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Phylogenetic Diversity of Bacteria in an Earth-Cave in Guizhou Province, Southwest of China

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The objective of this study was to analyze the phylogenetic composition of bacterial community in the soil of an earth-cave (Niu Cave) using a culture-independent molecular approach. 16S rRNA genes were amplified directly from soil DNA with universally conserved and *Bacteria*-specific rRNA gene primers and cloned. The clone library was screened by restriction fragment length polymorphism (RFLP), and representative rRNA gene sequences were determined. A total of 115 bacterial sequence types were found in 190 analyzed clones. Phylogenetic sequence analyses revealed novel 16S rRNA gene sequence types and a high diversity of putative bacterial community. Members of these bacteria included *Proteobacteria* (42.6%), *Acidobacteria* (18.6%), *Planctomycetes* (9.0%), *Chloroflexi* (Green nonsulfur bacteria, 7.5%), *Bacteroidetes* (2.1%), *Gemmatimonadetes* (2.7%), *Nitrospirae* (8.0%), *Actinobacteria* (High G+C Gram-positive bacteria, 6.4%) and candidate divisions (including the OP3, GN08, and SBR1093, 3.2%). Thirty-five clones were affiliated with bacteria that were related to nitrogen, sulfur, iron or manganese cycles. The comparison of the present data with the data obtained previously from caves based on 16S rRNA gene analysis revealed similarities in the bacterial community components, especially in the high abundance of *Proteobacteria* and *Acidobacteria*. Furthermore, this study provided the novel evidence for presence of *Gemmatimonadetes*, *Nitrosomonadales*, *Oceanospirillales,* and *Rubrobacterales* in a karstic hypogean environment.

Keywords: 16S rRNA gene, karst, cave, bacterial community, microbial ecology

The term of "cave" is defined as any natural space below the Earth's surface that extends beyond the twilight zone, and that is accessible to humans (Gillieson, 1996). Most of common types of caves in karst regions are those formed in limestone and other calcareous rocks, and as lava tubes in basaltic rock. Remaining types, including those formed in gypsum, granite, talus, quartzite, ice, and sandstone are usually limited in extent. Caves, with relatively limited nutrient in organic matter, stable and low temperatures, high humidity and mineral concentrations, can be considered extreme environments for life and provide ecological niches for highly specialized microorganisms (Schabereiter-Gurtner *et al*., 2003). Dripping water, visitors and animals can provide organic input that facilitates life of heterotrophic microorganisms in some caves (Groth and Saiz-Jimenez, 1999; Groth *et al*., 1999, 2001).

Microorganism in caves is the main biological habitant and remarkably contributes to cave ecology (Northup and Lavoie, 2001). Presences of microorganisms in terrestrial and aquatic cave environments around the world have been reported using the cultivation method (Cunningham *et al*., 1995; Gonzalez *et al*., 1999; Laiz *et al*., 1999, 2000; Groth *et al*., 1999, 2001; Cañaveras *et al*., 2001). Many microbes have been identified to be related to dissolution and precipitation reactions that involves carbonates, moonmilk, silicates, clays, iron, manganese, sulfur, and saltpeter (Northup and Lavoie, 2001). Enrichment-based and cultural investigations on typical heterotrophic microbes have shown that microbes grow in proportion to less than 1% in an environment (Amann *et al*., 1995).

Culture-independent 16S rRNA gene sequence analysis has been employed to study bacterial communities in environmental samples without prior cultivation. It has significantly revealed a broader diversity of 16S rRNA gene sequence types than culture-based studies (Amann *et al*., 1995; Head *et al*., 1998; Hugenholtz *et al*., 1998). The combination of phylogenetic sequence analysis with restriction fragment length polymorphisms (RFLPs) of PCR-amplified bacterial 16S rRNA genes has become a powerful tool to investigate natural bacterial communities. However, the 16S rRNA gene-based analysis of bacterial colonization in caves has been restricted to the samples of rocks, paintings, dripping waters, springs, and underwater passages, but not on soil (Northup *et al*., 2000; Vlasceanu *et al*., 2000; Engel *et al*., 2001, 2004; Holmes *et al*., 2001; Schabereiter-Gurtner *et al*., 2002a, 2002b, 2003).

Guizhou, a province in southwest of China, is the center of East-Asia developing karst area. As one of the three developing karst areas, this area has a karst area of over 5.5×10^5 km^2 and is the largest and the most complex developing karst area in the world (Smart *et al*., 1986; Zhang *et al*., 1992). Niu Cave, an unusual cave in this karst area, is composed of soil other than usual limestone or alcareous rocks. So far we have limited knowledge to understand the microbial community in an earth-cave. The aim of the present study was to investigate the bacterial diversity in Niu

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Cave by culture-independent method and present the first knowledge to understand microbial composition in such environment.

Materials and Methods

Site description and environmental sample collection

Niu Cave (25° 57′ N, 107° 48′ E) is located in Dushan county, south of Guizhou province, southwest of China. As a "karst province", Guizhou has a karst area of 1.3×10^5 km^2, comprising 73.8% of its total area. Niu Cave is formed by soil, and with 5-10 m height, 4-50 m width. After a distance of about 2 km from the entrance, the cave is divided into three layers. Each layer has a long distance more than 5 km. Thirty soil samples, 10 from each layer, were sampled and mixed thoroughly for bacterial community analysis. Soil samples were stored at -20°C and analyzed within a month.

Soil DNA extraction, PCR amplification, and cloning

Soil DNA was extracted with a soil DNA isolation kit (Catalog #12800-50, MO BIO Laboratories, USA) following the manufacturer's instructions. Bacterial 16S rRNA genes were amplified by PCR using the combination of universal primer 1492r and bacterial primer 27f (Lane, 1991). The PCR reaction was performed with a thermal program, which comprised preheating at 95°C for 2 min, 25 cycles at 98°C for 1 min, 50°C for 40 s, 72°C for 2 min and a final extension of at 72°C for 10 min. The amplified products were purified using an agarose gel DNA purification kit (No. DV805A, Takara, Japan). Bacterial 16S rRNA gene amplicon (ca. 1500 bases) was then excised from a 1% agarose gel and eluted with the same kit. Finally, the purified product was ligated into the pMD 18 T-vector (Takara) and the ligation product was transformed into *Escherichia coli* DH5α competent cells with ampicillin and blue/white screening following manufacturer's instructions.

Screening of rRNA gene clones

Inserts of rRNA genes from recombinant clones were reamplified with vector primers M13-M3 and M13-RV. The amplifications were subjected to restriction fragment length polymorphism (RFLP) by separate enzymatic digestions with *Hha*I (Takara) and *Msp*I (Takara) endonucleases following the manufacturer's instructions, and the digested DNA fragments were electrophoresed in 3% agarose gels. After staining with ethidium bromide, the gels were photographed using an image-capture system UVITEC DBT-08, and scanning image analyses were performed manually.

DNA sequencing and phylogenetic analysis

One to three representative clones from each unique RFLP type were selected for sequencing. The 16S rRNA gene inserts were sequenced using plasmid DNA as template and M13-20 or M13-RV-P as sequencing primer. Sequencing was done on an automated ABI 3730 sequencer by Beijng Genomics Institute. The resulting sequences (next to the primer 1492r and at least 600 bp) were compared with those available in GenBank by use of the BLAST method to determine their approximate phylogenetic affiliation and 16S rRNA gene sequence similarities (Altschul *et al.*, 1990; Engel *et al.*, 2004). Chimeric sequences were identified by use of the CHECK-CHIMERA program of the Ribosomal Database Project (Maidak *et al.*, 1997), and by independently comparing the alignments at the beginning and the end of each sequence and the alignments of the entire sequence with sequences from public databases. Sequences differing only slightly (≤3%) were considered as a phylotype, and each phylotype was represented by a sequence (Huang *et al.*, 2004). Nucleotide sequences were initially aligned using CLUSTAL X (Thompson *et al.*, 1997) and then manually

Table 1. Distribution of clones and phylotypes from the bacterial 16S rRNA gene library

Putative phylogenetic affiliation[b]	No. of clones	% of clones	No. of phylotypes[a]	% of phylotypes	% of sequence similarity to its closest relatives[b]	No. of phylotypes that exhibit <90% similarities to its closest relatives[b]
1. *Proteobacteria*	80	42.6	43	37.4	89-100	3
1.1 *Alphaproteobacteria*	21	11.2	12	10.4	91-98	-
1.2 *Betaproteobacteria*	14	7.5	7	6.1	94-100	-
1.3 *Deltaproteobacteria*	20	10.6	11	9.6	89-98	3
1.4 *Gammaproteobacteria*	25	13.3	13	11.3	92-99	-
2. *Acidobacteria*	35	18.6	21	18.3	91-98	-
3. *Planctomycetes*	17	9.0	14	12.2	87-95	1
4. *Chloroflexi*	14	7.5	8	7.0	88-94	2
5. *Bacteroidetes*	4	2.1	4	3.5	92-96	-
6. *Gemmatimonadetes*	5	2.7	5	4.4	92-99	-
7. *Nitrospirae*	15	8.0	6	5.2	89-98	1
8. *Actinobacteria*	12	6.4	10	8.7	90-99	-
9. Candidate division	6	3.2	4	3.5	89-95	2

[a] Sequences of RFLP types differing only slightly (≤3%) were considered as a phylotype (Huang *et al.*, 2004).
[b] Closest relatives as determined by the BLAST method (Altschul *et al.*, 1990; Engel *et al.*, 2004).

adjusted. Distance matrices and phylogenetic trees were calculated according to the Kimura two-parameter model (Kimura, 1980) and neighbor-joining (Saitou *et al.*, 1987) algorithms using the MEGA (version 3.1) software packages (Kumar *et al.*, 2004). One thousand bootstraps were performed to assign confidence levels to the nodes in the trees. The 16S rRNA gene sequences have been deposited in the GenBank nucleotide sequence database under accession numbers EF141837-EF141978.

Results

A total of 190 recombinant clones were randomly selected, and their rRNA gene inserts were subjected to restriction endonuclease analysis (RFLP), resulting in 142 different RFLP types. One to three representative clones of each unique RFLP type were partially sequenced. The clones showing sequence dissimilarity less than 3% among cloned library were considered as a phylotype. As a result, 117 phylotypes were generated. Two chimeric sequences were identified and excluded from subsequent analyses. Both of them belonged to unique RFLP types represented by a single clone. It was determined that most relatives of phylotypes (87 sequences representing 133 clones) were related to environmental clones and 9 phylotypes had relatively low levels of similarity (<90%) with their closest counterparts in the GenBank databases (Table 1). None of phylotypes were closely related to bacterial sequences detected in other caves or karst areas in the public databases except the clone CV76 (DQ499315), relative of NC123. These indicated that the bacterial community associated with Niu Cave was novel and complex.

Phylogenetic analyses placed the 115 phylotypes in the following 9 groups of the domain Bacteria: *Proteobacteria*, *Acidobacteria*, *Planctomycetes*, *Chloroflexi* (Green nonsulfur bacteria), *Bacteroidetes*, *Gemmatimonadetes*, *Nitrospirae*, *Actinobacteria* (High G+C Gram-positive bacteria), and candidate divisions (including the OP3, GN08, and SBR 1093). Among them, the *Proteobacteria* was the largest group including 80 clones, followed by *Acidobacteria* (35 clones) and *Planctomycetes* (17 clones).

Proteobacteria

A total of 80 clones, represented by 43 phylotypes and accounting for 42.6% of the clone library, were phylogenetically associated with 4 classes of *Proteobacteria* with similarities between 89%-100%: *Alphaproteobacteria* (number of phylotypes, np=12, number of clones, nc=21), *Betaproteobacteria* (np=7, nc=14), *Deltaproteobacteria* (np=11, nc=20), and *Gammaproteobacteria* (np=13, nc=25) (Table 1). Thirty-eight clones were represented by 10 phylotypes each including at least 3 clones, while 24 clones were represented by phylotypes of which each included a single clone.

Eight phylotypes of *Deltaproteobacteria* revealed less than 94% similarity. Three phylotypes (NC002, NC039, and NC 141), sharing less than 90% similarity to known sequences, seemed to be representatives of novel taxa within *Deltaproteobacteria* subdivisions, respectively (Table 1, Fig. 1).

Thirty-six clones, represented by 19 phylotypes, were related to cultured members and belonged to putatively

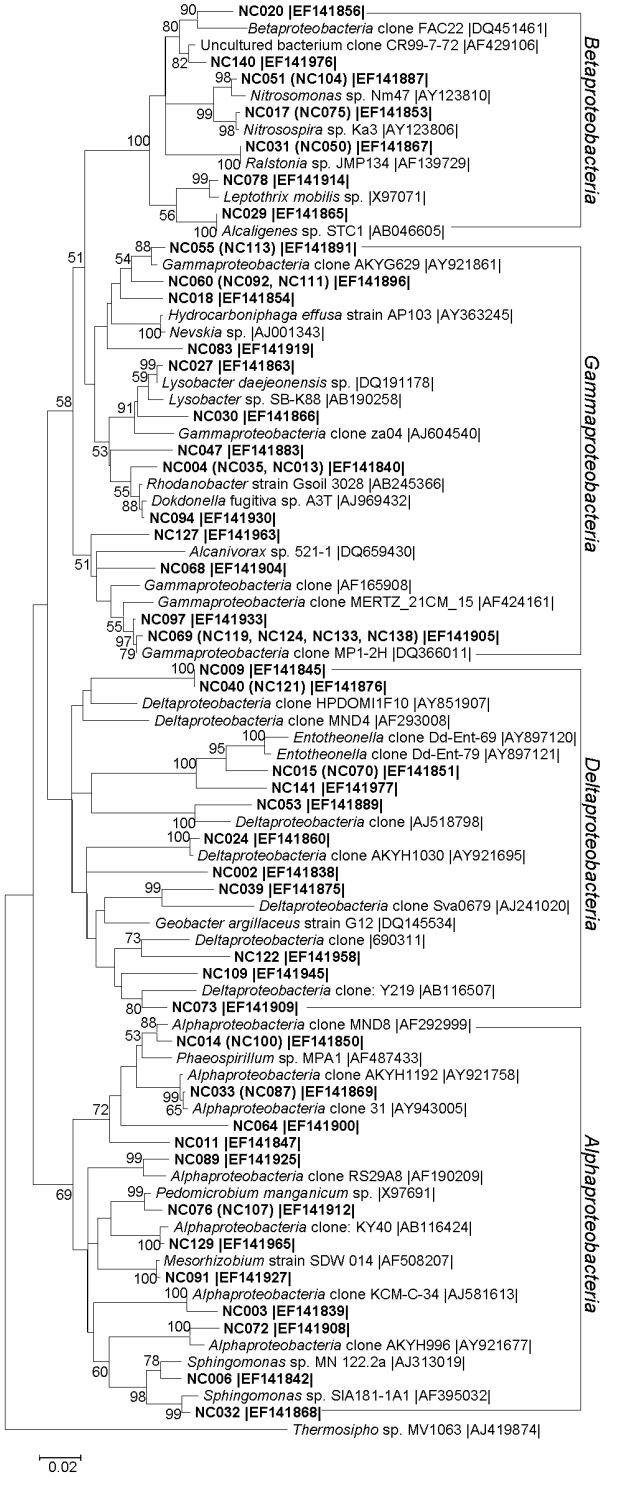

Fig. 1. 16S rRNA gene-based dendrogram showing phylogenetic relationships of bacterial phylotypes from Niu Cave (shown in bold) to members of the *Proteobacteria* from public database. Bootstrap values (n=1000 replicates) of ≥50% are reported as percentages. The scale bar represents the number of changes per nucleotide position. *Thermosipho* sp. MV1063 (AJ419874) was used as outgroup. Sequences of RFLP types differing only slightly (≤3%) are shown in parentheses. Accession numbers are given at the end of each sequence.

Sphingomonadales (NC006 and NC032), *Rhizobiales* (NC091 and NC076), *Rhodospirillales* (NC011), *Nitrosomonadales* (NC017 and NC051), *Burkholderiales* (NC029, NC031, and NC078), *Desulfuromonales* (NC002), *Xanthomonadales* (NC004, NC018, NC027, NC030, NC060, NC083, and NC094) and *Oceanospirillales* (NC127). The closest relatives of NC006 and NC032 were strains of *Sphingomonas* (Fig. 1). NC015, NC141 affiliated to the members of *Entotheonella* (similarity >95%) which is a new genus belonging to unclassified *Deltaproteobacteria* (Schirmer *et al.*, 2005).

Acidobacteria

Thirty-five clones, represented by 21 phylotypes and accounting for 18.6% of the clone library, were clustered with the uncultivated bacterial sequences of *Acidobacteria* with similarities between 91%-98% (Table 1, Fig. 2). Fig. 2 showed a phylogenetic tree of *Acidobacteria*, which was grouped into at least 4 acidobacterial clusters. *Acidobacteria* form a newly devised division of *Bacteria*, probably as diverse as *Proteobacteria* or Gram-positive bacteria. The definition of this phylum was based on the analysis of 16S rRNA gene sequences retrieved from cloned rRNA genes and phylogenetically related to several cultivated species such as the Fe(III)-reducing *Geothrix fermentans* (Ludwig *et al*., 1997; Quaiser *et al*., 2003). The clones Y72 (AB116490) and Y190 (AB116442), relatives of NC065 and NC099 respectively, were detected in coastal marine sediment beneath areas of intensive shellfish aquaculture where sulfur cycle was accelerated (Asami *et al*., 2005).

Planctomycetes

Fourteen phylotypes, representing 17 clones and accounting for 9.0% of the clone library, were grouped into at least 3 clusters with *Planctomycetes*. These clones were related with relatively low similarities (in the range of 87%-95%) to cultured and uncultured bacterial sequences listed in the GenBank database (Table 1, Fig. 2). Molecular microbial ecology has provided new evidence showing that *Planctomycetes* bacteria are ubiquitous and constitute a representative part of the natural bacteria population (Hugenholtz *et al*., 1998; Neef *et al*., 1998). The clone 018 (AB252879), relative of NC057, was a member detected in an iron-oxidation biofilm at Shibayama lagoon. The retrieved three sequences NC010, NC038, and NC052 were clustered with *Pirellula staleyi* strain ATCC 35122 (AF399914) which can outlast periods of nutrient depletion with the expression of genes for carbon starvation (Glöckner *et al*., 2003).

Chloroflexi (Green nonsulfur bacteria)

Fourteen clones, represented by 8 phylotypes and accounting for 7.5% of the clone library were related to the members of the *Chloroflexi* phylum (88% < similarity < 94%) (Table 1, Fig. 3). The low similarities to closest members indicated that the corresponding bacteria detected in Niu Cave belonged to putatively new taxonomic groups. The clone H5 (AF234688), close to NC130, was found in a nitrifying-denitrifying activated sludge (Juretschko *et al*., 2002).

Bacteroidetes, Gemmatimonadetes, and Nitrospirae

Four phylotypes, each representing a single clone, were clustered with *Bacteroidetes*. Five clones, each represented by a phylotype and accounting for 2.7% of the clone library, were grouped within *Gemmatimonadetes* phylum. These clones were related to only uncultured bacterial sequences listed in the GenBank database (Table 1, Fig. 3). Fifteen clones, represented by 6 phylotypes and accounting for 8.0% of the clone library, were grouped with *Nitrospirae*. Among them, 10 of the 15 clones were represented by 2 phylotypes (NC022 and NC132).

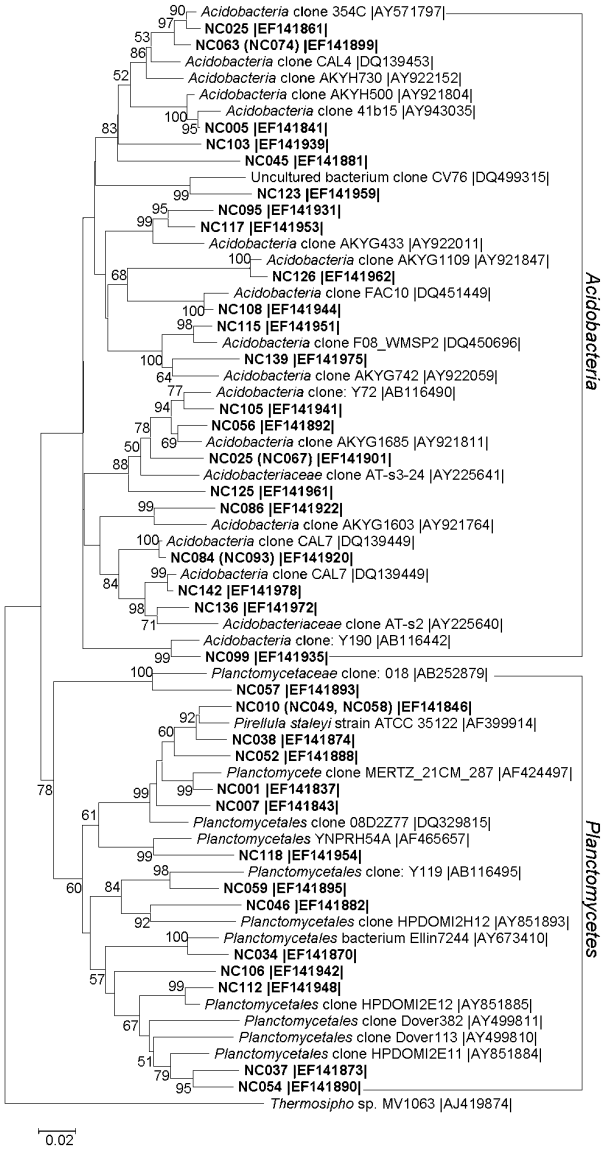

Fig. 2. 16S rRNA gene-based dendrogram showing phylogenetic relationships of bacterial phylotypes from Niu Cave (shown in bold) to members of the *Acidobacteria* and *Planctomycetes* from public database. Bootstrap values (n=1000 replicates) of ≥50% are reported as percentages. The scale bar represents the number of changes per nucleotide position. *Thermosipho* sp. MV1063 (AJ419874) was used as outgroup. Sequences of RFLP types differing only slightly (≤3%) are shown in parentheses. Accession numbers are given at the end of each sequence.

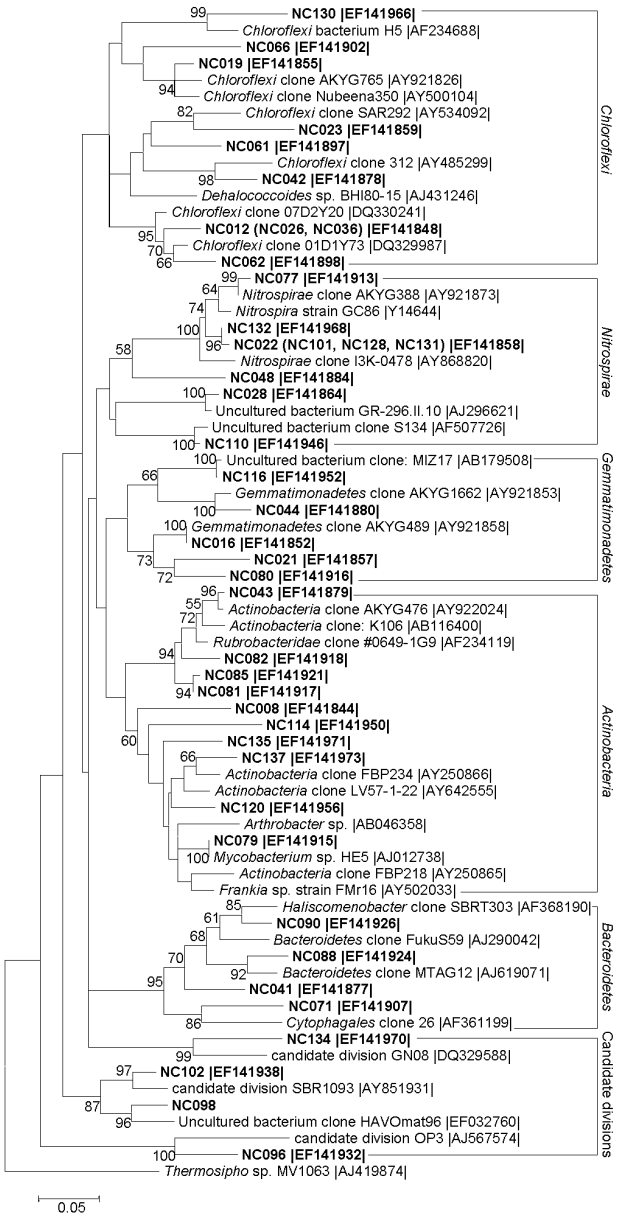

Fig. 3. 16S rRNA gene-based dendrogram showing phylogenetic relationships of bacterial phylotypes from Niu Cave (shown in bold) to members of the *Chloroflexi, Nitrospirae, Gemmatimonadetes, Actinobacteria, Bacteroidetes* and Candidate divisions from public database. Bootstrap values (n=1000 replicates) of ≥50% are reported as percentages. The scale bar represents the number of changes per nucleotide position. *Thermosipho* sp. MV1063 (AJ419874) was used as outgroup. Sequences of RFLP types differing only slightly (≤3%) are shown in parentheses. Accession numbers are given at the end of each sequence.

Actinobacteria (High G+C Gram-positive bacteria)

Ten phylotypes, representing 12 clones and accounting for 6.4% of the clone library, were associated with *Actinobacteria* with 90%-99% similarities (Table 1, Fig. 3). Five phylotypes were related to members of *Actinomycetales* (NC008, NC 079, and NC114) and *Rubrobacterales* (NC081 and NC085).

Discussion

Terrestrial subsurface environments are often inaccessible for study, limiting our understanding of ecosystem structure and dynamics, elemental cycling, and the impacts to earth and atmospheric biogeochemical processes. Geomicrobiological activities in caves are no longer underestimated, since studies showed that bacterial metabolism remarkably contributes to cave ecology (Northup and Lavoie, 2001). However, no study has been initiated to investigate the phylogenetic diversity of bacteria in caves formed in soil, such as Niu Cave. We firstly presented the knowledge on bacterial community and revealed a considerable number of novel and unknown bacterial sequences and a high diversity of putative bacterial communities in this environment.

Using culture-independent methods, previous studies (Holmes *et al*., 2001; Schabereiter-Gurtner *et al*., 2002a, 2002b, 2003; Northup *et al*., 2003; Engel *et al*., 2004) have revealed that bacteria belonging to *Proteobacteria*, *Bacteroidetes,* and *Actinobacteria* were usually found in different substances of caves (Table 2). Among them, *Proteobacteria* was reported to be the dominant bacteria and could act as a key role in the processes of biogeochemical cycle in caves. In Tito Bustillo Cave, Altamira Cave and La Garma Cave, *Acidobacteria* was the second dominating phylogenetic group after *Proteobacteria*. Our result supported this conclusion. Higher than 7.0% of *Chloroflexi* was found in NC and Tito Bustillo Cave. And *Firmicutes* had been found in Llonín Cave, La Garma Cave and Lechuguilla and Spider Caves. In our study, there were no phylotypes belonging to *Firmicutes*. Orders *Rhizobiales*, *Sphingomonadales*, *Rhodospirillales*, *Burkholderiales*, *Rhodocyclales*, *Desulfuromonales*, *Pseudomonadales* and *Actinobacterales* were reported to be present in caves, including Niu Cave. However, *Myxococcales* was found only in Altamira Cave, and *Xanthomonadales* only in Llonín Cave.

The phylum *Gemmatimonadetes*, and the orders *Nitrosomonadales* (*Betaproteobacteria*), *Oceanospirillales* (*Gammaproteobacteria*), and *Rubrobacterales* (*Actinobacteria*) found in Niu Cave were never reported in the previous studies.

Six clones, NC091, NC031, NC051, NC017, NC029, and NC022, were related to *Mesorhizobium*, *Ralstonia*, *Nitrosomonas*, *Nitrosospira*, *Alcaligenes,* and *Nitrospira* with similarities higher than 97%, respectively (Fig. 1, 3). These genera were reported to involve in putatively nitrogen cycle. *Mesorhizobium* belongs to members of nitrogen-fixing bacteria known as rhizobia (Bottomley, 1992). *Ralstonia taiwanensis* was reported being capable of nitrogen fixation (Chen *et al*., 2001), and *Ralstonia eutropha* containing two nitric oxide reductases (Cramm *et al*., 1997). *Nitrosomonas* and *Nitrosospira* are members of the ammonia-oxidizing proteobacteria which can convert ammonia to nitrite (Suzuki *et al*., 1974; Hiorns *et al*., 1995). *Alcaligenes* sp. STC1 (AB046605) was a C1-using aerobic denitrifier (Ozeki *et al*., 2001). *Nitrospira* was the dominant nitrite oxidizers in most environmental samples tested so far (Burrell *et al*., 1998). The bacteria involved in nitrogen cycle were reported in all studies on bacterial diversity in caves.

NC076 and NC078 were related to *Pedomicrobium* and *Leptothrix* respectively (Fig. 1). *Pedomicrobium* plays an important role in iron- and manganese-oxidization. Ghiorse and Hirsch (1979) observed that two *Pedomicrobium*-like

Table 2. Distribution of bacteria in caves investigated by culture-independent molecular method

Putative phylogenetic affiliation	% of clones							
	Nullarbor Caves (Holmes *et al.*, 2001)	Altamira Cave (Schabereiter-Gurtner *et al.*, 2002a)	Tito Bustillo Cave (Schabereiter-Gurtner *et al.*, 2002b)	Llonín Cave (Schabereiter-Gurtner *et al.*, 2003)	La Garma Cave (Schabereiter-Gurtner *et al.*, 2003)	Lechuguilla and Spider Caves (Northup *et al.*, 2003)	Lower Kane Cave (Engel *et al.*, 2004)	Niu Cave (this study)
Proteobacteria	40.0	52.3	48.8	59.3	32.8	35.0	92.7	42.6
Acidobacteria		23.8	29.2		24.1		5.6	18.6
Planctomycetes	5.7	4.8	2.4					9.0
Bacteroidetes	8.6	9.5	2.4	11.1	3.5		1.7	2.1
Chloroflexi		4.8	7.3		1.7			7.5
Nitrospirae	5.7			3.7	3.5	15.5		8.0
Actinobacteria	5.7	4.8	9.8	22.2	19.0	11.7		6.4
Firmicutes				3.7	13.8	37.9		
Gemmatimonadetes								2.7

budding bacteria deposit Fe and Mn ions on their cell walls. Peck (1986) reported iron-impregnated sheaths of *Leptothrix* sp., which was inoculated with mud from Level Crevice Cave. Moore (1981) found manganese-oxidizing bacteria *Leptothrix* sp. in a stream in Matts Black Cave, West Virginia, and attributed the formation of birnessite in this cave to the precipitation of manganese around sheaths of the bacteria. The concentrations of iron, manganese and other elements have been found in Tito Bustillo and other Spanish and Italian caves (Schabereiter-Gurtner *et al*., 2002a, 2002b, 2003).

The order *Desulfuromonales* included *Desulfuromonas* (Liesack and Finster, 1994) related with sulfate/sulfur reduction and *Geobacter* with Fe(III)/Mn(IV) reduction (Nealson and Saffarini, 1994). A pioneering study of Movile Cave, Romania, by Sarbu *et al*. (1996) revealed sulfide/ sulfur oxidation bacteria including species of *Thiobacillus* and *Beggiatoa*, and sulfate-reducers including species of *Desulfovibrio*. Up to now, sulfur and sulfide oxidizers, sulfate reducers, appear abundant in caves (Schabereiter-Gurtner *et al*., 2003).

Acidobacteria and *Planctomycetes* were the second and third dominating phylogenetic groups in Niu Cave respectively. It confirmed members of the divisions are ecologically significant constituents of Niu Cave. Representatives of the poorly studied phylogenetic divisions have been detected in many clonal analyses and are thought to be of great ecological significance to many ecosystems (Kuske *et al*., 1997; Ludwig *et al*., 1997; Holmes *et al*., 2001). As limited cultivated species, *Acidobacteria* and *Planctomycetes*' ecological functions, and possible impacts on caves remain unclear at present. Two novel genera of *Planctomycetes*, Candidatus *Kuenenia stuttgartiensis,* and Candidatus *Brocadia annamoxidans* are capable of catalyzing the anaerobic oxidation of ammonium (Schmid *et al*., 2000; Jetten *et al*., 2001).

All above-mentioned results in our study were ascribed to culture-independent techniques including PCR amplification of bacterial 16S rRNA genes and restriction fragment length polymorphism (RFLP). The applied approach allows the analysis of poorly studied environments where nothing or little is known about bacterial diversity and natural growing conditions. Our present study gave insight into the great bacterial taxonomic diversity in Niu Cave, overcoming some of the limiting factors of cultivation methods and allowing the detection of putatively uncultivable and unexpected bacteria.

However, studies based on culture-independent methods make more difficult valid statements about the ecological role that clones might play in the environment. To determine microbial contribution to cave ecology, we need to draw on additional wealth of rigorous research done on this system. Stable isotope techniques can provide information on microbial contribution to mineral formation (Hose *et al*., 2000) and ecosystem bioenergetics (Sarbu *et al*., 1996). The PCR- and RFLP-based investigation of bacterial diversity is not free from bias. Limitations such as inefficient cell lysis, and the preferential and selective amplification of 16S rRNA gene fragments may lead to an underestimation of bacterial diversity (Liesack *et al*., 1997). Potentially, with the development of molecular sequence-based techniques and accumulation of information on the diversity and structure of bacterial communities in caves, more explanations concerning contribution of microorganisms to cave ecosystem will be presented.

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