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# Phylogenetic analysis of 16S rRNA gene reveals high species diversity of *Halorubrum* in China

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**Haloarchaea such as *Halorubrum* are excellent models for field and laboratory study. Almost half of the new species of *Halorubrum* were identified from the samples collected from China. The aim of this study is to determine whether some species reported elsewhere are distributed in China. Hypersaline environments-a special niche, are distributed all over the world. The genus *Halorubrum* frequently dominates the microbial communities in hypersaline environment. We collected tens of salt mine or saline soil samples from China's Yunnan, Hainan and Jiangsu provinces (China) for *Halorubrum* isolation. Tens of haloarchaea strains with different morphological properties were isolated. The 16S rRNA genes were amplified by using *Halorubrum* specific primers and cloned into the vector for sequencing. The raw classifications were based on the phylogenetic analyses, and the results showed that 29 strains were affiliated with genus *Halorubrum* and isolated 29 strains of haloarchaea affiliated with *Halorubrum*. The 16S rRNA gene sequences were amplified by *Halorubrum* specific primers. Additional sequences available in GenBank were downloaded. Phylogenetic analyses of 16S rRNA gene sequences revealed: 1) Nineteen lineages, of which eleven shared a low sequence similarity (less than 99%) with the validly described species and, thus, may represent new taxa; 2) new records of five known species also distributed in China; 3) at least two separated lineages may represent new species. This is the first report of such high species diversity of *Halorubrum* in China.**

**Key word:** Halorubrum, rRNA gene sequences, hypersaline, haloarchaea strains.

## INTRODUCTION

Numerous Haloarchaea always found in hypersaline environments, such as salt lakes, soda lats, hypersaline soils, underground salt deposits and evaporation ponds, solar salterns, salt lakes, soda lakes, salt pans, saltern crystallizer ponds among others are which widely distributed all overacross the world (Oren, 2002; McGenity and Oren, 2012). Furthermore, they are the dominant members in those communities, and they are physiologically diverse, have dynamic genomes, and show ''island biogeography'' because of the patchy distribution of hypersaline waters (Papke et al., 2007). The first representatives of the family Halobacteriaceae were isolated more than a hundred years ago, and currently (November 2011) the family encompasses 36 genera with 129 species (Arahal et al., 2011 and later updates) (Oren, 2012). To date, genus *Halorubrum* contains the largest number of species within the family Halobacteriaceae (May 2013).

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Genus *Halorubrum*, a wide distributed haloarchaeal group, was proposed by McGenity and Grant (1995) based on the phylogenetic analysis of 16S rRNA gene sequences. Twelve new species (25 in total) of genus *Halorubrum* were reported based on the samples collected from China land. However, whether the left 13 species are distributed in China is unclear to us. Microbial diversity of these hypersaline environments were reported base on the phylogenetic analysis of 16S rRNA gene sequences (Wani et al. 2006; Baati et al., 2008; Mutlu et al., 2008; Ahmad et al., 2008; Rohban et al., 2009; Baati et al., 2010; Ahmad et al., 2011; Luque et al., 2012). Genus *Halorubrum* was proposed by McGenity and Grant (1995). *Halorubrum* was then the largest genus within the family Halobacteriaceae, with 25 validly described species (http://www.bacterio.cict.fr/h/*Halorubrum*.html).

Although many investigations have been carried out to study the species diversity of prokaryotesic and archaeal diversity in hypersaline environments of China (Chen et al. 2007; Xiao et al. 2007; Zhang and Kong, 2010; Zhu et al., 2012), lateral gene transfer (Fröls et al., 2012; Chen et al. 2012), the species diversity of genus *Halorubrum* in the vast land of China has not yet been surveyed. In order to investigate the species diversity and distribution of genus *Halorubrum* in China, we collected samples from three provinces (Jiangsu, Hainan and Yunnan) (Table 1) of China for haloarchaeal strains. Chromosomal DNA were extracted and purified for polymerase chain reaction (PCR) amplification. DNA cloning and sequencing were used to obtain the 16S rRNA gene sequence (Sambrook and Russell, 2001). 16S rRNA gene sequences were assembled by using Seqman (http://www.dnastar.com/t-sub-products-lasergene-seqmanpro.aspx) and used for homologous searching (http://blast.ncbi.nlm.nih.gov/). Other 16S rRNA gene sequences of which strains isolated from other provinces were retrieved from GenBank and concatenated with which we got phylogenetic analysis by using MEGA 5.1 Beta 2 (http://www.megasoftware.net/).

## MATERIALS AND METHODS

### Sample collection

Samples were collected from Yunnan (Mohei, Yuanyongjing, Anning, Yipinglang and Qiaohou salt mines), Hainan and Jiangsu province (China) at June 2004, July 2006 and July 2008, respectively (Table 1). Salt mine sample was salt crystal collected from the soil-covered surface, and others were saline enriched from solar salterns. Sample solution was prepared by adding 10 g of sample (salt crystal or saline) in 100 ml sterilized 15% (W/V) salt solution with stirring; and then, filtered with filter paper for plating.

### Culture medium, conditions and purification

Modified growth medium (MGM) was prepared as described previously in the Halohandbook (http://www.haloarchaea.com/resources/halohandbook/Dyall-Smith 2001). The sample solution (equivalent to 1% volume of culture medium) was inoculated into MGM, and cultured at 37°C for one or two weeks with shaking at 180 rpm. For preparing solid agar plate, 1.5% agar was added. Strains were purified by subsequent plating of the enriched cultures on agar medium, and cultured at 37°C for more than two weeks. Orange-red single colonies were picked for preparing pure cultures.

### DNA extraction, PCR amplification and cloning of 16S rRNA genes

Genomic DNA of haloarchaeal strains were extracted and purified according to the method in the Halohandbook (http://www.haloarchaea.com/resources/halohandbook/) of Sambrook and Russell, (2001). 16S rRNA genes were amplified by PCR with a primer set designed to complement the highly conserved regions of the Halobacterium salinarum and *Halorubrum* species 16S rRNA genes: forward primer F8 (5'-TTGATCCTGCCGGAGGCCATTG-3') and reverse primer R1462 (5'-ATCCAGCGCAGATTCCCCTAC-3'), corresponding to positions 8-30bp and 1462-1441bp, respectively (Kharroub et al., 2006). The PCR procedure was conducted as follows: One cycle of 94°C for 5 min, 35 cycles of 94°C for 50s, 55°C for 50s, and 72°C for 90 s; plus an extension step of 72°C for 10 min. Negative controls were included with no addition of template DNA. The PCR products (~1.5Kb) were detected by 1% agarose gels in 1× Tris-acetic - EDTA (TAE) buffer, and purified by Gel Extraction Kit (OMEGA Bio-Tek, USA). The purified DNA fragments were cloned into pMD-18T vector (TaKaRa, Japan) in accordance with the product manual, and then, transformed into *Escherichia coli* DH5α (Invitrogen, USA) using the method of Sambrook and Russell (2001). Positive clones were picked by Blue/White selection and checked for size of the right insert by colony PCR. The sequences of primers used in colony PCR were shown as follows: M13 Forward 5'-GTTTTCCCAGTCACGAC-3' and M13 Reverse 5'-CAGGAAACAGCTATGAC-3'.

### DNA sequencing and sequence assemblyanalysis

DNA sequencing was performed on both the strands using vector specific M13 forward (5'-GTTTTCCCAGTCACGAC-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers on a 3730 DNA analyzer (Applied BioSystems, USA) using the ABI Big-Dye version 3.1 sequencing kit as per the manufacturer's instructions (BGI, Beijing). The SeqMan program of Lasergene version 10.1 (http://www.dnastar.com/t-sub-products-lasergene-eqmanpro.aspx) was used for sequence assembly (DNASTAR Inc., USA).

### Phylogenetic analysis

The 16S rRNA gene sequences were compared to those of the GenBank and EMBL databases by Basic Alignment Search Tool (BLAST) searches from the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Relative 16S rRNA gene sequences were downloaded for multiple sequence alignment. Multiple sequence alignments were performed using Clustal W 1.8 (Thompson et al., 1994). A phylogenetic tree was constructed using the Neighbor-Joining (NJ) method with the MEGA5.1 Beta 23 program package under the bootstrap method with 1000 replications (http://www.megasoftware.net/Kumar et al., 2004).

### Nucleotide sequence accession numbers

Sequences reported in this study have been deposited in GenBank,

**Table 1.** Sampling location and similarity of 16S rRNA gene sequences.

| Name of strain | Accession number | Source of sample | Closest relative | % Similarity[a] |
|---|---|---|---|---|
| *Halorubrum* sp. YC-11 | JN216856 | Yuncheng salt lake, Shanxi, China | *Halorubrum chaoviator* Halo-G(T) | 99.444 |
| *Halorubrum* sp. YC-3 | JN216848 | Yuncheng salt lake, Shanxi, China | *Halorubrum chaoviator* Halo-G(T) | 99.305 |
| *Halorubrum* sp. YC-X7 | JN216863 | Yuncheng salt lake, Shanxi, China | *Halorubrum xinjiangense* BD-1(T) | 98.979 |
| *Halorubrum* sp. YC-4 | JN216849 | Yuncheng salt lake, Shanxi, China | *Halorubrum chaoviator* Halo-G(T) | 98.538 |
| *Halorubrum* sp. YC-2 | JN216847 | Yuncheng salt lake, Shanxi, China | *Halorubrum chaoviator* Halo-G(T) | 98.887 |
| *Halorubrum* sp. YC-7 | JN216852 | Yuncheng salt lake, Shanxi, China | *Halorubrum chaoviator* Halo-G(T) | 98.748 |
| *Halorubrum* sp. YC-X4 | JN216862 | Yuncheng salt lake, Shanxi, China | *Halorubrum chaoviator* Halo-G(T) | 99.305 |
| *Halorubrum* sp. YC-12 | JN216857 | Yuncheng salt lake, Shanxi, China | *Halorubrum chaoviator* Halo-G(T) | 99.374 |
| *Halorubrum* sp. YC-X1 | JN216860 | Yuncheng salt lake, Shanxi, China | *Halorubrum chaoviator* Halo-G(T) | 98.887 |
| *Halorubrum* sp. YC-8 | JN216853 | Yuncheng salt lake, Shanxi, China | *Halorubrum litoreum* Fa-1(T) | 98.364 |
| *Halorubrum* sp. HMC-1 | JX188259 | Huamachi salt lake, Shaanxi, China | *Halorubrum chaoviator* Halo-G(T) | 99.305 |
| *Halorubrum* sp. GC-5 | JX188262 | Gouchi salt lake, Shaanxi, China | *Halorubrum chaoviator* Halo-G(T) | 98.887 |
| *Halorubrum* sp. GC-4 | JX188261 | Gouchi salt lake, Shaanxi, China | *Halorubrum chaoviator* Halo-G(T) | 99.166 |
| *Halorubrum* sp. LYG-2 | JX188266 | Tainan solar salterns, Lianyungang, Jiangsu, China | *Halorubrum xinjiangense* BD-1(T) | 99.183 |
| *Halorubrum* sp. LYG-7 | JX188271 | Tainan solar salterns, Lianyungang, Jiangsu, China | *Halorubrum litoreum* Fa-1(T) | 99.523 |
| *Halorubrum* sp. LYG-6 | JX188270 | Tainan solar salterns, Lianyungang, Jiangsu, China | *Halorubrum chaoviator* Halo-G(T) | 98.446 |
| *Halorubrum* sp. LYG-9 | JX188273 | Tainan solar salterns, Lianyungang, Jiangsu, China | *Halorubrum chaoviator* Halo-G(T) | 98.371 |
| *Halorubrum* sp. LYG-5 | JX188269 | Tainan solar salterns, Lianyungang, Jiangsu, China | *Halorubrum litoreum* Fa-1(T) | 99.523 |
| **Halorubrum* sp. YZL-3 | JX972113 | Lianyungang Solar Salterns, Jiangsu, China | *Halorubrum chaoviator* Halo-G(T) | 99.235 |
| **Halorubrum* sp. YZL-9 | JX972119 | Lianyungang Solar Salterns, Jiangsu, China | *Halorubrum chaoviator* Halo-G(T) | 99.166 |
| **Halorubrum* sp. YZL-10 | JX972120 | Lianyungang Solar Salterns, Jiangsu, China | *Halorubrum chaoviator* Halo-G(T) | 99.095 |
| **Halorubrum* sp. LYG2-BQ-3 | JX972131 | Lianyungang Solar Salterns, Jiangsu, China | *Halorubrum litoreum* Fa-1(T) | 98.912 |
| **Halorubrum* sp. LYG2-BQ-2 | JX972130 | Lianyungang Solar Salterns, Jiangsu, China | *Halorubrum chaoviator* Halo-G(T) | 98.818 |
| **Halorubrum* sp. WJK-3 | JX972133 | Lianyungang Solar Salterns, Jiangsu, China | *Halorubrum chaoviator* Halo-G(T) | 99.166 |
| **Halorubrum* sp. WJK-P | JX972134 | Lianyungang Solar Salterns, Jiangsu, China | *Halorubrum kocurii* BG-1(T) | 98.464 |
| *Halorubrum* sp. LYGTB-6 | HM132877 | Taibei salt ponds, Lianyungang, Jiangsu, China | *Halorubrum chaoviator* Halo-G(T) | 99.234 |
| *Halorubrum* sp. CS4-4 | GQ478078 | Salt brine, Daying Salt Lake, Hebei, China | *Halorubrum xinjiangense* BD-1(T) | 99.167 |
| *Halorubrum* sp. AS2-1 | GQ478056 | Salt brine, Daying Salt Lake, Hebei, China | *Halorubrum chaoviator* Halo-G(T) | 98.609 |
| *Halorubrum* sp. AS2-5 | GQ478052 | Salt brine, Daying Salt Lake, Hebei, China | *Halorubrum litoreum* Fa-1(T) | 98.960 |
| *Halorubrum* sp. AS2-7 | GQ478051 | Salt brine, Daying Salt Lake, Hebei, China | *Halorubrum litoreum* Fa-1(T) | 98.823 |
| *Halorubrum* sp. AS2-6 | GQ478053 | Salt brine, Daying Salt Lake, Hebei, China | *Halorubrum litoreum* Fa-1(T) | 98.623 |
| **Halorubrum* sp. CJ-2 | JX972125 | Hainan Solar Salterns, Hainan, China | *Halorubrum chaoviator* Halo-G(T) | 98.818 |
| **Halorubrum* sp. HII-4 | JX972128 | Hainan Solar Salterns, Hainan, China | *Halorubrum litoreum* Fa-1(T) | 98.980 |
| *Halorubrum* sp. AJ201 | EF108328 | Ayakekumu Salt Lake, Xinjiang, China | *Halorubrum chaoviator* Halo-G(T) | 98.679 |
| **Halorubrum* sp. YZL-6 | JX972116 | Mohei Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 99.166 |
| **Halorubrum* sp. YZL-7 | JX972117 | Mohei Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 99.235 |

**Table 1.** Contd

| | | | | |
|---|---|---|---|---|
| **Halorubrum* sp. YZL-8 | JX972118 | Mohei Salt Mine, Yunnan, China | *Halorubrum kocurii* BG-1(T) | 99.049 |
| **Halorubrum* sp. LLL | JX972129 | Mohei Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 98.748 |
| **Halorubrum* sp. YYJ-16 | JX972135 | Yuanyongjing Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 99.305 |
| **Halorubrum* sp. YYJ-7 | JX972136 | Yuanyongjing Salt Mine, Yunnan, China | *Halorubrum litoreum* Fa-1(T) | 98.912 |
| **Halorubrum* sp. YZL-11 | JX972121 | Yuanyongjing Salt Mine, Yunnan, China | *Halorubrum kocurii* BG-1(T) | 98.610 |
| **Halorubrum* sp. YZL-12 | JX972122 | Yuanyongjing Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 98.887 |
| **Halorubrum* sp. YZL-4 | JX972114 | Yuanyongjing Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 99.094 |
| **Halorubrum* sp. T2R | JX972132 | Yuanyongjing Salt Mine, Yunnan, China | *Halorubrum coriense* Ch2(T) | 99.373 |
| **Halorubrum* sp. YYJ0 | JX972111 | Yuanyongjing Salt Mine, Yunnan, China | *Halorubrum kocurii* BG-1(T) | 98.682 |
| **Halorubrum* sp. YZL-2 | JX972112 | Yuanyongjing Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 98.929 |
| **Halorubrum* sp. HMX-II | JX972123 | Yuanyongjing Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 99.026 |
| **Halorubrum* sp. YYJ-8 | JX972118 | Yuanyongjing Salt Mine, Yunnan, China | *Halorubrum aidingense* 31-hong(T) | 98.621 |
| **Halorubrum* sp. T3 | JQ936845 | Yuanyongjing Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 99.217 |
| **Halorubrum* sp. CY | FJ267614 | Yipinglang Salt Mine, Yunnan, China | *Halorubrum litoreum* Fa-1(T) | 99.524 |
| **Halorubrum* sp. CY-16R2 | JX972126 | Yipinglang Salt Mine, Yunnan, China | *Halorubrum kocurii* BG-1(T) | 98.610 |
| **Halorubrum* sp. CY-42W | JX972127 | Yipinglang Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 99.583 |
| **Halorubrum* sp. ZM5 | JX972138 | Qiaohou Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 99.513 |
| **Halorubrum* sp. YZL-5 | JX972115 | Anning Salt Mine, Yunnan, China | *Halorubrum chaoviator* Halo-G(T) | 99.096 |

*, Strains isolated and sequenced in our laboratory.

EMBL databases under accession numbers from JX972111 to JX972123 and JX972125 to JX972138 (Table 1).

## RESULTS

### Distribution pattern and habitats of genus *Halorubrum* in China

The distribution pattern of the validly described twelve species of *Halorubrum* from China is as shown in Figure 1A. Nearly half of the total numbers well-documented of species were reported from China's samples (Table 2). Recently, additional new strains of *Halorubrum* were isolated from China (Table 1). The new isolates and validly described species, and the distribution of *Halorubrum* in China is shown in Figure 2. Most of the species reported previously were isolated from northwest (NW) of China, however, new taxa in this study were isolated from southeast (SE) of China (Figure 2). Generally, the genus *Halorubrum* was widely distributed in China (Figure 2). The habitats of twenty four 25 validly described species of *Halorubrum* worldwide are shown in Figure 1A. Among the species, more than 60% of them were isolated from salt lake or solar saltern (Figure 1). However, most of strains from our laboratory were isolated from the surface of some salt mine (Table 1). It demonstrated that salt mine was another important source for isolation of haloarchaea.

### Phylogenetic analysis

A total of 54 16S rRNA gene sequences (29 newly generated and 25 from GenBank) were used to construct the Neighbor-Joining phylogenetic tree (Figure 3). The result showed that they clustered into nineteen lineages, labeled as sp. 1 to sp. 19, respectively. Among them, eleven lineages containing one or more strains with a similarity of less than 99% with the validly described species

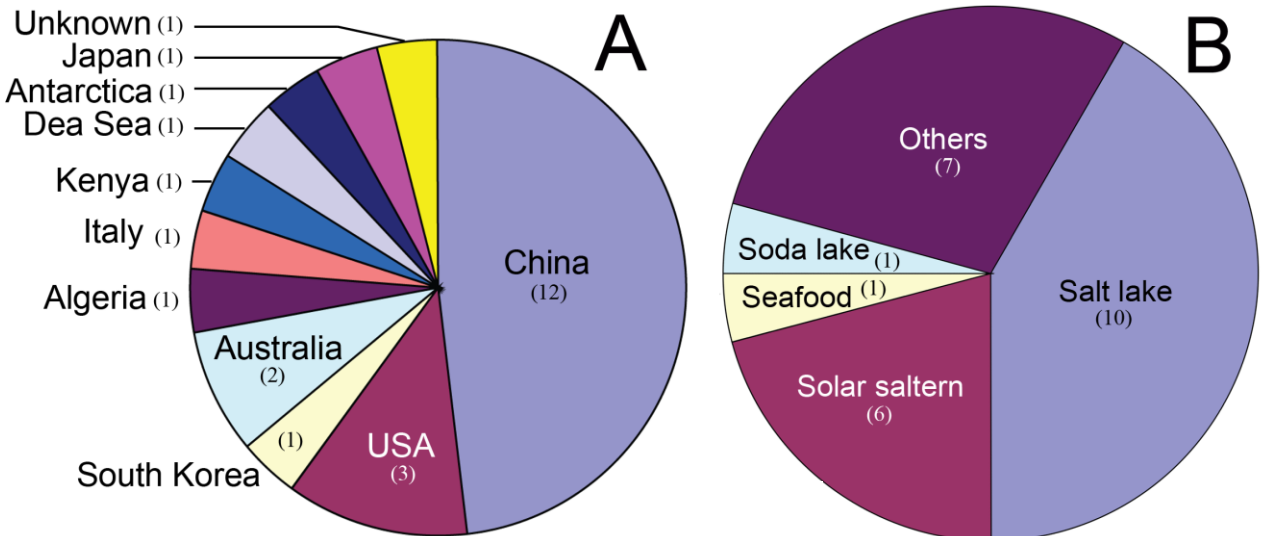


**Figure 1**. Distribution and niches of reported species from *Halorubrum*. The numbers in brackets represented the quantity of validly described species.

**Table 2.** Location of validly described species of *Halorubrum* isolated from China.

| Name of type strain | Accession numbers | Source |
|---|---|---|
| *Halorubrum tibetense* 8W8 | AY149598 | Lake Zabuye, Tibet, China |
| *Halorubrum xinjiangense* BD-1 | AY510707 | Xiao-Er-Kule Lake, Xinjiang, China |
| *Halorubrum alkaliphilum* DZ-1 | AY510708 | A soda lake, Xinjiang, China |
| *Halorubrum orientale* EJ-52T | AM235786 | Lake Ejinor, Inner Mongolia, China |
| *Halorubrum lipolyticum* 9-3 | DQ355814 | Aibi salt lake, Xinjiang, China |
| *Halorubrum aidingense* 31-hong | DQ355813 | Aiding salt lake, Xinjiang, China |
| *Halorubrum arcis* AJ201 | DQ355793 | A saline lake, Qinghai–Tibet Plateau, China |
| *Halorubrum ejinorense* EJ-32T | AM491830 | Lake Ejinor, Inner Mongolia, China |
| *Halorubrum litoreum* JCM 13561 | EF028067 | A marine solar saltern, Fujian, China |
| *Halorubrum luteum* CGSA15 | DQ987877 | Lake Chagannor, Inner Mongolia, China |
| *Halorubrum kocurii* BG-1T | AM900832 | Lake Bagaejinnor, Inner Mongolia, China |
| *Halorubrum aquaticum* EN-2 | AM268115/FN691473 | Saline lakes Erliannor/Shangmatala, Inner Mongolia, China |

may well be unnamed taxa (Figure 3). Nine lineages were first reported from our laboratory. Sp. 1, sp. 2, sp. 3, sp. 4, sp. 9, sp. 10, sp. 17 and sp. 18 were closely related to the published described species isolated from China, while sp. 5 and sp. 19 were clustered with the species from Australia, sp. 11 is close to the species from Algeria or from the USA, sp. 13 is close to a species from the USA, while sp. 14 and sp. 15 were sister to the species from the Dead Sea.

## DISCUSSION

*Halorubrum* are always found in hypersaline environments, and they are the dominant members in those environments (Park et al., 2009; Oh et al., 2010). Salt mine is one of the typical hypersaline environments in the world, however, there are few previous described species isolated from a salt mine. There are few reports on microbial diversity of samples from a salt mine, whereas salt mine is one of the typical hypersaline environments in the world. In hypersaline environment where *Haloquadratum* is not dominant, other haloarchaeal groups dominate, such as *Halorubrum*, *Haloarcula*, or *Halobacterium* (Pašić et al. 2007; Park et al. 2009; Oh et al. 2010). In this study, we isolated more than 20 haloarchaeal strains belonging to genus *Halorubrum* from several salt mines (Table 1), which suggested that almost all the haloarchaeal strains isolated from these salt mines in China were affiliated with the *Halorubrum*. Hence, *Halorubrum* may also dominatin the salt mines in China, and could survive in almost all kinds of hpyersaline

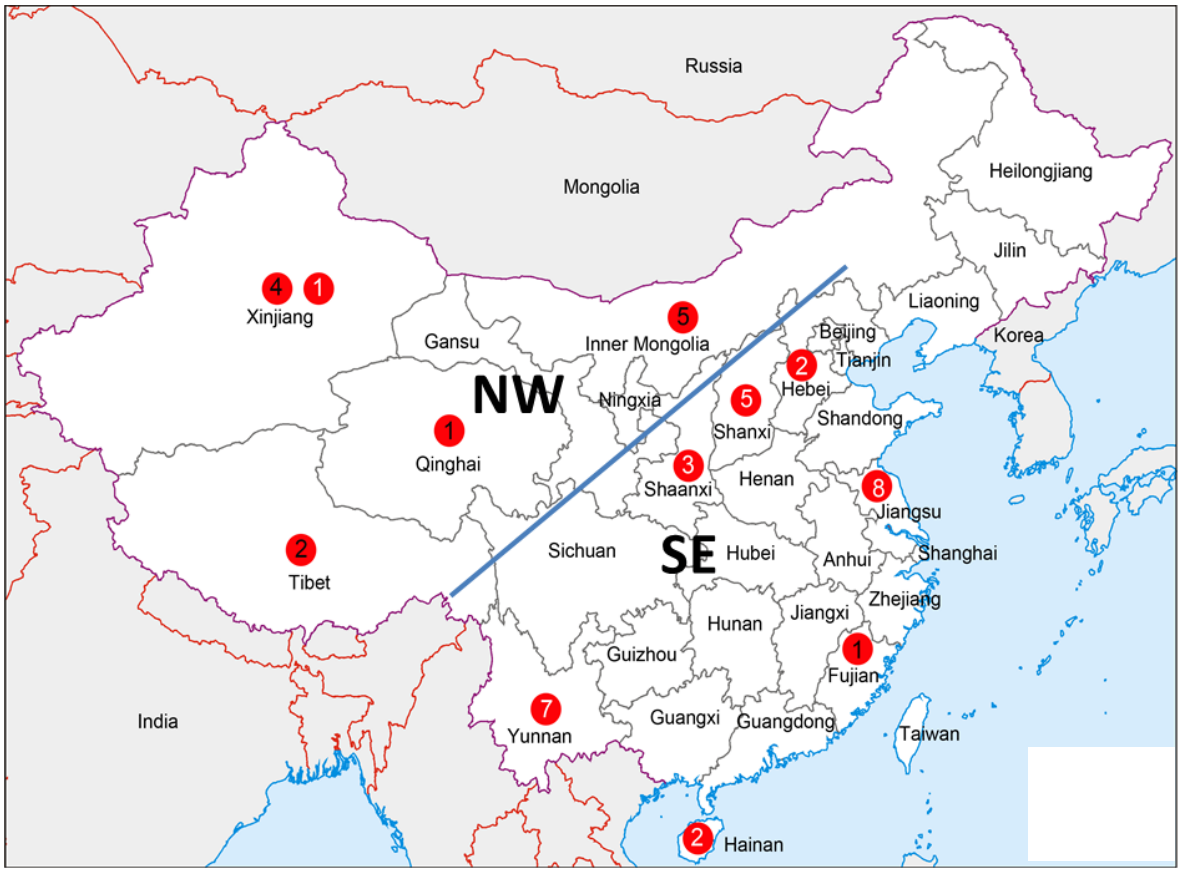


**Figure 2.** Sampling locations in China. Black numbers in the circles represented the quantity of validly described species. White numbers represented lineages reported in this study. The sum of the white numbers is greater than 19, because some lineages were distributed in several different locations.

environments (Figure 1B).

To date, 12 of 25 validly described *Halorubrum* species (Figure 1A) were isolated from the northwest (NW) of China (Figures 1A and 2), combined with other 54 strains isolated from the southeast (SE) of China (Figure 2), which suggests that *Halorubrum* are widely distributed in China land. In this study, based on the analysis of 16S rRNA gene sequences, 54 strains of *Halorubrum* isolated from southeast part of China were grouped into 19 lineages (Figure 3). Furthermore, 11 lineages (sp.1, sp.2, sp.4, sp.10, sp.11, sp.13, sp.15, sp.16, sp.17 and sp.18) contained strains with < 99% sequence identity to the known taxa (Figure 3) and probably represented novel species.

Unexpectedly, we did not isolate any haloarchaeal strains affiliated with other genera except two strains of *Haloferax* from Hainan (data not shown). The reason for this remains unknown. Five lineages, sp.5, sp.11, sp.13, sp.14 and sp.19, shared a high similarity (>98%) of 16S rRNA gene sequence with typical strain *Halorubrum* litoreum Fa-1 (Hrr.litoreum Fa-1) isolated from Australia, *Halorubrum ezzemoulense* 5.1 isolated from Algeria, *Halorubrum chaoviator* HALO-G isolated from the USA, *Halorubrum sodomense* ATCC33755 isolated from Dea Sea and *Halorubrum* saccharovorum JCM8865, respectively. This is the first report of that these five species of *Halorubrum* distributed in China land. *Halorubrum distributum* JCM9100, a wide distributed haloarchaeal strain, are also found in China. It is announced that most (18 out of 25) species of genus *Halorubrum* are distributed in China land.

On the other hand, *Halorubrum* DNA was found in 23 MYA salt, and *Halorubrum* appear to be a dominant group in relatively modern hypersaline habitats (Park et al., 2009; Srivastava and Kowshik, 2013). It is possible that dozens of millions of years ago, *Halorubrum* originated from ocean. At that period, the earth movements associated with the orogeny triggered the formation of inland salt lake. Some of natural salt lakes often dry out completely because of the more evaporation and less precipitation, and the crystallizers of marine solar salterns are become a big salt mine (Oh et al., 2010). *Halorubrum* constantly evolved to adapt to haypersaline environment and the global dispersal system was largely blocked by land. The lowest NaCl concentration that *Halorubrum* cells can endure was about 8% (w/v), which is much higher

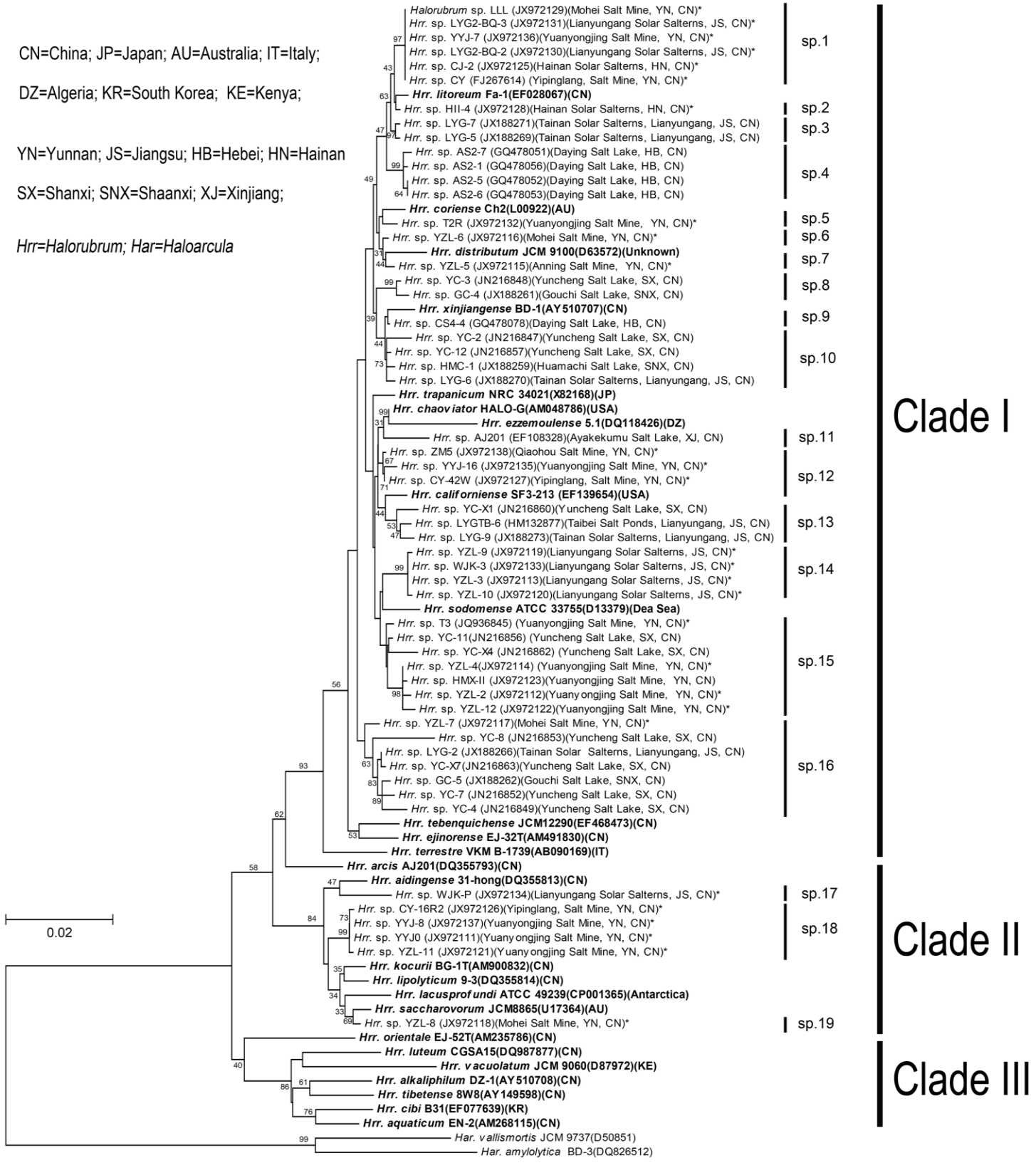


**Figure 3.** Neighbour-Joining phylogenetic tree based on 16S rRNA gene sequences. Sequences from our laboratory were marked with a star (*) behind, with reference sequences obtained available from GenBank. Formally published sequences were bolded. The scale bar corresponds to a 2% sequence divergence in nucleotide sequence position. *Haloarcula vallismortis* JCM 9737 (D50851) and *Haloarcula amylolytica* BD-3 (DQ826512) were used as out group. GenBank/EMBL/DDBJ accession numbers were given in brackets.

than the salt concentration of normal seawater (Chen et al., 2012). *Halorubrum* has been unable to go back to the ocean, and distributed across the hypersaline environments, and speciation of *Halorubrum* occurred after a long term segregation, although some evidences shown that wind or the migratory birds could help their dispersing (Brito-Echeverría et al., 2009).

Based on the molecular phylogenetic tree, the reported species of genus *Halorubrum* were divided into three major clades (Figure 3). In addition, phylogenetic tree showed that the widespread Clade (Clade I) on the phylogenetic tree were rich species (from sp. 1 to sp. 16), with few divergence (Figure 3). We further speculated that fragmentation of ancestral species ranges resulting from geographic and habitat segregation may have contributed to the explosive radiation of this species-rich Clade. Most of the reported species in the base group (Clade III) were isolated from East Asia. All 16S rRNA gene sequences from genus *Halorubrum* deposited in the GenBank were used to construct a large phylogenetic tree (data not shown), it revealed the same result. Hence we proposed that genus *Halorubrum* originated in East Asia.

In conclusion, our study indicates that: 1) Strains of *Halorubrum* can survive almost all kinds of hypersaline environment; 2) five new record species of genus *Halorubrum* reported out of East Asia, are found in China land; 3) two separated lineages (sp.16 and sp.18) are recognized on the 16S rRNA gene sequence-dependent phylogenetic tree.

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