptomyces spongiae
Streptomyces platensis
ST_30_C
ii
ST_577_C
ST_169_F
ST_610_A
ST_648_C
ST_676_A
ST_617_D
ST_619_D
ST_665_C
IV
ST_565_A
ST_207_C
V
Streptomyces somaliensis
Streptomyces coeruleoprunus
Streptomyces fradiae
Streptomyces rubrolavendulae
Streptomyces thermolilacinus
Streptomyces sulfonofaciens
Streptomyces deserti
Streptomyces bullii
Streptomyces hebeiensis
Streptomyces sannanensis
ST_627_E
iii
ST_186_F
ST_123_C
Streptomyces olivaceus
ST_209_E
ST_403_D
ST_409_C
VI
Streptomyces malachitospinus
Streptomyces alboflavus
Streptomyces variegatus
ST_699_E
Streptomyces anthocyanicus
Streptomyces coelescens
Streptomyces odorifer
Streptomyces felleus
Streptomyces albidoflavus
Streptomyces coelicolor
ST_407_F
ST_53_A
Streptomyces pseudogriseolus
ST_26_A
iv
Streptomyces mutabilis
ST_580_B
Streptomyces vinaceusdrappus
Streptomyces rochei
ST_602_E
v
Streptomyces plicatus
Streptomyces viridosporus
Streptomyces alkaliphilus
Streptomyces chumphonensis
ST_649_E
ST_698_E
Streptomyces xinghaiensis
Streptomyces tateyamensis
Streptomyces yanglinensis
Streptomyces antioxidans
Streptomyces marinus
Streptomyces hallclonae
ST_695_C
vi
Streptomyces nanshensis
Streptomyces pharmamarensis
Streptomyces artemisiae
Streptomyces iconiensis
Streptomyces hoynatensis
Streptomyces atacamensis
Streptomyces glaucosporus
Streptomyces megasporus
ST_500_A
vii
Streptomyces smyrnaeus
Streptomyces cacaoi_subsp._cac
Streptomyces almquistii
Streptomyces rangoonensis
Streptomyces floculus
Streptoalloteichus tenebrarius
0.03

Figure 5. Phylogenetic tree of Streptomycetaceae family based on nearly full-lenght 16s rRNA gene sequences and their closely related type strains based on the maximum likelihood (ML) method, constructed by Tamura–Nei I + G evolutionary model with 1000 bootstrap replicates. Bootstrap values for ML in the range from 0.7 to 1 were marked with black circles. Bayesian supports at nodes in ranges 0.95 to 1 were marked with a red triangles and Bootstrap values for neighbor-joining at ranges 0.6 to 1 in blue squares.

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Bibliography

Alcaraz, L. D., G. Olmedo, G. Bonilla, R. Cerritos, G. Hernandez, A. Cruz, E. Ramirez, C. Putonti, B. Jimenez, E. Martinez, V. Lopez, J. L. Arvizu, F. Ayala, F. Razo, J. Caballero, J. Siefert, L. Eguiarte, J. P. Vielle, O. Martinez, V. Souza, A. Herrera-Estrella and L. Herrera-Estrella (2008). "The genome of Bacillus coahuilensis reveals adaptations essential for survival in the relic of an ancient marine environment." *Proc Natl Acad Sci U S A* 105(15): 5803-5808 DOI: 10.1073/pnas.0800981105.

Allen, M. A., F. Goh, B. P. Burns and B. A. Neilan (2009). "Bacterial, archaeal and eukaryotic diversity of smooth and pustular microbial mat communities in the hypersaline lagoon of Shark Bay." *Geobiology* 7(1): 82-96 DOI: 10.1111/j.1472-4669.2008.00187.x.

Andam, C. P., J. R. Doroghazi, A. N. Campbell, P. J. Kelly, M. J. Choudoir and D. H. Buckley (2016). "A Latitudinal Diversity Gradient in Terrestrial Bacteria of the Genus Streptomyces." *MBio* 7(2): e02200-02215 DOI: 10.1128/mBio.02200-15.

Antony-Babu, S., J. E. Stach and M. Goodfellow (2008). "Genetic and phenotypic evidence for Streptomyces griseus ecovars isolated from a beach and dune sand system." *Antonie Van Leeuwenhoek* 94(1): 63-74 DOI: 10.1007/s10482-008-9246-y.

Avitia, M., A. E. Escalante, E. A. Rebollar, A. Moreno-Letelier, L. E. Eguiarte and V. Souza (2014). "Population expansions shared among coexisting bacterial lineages are revealed by genetic evidence." *PeerJ* 2: e696 DOI: 10.7717/peerj.696.

Baltz, R. H. (2016). "Gifted microbes for genome mining and natural product discovery." *J Ind Microbiol Biotechnol* DOI: 10.1007/s10295-016-1815-x.

Barka, E. A., P. Vatsa, L. Sanchez, N. Gaveau-Vaillant, C. Jacquard, H. P. Klenk, C. Clement, Y. Ouhdouch and G. P. van Wezel (2016). "Taxonomy, Physiology, and Natural Products of Actinobacteria." *Microbiol Mol Biol Rev* 80(1): 1-43 DOI: 10.1128/MMBR.00019-15.

Cano-Prieto, C., R. Garcia-Salcedo, M. Sanchez-Hidalgo, A. F. Brana, H. P. Fiedler, C. Mendez, J. A. Salas and C. Olano (2015). "Genome Mining of Streptomyces sp. Tu 6176: Characterization of the Nataxazole Biosynthesis Pathway." *Chembiochem* 16(10): 1461-1473 DOI: 10.1002/cbic.201500153.

Cerritos, R., L. E. Eguiarte, M. Avitia, J. Siefert, M. Travisano, A. Rodriguez-Verdugo and V. Souza (2011). "Diversity of culturable thermo-resistant aquatic bacteria along an environmental gradient in Cuatro Cienegas, Coahuila, Mexico." *Antonie Van Leeuwenhoek* 99(2): 303-318 DOI: 10.1007/s10482-010-9490-9.

Chen, P., L. Zhang, X. Guo, X. Dai, L. Liu, L. Xi, J. Wang, L. Song, Y. Wang, Y. Zhu, L. Huang and Y. Huang (2016). "Diversity, Biogeography, and Biodegradation Potential of Actinobacteria in the Deep-Sea Sediments along the Southwest Indian Ridge." *Front Microbiol* 7: 1340 DOI: 10.3389/fmicb.2016.01340.

Claverias, F. P., A. Undabarrena, M. Gonzalez, M. Seeger and B. Camara (2015). "Culturable diversity and antimicrobial activity of Actinobacteria from marine sediments in Valparaiso bay, Chile." *Front Microbiol* 6: 737 DOI: 10.3389/fmicb.2015.00737.

Coghill, L. M., C. D. Hulsey, J. Chaves-Campos, F. J. Garcia de Leon and S. G. Johnson (2013). "Phylogeography and conservation genetics of a distinct lineage of sunfish in the Cuatro Cienegas valley of Mexico." *PLoS One* 8(10): e77013 DOI: 10.1371/journal.pone.0077013.

Colwell, R. K. and J. E. Elsensohn (2014). "EstimateS turns 20: statistical estimation of species richness and shared species from samples, with non-parametric extrapolation." *Ecography* 37(6): 609-613 DOI: 10.1111/ecog.00814.

Colwell, R. K., C. X. Mao and J. Chang (2004). "Interpolating, extrapolating, and comparing incidence-based species accumulation curves." *Ecology* 85(10): 2717-2727 DOI: 10.1890/03-0557.

Coombs, J. T. and C. M. Franco (2003). "Isolation and identification of actinobacteria from surface-sterilized wheat roots." *Appl Environ Microbiol* 69(9): 5603-5608.

Davelos, A. L., K. Xiao, D. A. Samac, A. P. Martin and L. L. Kinkel (2004). "Spatial variation in Streptomyces genetic composition and diversity in a prairie soil." *Microb Ecol* 48(4): 601-612 DOI: 10.1007/s00248-004-0031-9.

Duncan, K. R., B. Haltli, K. A. Gill, H. Correa, F. Berrue and R. G. Kerr (2015). "Exploring the diversity and metabolic potential of actinomycetes from temperate marine sediments from Newfoundland, Canada." *J Ind Microbiol Biotechnol* 42(1): 57-72 DOI: 10.1007/s10295-014-1529-x.

Duran, R., A. Bielen, T. Paradzik, C. Gassie, E. Pustijanac, C. Cagnon, B. Hamer and D. Vujaklija (2015). "Exploring Actinobacteria assemblages in coastal marine sediments under contrasted Human influences in the West Istria Sea, Croatia." *Environ Sci Pollut Res Int* 22(20): 15215-15229 DOI: 10.1007/s11356-015-4240-1.

Elser, J. J., J. H. Schampel, F. Garcia-Pichel, B. D. Wade, V. Souza, L. Eguiarte, A. N. A. Escalante and J. D. Farmer (2005). "Effects of phosphorus enrichment and grazing snails on modern stromatolitic microbial communities." *Freshwater Biology* 50(11): 1808-1825 DOI: 10.1111/j.1365-2427.2005.01451.x.

Escalante, A. E., J. Caballero-Mellado, L. Martinez-Aguilar, A. Rodriguez-Verdugo, A. Gonzalez-Gonzalez, J. Toribio-Jimenez and V. Souza (2009). "Pseudomonas cuatrocienegasensis sp. nov., isolated from an evaporating lagoon in the Cuatro Cienegas valley in Coahuila, Mexico." *Int J Syst Evol Microbiol* 59(Pt 6): 1416-1420 DOI: 10.1099/ijs.0.006189-0.

Farris, M. H. and J. B. Olson (2007). "Detection of Actinobacteria cultivated from environmental samples reveals bias in universal primers." *Lett Appl Microbiol* 45(4): 376-381 DOI: 10.1111/j.1472-765X.2007.02198.x.

Felsenstein, J. (1985). "Confidence Limits on Phylogenies: An Approach Using the Bootstrap." *Evolution* 39(4): 783-791 DOI: 10.2307/2408678.

Fenical, W. (1993). "Chemical studies of marine bacteria: developing a new resource." *Chemical Reviews* 93(5): 1673-1683 DOI: 10.1021/cr00021a001.

Gonzalez-Gonzalez, A., L. L. Sanchez-Reyes, G. Delgado Sapien, L. E. Eguiarte and V. Souza (2013). "Hierarchical clustering of genetic diversity associated to different levels of mutation and recombination in Escherichia coli: a study based on Mexican isolates." *Infect Genet Evol* 13: 187-197 DOI: 10.1016/j.meegid.2012.09.003.

Goodfellow, M. and S. T. Williams (1983). "Ecology of actinomycetes." *Annu Rev Microbiol* 37: 189-216 DOI: 10.1146/annurev.mi.37.100183.001201.

Gremion, F., A. Chatzinotas and H. Harms (2003). "Comparative 16S rDNA and 16S rRNA sequence analysis indicates that Actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil." *Environ Microbiol* 5(10): 896-907.

Grossart, H. P., A. Schlingloff, M. Bernhard, M. Simon and T. Brinkhoff (2004). "Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea." *FEMS Microbiol Ecol* 47(3): 387-396 DOI: 10.1016/S0168-6496(03)00305-2.

Hansen, M. C., T. Tolker-Nielsen, M. Givskov and S. Molin (1998). "Biased 16S rDNA PCR ampli¢cation caused by interference from DNA flanking the template region " *FEMS Microbiology Ecology* 26 141-149.

Higgins, D. G. (1994). "CLUSTAL V: multiple alignment of DNA and protein sequences." *Methods Mol Biol* 25: 307-318 DOI: 10.1385/0-89603-276-0:307.

Huguet-Tapia, J. C., T. Lefebure, J. H. Badger, D. Guan, G. S. Pettis, M. J. Stanhope and R. Loria (2016). "Genome Content and Phylogenomics Reveal both Ancestral and Lateral Evolutionary Pathways in Plant-Pathogenic Streptomyces Species." *Appl Environ Microbiol* 82(7): 2146-2155 DOI: 10.1128/AEM.03504-15.

Iftime, D., A. Kulik, T. Hartner, S. Rohrer, T. H. Niedermeyer, E. Stegmann, T. Weber and W. Wohlleben (2016). "Identification and activation of novel biosynthetic gene clusters by genome mining in the kirromycin producer Streptomyces collinus Tu 365." *J Ind Microbiol Biotechnol* 43(2-3): 277-291 DOI: 10.1007/s10295-015-1685-7.

Jami, M., M. Ghanbari, W. Kneifel and K. J. Domig (2015). "Phylogenetic diversity and biological activity of culturable Actinobacteria isolated from freshwater fish gut microbiota." *Microbiol Res* 175: 6-15 DOI: 10.1016/j.micres.2015.01.009.

Jensen, P. R., R. Dwight and W. Fenical (1991). "Distribution of actinomycetes in near-shore tropical marine sediments." *Appl Environ Microbiol* 57(4): 1102-1108.
Jensen, P. R. and C. Mafnas (2006). "Biogeography of the marine actinomycete Salinispora." *Environ Microbiol* 8(11): 1881-1888 DOI: 10.1111/j.1462-2920.2006.01093.x.
Johnson, S. G. (2005). "Age, phylogeography and population structure of the microendemic banded spring snail, Mexipyrgus churinceanus." *Mol Ecol* 14(8): 2299-2311 DOI: 10.1111/j.1365-294x.2005.02580.x.
Katz, L. and R. H. Baltz (2016). "Natural product discovery: past, present, and future." *J Ind Microbiol Biotechnol* 43(2-3): 155-176 DOI: 10.1007/s10295-015-1723-5.
Kim, K., J. J. Kim, R. Masui, S. Kuramitsu and M. H. Sung (2011). "A commensal symbiotic interrelationship for the growth of Symbiobacterium toebii with its partner bacterium, Geobacillus toebii." *BMC Res Notes* 4: 437 DOI: 10.1186/1756-0500-4-437.
Kinkel, L. L., D. C. Schlatter, K. Xiao and A. D. Baines (2014). "Sympatric inhibition and niche differentiation suggest alternative coevolutionary trajectories among Streptomycetes." *ISME J* 8(2): 249-256 DOI: 10.1038/ismej.2013.175.
Krogius-Kurikka, L., A. Kassinen, L. Paulin, J. Corander, H. Makivuokko, J. Tuimala and A. Palva (2009). "Sequence analysis of percent G+C fraction libraries of human faecal bacterial DNA reveals a high number of Actinobacteria." *BMC Microbiol* 9: 68 DOI: 10.1186/1471-2180-9-68.
Kuang, W., J. Li, S. Zhang and L. Long (2015). "Diversity and distribution of Actinobacteria associated with reef coral Porites lutea." *Front Microbiol* 6: 1094 DOI: 10.3389/fmicb.2015.01094.
Kuhn, E., A. S. Ichimura, V. Peng, C. H. Fritsen, G. Trubl, P. T. Doran and A. E. Murray (2014). "Brine assemblages of ultrasmall microbial cells within the ice cover of Lake Vida, Antarctica." *Appl Environ Microbiol* 80(12): 3687-3698 DOI: 10.1128/AEM.00276-14.
Kumar, S., G. Stecher and K. Tamura (2016). "MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets." *Mol Biol Evol* 33(7): 1870-1874 DOI: 10.1093/molbev/msw054.
Kumar, Y., P. Aiemsum-Ang, A. C. Ward and M. Goodfellow (2007). "Diversity and geographical distribution of members of the Streptomyces violaceusniger 16S rRNA gene clade detected by clade-specific PCR primers." *FEMS Microbiol Ecol* 62(1): 54-63 DOI: 10.1111/j.1574-6941.2007.00374.x.
Labeda, D. P., C. A. Dunlap, X. Rong, Y. Huang, J. R. Doroghazi, K. S. Ju and W. W. Metcalf (2017). "Phylogenetic relationships in the family Streptomycetaceae using multi-locus sequence analysis." *Antonie Van Leeuwenhoek* 110(4): 563-583 DOI: 10.1007/s10482-016-0824-0.
Labeda, D. P., M. Goodfellow, R. Brown, A. C. Ward, B. Lanoot, M. Vanncanneyt, J. Swings, S. B. Kim, Z. Liu, J. Chun, T. Tamura, A. Oguchi, T. Kikuchi, H. Kikuchi, T. Nishii, K. Tsuji, Y. Yamaguchi, A. Tase, M. Takahashi, T. Sakane, K. I. Suzuki and K. Hatano (2012). "Phylogenetic study of the species within the family Streptomycetaceae." *Antonie Van Leeuwenhoek* 101: 32 DOI: 10.1007/s10482-011-9656-0).
Lee, L. H., N. Zainal, A. S. Azman, S. K. Eng, B. H. Goh, W. F. Yin, N. S. Ab Mutalib and K. G. Chan (2014). "Diversity and antimicrobial activities of actinobacteria isolated from tropical mangrove sediments in Malaysia." *ScientificWorldJournal* 2014: 698178 DOI: 10.1155/2014/698178.
Liu, L., N. Salam, J. Y. Jiao, H. C. Jiang, E. M. Zhou, Y. R. Yin, H. Ming and W. J. Li (2016). "Diversity of Culturable Thermophilic Actinobacteria in Hot Springs in Tengchong, China and Studies of their Biosynthetic Gene Profiles." *Microb Ecol* 72(1): 150-162 DOI: 10.1007/s00248-016-0756-2.
Lopez-Lozano, N. E., K. B. Heidelberg, W. C. Nelson, F. Garcia-Oliva, L. E. Eguiarte and V. Souza (2013). "Microbial secondary succession in soil microcosms of a desert oasis in the Cuatro Cienegas Basin, Mexico." *PeerJ* 1: e47 DOI: 10.7717/peerj.47.
Mahmoud, H. M. and A. A. Kalendar (2016). "Coral-Associated Actinobacteria: Diversity, Abundance, and Biotechnological Potentials." *Front Microbiol* 7: 204 DOI: 10.3389/fmicb.2016.00204.
Maldonado, L. A., D. Fragoso-Yanez, A. Perez-Garcia, J. Rosellon-Druker and E. T. Quintana (2009). "Actinobacterial diversity from marine sediments collected in Mexico." *Antonie Van Leeuwenhoek* 95(2): 111-120 DOI: 10.1007/s10482-008-9294-3.

Marri, P. R., W. Hao and G. B. Golding (2006). "Gene gain and gene loss in streptococcus: is it driven by habitat?" *Mol Biol Evol* 23(12): 2379-2391 DOI: 10.1093/molbev/msl115.
Milne, I., D. Lindner, M. Bayer, D. Husmeier, G. McGuire, D. F. Marshall and F. Wright (2009). "TOPALi v2: a rich graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multi-core desktops." *Bioinformatics* 25(1): 126-127 DOI: 10.1093/bioinformatics/btn575.
Minckley, W. L. and G. A. Cole (1968). "Preliminary Limnologic Information on Waters of the Cuatro Cienegas Basin, Coahuila, Mexico." *The Southwestern Naturalist* 13(4): 421-431 DOI: 10.2307/3668909.
Mohammadipanah, F. and J. Wink (2015). "Actinobacteria from Arid and Desert Habitats: Diversity and Biological Activity." *Front Microbiol* 6: 1541 DOI: 10.3389/fmicb.2015.01541.
Okoro, C. K., R. Brown, A. L. Jones, B. A. Andrews, J. A. Asenjo, M. Goodfellow and A. T. Bull (2009). "Diversity of culturable actinomycetes in hyper-arid soils of the Atacama Desert, Chile." *Antonie Van Leeuwenhoek* 95(2): 121-133 DOI: 10.1007/s10482-008-9295-2.
Pace, N. R. (2009). "Mapping the tree of life: progress and prospects." *Microbiol Mol Biol Rev* 73(4): 565-576 DOI: 10.1128/MMBR.00033-09.
Pajares, S., L. E. Eguiarte, G. Bonilla-Rosso and V. Souza (2013). "Drastic changes in aquatic bacterial populations from the Cuatro Cienegas Basin (Mexico) in response to long-term environmental stress." *Antonie Van Leeuwenhoek* 104(6): 1159-1175 DOI: 10.1007/s10482-013-0038-7.
Pajares, S., V. Souza and L. E. Eguiarte (2015). "Multivariate and phylogenetic analyses assessing the response of bacterial mat communities from an ancient oligotrophic aquatic ecosystem to different scenarios of long-term environmental disturbance." *PLoS One* 10(3): e0119741 DOI: 10.1371/journal.pone.0119741.
Parte, A., W. Whitman, M. Goodfellow, P. Kämpfer, H. J. Busse, M. Trujillo, W. Ludwig and K. Suzuki (2012). *Bergey's Manual of Systematic Bacteriology: Volume 5: The Actinobacteria*, Springer New York.
Prieto-Davo, A., L. J. Villarreal-Gomez, S. Forschner-Dancause, A. T. Bull, J. E. Stach, D. C. Smith, D. C. Rowley and P. R. Jensen (2013). "Targeted search for actinomycetes from nearshore and deep-sea marine sediments." *FEMS Microbiol Ecol* 84(3): 510-518 DOI: 10.1111/1574-6941.12082.
Qin, S., W. J. Li, S. G. Dastager and W. N. Hozzein (2016). "Editorial: Actinobacteria in Special and Extreme Habitats: Diversity, Function Roles, and Environmental Adaptations." *Front Microbiol* 7: 1415 DOI: 10.3389/fmicb.2016.01415.
Rajendhran, J. and P. Gunasekaran (2011). "Microbial phylogeny and diversity: small subunit ribosomal RNA sequence analysis and beyond." *Microbiol Res* 166(2): 99-110 DOI: 10.1016/j.micres.2010.02.003.
Rateb, M. E., W. E. Houssen, W. T. Harrison, H. Deng, C. K. Okoro, J. A. Asenjo, B. A. Andrews, A. T. Bull, M. Goodfellow, R. Ebel and M. Jaspars (2011). "Diverse metabolic profiles of a Streptomyces strain isolated from a hyper-arid environment." *J Nat Prod* 74(9): 1965-1971 DOI: 10.1021/np200470u.
Rebollar, E. A., M. Avitia, L. E. Eguiarte, A. Gonzalez-Gonzalez, L. Mora, G. Bonilla-Rosso and V. Souza (2012). "Water-sediment niche differentiation in ancient marine lineages of Exiguobacterium endemic to the Cuatro Cienegas Basin." *Environ Microbiol* 14(9): 2323-2333 DOI: 10.1111/j.1462-2920.2012.02784.x.
Ronquist, F., M. Teslenko, P. van der Mark, D. L. Ayres, A. Darling, S. Hohna, B. Larget, L. Liu, M. A. Suchard and J. P. Huelsenbeck (2012). "MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space." *Syst Biol* 61(3): 539-542 DOI: 10.1093/sysbio/sys029.
Seth, E. C. and M. E. Taga (2014). "Nutrient cross-feeding in the microbial world." *Front Microbiol* 5: 350 DOI: 10.3389/fmicb.2014.00350.
Smanski, M. J., D. C. Schlatter and L. L. Kinkel (2016). "Leveraging ecological theory to guide natural product discovery." *J Ind Microbiol Biotechnol* 43(2-3): 115-128 DOI: 10.1007/s10295-015-1683-9.
Souza, V., L. E. Eguiarte, M. Travisano, J. J. Elser, C. Rooks and J. L. Siefert (2012). "Travel, sex, and food: what's speciation got to do with it?" *Astrobiology* 12(7): 634-640 DOI: 10.1089/ast.2011.0768.
Souza, V., J. L. Siefert, A. E. Escalante, J. J. Elser and L. E. Eguiarte (2012). "The Cuatro Cienegas Basin in Coahuila, Mexico: an astrobiological Precambrian Park." *Astrobiology* 12(7): 641-647 DOI: 10.1089/ast.2011.0675.

Stach, J. E., L. A. Maldonado, A. C. Ward, M. Goodfellow and A. T. Bull (2003). "New primers for the class Actinobacteria: application to marine and terrestrial environments." *Environ Microbiol* 5(10): 828-841.
Subramani, R. and W. Aalbersberg (2013). "Culturable rare Actinomycetes: diversity, isolation and marine natural product discovery." *Appl Microbiol Biotechnol* 97(21): 9291-9321 DOI: 10.1007/s00253-013-5229-7.
Tamura, K. (1992). "Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases." *Mol Biol Evol* 9(4): 678-687.
Tanaka, Y., S. Hanada, A. Manome, T. Tsuchida, R. Kurane, K. Nakamura and Y. Kamagata (2004). "Catellibacterium nectariphilum gen. nov., sp. nov., which requires a diffusible compound from a strain related to the genus Sphingomonas for vigorous growth." *Int J Syst Evol Microbiol* 54(Pt 3): 955-959 DOI: 10.1099/ijs.0.02750-0.
Tang, J., X. Liu, J. Peng, Y. Tang and Y. Zhang (2015). "Genome sequence and genome mining of a marine-derived antifungal bacterium Streptomyces sp. M10." *Appl Microbiol Biotechnol* 99(6): 2763-2772 DOI: 10.1007/s00253-015-6453-0.
Tian, X., Z. Zhang, T. Yang, M. Chen, J. Li, F. Chen, J. Yang, W. Li, B. Zhang, Z. Zhang, J. Wu, C. Zhang, L. Long and J. Xiao (2016). "Comparative Genomics Analysis of Streptomyces Species Reveals Their Adaptation to the Marine Environment and Their Diversity at the Genomic Level." *Front Microbiol* 7: 998 DOI: 10.3389/fmicb.2016.00998.
Tiwari, K. and R. K. Gupta (2012). "Rare actinomycetes: a potential storehouse for novel antibiotics." *Crit Rev Biotechnol* 32(2): 108-132 DOI: 10.3109/07388551.2011.562482.
Tiwari, K. and R. K. Gupta (2013). "Diversity and isolation of rare actinomycetes: an overview." *Crit Rev Microbiol* 39(3): 256-294 DOI: 10.3109/1040841X.2012.709819.
Trujillo, M. E., R. Riesco, P. Benito and L. Carro (2015). "Endophytic Actinobacteria and the Interaction of Micromonospora and Nitrogen Fixing Plants." *Front Microbiol* 6: 1341 DOI: 10.3389/fmicb.2015.01341.
Undabarrena, A., F. Beltrametti, F. P. Claverias, M. Gonzalez, E. R. Moore, M. Seeger and B. Camara (2016). "Exploring the Diversity and Antimicrobial Potential of Marine Actinobacteria from the Comau Fjord in Northern Patagonia, Chile." *Front Microbiol* 7: 1135 DOI: 10.3389/fmicb.2016.01135.
Valdivia-Anistro, J. A., L. E. Eguiarte-Fruns, G. Delgado-Sapien, P. Marquez-Zacarias, J. Gasca-Pineda, J. Learned, J. J. Elser, G. Olmedo-Alvarez and V. Souza (2015). "Variability of rRNA Operon Copy Number and Growth Rate Dynamics of Bacillus Isolated from an Extremely Oligotrophic Aquatic Ecosystem." *Front Microbiol* 6: 1486 DOI: 10.3389/fmicb.2015.01486.
Ward, A. C. and N. Bora (2006). "Diversity and biogeography of marine actinobacteria." *Curr Opin Microbiol* 9(3): 279-286 DOI: 10.1016/j.mib.2006.04.004.
Winsborough, B. M., E. Theriot and D. B. Czarnecki (2009). "Diatoms on a continental "island": Lazarus species, marine disjuncts and other endemic diatoms of the Cuatro Cienegas basin, Coahuila, Mexico." *Nova Hedwigia, Suppl* 135: 257-274.
Wolaver, B. D., L. J. Crossey, K. E. Karlstrom, J. L. Banner, M. B. Cardenas, C. G. Ojeda and J. M. Sharp (2012). "Identifying origins of and pathways for spring waters in a semiarid basin using He, Sr, and C isotopes: Cuatrocienegas Basin, Mexico." *Geosphere* 9(1): 113-125 DOI: 10.1130/ges00849.1.
Yang, J., X. Li, L. Huang and H. Jiang (2015). "Actinobacterial Diversity in the Sediments of Five Cold Springs on the Qinghai-Tibet Plateau." *Front Microbiol* 6: 1345 DOI: 10.3389/fmicb.2015.01345.
Yoon, J.-H., S. T. Lee, S.-B. Kim, M. Goodfellow and Y. Park (1997). "Inter- and Intraspecific Genetic Analysis of the Genus *Saccharomonospora* with 16S to 23S Ribosomal DNA (rDNA) and 23S to 5S rDNA Internally Transcribed Spacer Sequences." *International Journal of Systematic Bacteriology* 47(3): 9.
Zhao, J., L. Guo, Z. Li, C. Piao, Y. Li, J. Li, C. Liu, X. Wang and W. Xiang (2016). "Streptosporangium jiaoheense sp. nov. and Streptosporangium taraxaci sp. nov., actinobacteria isolated from soil and dandelion root (Taraxacum mongolicum Hand.-Mazz.)." *Int J Syst Evol Microbiol* 66(6): 2370-2376 DOI: 10.1099/ijsem.0.001040.

731 Zotchev, S. B. (2012). "Marine actinomycetes as an emerging resource for the drug development
732 pipelines." *J Biotechnol* 158(4): 168-175 DOI: 10.1016/j.jbiotec.2011.06.002.

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734