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Starch- and cellulose-related microbial diversity of soil sown with sugarcane crops in the Papaloapan Basin, a megadiverse region of Mexico
Diversidad microbiana de suelos cultivados con caña de azúcar relacionados con almidón y celulosa en la cuenca del Papaloapan, una región megadiversa de México

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Palabras clave: biodiversidad bacteriana; caña de azúcar; cultivos enriquecidos; unidades taxonómicas operacionales
Keywords: bacterial biodiversity; sugarcane; enriched culture; OTUs

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Resumen

Introducción: La caña de azúcar es un producto agrícola esencial para producir bioetanol en México. El conocimiento de la comunidad bacteriana asociada a este cultivo y el estado del suelo es un paso decisivo para comprender cómo los microorganismos influyen en la productividad de los cultivos. Los cultivos enriquecidos con sustrato es una técnica que permite conocer la biodiversidad de muestras biológicas. El objetivo de esta investigación fue identificar a través de cultivos enriquecidos la biodiversidad bacteriana relacionada con dos fuentes complejas de carbohidratos, almidón y celulosa, en suelos de cultivo con caña de azúcar en la cuenca del Papaloapan en Oaxaca, México.

Método: El contenido del suelo fue analizado químicamente. Medios de LB, LB-almidón y LB-carboximetilcelulosa 1% se inocularon con 2 g de suelo y se mantuvieron a 180 rpm y 37°C durante 48 h. A partir de la biomasa recolectada, se amplificó el gen 16S rDNA y se construyó una librería que fue analizada por secuenciación.

Resultados: Los contenidos de materia orgánica y N, K y Zn mostraron valores moderadamente altos, a diferencia del fosfato asimilable y el Na, los cuales fueron menores al promedio. En la librería, se encontraron 35 unidades taxonómicas operacionales (OTUs) relacionados con los géneros *Clostridium, Bacillus, Enterococcus, Lysinibacillus* y *Citrobacter*, que podrían tener genes para romper la celulosa y el almidón.

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Discusión o Conclusión: Esta es la primera aproximación de diversidad relacionada con la hidrólisis del almidón y celulosa en la región de Papaloapan, donde los principales géneros detectados fueron *Clostridium, Bacillus, Enterococcus, Citrobacter* y *Lysinibacillus* en un suelo moderadamente alto en materia orgánica.

Abstract

Introduction: Sugarcane is an essential agricultural product for bioethanol production in Mexico. The discovery of both the bacterial community associated with this crop and the soil status is a decisive step towards understanding how microorganisms influence crop productivity. Culture enrichment allows for the identification of the biodiversity of biological samples. The objective of this research was to identify the bacterial biodiversity related with two complex carbohydrate sources (starch and cellulose) in soils sown with sugarcane in the Papaloapan Basin in Oaxaca, Mexico via a metagenomic approach.

Method: Soil content was analyzed chemically. Liquid LB, LB-starch and LB-1% carboximetilcellulose media were inoculated with 2 g soil and cultured at 180 rpm, 37°C for 48 h. The biomass was collected and the 16S rDNA gene was amplified and a library was constructed which was analyzed by sequencing.

Results: N, K and Zn content of organic matter showed higher values than average, as opposed to P and Na, which were lower than average. In the library, 35 OTUs related to *Clostridium*, *Bacillus, Enterococcus, Lysinibacillus* and *Citrobacter* genera were found which could contain genes for breaking cellulose and starch.

Discussion or Conclusion: This is the first approach to identify the diversity related to starch and cellulose hydrolysis in the Papaloapan region, where the principal genera detected were *Clostridium, Bacillus, Enterococcus, Citrobacter* and *Lysinibacillus* in a soil moderately rich in organic matter.

Introduction

Soil is the most important biological matrix on Earth, where a broad microbial diversity is developed including prokaryotes, eukaryotes and virus. Microbiota play important roles in the edaphogenesis process, biogeochemical cycles, xenobiotics degradation (herbicides, insecticides and hydrocarbons), but above all in plant growth, carried out by the so called plant-growth

promoting bacteria (PGPB) (Hillel, 1998; Jaramillo *et al.*, 1994; Tarbuck *et al.*, 2005). The PGPBs can be free-living or symbiotic relationship, they have mainly isolated from grasses soils, such as corn (Loredo-Osti *et al*., 2004). It is estimated that microbial biodiversity in soil ecosystems is mainly represented by eukaryotic organisms. For example, one gram of rhizospheric soil can contain up to 10 billion microorganisms and more than 30,000 prokaryotes species (Egamberdieva *et al*., 2008; Mendes *et al*., 2011). Soil microorganism study not only lies in ascertaining their importance in product generation, metabolic processes and biotechnology capacities, but also in their direct relationship with nutrient utilization. A few years ago, soil bioprospecting only consisted of microorganism culture through traditional microbiological techniques (Handelsman *et al*., 2002; Torsvik and Øvreås, 2002). However, these culture methods only allow a recovery of between 0.1-10% total microorganisms (Escalante-Lozada *et al*., 2004). This can be explained because the nutrient requirements of all microorganisms are unknown. In addition, the precise physicochemical conditions of their natural environment have not been documented, as well as the information on the symbiotic, commensal or parasitic relationships that are maintained in a microbial community. For this reason, soil ecosystems are largely unknown (Keller and Zengler, 2004; Zengler *et al*., 2002). Schloss and Handelsman (2003) found that most of strains in the soil belong to four phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*, which represent 20% of the bacterial community in the soil.

An alternative to microorganism recovery related to the degradation of some metabolite or substrate is culture enrichment. Enrichment can be of two types: in the laboratory adding some specific substrate or media and by *in situ* enrichment before isolation (Sar and Islam, 2012). Thus, diverse microorganisms, metabolic pathways and enzymes related to the degradation of complex carbohydrates (chitin, lignocellulosic residues) and simple, fats and oils among others have been able to be identified (Beloqui *et al*., 2009; Jacquiod *et al*., 2013; Peña-García *et al*., 2016; Wang *et al*., 2016).

Sugarcane is a perennial grass grown in many tropical countries. Globally, it is one of the most important staple crops, both in terms of total production (ranked #1 at 1,685 million tons) and area cultivated (#13 at 23.8 million ha) (2010 data; http://faostat.fao.org). In many tropical countries, sugarcane production represents the most important land-use and agricultural commodity; in countries such as Brazil, its importance is due to ethanol production.

The Papaloapan Basin is located in southeast Mexico. It is the second most important hydric region of Mexico with warm humid weather. Its soils are so fertile that sugarcane is the main crop harvest in the region with 30% coverage (OEIDRUS, 2005). Main soil types are ferrosol, fluvisol and phaeozems, which have particular characteristics that allow the development of the microorganisms (CODICE, 2010). Until now, diversity studies using enriched cultures in soils sown with sugarcane in the Papaloapan Basin (a megadiverse zone) have not been carried out. The objective of this research was to obtain a preliminary estimate of soil biodiversity related to starch and cellulose hydrolysis in soils sown with sugarcane in the Papaloapan Basin via enriched cultures. The results represent a preliminary estimate of the microbiota present and to associate them with crop type, in order to generate useful knowledge for future research or for the industries and institutions related to sugarcane culture and biofuel production from lignocellulosic residues.

Method

Soil sampling protocol

Samples were collected (April 2013) from four different sugarcane sites (CP 72-2086 and Mex 69-290 varieties) in rhizospheric soil, belonging to the same plot (lat. 18.00˚N, 96.07˚W and 46 m.a.s.l.). Vegetable matter was removed, and the samples were placed in plastic bags and stored at 4°C until its use and chemical analysis.

Soil physico-chemical characterization

All soil samples collected were mixed and an aliquot was characterized. Saturated soil paste extract pH, electrical conductivity (EC) of soluble ions (dSm^{-1}) (Jenway Model 4520 Laboratory Conductivity/TDS Meter), saturation percent (SP), cations (Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}) and anions (SO_4^{2-}, Cl^{-}, HCO^{3-}, CO_3^{2-}) were determined for each sample (Jackson, 1973). Soil calcium carbonate was measured by the calcimeter method (Nelson, 1982). Mechanical analysis was carried out by the international pipette method (Piper, 1966), and the textural class was ascertained from SP and confirmed by textural triangle.

Enriched cultures

Soil samples were homogenized, and 2 g soil sample were placed on 200 mL Luria-Bertani medium alone (Sigma-Aldrich), with carboximetilcellulose (Sigma-Aldrich) (LB-CMC-1%, W/V), and

starch (Jalmek) (LB-starch 1%, W/V). Liquid cultures were maintained at 37°C, 180 rpm for 48 h. Subsequently, biomass was collected by centrifugation at 5000 rpm (Eppendorf 5418R) and stored at -20°C until its analysis.

DNA extraction and amplification of 16S rDNA gene

From the collected biomass, metagenomic DNA was extracted using Microbial DNA Isolation Kit (MOBIO) following the instructions of the supplier. A polymerase chain reaction (PCR) assay was designed to amplify a 1.6 kb region of the 16S gene. For the PCR, 35-50 ng extracted DNA were used with a total volume of 50 µL using 0.5 - 1 µL (10 mM) of the fD1 primers (5'-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G-3') and rD1 (5'-CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC-3'), 1.4 µL (5 U/µL) of Platinum Taq DNA polymerase (Invitrogen, cat. 10966-026) and 3 mM $MgCl_2$ under the following conditions: 3 min at 94˚C, 30 cycles of 30 seconds at 94˚C, 30 seconds at 56˚C, and 2 min at 72˚C, plus an additional 5 min cycle at 72˚C (Gutiérrez-Lucas *et al*., 2014). PCR products were analyzed on 1% agarose gels.

Purification and cloning of the PCR products

PCR products were purified using a GeneJET PCR Purification kit (Thermo Scientific, cat. K0701, USA) following the indications of the supplier. PCR products were cloned on the pCR-XL-TOPO vector of the TOPO XL PCR Cloning kit (Invitrogen, cat. K4700-20, USA) according to supplier specifications, (80 ng/µl DNA, 10 ng/µl vector). PCR products were analyzed by sequencing (Macrogen Inc, South Korea).

OTUs and Phylogenetic analysis

Operational taxonomic units (OTUs) and diversity indexes were obtained with the typically implemented mothur software pipeline (Schloss *et al*., 2009). The program was run with the 35 obtained sequences. No chimeric sequences were found through the "chimera.uchime" algorithm incorporated in the platform. A distance matrix was built using "dnadist", included in PHYLIP software (v.3.69 Felsenstein, USA, 2005) and, with the cluster command included in mothur; the sequences were assigned to 20 OTU's. The cluster method used the "furthest neighbor" in which all the sequences within an OTU are at the most 0.03 distant (or 97% similar) from all of the other sequences with the OTU. After clustering, the sampling effort was evaluated by a rarefaction curve

and the diversity among the sequences was calculated with the Chao1, Shannon and Simpson indexes (Figure 6).

Subsequently, sequence homology and analysis were performed using a BLASTn tool program available on-line (ncbi.nlm.nih.gov); the sequences of the species of the resulting genus were obtained (img.jgi.doe.gov) and aligned with the MUSCLE program (ebi.ac.uk/Tools/msa/muscle/) to obtain a profile. With the alignment of the profile, a search was made for the best method of phylogenetic inference with the FindModel program (hiv.lanl.gov). For all inferences, the maximum likelihood method was GRT + G (General Time Reversible plus Gamma distribution). The sequences obtained from the isolates were aligned with the profile obtained from GenBank with the Clustal_X program in the profile alignment mode. After the final alignment, phylogenetic inference was carried out with the above-mentioned method and 500 bootstrap replicates with the program MEGA6 program. The phylogenetic tree was constructed using the closest genera.

Results and discussion

Soil physico-chemical characteristics

Analyzed soil was composed of 51.96% clay, 30% silt and 18.04% sand; its textural class was clay, with 2.06 g/cm^3 density, pH almost neutral (6.32) and the EC value was 0.147 dSm^{-1}. Organic material and nitrogen content were moderately high (Table 1). Analyzed soil satisfied the physico-chemical conditions required for sugarcane harvest and showed similar parameters to Brazilian soil sown with this crop (Rachid *et al*., 2012; Rachid *et al*., 2016). Therefore, these data could be used as reference in sugarcane production in Mexico.

Table 1. Chemical characterization of the soil sample from the studied area.

Parameter	Result	Unit	Observations
Organic material	3.09	%	Moderately high
Total Nitrogen	0.177	%	Moderately high
Assimilable Phosphate	6.20	mg.kg^{-1}	Low
Ca^{+2}	7216.80	mg.kg^{-1}	High
Mg^{+2}	304.88	mg.kg^{-1}	Medium
Na^{+}	44.85	mg.kg^{-1}	Very low

K^+	149.37	$mg.kg^{-1}$	Moderately low
Fe	38.70	$mg.kg^{-1}$	High
Cu	2.67	$mg.kg^{-1}$	High
Zn	1.26	$mg.kg^{-1}$	Moderately low
Mn	20.96	$mg.kg^{-1}$	Moderately high
B	0.35	$mg.kg^{-1}$	Very High
CIC (Cationic Interchange Capacity)	36.80	$cmol.kg^{-1}$	Moderately high

Diversity analysis

In this research, 35 OTUs were obtained 33 of which were identified from the LB-starch library, two OTUs from the LB-CMC library and none from the LB library. Sequence analysis indicates that 17 OTUs are related to *Clostridium*, nine to *Bacillus*, six to *Enterococcus*, two to *Lysinibacillus* (one in LB-starch and the another in LB-CMC libraries) and one to *Citrobacter* (found exclusively in LB-CMC library) (Table 2). In a previous study carried out in soil sown with sugarcane in the Papaloapan Basin, 12 and 6 strains with amylase and cellulase activity respectively were isolated and identified by biochemical and molecular tests (16S rDNA gene sequencing). Isolated strains were related to *Bacillus* (phylum *Firmicutes*, class *Bacilli*), *Arthrobacter* (phylum *Actinobacteria*, class *Actinobacteria*) and *Pseudomonas* (phylum *Proteobacteria*, class *Gammaproteobacteria*). Some of these could be new species of certain genera, due to the low similarity they showed with the closest strains (Montor *et al*., 2011).

In this work, OTUs related to *Bacillus* and *Lysinibacillus* genera were found, similar to Montor-Antonio *et al*., 2014, although, in this case also OTUs related to *Enterococcus*, *Clostridium* and *Citrobacter* were also found. In deeper studies performed on Brazilian soils, a higher diversity, (43 genera) has been found (Pisa *et al*., 2011). However, in that research, DNA was isolated directly from soil samples. Due to the difference between libraries in the number of OTUs, the PCR products from LB-CMC and LB were cloned twice more, varying the insert concentration: however, the result remained the same (only two clones were obtained). The low number of OTUs found in the LB-CMC medium was possibly due to the brief incubation time (48 h), the substrate complexity (higher than starch), the enrichment adaptation phase (lag phase) and the number of sequences obtained from the library. Using microorganisms with cellulase genes or a cellulosome complex sometimes requires 10 days only to break down 50% of the cellulose (Yang *et al*., 2015).

In this work, growth was stopped at 48 h. Yet, *Citrobacter* represents an important OTU in the degradation of cellulose with biotechnological relevance, as previous studies have found for H_2 production in *Citrobacter* sp. in media enriched with cellobiose (Mangayil *et al*., 2011).

Table 2. OTUs obtained from enrichment media with starch and cellulose.

Culture media	OTU	Genera	ID_NCBI	Best-hit/coverage
LB-1% starch	ANC3	*Bacillus*	CP020002.1	100/99
	ANC7	*Bacillus*	CP020002.1	100/99
	ANC8	*Enterococcus*	CP019512.1	100/99
	ANC9	*Clostridium*	KM497177.1	98/99
	ANC14	*Clostridium*	EU773775.1	92/96
	ANC15	*Enterococcus*/Unc	EU723856.1	97/91
	ANC16	*Bacillus*	CP019726.1	100/99
	ANC17	*Clostridium*	X73446.1	99/94
	ANC18	*Clostridium*/Unc	HG917272.1	100/94
	ANC19	*Clostridium*	CP010994.1	100/99
	ANC22	*Lysinibacillus*	KY643638.1	100/99
	ANC23	*Bacillus*	KP209387.1	100/97
	ANC26	*Bacillus*	CP018935.1	100/99
	ANC28	*Clostridium*	AB161374.1	100/99
	ANC30	*Clostridium*	AB161369.1	100/99
	ANC34	*Clostridium*	AB161372.1	99/99
	ANC35	*Enterococcus*	CP003726.1	100/96
	ANC36	*Clostridium*	AB161372.1	100/98
	ANC42	*Clostridium*	EU775706.1	92/97
	ANC43	*Bacillus*	CP014486.1	100/99
	ANC46	*Clostridium*	AP017630.1	100/99
	ANC50	*Bacillus*	CP018935.1	100/100
	ANC52	*Clostridium*	JF428988.1	99/94
	ANC53	*Clostridium*	KC835992.1	99/96
	ANC55	*Clostridium*/Unc	HG917272.1	100/95

	ANC60	*Clostridium*	CP010994.1	100/93
	ANC61	*Enterococcus*	JF772098.1	99/97
	ANC65	*Clostridium*	CP010994.1	100/99
	ANC67	*Enterococcus*	CP019512.1	100/99
	ANC68	*Enterococcus*	CP003726.1	100/96
	ANC69	*Clostridium*	AB161372.1	99/99
	ANC71	*Bacillus*	KP209387.1	100/97
	ANC73	*Bacillus*	CP009600.1	97/99
LB-1% CMC	CNC1	*Citrobacter*	EU775609.1	100/92
	CNC4	*Lysinibacillus*	KT254135.1	100/99

Our comparison is also limited by the methodology used, focussed on obtaining starch-and cellulose-related microbial diversity of soil with sugarcane crops, which can explain the low rates of diversity indexes and the specificity of the observed OTUs (Figure 6). However, the sequencing effort was adequate, since it was observed that at the end of the rarefaction curve it entered its plateau phase, indicating that it will be difficult to find more OTUs (Schloss and Handelsman, 2003). These results are expected because the cultivation conditions, rich in starch and cellulose, are not optimal for most microorganisms that inhabit the soil, more so for those specialized in their degradation (Montor-Antonio *et al*., 2014).

Phylogenetic classification of the library sequences obtained from the enrichment of soil samples

Ribotyping was used for the classification of the obtained OTUs that were assigned to a species level in some cases, or at least to a complex of really close species (Figures 1, 2, 3, 4 and 5). The most representative bacterial group was *Clostridium* with seventeen sequences, which form a new branch in some cases (Figure 4). *Bacillus* was represented by nine sequences, all in the *B. anthracis, B. cereus* and *B. mycoides* complex. In the *Enterococcus* genera, six sequences were found in the *E. faecalis* complex. In the *Lysinibacillus* genera, two sequences in the *L. kistanensis, L. macroides* and *L. borotolerans* complex were found, and one sequence was present in the *Citrobacter* genera phylogenetically related to *C. werkmanii*. *Clostridium, Bacillus, Lysinibacillus* and *Enterococcus* genera belong to the *Firmicutes* phylum; 97% of sequences obtained appertain to this phylum and

only 3% to the *Proteobacteria* phylum. Some reports show that *Firmicutes* is the dominant phylum in sugarcane soil, mainly the *Bacillus* genus; however other research shows *Proteobacteria* (30%), *Acidobacteria* (23%), *Bacteroides* (12%) and *Firmicutes* (10%) as the principal reported phylum proportion (Pisa *et al*., 2011; Sharmin *et al*., 2013). Differences could exist due to type of soil and the methodology used.

The *Firmicutes* phylum including *B. licheniformis, B. cereus, B. amyloliquefaciens* and *B. subtilis,* produce amylolytic enzymes (α-amylase, α-glucosidase, amyloglucosidase) to break starch in the environment, to reduce Fe ions in anaerobiosis, and to use oxygen and nitrates as final electron acceptors (de Souza and Magalhães, 2010; Illmer and Schinner, 1992). *Bacillus* genus strains are related with phosphorus fixing, using several strategies, one of them being to produce acids to solubilize non-soluble phosphorus (Banik and Dey, 1982; Illmer and Schinner, 1992). Strains of *B. licheniformis* and *B. amyloliquefaciens* were found to produce mixtures of lactic, isovaleric, isobutyric, and acetic acids for that activity (Loredo-Osti, *et al*., 2004; Sivaramakrishnan *et al*., 2006). The *Clostridium* genus, for example *C. acetobutylicum, C. butyricum* and *C. thermoamylolyticum,* also includes microorganisms with α-amylase, amyloglucosidase genes able to degrade starch. *Clostridium* are obligatory anaerobic heterotrophs only capable of fixing N_2 in the complete absence of oxygen (Kennedy *et al.*, 2004; Kennedy and Tchan, 1992) and some *Clostridium* strains can reduce phosphate to phosphite in the soil (Almeida *et al*., 2011; Falkowski *et al.*, 2008).

The *Lysinibacillus* genus is an endemic soil strain characterized by toxin production; genetically, it is related to the *Bacillus* genus and some strains, such as *L. sphaericus* show α-amylase activity (Kumar *et al*., 2012; Montor-Antonio *et al*., 2014; Tambekar *et al*., 2016). *Enterococcus* is part of the intestinal microbiota of animals and has been used as an indicator of fecal contamination in environmental samples; in fermented corn mass, a strain was identified with low α-amylase activity (Kumar *et al*., 2012; Mazzucotelli *et al*., 2013; Montor-Antonio *et al*., 2014; Tambekar *et al*., 2016). It should be noted that all these genera have strains which produce amylolytic enzymes. It is possible that the OTUs isolated in this work are related to starch degradation. The *Citrobacter* genus is important in the production of cellulases and H_2 from lignocellulosic residues; in the soil, it is responsible for reducing nitrate to nitrite in the environment and phosphorus solubilization (Sprocati *et al*., 2014; Zhang *et al*., 2017). In this work, an OTU related to this species was only found in LB-CMC.

The bacteria genus here identified by OTUs belong to free-PGPB, demonstrated as having a symbiotic and enhanced relationship with rhizobacteria (Bashan *et al.*, 1996; Reverbel-Leroy *et al*., 1996). Studies of bacteria such as *Azotobacter, Azospirillum, Bacillus*, and *Klebsiella* sp. are also used to inoculate a large area of arable land in the world with the aim of enhancing plant productivity (Lynch, 1983). In addition, phosphate solubilizing bacteria, such as the *Bacillus* and *Paenibacillus* species (formerly *Bacillus*), have been applied to soils to specifically enhance the phosphorus status of plants (Brown, 1974; Hayat *et al*., 2010). Therefore, isolating bacteria related to the *Bacillus, Lysinibacillus* and *Clostridium* genera can improve soil quality, as many belong to the free-PGPB group and are able to fix nitrogen, solubilize phosphate as well as degrading starch and cellulose, some of the most abundant polymers in the world.

Operational taxonomical units and the diversity indexes of the obtained sequences

The mothur program was run with the 35 obtained sequences, no chimera sequences were found, and 20 OTUs were observed at 97% of cutpoint (Figure 6). In general, in the rarefraction curve, a low diversity that agrees with the Chao, Shannon and Simpson indexes was observed (Figure 6).

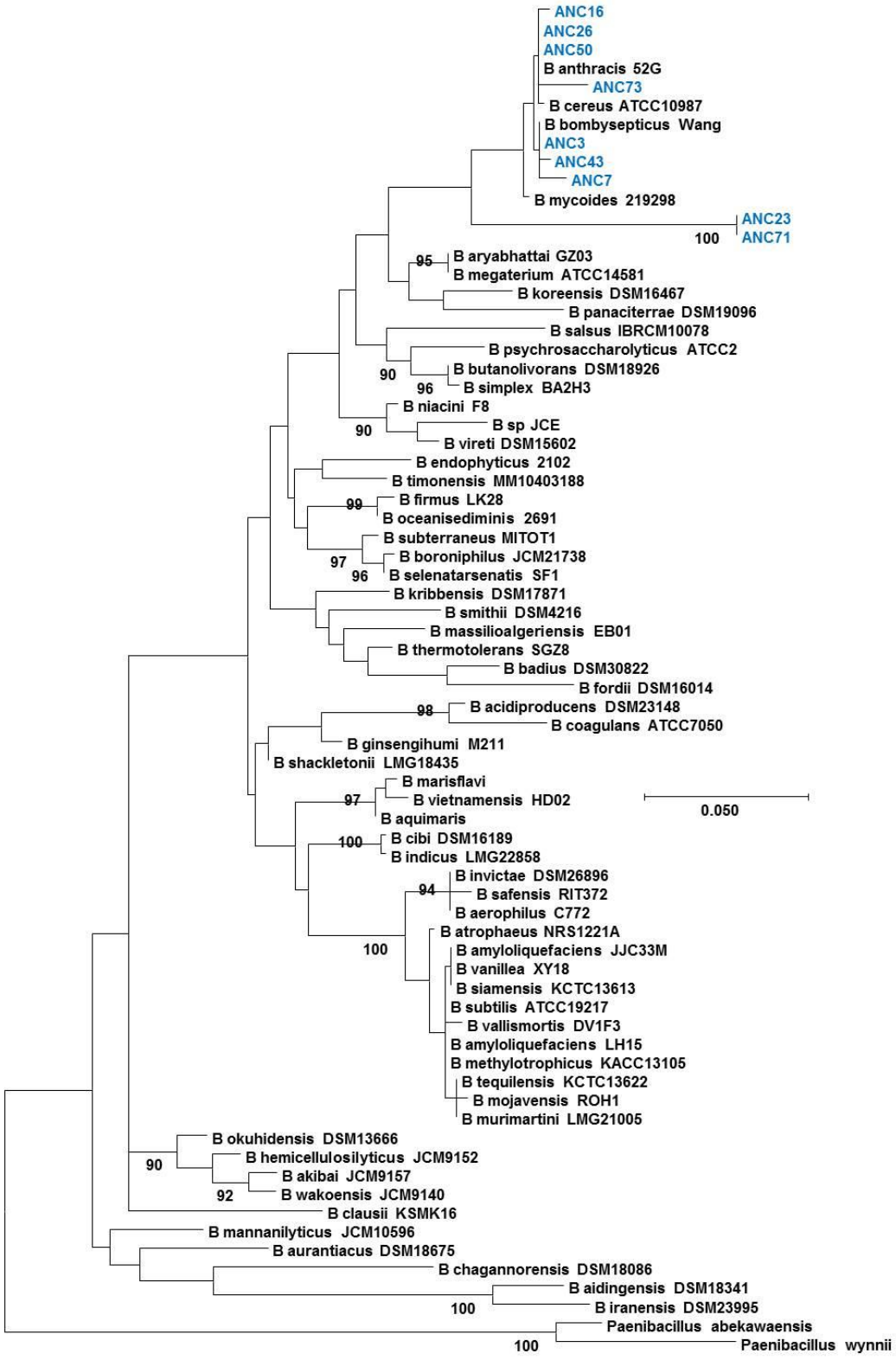

Figure 1. Phylogenetic tree of the *Bacillus* genus. Evolutionary inference was carried out following the maximum likelihood method, with the GTR + G (General Time Reversible Model plus Gamma distribution) model, and 500 bootstrap replicates. The *Paenibacillus* genus was used as the nearest external source.

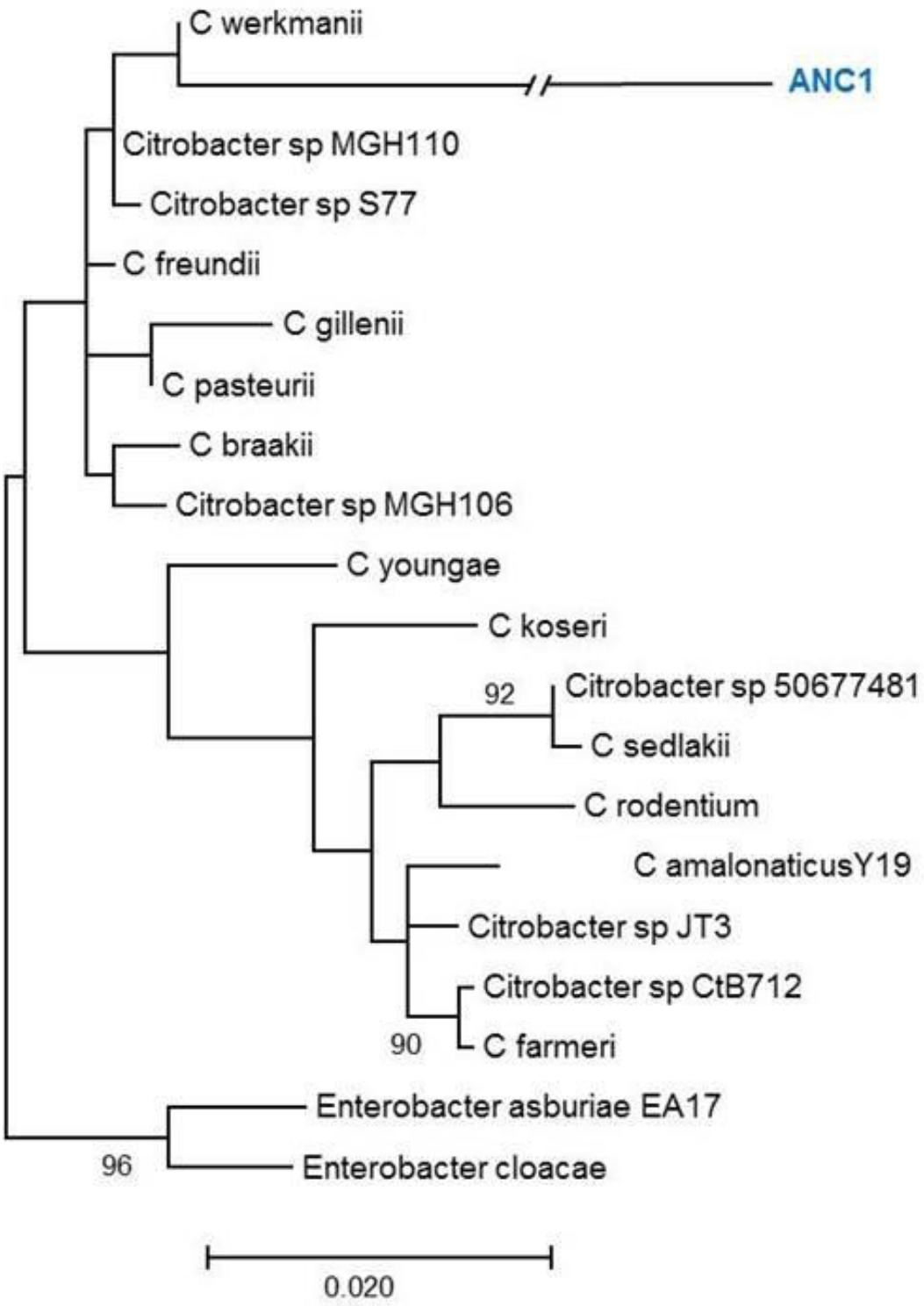

Figure 2. Phylogenetic tree of the *Citrobacter* genus. Evolutionary inference was carried out following the maximum likelihood method, with the GTR + G (General Time Reversible Model plus Gamma distribution) model, and 500 bootstrap replicates. The *Enterobacter* genus was used as the nearest external source.

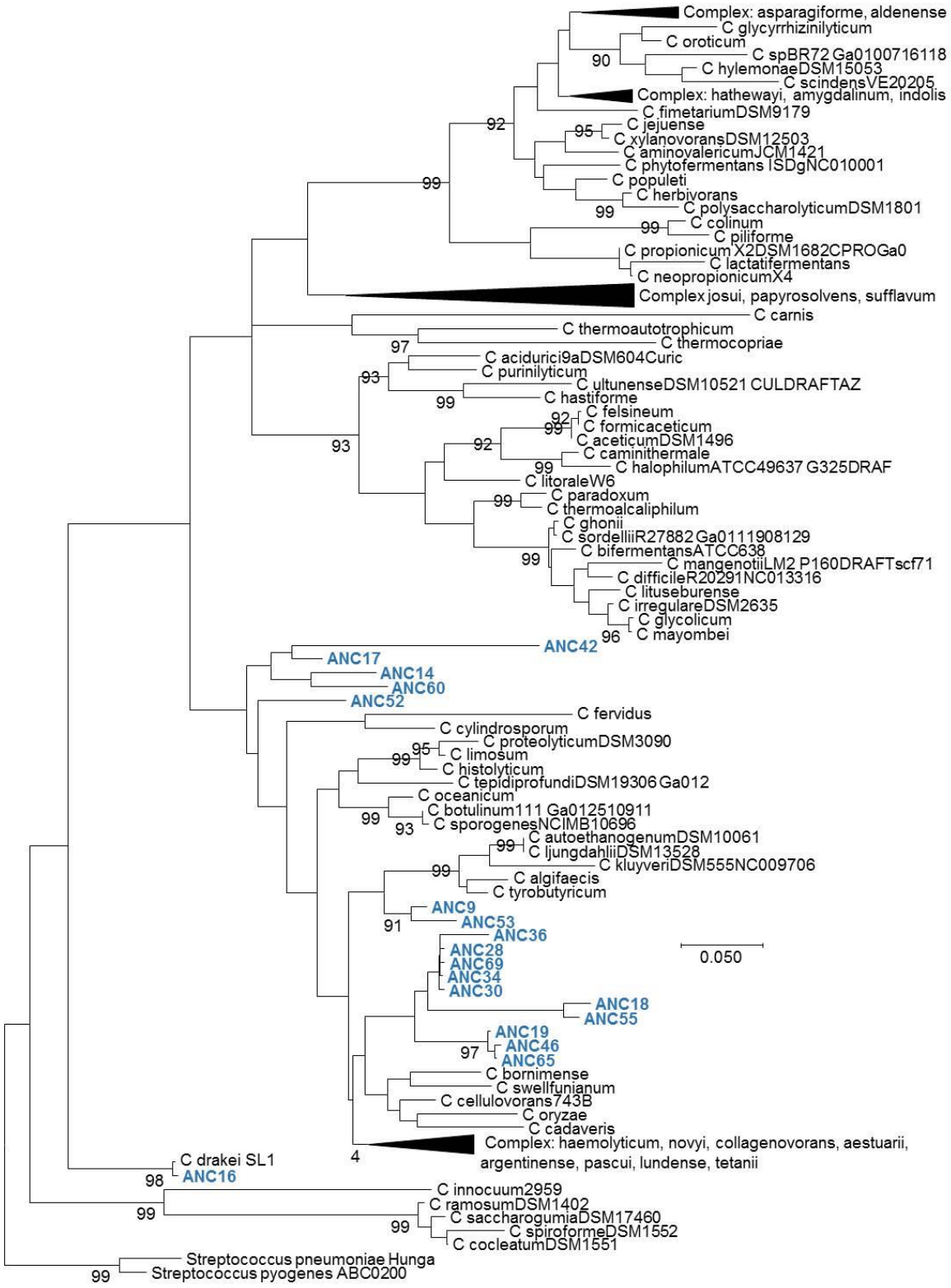

Figure 3. Phylogenetic tree of the *Clostridium* genus. Evolutionary inference was carried out following the maximum likelihood method, with the GTR + G (General Time Reversible Model plus Gamma distribution) model, and 500 bootstrap replicates. The *Streptococcus* genus was used as the nearest external source.

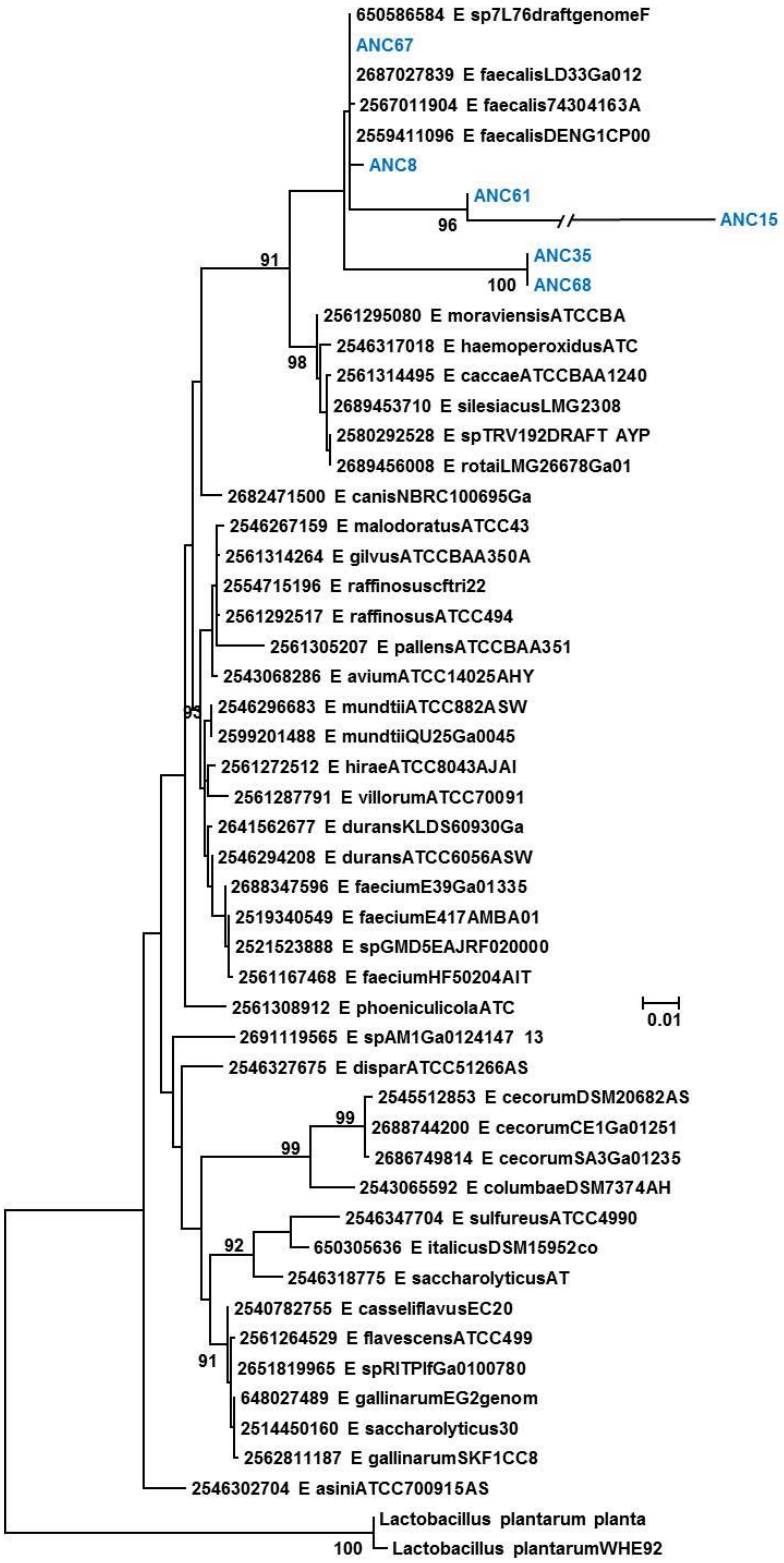

Figure 4. Phylogenetic tree of the *Enterococcus* genus. Evolutionary inference was carried out following the maximum likelihood method, with the GTR + G (General Time Reversible Model plus Gamma distribution) model, and 500 bootstrap replicates. The *Lactobacillus* genus was used as the nearest external source.

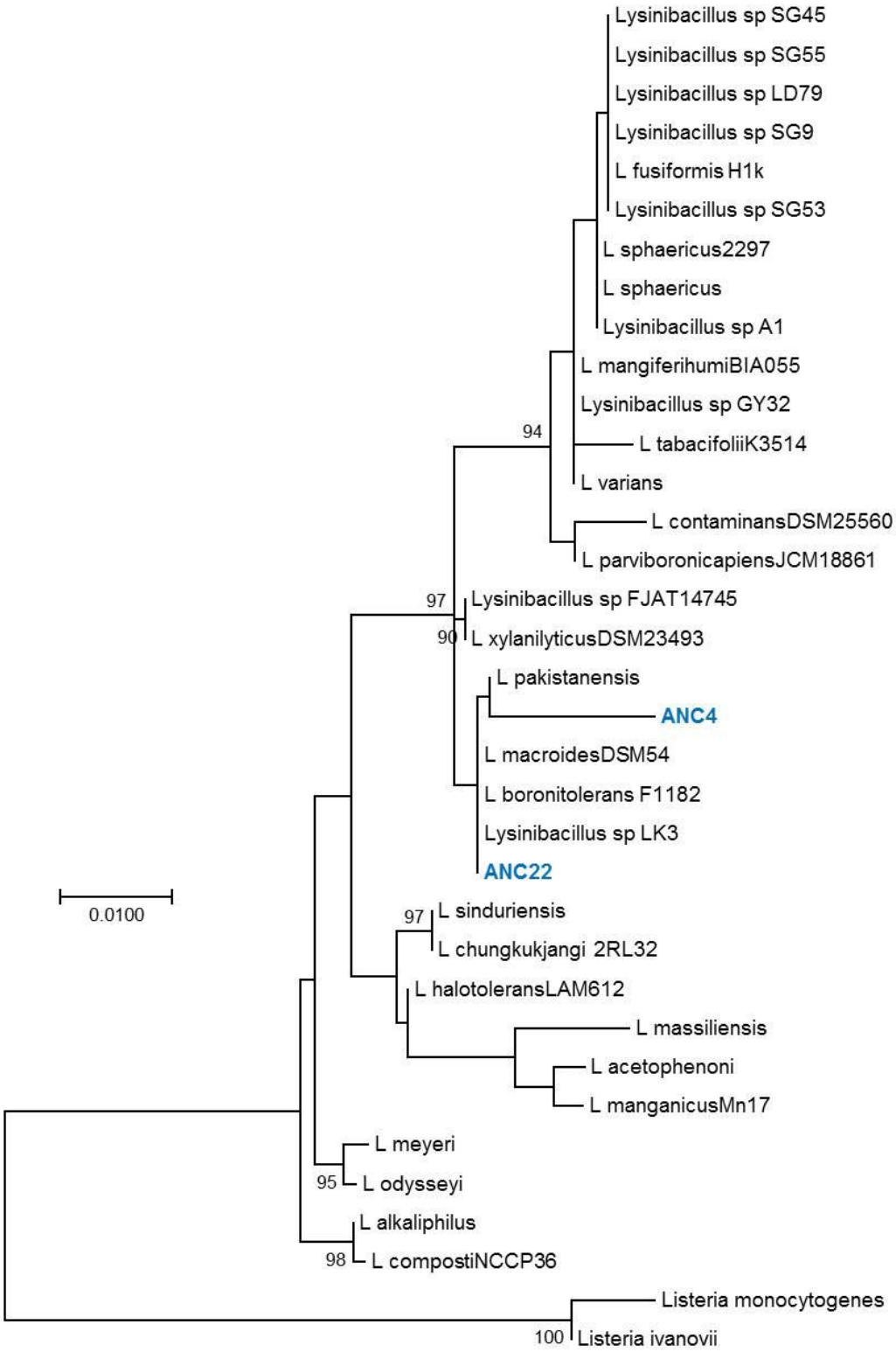

Figure 5. Phylogenetic tree of the *Lysinibacillus* genus. Evolutionary inference was carried out following the maximum likelihood method, with the GTR + G (General Time Reversible Model plus Gamma distribution) model, and 500 bootstrap replicates. The *Listeria* genus was used as the nearest external source.

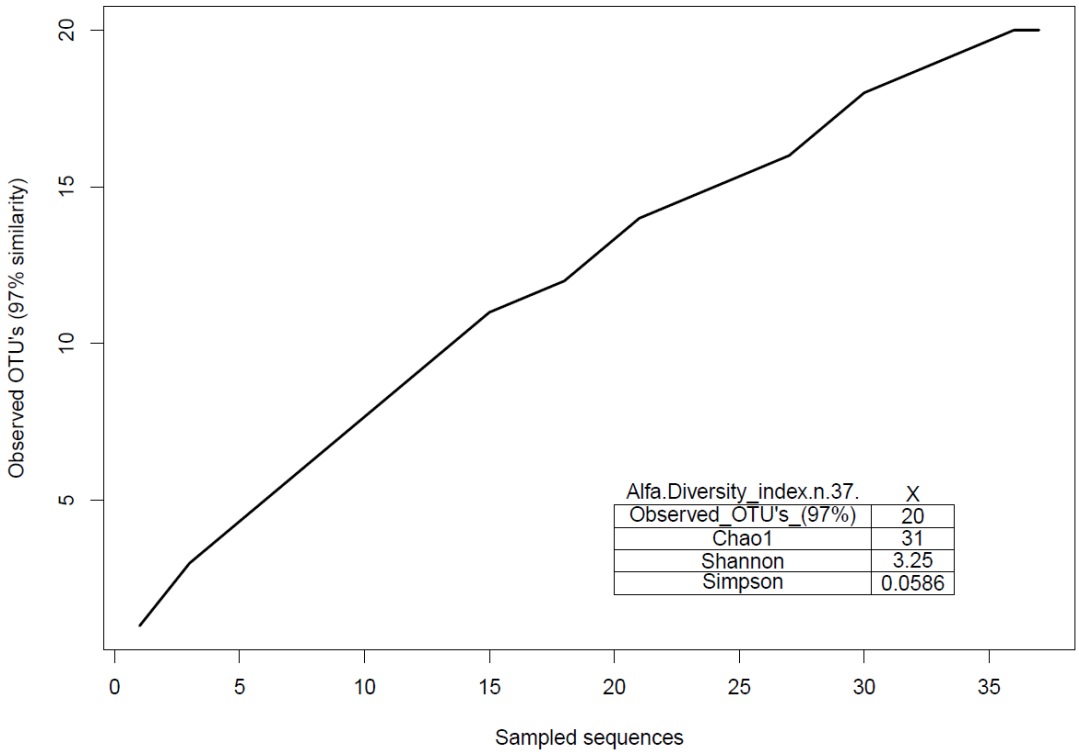

Figure 6. OTU rarefaction curve identified.

Conclusions

With enriched metagenomic cultures, 35 OTUs were obtained, mainly from LB-starch; these are related to the *Clostridium*, *Bacillus*, *Lysinibacillus* and *Enterococcus* genera (*Firmicutes*). Also, an OTU related to *Citrobacter* was found, a genus important in cellulose degradation to H_2 production. Soil tends to have a high organic material content, but according to the rarefaction curve it is not diverse, due to the use of complex substrates. Principally bacteria genera found in this study are related to free-living-PGPB. Some *Bacillus* and *Citrobacter* strains are highly involved with phosphorus solubilization and nitrogen fixing that correlates with the high content of phosphorus and nitrogen in the analyzed soil. Besides, the OTUs found are related to microorganisms able to break down starch and cellulose residues. The information acquired could be used by institutions or organizations related to sugarcane cultivation and commercialization to improve the soil quality via exogenous inoculation of the species here mentioned and for biotechnological applications in biofuel production.

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