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* (Jensen, Dwight & Fenical 1991; Johnson 2005; Jensen & Mafnas 2006; Winsborough, Theriot & Czarnecki 2009; Coghill, Hulsey, Chaves-Campos 2013; Prieto-

77 Davo, Villarreal-Gomez, Forschner-Dancause 2013). Nevertheless, to unambiguously accept the idea
78 of unlimited dispersal of microorganisms, we need data from studies employing good sampling.
79 Such is the case, for example, of *Escherichia coli,* human-related strains of which travel with
80 their host all around the world, or the case of *Bacillus subtilis* that can form endospores and
81 travel with the air (Souza, Eguiarte, Travisano 2012). Even in such cosmopolitan bacteria, there
82 are local ecotypes that are unrelated to any other known strains (Gonzalez-Gonzalez, Sanchez-
83 Reyes, Delgado Sapien 2013; Avitia, Escalante, Rebollar 2014; Valdivia-Anistro, Eguiarte-
84 Fruns, Delgado-Sapien 2015). *Streptomyces*, a filament and spore producer, and the most
85 extensively studied genera of Actinomycetes, has been studied and it had shown environmental
86 gradients and regional endemism in some localities (Davelos, Xiao, Samac 2004; Antony-Babu,
87 Stach & Goodfellow 2008; Kinkel, Schlatter, Xiao 2014; Andam, Doroghazi, Campbell 2016).
88 Actinobacterial diversity and community structure have been thoroughly investigated, not
89 only for their ecological importance, but also by virtue of their unique biotechnological potential
90 due to their robust secondary metabolism and incomparable ability to produce a plethora of
91 bioactive molecules with extensive medical, industrial and agricultural applications.
92 Actinomycetes, are the source of most clinically relevant antibiotics in use today (Barka, Vatsa,
93 Sanchez 2016). Nevertheless, the growing emergence of antibiotic multirresistant pathogenic
94 strains, challenges the scientific community to overcome the problem of rediscovery of known
95 compounds. Recent studies have concluded that discovery of unkown bioactive molecules will
96 be facilitated by focusing heavily on "gifted" (secondary-metabolites-rich), readily culturable
97 microbes that have been isolated from untapped environments, such as marine ecosystems,
98 which enhance the isolation of large-genome (>8 Mb), thus, rare culturable bacteria (Tiwari &
99 Gupta 2012; Zotchev 2012; Subramani & Aalbersberg 2013; Tiwari & Gupta 2013; Baltz 2016;

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

101 Correspondingly, efforts towards describing the extent of the diversity of culturable
102 actinomycetes on different conditions and extreme environments have been done, as evidenced
103 by recent reports of bioprospecting and diversity studies of actinobacteria on deserts, marine
104 sediments and vents, coral reefs, glaciers, as well as in symbiotic relationships (Maldonado et al.,
105 2009; Rateb, Houssen, Harrison 2011; Lee, Zainal, Azman 2014; Duncan, Haltli, Gill 2015;
106 Duran et al., 2015; Jami, Ghanbari, Kneifel 2015; Kuang, Li, Zhang 2015; Mohammadipanah &
107 Wink 2015; Trujillo, Riesco, Benito 2015; Yang, Li, Huang 2015; Andam et al., 2016; Chen et
108 al., 2016; Liu et al., 2016; Mahmoud & Kalendar 2016; Undabarrena et al., 2016).

109 To assess the extent of morphological and metabolic diversity and the distribution of
110 culturable actinobacteria populations on a local scale, we chose the extremely oligotrophic and
111 biodiverse Cuatro Cienegas Basin (CCB), an endangered oasis in the Chihuahuan desert (Souza,
112 Siefert, Escalante 2012). This is a site where endemic *Bacillus* (Alcaraz, Olmedo, Bonilla 2008;
113 Cerritos, Eguiarte, Avitia 2011), *Pseudomonas* (Escalante, Caballero-Mellado, Martinez-Aguilar
114 2009) and *Exiguobacterium* (Rebollar, Avitia, Eguiarte 2012) have been described. Particularly,
115 within the CCB, the Churince System has been studied with more intensity by a large team of
116 scientists since it is the most endangered hydrological system due to its relatively high altitude
117 within the valley (730 m above sea level, compared to, ca. 700 m above sea level which is the
118 average of most of the CCB), and because the San Marcos Sierra near this site of the basin is too
119 step to efficiently recharge the aquifer locally. Hence, the system depends mostly on deep
120 ancient water with a magmatic influence (Wolaver, Crossey, Karlstrom 2012). This, together
121 with the calcium sulfate soil matrix, and extreme oligotrophy in terms of phosphorus-limitation
122 (Elser, Schampel, Garcia-Pichel 2005), makes Churince the most unusual site within the CCB

123 (Minckley & Cole 1968). This analysis is relevant not only for understanding the extensive
124 biodiversity of this bacteria in such a peculiar environment, but also, for allowing us the
125 biological material for the elucidation of biochemical strategies for survival in conditions of
126 scarcity, future experimentation of bioactive molecules, as well as studies of ecological
127 interactions, including cooperation and competition analyses to understand the processes that are
128 relevant to structure these complex bacterial communities. In contrast to what is commonly
129 expected in an extremely oligotrophic site, we found high morphological and unique taxonomic
130 diversity of culturable Actinobacteria, and we were able to isolate enriched abundance of the
131 genus *Streptomyces.* When compared to available databases, we observed six novel
132 monophyletic clades and seven single-member clusters, containing a total of 31 OTUs of the
133 genus *Streptomyces* that are presumably different from other species previously described, and
134 thus, good candidates for consideration as endemic to the CCB. These unique groups of
135 *Streptomyces* strains represent key clades in evolutionary history of an anciently divergent
136 Phylum of the Bacteria domain.
137

138 **MATERIALS AND METHODS**

139 **Study Site and Sampling**

140 The Churince hydrological system (Figure 1) is located in the western part of the CCB, at 740 m
141 above sea level, surrounded by large and mostly pure gypsum dunes. This system consists of
142 three main zones connected by small water causeways: a spring, an Intermediate Lagoon (IL),
143 and a desiccation lagoon (Lopez-Lozano, Heidelberg, Nelson 2013). The Intermediate Lagoon
144 (IL), where sampling took place, has low seasonal variations such as: salinity ranging ~1.5–
145 7.1 ppt, pH 7.6 to 8, and water temperature fluctuation from 14-20 °C in winter and 20 to 30 °C
146 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,

170 5g Yeast extract, 16g agar, 25g Reef salt mix); **AIM9** ([per liter]: 100µl humic acid, 0.02 g
171 CaCO3, 0.5 g Na2HPO4, 0.5 g MgSO4, 1.7 g KCl, 0.01 g FeSO4, 0.5 mg Vitamin B12, 18g
172 agar, 25g Reef salt mix).

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

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

182 **Nucleic acid extraction**

183 To confirm Actinobacteria identity and further phylogenetic analysis of isolates, after testing
184 several techniques, genomic DNA was prepared using a modified phenol/ chloroform method
185 that yielded the best quality DNA for our isolates: colonies of putative Actinobacteria were
186 carefully scraped from agar plates and placed in centrifuge tubes; cell pellets were washed 2×
187 10mL of 10% (w/v) with sucrose and resuspended in 400µl of lysis solution (4% Triton x-100,
188 20% SDS, 5M NaCl, 2M Tris-HCl pH 8, 500mM EDTA pH 8). After resuspension, 400 µl of
189 Phenol/Chloroform and 0.1mm glass beads were added to lysis mix and this was mechanically
190 disrupted for 2 minutes. The lysates were centrifuged (12,000 x rpm, 15 min) and DNA in
191 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

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

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

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

237 I + G parameter as an evolutionary model. The reliability of nodes was estimated by ML
238 bootstrap percentages (Felsenstein 1985) obtained after 1,000 replications. A total of 41 16S
239 sequences obtained in this study were included, and 73 reference strains belonging to the genera
240 *Streptomyces,* 6 of *Kitasatospora* and 3 *Streptoacidophilus,* which were the most closely related
241 to our isolates, were selected (trimmed to 1074 bp).
242 To provide support to ML tree, we conducted a Bayesian analysis employing MrBayes v3.2.5
243 (Ronquist, Teslenko, van der Mark 2012) with 10,000,000 Markov chain Monte Carlo
244 generations and the GTR+ G model of evolution with a nucmodel= 4by4, nruns = 2, nchains = 4,
245 and sampled freq = 100. The average standard deviation of split frequences was below 0.001.
246 The nodes that had posterior probabilities greater than 95 % (Bayesian), were considered well-
247 supported and were shown in the resulting tree.
248

249 **Estimation of diversity of Actinobacteria in CCB**

250 To estimate species richness in the IL in the Churince system, we performed a rarefaction
251 analysis for total Actinobacteria isolates, and another for only *Streptomyces* isolates. The
252 definition of operational taxonomic units (OTUs) was conducted with MEGA software v. 7 at
253 97% cutoff according to their pairwise distances. Then we conducted the rarefaction curve using
254 the EstimateS 9.1.0 software package (Colwell & Elsensohn 2014) at the 95% confidence level.
255
256

257 **RESULTS**

258 **Diversity of culturable Actinobacteria within the Churince system in CCB**

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

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

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

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

281

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

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

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

302

303 **DISCUSSION**

304 ***Actinobacteria from oligotrophic CCB are diverse and abundant.***

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

319 Although gram-positive bacteria are more commonly observed in organic rich habitats
320 (Fenical 1993), isolated strains from the extremely oligotrophic Churince IL encompass 11
321 genera of Actinobacteria (Figure 2), which is comparable to the culturable diversity found in rich
322 marine environments (Duncan et al., 2015; Duran et al., 2015; Kuang et al., 2015; Chen et al.,
323 2016; Undabarrena et al., 2016). Interestingly, *Streptomyces* was the most abundant taxa,
324 representing over 50% of the total sequenced isolates varying in relation to sampling point within
325 the Churince system (figure 2). This result is comparable to the *Streptomyces*-enriched isolation
326 in extreme environments such as the Atacama Desert (Okoro et al., 2009), nonetheless CCB
327 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

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

359 Another possible factor that could explain differences between our study and Cerritos et
360 al. (2011) is the years which passed between sampling periods, including possible temporal
361 variation in the community structure. Notably in the CCB, after the time of the initial isolations
362 described in Cerritos et al. (2011), a decline of the Churince aquifer occurred. As shown in
363 experiments with UV and temperature increase in mesocosms (Pajares, Eguiarte, Bonilla-Rosso
364 2013; Pajares, Souza & Eguiarte 2015), endemic CCB Actinobacteria are particularly susceptible
365 to perturbation. Hence, it is possible that enrichment of *Streptomyces* after 2010 is a succession
366 response to the shrinkage and concomitant changes in the Churince aquifer system.

367

368 ***Endemicity of* Streptomyces *in CCB***

369 As expected from previous studies finding endemic microorganisms at CCB (Alcaraz et al.,
370 2008; Rebollar et al., 2012), we found 38 unique operational taxonomic units (OTU´s) for
371 *Streptomyces*. Moreover, these 38 novel OTUs are in six new monophyletic clades in a deeply
372 represented and well-supported phylogeny of the family Streptomycetaceae, which is a sign of
373 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

397 morphologies that clearly reflect the uniqueness of each isolate. Hence, this collection of
398 Actinobacteria from Cuatro Cienegas represents an invaluable source of great diversity for
399 microbial ecology and biotechnology studies considering that: *i)* phylogenies constructed with
400 the sequenced portion of our collection indicate six novel clades of *Streptomyces,* but they only
401 represent a third of the successfully cultured collection; *ii)* this collection has been isolated from
402 an environment of a diversity and endemicity, that has previously been considered comparable to
403 that in the Galápagos Island (Souza et al., 2012), and as revealed by our six clades cointaining
404 only CCB isolates (Fig. 5), it is quite likely that we have cultured several unique species yet to be
405 described; *iii)* the great diversity shown here has been calculated using the conserved 16S rDNA
406 marker, but it is well known that single-gene phylogenies might not always reflect the
407 evolutionary history of a species due to the high degree of horizontal gene transfer (Marri, Hao
408 & Golding 2006), a phenomenon particularly common in *Streptomyces* (Huguet-Tapia, Lefebure,
409 Badger 2016; Tian, Zhang, Yang 2016).
410 In conclusion, we can mention that our findings suggest a very high, albeit still
411 uncalculated richness in microbial diversity in CCB, as well as suggested endemism. Our main
412 result show that the CCB is not only a special place to study community structure where
413 Actinobacteria diversity plays a major ecological role in such an oligotrophic environment, but it
414 also represents a promising area for bioprospecting studies that will require concerted long-term
415 efforts to search for genuine and substantial contributions to the discovery of natural products.
416
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420

421 **FIGURES**

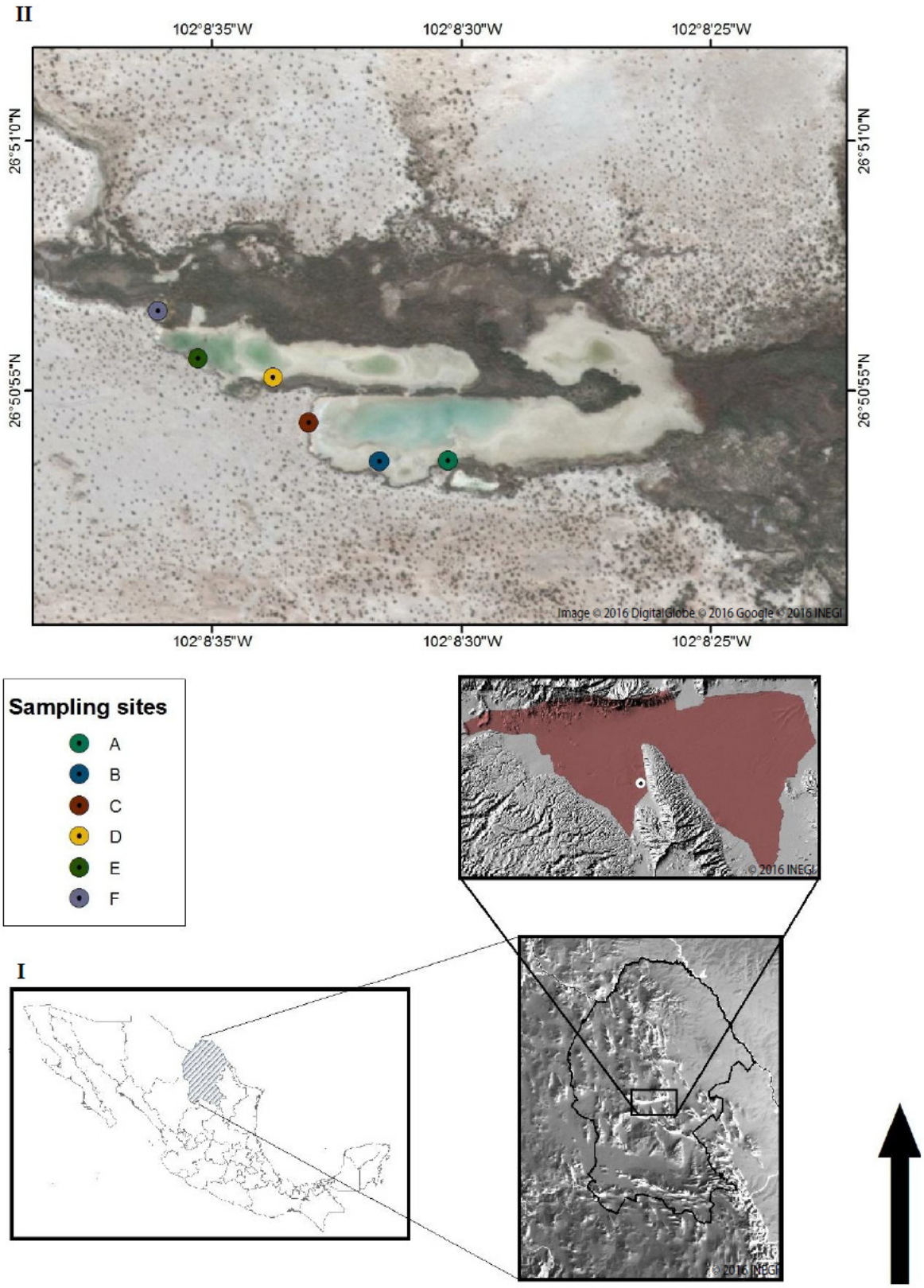
II
102°8'35"W
102°8'30"W
102°8'25"W
26°51'0"N
26°50'55"N
Image © 2016 DigitalGlobe © 2016 Google © 2016 INEGI
Sampling sites
A
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Figure 1. The Churince hydrological system. (I) Map of Mexico displaying the State of Coahuila and the location of the Cuatro 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.

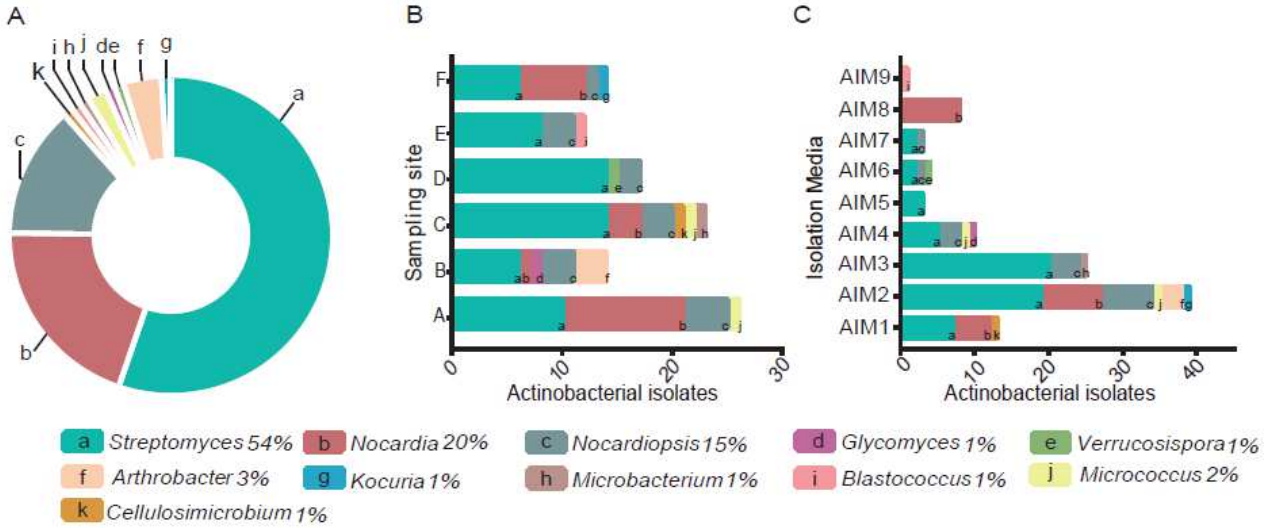


Figure 2. (A) Pie chart of the percentage of Actinobacteria genera isolated from the intermediate lagoon in Churince system. (B) Number of Actinobacteria isolated according to the sampling sites. (C) Number of Actinobacterial isolated according to the culture media used.

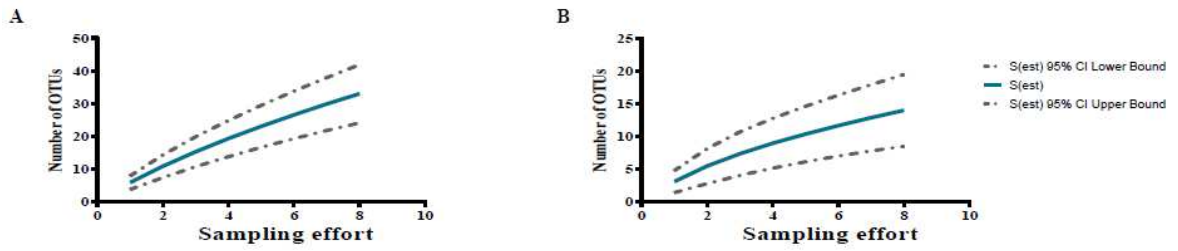


Figure 3. Rarefaction curves show sampling effort on the estimation of the numbers of OTUs at 97% sequence identity from cultured Actinobacteria (A), and total isolated *Streptomyces* (B) from CCB.

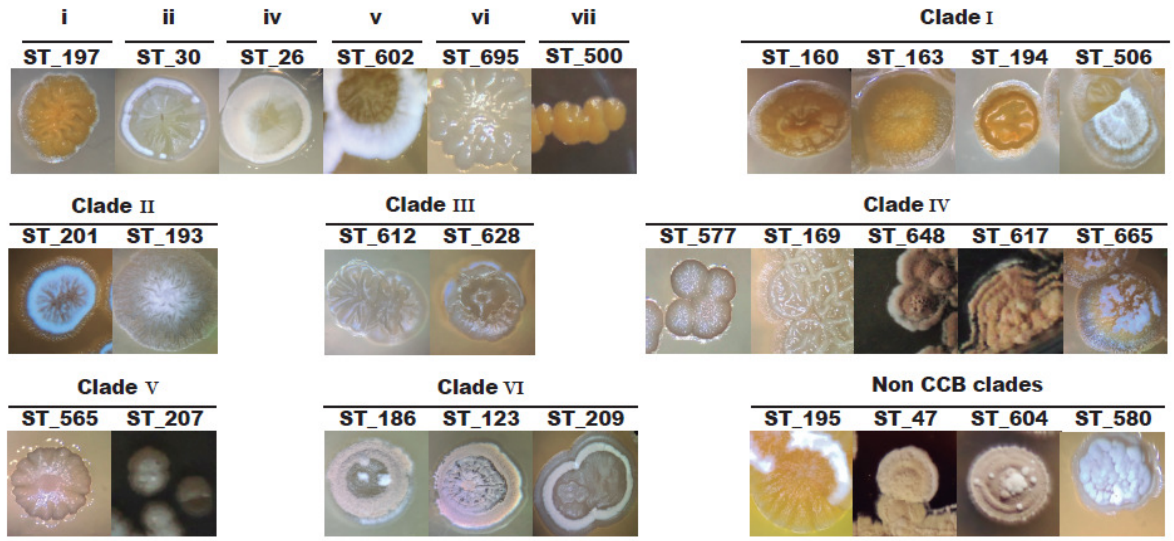
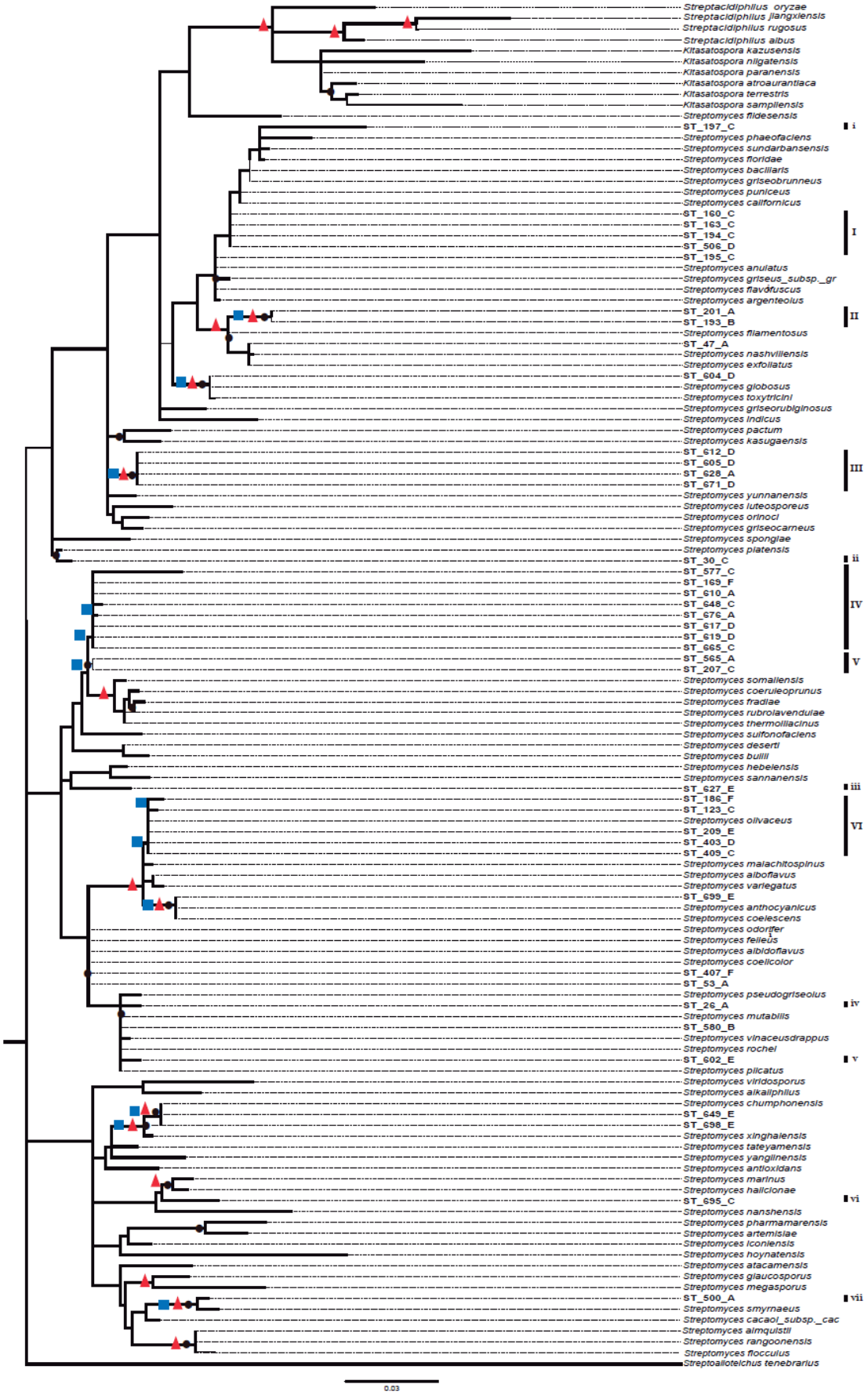


Figure 4. Colony morphological diversity of *Streptomyces* isolated from CCB within clades.


Streptacidiphilus oryzae
Streptacidiphilus jiangxiensis
Streptacidiphilus rugosus
Streptacidiphilus albus
Kitasatospora kazusensis
Kitasatospora niigatensis
Kitasatospora paranensis
Kitasatospora atroaurantiaca
Kitasatospora terrestris
Kitasatospora sampliensis
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 thermoviolaceus
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 odoriferi
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 pilcatus
Streptomyces viridosporus
Streptomyces alkaliphilus
Streptomyces chumphonensis
ST_649_E
ST_698_E
Streptomyces xinghaiensis
Streptomyces tateyamensis
Streptomyces yangjiensis
Streptomyces antioxidans
Streptomyces marinus
Streptomyces halicionae
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 flocculus
Streptoalloteichus tenebrarius
0.03

454

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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