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ORIGINAL PAPER

Microbial diversity in acid mine drainage of Xiang Mountain sulfide mine, Anhui Province, China

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Abstract To understand the composition and structure of microbial communities in acid (pH 3.0) mine drainage (AMD) associated with pyrite mine tailings in Anhui Province, China, molecular diversities of 16S rRNA and 18S rRNA genes were examined using a PCR-based cloning approach. Bacterial, archaeal and microeukaryotic clone libraries were constructed. In contrast to typical dominance of autotrophic acidophiles, genus *Acidiphilium*, which consists of mixotrophic acidophiles capable of chemoorganotrophic and photosynthetic metabolisms, was the largest group in the bacterial clone library. These mixotrophic organisms may be advantageous in the oligotrophic AMD environment of the study site (certain amounts of dissolved organic carbon and light) by switching between two modes of metabolisms. Unexpectedly, a large fraction of bacterial clones (12.7%) were related to the neutrophilic genus *Legionella*, which can cause Legionnaires' disease, a potentially lethal pneumonia. The eukaryotic 18S rRNA gene sequences were mostly related to *Oxytricha*, *Nuclearia*, and *Penicillium*. In the archaeal clone library, all the sequences were affiliated to the phylum *Crenarchaeota*, while the *Euryarchaeota* was not present.

Keywords Acid mine drainage · Acidophile · 16S rRNA · Clone library · *Acidiphilium* · *Legionella*

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Introduction

Mining of metal sulfide ores constitutes an important global industry. However, closure and abandonment of mine sites usually results in a legacy of pollution of local environments that may persist for decades and even centuries after mining activity ceased (Younger 1997). One of the most serious ecological problems caused by mining industry is the occurrence of acid mine drainage (AMD). The mining wastewaters typically contain high levels of metal ions such as iron, copper, aluminum and manganese, as well as metalloids, of which arsenic is generally of the greatest concern. AMD generates when metal sulfide minerals, particularly pyrite (FeS_2), come in contact with oxygen and water. The overall reaction can be written as follows: $FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$; Fe^{3+} is the predominant oxidant at low pH and is usually the limiting agent. The abiotic oxidation rate of ferrous ion (Fe^{2+}) below pH 3 is slow. Acidophilic organisms, however, can generate energy by converting ferrous to ferric iron in acidic environment. Thus, the oxidation of pyrite and acidification of the AMD is greatly increased in the presence of iron-oxidizing species such as *Acidithiobacillus ferrooxidans* (Johnson 1998).

Many workers have used the rRNA gene analysis approach to detect acidophile populations and to describe the structures of microbial communities in various acidic

environments, such as AMD sites, bio-oxidation reactors, and bioleaching heaps, without isolating actual microorganisms (Gonzalez-Toril et al. 2003; Rzhepishevska et al. 2005; Demergasso et al. 2005; Hao et al. 2010). By the application of these cultivation-independent molecular techniques, our understanding of the biodiversity of acidophiles has been rapidly expanding, which has given more comprehensive insights into the role of acidophilic microorganisms in acidic ecosystems. It has now been shown that some organisms such as *Ferroplasma* spp. and *Gallionella* spp. have been found to be dominant in various acidic mine environments, and they probably play important roles in AMD generation (Bond et al. 2000; Bruneel et al. 2006; Hallberg et al. 2006; He et al. 2007).

In order to understand how a microbial ecosystem is structured and how it functions, the first step is to address the diversity and composition of the whole community. In this report, we present molecular results of the acidophilic community in water samples collected from an AMD lake associated with a pyrite-dominated ore body at the Xiang Mountain, Anhui Province, China. Our results considerably extend our knowledge of the diversity of acidophiles and shed some light on the relationship and interaction between prokaryotes and eukaryotes in AMD environments.

Materials and methods

Sample collection and geochemical measurements

Samples were collected from an AMD lake at Xiang Mountain, Anhui Province, China in June, 2009. The dominant ore body of Xiang Mountain is composed of pyrite (>90%), which has been mined for more than 50 years. Waste products are disposed of in a nearby refuse dump. The exposed waste ore minerals are oxidized by acidophiles and large amounts of Fe^{3+}, H^+ and SO_4^{2-} are released, which have resulted in the formation of the AMD lake since 1970s. The AMD lake is approximately 700 m long and 300 m wide.

Geochemistry of lake water was measured in several locations using a Hanna water test field analyzer (VWR, UK) and the results indicated that the physico-chemical conditions around the lake were nearly the same. Therefore, one surficial water sample from the southwestern corner of the lake was collected into 2.5-l bottles for microbial analysis. The sample was kept on ice until it was filtered through a Millex-GS Millipore filter (pore size 0.22 μm, diameter 50 mm). The filters were stored at −20°C until processing.

Temperature, pH, redox potential, dissolved oxygen (DO), and conductivity of lake water were measured in situ using a Hanna field analyzer (VWR, UK). Additional water samples were frozen at −20°C and shipped to the Geomicrobiology Laboratory of China University of Geosciences in Beijing on the same day. The following analyses were conducted within 2 days of collection. Sulfate concentration was determined by ion chromatography; dissolved metals by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, JYl-ULTIMA, France); dissolved ferrous iron titrated with ceric sulfate; and total organic carbon (TOC) measured by a TOC analyzer (Shimadzu, Japan). Chemical oxygen demand (COD), total nitrogen (TN), total phosphorous (TP), and NH_4^+-N were determined in accordance with standard methods (Ministry of Environmental Protection 2002).

DNA extraction and purification

The method used for extraction of nucleic acids from the AMD sample was based on a protocol described previously (Zhou et al. 1996) and included initial wash steps in order to exclude iron and to raise the pH to 8.0 prior to cell lysis.

Each Millex-GS Millipore filter was cut and placed into a 15-ml Falcon tube. After thawing, the filter samples were washed twice with 5 ml 0.02 M H_2SO_4 and then with 5 ml STE buffer (sucrose 100 mg/ml, Tris–HCl 50 mM, EDTA 10 mM, NaCl 100 mM, pH 8.0). Subsequently, the samples were resuspended in 3 ml DNA extraction buffer (sucrose 100 g/l, Tris–HCl 50 mM, EDTA 10 mM, NaCl 100 mM, 10 g/l CTAB, pH 8.0), and 50 μl lysosyme (10 mg/l) were added to the suspension. This mixture was incubated at 37°C for 40 min. After incubation, 50 μl Proteinase K (10 mg/ml) and 1 ml 10 g/l SDS solutions were added to the mixture followed by incubation at 50°C for 45 min. The supernatants were collected after centrifugation at 6,000×*g* for 10 min at room temperature and transferred into new centrifuge tubes. The cell lysates were extracted twice with an equal volume of chloroform:isoamylalcohol (24:1 vol/vol). The nucleic acids were precipitated from the aqueous phase with two volumes of ethanol at −20°C for 2 h. The pellet of nucleic acids was obtained by centrifugation at 12,000×*g* for 20 min at 4°C, washed with ice cold 70% ethanol, and dissolved in 50 μl sterile deionized water.

PCR amplification and clone library construction

PCR of 16S/18S rRNA gene fragments of bacteria, archaea, and microeukaryotes were all performed with Applied Biosystem Gene Amp PCR system 9700. In the reactions, bacterial primer pairs were universal 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′, M=C or A) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′, Y=C or T). Archaea-specific primers were Arch-21F (5′-TTC YGG TTG ATC CYG CCR GA-3′, R=A or C) and

Arch-958R (5′-YCC GGC GTT GAM TCC AWT T-3′, W=A or T). Primers for eukaryotic microbes were Euk-817F (5′-TTA GCA TGG AAT AAT RRA ATA GGA-3′) and Euk-1536(5′-ATT GCA ATG CYC TAT CCC CA-3′). PCR amplification reaction conditions were the same as previously described (Yin et al. 2008; Bruneel et al. 2006; Borneman and Hartin 2000).

PCR products were purified using the EZ-10 spin column DNA Gel Extraction kit (BBI, Canada), quantified by Nanodrop (Thermo, USA), and ligated into the pGEM-T easy vector (Promega, USA). The resulting plasmids were transformed into *E. coli* DH5α cells following the manufacturer's instructions.

Amplified ribosomal DNA restriction analysis and sequencing of inserted 16S rRNA genes

For amplified ribosomal DNA restriction analysis (ARDRA) and sequencing, the inserted fragment was amplified by PCR and template DNA was provided by contact of a small pipette tip with a colony of cloned host cells which were immersed in the PCR mixture. Primers for the PCR were the vector-specific T7 (5′-TAA TAC GAC TCA CTA TAG GGC-3′) and SP6 (5′-ATT TAG GTG ACA CTA TAG AAT ACT C-3′). The PCR products were digested with two four-base-specific restriction enzymes (*Hha*I and *Msp*I) (Promega, USA). The procedure of restriction enzyme digestion of the unpurified PCR products and observation of restricted fragments were the same as previously described (Picard et al. 2000). ARDRA patterns were grouped, and unique clones were sequenced by Shanghai Sangon Co., Ltd, China.

Phylogenetic analysis

The clone sequences obtained in this research were checked for chimeric artifacts by the check-chimera program of the Ribosomal Database Project (RDP) and a new program, Mallard, which can screen whole libraries of 16S rRNA gene sequences simultaneously. These sequences were also compared with 16S/18S rRNA gene sequences deposited in public database GenBank using the BLAST search program. The 16S/18S rRNA gene sequences of various microbes obtained from the GenBank database were aligned with the new sequences using BioEdit 7.0. Phylogenetic trees were constructed by the neighbor-joining method with robustness of 1,000 bootstrapping value in MEGA 4.0.

Nucleotide sequence accession numbers

Sequences reported in this paper have been submitted to GenBank with accession numbers GU979537-GU97956, GU979558-GU979562, and GU979564-GU979567.

Results

Aqueous chemistry

The temperature and pH of the AMD water associated with the tailings were 30.0°C and pH 3.0, respectively (Table 1). The acidic solution contained high levels of total Fe (mean 100.6 mg/l) and sulfate (mean 1,183.6 mg/l), and had a high Eh value (532 mV). The Fe^{2+} concentration was only 0.5 mg/l, indicating that ferric iron was the dominant form of dissolved iron in this ecosystem. The AMD at this site was highly acidic and red-brown colored throughout the year (data not shown). Unexpectedly, the concentration of Al was the highest among all measured metals and reached 1,186 mg/l, which demonstrated that some Al-rich mineral may be associated with pyrite in the tailing. Concentrations of other metals, such as Ca, Mn, Na, and Cu, were 488.1, 433.1, 69.1, and 51.8 mg/l, respectively. COD, TOC, TN, NH_4^+-N and TP were 38.8, 3.1, 14.8, 11.6, and 0.6 mg/l, respectively.

Cloning and sequencing

In this study, 140, 50, and 30 white colonies with inserted small-subunit ribosomal genes were chosen to construct bacterial, archaeal and microeukaryotic libraries, respectively. The results of the in situ PCR indicated that 130 out of 140 chosen clones (prefix AMD-Bac) had the correct size fragments for the bacterial library. Similarly, 45 archaeal clones and 26 microeukaryotic clones were proved valid, and they were given the prefixes AMD-Arc and AMD-Euk, respectively.

ARDRA banding patterns were clearly distinguishable and representatives of each group were sequenced. Sequence similarity then reduced the ARDRA groups to 19, 7, and 3 representative sequences for the bacterial, archaeal, and microeukaryotic library, respectively. Two of

Table 1 Physico-chemical characteristics of the AMD sample

Parameter	Value	Parameter	Value
Temperature (°C)	30.0	TP (mg/l)	0.6
pH	3.0	Fe_{tot} (mg/l)	100.6
Eh (mV)	532	Fe^{2+} (mg/l)	0.5
DO (mg/l)	4.7	Al (mg/l)	1,186
EC (mS/cm)	12.3	Ca (mg/l)	488.1
TDS (mg/l)	6,121	Cu (mg/l)	51.8
COD (mg/l)	38.8	Mn (mg/l)	433.1
TOC (mg/l)	3.1	Na (mg/l)	69.1
TN (mg/l)	14.8	K (mg/l)	6.8
NH_4^+-N (mg/l)	11.6	SO_4^{2-} (mg/l)	1,883.6

the bacterial sequences were removed since they were judged to be chimeras by Mallard.

Phylogenetic analysis of bacterial sequences

The 16S rRNA gene sequences of the bacterial library fell into seven phylogenetic divisions: *Alphaproteobacteria* (occupying 41.3% of total bacterial clones), *Betaproteobacteria* (6.3%), *Gammaproteobacteria* (14.3%), *Actinobacteria* (23.8%), *Acidobacteria* (1.6%), *Firmicutes* (11.1%), and *Cyanobacteria* (1.6%). A neighbor-joining tree was constructed with these and related sequences from the GenBank database (Fig. 1).

The *Alphaproteobacteria* group comprised the largest portion of the 16S rRNA gene library. The most abundant sequence AMD-Bac-11, representing 28.6% of the bacterial clones, formed a subdivision with *Acidiphilium* species in the phylogenetic tree (Fig. 1).

Sequences AMD-Bac-20, AMD-Bac-39, and AMD-Bac-49 constituted another abundant group within the *Alphaproteobacteria*, accounting for 11.1% of the total clones. The closest relative of these sequence types was the single sequence RCP2-17 retrieved from an acidic forested wetland (pH 3.0) impacted by reject coal. Furthermore, the closest cultured bacteria of the three sequence types were also accordant, e.g., a bacterium in the *Rickettsiaceae* family that inhabits the cytoplasm of the marine ciliate *Diophrys appendiculata* (*Ciliophora*, *Hypotrichia*), displaying over 95% homology (Vannini et al. 2005).

The sequences in the *Betaproteobacteria* represented 6.3% of the total clones. Of the three sequence types identified, AMD-Bac-29 and AMD-Bac-99 (4.8% of the total clones) were affiliated with *Ferrovum myxofaciens*, which was recently isolated from an abandoned copper mine (unpublished data). The species designated as *F. myxofaciens* in Genbank (accession number EF133508) has not been formally described, and it can not be phylogenetically associated with cultivated acidophiles. Limited physiological characterization carried out to date for this isolate confirmed that it can solely use ferrous iron as an

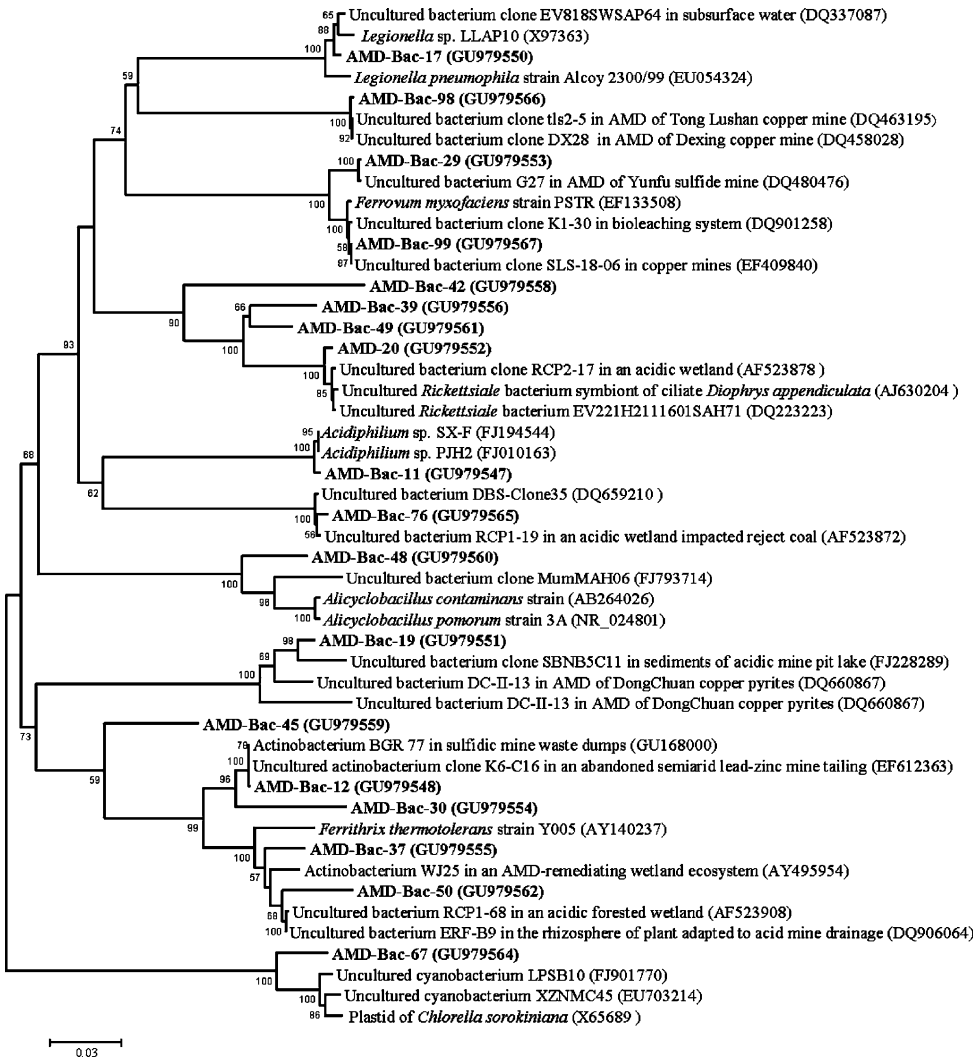

Fig. 1 Neighbor-joining phylogenetic tree based on analysis of 16S rRNA gene sequences of bacterial clones obtained from the AMD lake at Xiang Mountain in relation to reference sequences from the GenBank database. The sequences obtained in this study are indicated in bold. Scale bar indicates the Jukes-Cantor distances. Bootstrap values of >50% (for 1,000 iterations) are shown

electron donor, has an obligately autotrophic metabolism, and appears to be less acid tolerant than well-studied species *Leptospirillum ferrooxidans* and *A. ferrooxidans* (Rowe and Johnson 2008). The conspicuous dominance of this species in microbial community was recently reported in metal-rich mine waters in North Wales and a mine water treatment plant in Germany (Hallberg et al. 2006; Heinzel et al. 2009). Another clone type AMD-Bac-42 was a novel sequence, sharing low identity (<90%) to its closest relative in the GenBank database. The phylogenetically novel rRNA gene sequence was individually branched in the phylogenetic tree. Because members in this group were not closely related to any well-characterized organisms, neither their physiological characteristics nor their phylogenetic placement is currently known.

Another significant phylogenetic group was in the *Gammaproteobacteria*, including two sequence types. The majority of the *Gammaproteobacteria* sequences, represented by AMD-Bac-17, were related to the *Legionella* species. This sequence type was the third most abundant (12.7%) of the total bacterial clones. *Legionella* species are facultative intracellular gram-negative bacilli and ubiquitous in natural and man-made aqueous environments.

The second most abundant sequence type AMD-Bac-12, accounting for 15.9% of total clones, is positioned within the *Actinobacteria* group. AMD-Bac-12 and three other sequence types (AMD-Bac-30, AMD-Bac-37 and AMD-Bac-50) formed a branch in the phylogenetic tree with the iron-oxidizing acidophile *Ferrithrix thermotolerans* recently isolated from a geothermal site in Yellowstone National Park, USA. This species is an obligately heterotrophic, moderate thermophile and grows as long filaments, forming macroscopic flocs in liquid media. This organism can accelerate oxidative dissolution of pyrite in yeast extract-amended cultures, but is not able to oxidize reduced forms of sulfur (Johnson et al. 2009).

The phylum *Firmicutes* included two sequence types. The most abundant sequence type in this group (9.5% of the total clones), AMD-Bac-19, shared a high gene sequence identity with uncultured bacterium clone SBNB5C11 from sediments of acidic mine pits and some other clones detected in acidic environments. Another sequence type AMD-Bac-48 showed a high similarity value (>95%) with bacteria in the genus *Alicyclobacillus* and clustered with these species in the phylogenetic tree. *Alicyclobacillus* species are obligately heterotrophic iron-oxidizing acidophile, the presence of which in acidic mine waters is quite common and has been reported in various studies (Johnson and Hallberg 2009).

The *Cyanobacteria* were represented in the microbial community by only one sequence type, AMD-Bac-67, and accounted for 1.6% of the total clones. This sequence type was phylogenetically associated with uncultured cyanobacterial clones detected in lakes and sinkholes.

Phylogenetic analysis of archaeal sequences

The phylogenetic relationship of the seven archaeal representative sequences in the archaeal library was established with the bootstrap neighbor-joining method (Fig. 2). Clone analysis revealed that all the sequences were affiliated with the phylum *Crenarchaeota*, while *Euryarchaeota* was not represented. The two most abundant archaeal sequence types present in the AMD water, AMD-Arc-72 and AMD-Arc-70, representing 35.7 and 17.9% of the total clones, respectively, had 96 and 97% homology with uncultured archaeon clone Arc-DS17 detected in an acidic red soil. The two sequence types formed a cluster with

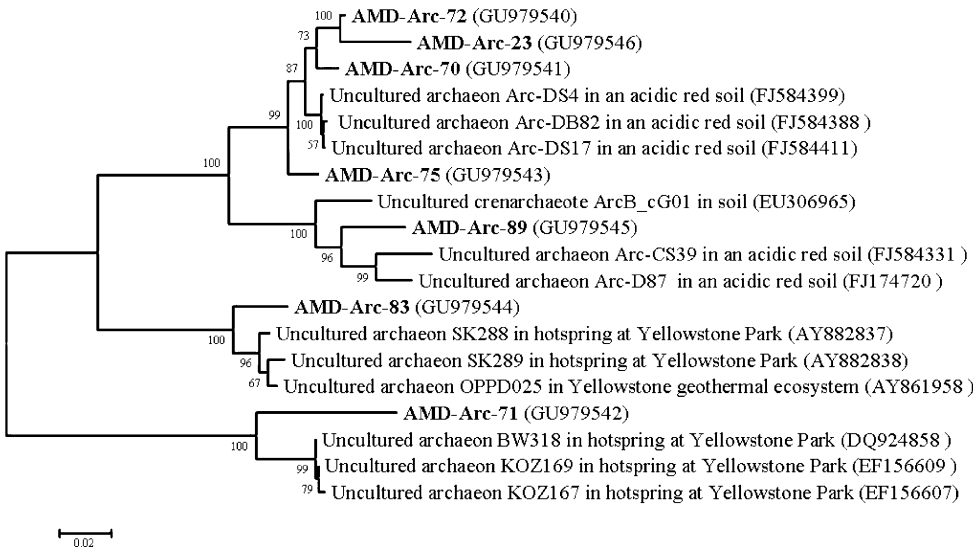

Fig. 2 Neighbor-joining phylogenetic tree based on analysis of 16S rRNA gene sequence of archaeal clones obtained from the AMD lake at Xiang Mountain in relation to reference sequences from the GenBank database. The sequences obtained in this study are indicated in bold. Scale bar indicates the Jukes-Cantor distances. Bootstrap values of >50% (for 1,000 iterations) are shown

AMD-Arc-23 (3.6% of the total clones) in the phylogenetic tree. Sequence AMD-Arc-75 had a relatively distant relation (<94% similarity) to the previous three sequence types, though all of them were positioned in the same branch in the phylogenetic tree (Fig. 2). Sequence AMD-Arc-89 had 94% homology with uncultured crenarchaeote clone ArcB_cG01 from an acidic soil and formed a cluster with its related sequences in the phylogenetic tree. Sequence AMD-Arc-71, representing 10.7% of the total clones, had a low sequence identity with sequences in the Genbank and its closest relative (93% similarity) was detected at Norris Geyser Basin in Yellowstone National Park. AMD-Arc-83, representing 7.1% of the total archaeal clones, shared a 96% gene sequence identity with uncultured archaeon clone SK289 (AY882838) detected in hot Spring in Yellowstone National Park. Other close relatives of AMD-Arc-83 are all from hot springs in Yellowstone National Park and other similar geothermal ecosystems.

Phylogenetic analysis of eukaryotic sequences

The eukaryotic clone library only generated three sequence types (Fig. 3). Sequence AMD-Euk-3, belonging to phylum *Alveolata*, was the predominant sequence type in the gene library and represented 83.3% of the total clones. AMD-Euk-3 formed an individual cluster with its closest relatives in the GenBank database *Oxytricha* sp. Ox_L1 (FN429124) isolated from acid mining lakes in Germany (unpublished data). The second most abundant sequence type was AMD-Euk-14 (11.1% of the total clones), which was positioned in phylum *Nucleariidae* and affiliated with amoeba *Nuclearia* species. One of its closely related species was *Nuclearia thermophila* (AB433328) isolated from the warm spring water of Yunoko Lake, Japan (Yoshida et al. 2009). The last sequence type was AMD-Euk-36 and represented only 5.6% of the total clones. This sequence type shared a high identity (>99%) with several strains of genus *Penicillium*, which were isolated from acidic or heavy metal-contaminated soils (Kawai et al. 2000).

Discussion

Acid mine drainage was caused by dissolution of sulfide ores, and the oxidation rate can be enhanced by several orders of magnitude by sulfur- and iron-oxidizing bacteria and archaea (Baker and Banfield 2003). Consequently, the most notably implicated in AMD generation are chemolithotrophic iron/sulfur-oxidizing acidophiles, such as *A. ferrooxidans* and *L. ferrooxidans*, and sometimes facultatively autotrophic *Sulfobacillus* species (Bond et al. 2000; Druschel et al. 2004; Kock and Schippers 2008). These chemolithotrophic or facultatively chemolithotrophic bacteria usually dominate all natural and man-made acidic environments. Surprisingly in the AMD Lake at Xiang Mountain, heterotrophic acidophiles were much more abundant than autotrophic ones.

The presence of *Acidiphilium* species in acidic ecosystem is quite common and has been reported in various studies (Tan et al. 2007; Xie et al. 2009), but its dominance in AMD water (pH < 3), as observed in this study, has not been reported previously. *Acidiphilium* spp. are aerobic, chemoheterotrophic bacteria having a respiratory type of metabolism with oxygen as terminal electron acceptor and optimal pH of around 3.0 (Hiraishi and Imhoff 2005), which is close to the pH of our AMD water. They use simple organic compounds as carbon and energy sources for growth. Some strains of *Acidiphilium* species are capable of coupling reduction of Fe(III) to oxidation of a variety of organic substrates under aerobic or anaerobic conditions (Küsel et al. 2002). They may co-respire oxygen and Fe(III) under toxic conditions (Hiraishi and Imhoff 2005).

All species of *Acidiphilium* exhibit a high resistance to heavy metals and are capable of growth on high

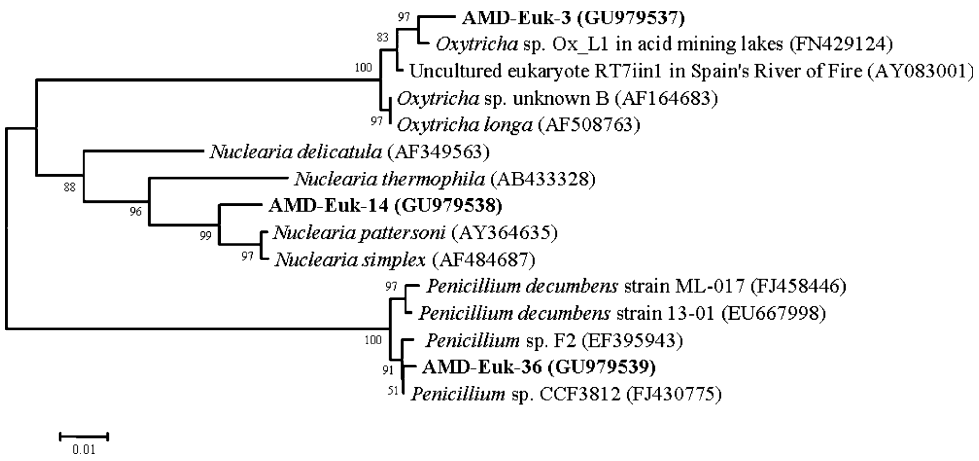

Fig. 3 Neighbor-joining phylogenetic tree based on analysis of 18S rRNA gene sequence of eukaryotic clones obtained from the AMD lake at Xiang Mountain in relation to reference sequences from the GenBank database. The sequences obtained in this study are indicated in bold. Scale bar indicates the Jukes-Cantor distances. Bootstrap values of >50% (for 1,000 iterations) are shown

concentrations of copper, nickel, and zinc (Hiraishi and Imhoff 2005). They possess the ability to accumulate and deposit some metals within the cells so as to remove their toxic effects (Matsuzawa et al. 2000). *A. cryptum* could be induced to be resistant to 300 mM $Al_2(SO_4)_3$ through a pre-cultivation with 50 mM $Al_2(SO_4)_3$ (Fischer et al. 2002). Indeed, the concentration of Al was the highest of the measured metals and reached 1,186 mg/l in the AMD water at the study site.

Furthermore, one of the most important characteristics of *Acidiphilium* species is the production of photopigments with Zn-BChl *a* as a major component (Hiraishi and Nagashima 1998). The ability of *Acidiphilium* to perform photosynthesis with Zn-BChl *a* (Wakao et al. 1996; Hiraishi and Imhoff 2005) may be an advantage to grow and survive in such oligotrophic and acidic environments. *Acidiphilium* species can rely on both heterotrophic use of organic compounds in the form of dissolved organic carbon (DOC) (chemoheterotroph) and use of light, inorganic carbon, and mineral nutrients for photosynthesis (photoautotroph). This dual strategy is termed as "mixotrophy". One of the characteristic properties of mixotrophic bacteria is their low maximum growth rate when compared with specialized photoautotrophs and chemoheterotrophs. Therefore, mixotrophs are expected to be inferior if they compete with specialist phototrophs for light or specialist chemoheterotrophs for organic matter (Rothhaupt 1996). However, there are also inherent advantages for being mixotrophic. For example, in a resource-limiting environment such as AMD waters, the bulk of light or organic matter is not sufficient for normal growth of specialist phototrophs or specialist chemoheterotrophs, however, mixotrophs can make use of both resources simultaneously for their optimal growth. The oligotrophic AMD environment allows mixotrophs to take a full advantage of this strategy and to function more than simple out-competition against their competitors. This strategy has been supported by some research in oligotrophic AMD and neutral lakes (Nixdorf et al. 1998; Rothhaupt 1996).

In addition to *Acidiphilium* species, sequences related to *Ferrithrix* spp., representing about 20% of the total clones, constituted the second most abundant group in bacterial population. *Ferrithrix* species, originally isolated from geothermal spring in Yellowstone National Park, are obligately chemoheterotrophic bacteria and capable of growth on yeast extract and a range of organic substrates. Similar to *Acidiphilium* species, they can oxidize organic matter using ferric iron as electron acceptor under anaerobic conditions. Furthermore, *Alicyclobacillus*, with which sequence AMD-Bac-48 (in *Firmicutes*) was affiliated, was another typical chemoheterotrophic acidophile.

In contrast to abundant heterotrophs, sequences related to photoautotrophic and chemoautrophic bacteria only accounted for 1.6% (AMD-Bac-67) and 4.8% (AMD-Bac-29 and AMD-Bac-99) of the total bacterial population, respectively. Moreover, photoautotrophic eukaryotic algae such as *Rhodophyta*, *Chrysophyte* and *Chlamydomonad* were not found in the Xiang Mountain AMD lake. These organisms usually thrive in AMD environments (Baker et al. 2004; Nixdorf et al. 1998; Rowe et al. 2007) and have also been found in acidic lake (Wollmann et al. 2000). There may be two reasons for the dominance of heterotrophic acidophiles and paucity of autotrophic organisms. First of all, the concentration of ferrous iron in the Xiang Mountain AMD lake may be too low (0.5 mg/l) to sustain normal growth of iron-oxidizing chemolithotrophs that are abundant in typical AMD environments such as Iron Mountain in USA, the Tinto River in southwestern Spain, and bioleaching system (Gonzalez-Toril et al. 2003; Druschel et al. 2004; Rzhepishevska et al. 2005). Second, low CO_2 concentration in the epilimnion of our AMD lake due to the low pH (Nixdorf et al. 1998; Satake and Saijo 1974) and reduced light transmission due to dissolved ferric iron potentially limit the growth of phytoplankton. However, allochthonous DOC from soils and grass community in the bank of the AMD lake may promote heterotrophic production of bacteria. Photolysis or acid hydrolysis of the complex organic matter, such as humic materials, leads to the formation of low molecular weight organic substances (such as acetate, formate, or pyruvate), which may enhance the blooming of heterotrophic acidophiles (Kamjunke et al. 2005).

In contrast to relatively high eukaryotic species richness in the Iron Mountain AMD in USA and the Tinto River in Spain (Baker et al. 2009; Zettler et al. 2002), the eukaryotic community in the Xiang Mountain AMD lake was so simple that only three sequence types were detected, which were related to the genera of *Oxytricha*, *Nuclearia* and *Penicillium*. Previous studies of the microbiology of AMD systems have focused on bacterial and archaeal populations, because they are known to directly impact acid generation rates via oxidation of iron and sulfur compounds. In comparison, studies of eukaryotic populations in AMD ecosystem are limited (Aguilera et al. 2006) and their identities and functions are relatively poorly understood. Fungi undoubtedly play an important role in community structure in AMD ecosystem, since most of them are metal resistant and can sequester specific metals. Such properties are important for the acidic ecosystem because metal sequestration can allow less tolerant species to survive where they might not be present otherwise. The metal sequestration mechanism may enhance the overall biodiversity in AMD areas (Gadanho et al. 2006). Fungi such as *Penicillium* detected in this study also may impact the community structure and function by the consumption of organic waste products and the production of secondary metabolites (Baker et al. 2004).

Protists are predominant in the eukaryotic community at the study site. Sequences related to the ciliate *Oxytricha* sp. and the ameba *Nuclearia* sp. represented 83.3 and 11.1% of the total eukaryotic library, respectively. Eukaryotic protozoa such as *Oxytricha* sp. feed on acidophilic bacteria and archaea and impact prokaryotic cell numbers, which would consequently impact the oxidation rate of pyrite and AMD generation rate. Furthermore, the prokaryotic population structure may change if acidophilic protists graze selectively on certain prokaryotic community members. Johnson and Rang (1993) reported that protozoa appeared to graze *A. ferrooxidans* in preference to *L. ferrooxidans* in mixed cultures, often causing *L. ferrooxidans* to become the dominant iron-oxidizer relative to corresponding protozoa-free controls. In acidic environment, a dynamic equilibrium between predator and prey should exist and the nature of the relationship changes depending on environmental conditions.

In addition to predation, there may be symbiotic relationships between acidophilic prokaryotes and protozoa. Baker et al. (2003) reported that acidophilic protists from AMD in Iron Mountain, CA, USA, hosted *Rickettsiales*-lineage endosymbionts. A further survey revealed that the bacterial endosymbiont appeared to be ubiquitous in this environment and approximately 4% of protists in the AMD samples contained endosymbionts, the average number of which per host cell was six (Baker et al. 2003). The symbiotic relationship between acidophilic bacteria and protists may have contributed to the relatively high abundance of *Rickettsiaceae* family in our AMD water (11.1% of the total bacterial population).

Unexpectedly, sequences related to genus *Legionella* constituted the third most abundant group in the bacterial population. This genus was established in 1977, following the isolation of *Legionella pneumophila* from patients with a form of pneumonia now known as Legionnaires' disease, a severe and sometimes deadly pneumonia, the fatality rate of which can approach as high as 50% in immuno-compromised patients (Diederen 2008). *Legionella* species are aerobic chemoorganotrophic, gram-negative bacilli. *Legionella* spp. are ubiquitous in surface waters, ponds, streams, soils, and other natural moist areas and man-made aqueous environments such as air conditioning cooling towers and evaporative condensers (Steinert et al. 2002). The relatively high abundance of *Legionalla*-related sequences (12.7% of the total bacterial clones) in the Xiang Mountain AMD lake is unexpected as *Legionella* spp. are typically known to grow best under neutral pH conditions (the optimum pH for growth is 6.8–7.0) (Winn 2005). Only two studies have found very small quantities of *Legionella* in acidic hot spring and mine water treatment plants (Sheehan et al. 2005; Heinzel et al. 2009). To our knowledge, Xiang Mountain is the first location where *Legionella* species were found as one of the dominant groups in an acidic habitat. The high abundance of *Legionella*-related sequences may be due to the dominance of ciliate and ameba (94.4% of total eukaryotic clones) in eukaryotic population. Legionellas have been reported to multiply in 13 species of amoebae and two species of ciliated protozoa (Murga et al. 2001). When *Legionella* species are grazed by protists, they can evade defense mechanisms of protozoa, avoid digestion, form endosymbionts, and replicate within vacuoles. The endosymbiotic *Legionella* cells are quite distinct from their free-living counterparts cultured on complex laboratory media by exhibiting decreased susceptibility to chemical inactivation and antibiotics and enhanced invasiveness for mammalian cells (Smith 2005; Berk et al. 2008). The endosymbiotic *Legionella* cells can also withstand desiccation and extreme temperature and pH such as in the AMD water in the present study (Sheehan et al. 2005). Therefore, the high abundance of *Legionalla*-related sequences in our AMD water may be a result of the presence of a large number of protozoa in eukaryotic population. Another potential reason for the abundance of *Legionella* was that the AMD waters contained as high as 100.6 mg/l iron, which is required for the growth of Legionellae (Winn 2005).

As for the archaeal population in the Xiang Mountain AMD lake, all of the archaeal sequences could not be closely related to order *Thermoplasmatales* and *Sulfolobales* (Hallberg and Johnson 2001), which are mostly commonly reported archaeal lineages from AMD environments, suggesting that there may be new taxa. It is impossible at the present time to assign any phenotypic traits to the archaea detected in the AMD water because of the absence of relatedness to characterized species. Most of the archaeal sequences from our AMD lake had a high identity to those of archaea in an acidic red soil, which may imply that the archaea population in the AMD waters had a close association with that in the grass soils in the bank of the AMD lake.

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