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Bacterial and archaeal diversities in Yunnan and Tibetan hot springs, China

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Summary

Thousands of hot springs are located in the northeastern part of the Yunnan–Tibet geothermal zone, which is one of the most active geothermal areas in the world. However, a comprehensive and detailed understanding of microbial diversity in these hot springs is still lacking. In this study, bacterial and archaeal diversities were investigated in 16 hot springs (pH 3.2–8.6; temperature 47–96°C) in Yunnan Province and Tibet, China by using a barcoded 16S rRNA gene-pyrosequencing approach. *Aquificae*, *Proteobacteria*, *Firmicutes*, *Deinococcus-Thermus* and *Bacteroidetes* comprised the large portion of the bacterial communities in acidic hot springs. Non-acidic hot springs harboured more and variable bacterial phyla than acidic springs. *Desulfurococcales* and unclassified *Crenarchaeota* were the dominated groups in archaeal populations from most of the non-acidic hot springs; whereas, the archaeal community structure in acidic hot springs was simpler and characterized by *Sulfolobales* and *Thermoplasmata*. The phylogenetic analyses showed that *Aquificae* and *Crenarchaeota* were predominant in the investigated springs and possessed many phylogenetic lineages that have never been detected in other hot springs in the world. Thus findings from this study significantly improve our understanding of microbial diversity in terrestrial hot springs.

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Introduction

Hydrothermal systems may have existed on Earth for billions of years (Gold, 1992). They are well-isolated habitats distributed globally in different regions, and the microorganisms inhabiting therein are extremophiles adapted to conditions quite different from the surrounding environments. Because the unique physical, chemical and geographical characteristics of hot springs make extremophiles physiologically distinct from other microorganisms in other environments, they become model ecosystems for research on the origin and evolution of life, biogeochemistry and biogeography (Pace, 1997; Whitaker *et al.*, 2003). Since the Brock's pioneering work in the 1960s and 1970s, extensive microbial investigations have been performed in hot springs of Yellowstone National Park (Barns *et al.*, 1994; 1996; Ferris *et al.*, 1996; Ward and Castenholz, 2000; Spear *et al.*, 2005; de la Torre *et al.*, 2008; Kan *et al.*, 2011), the Great Basin in the USA (Huang *et al.*, 2007; Zhang *et al.*, 2008; Costa *et al.*, 2009), Iceland (Hjorleifsdottir *et al.*, 2001; Marteinsson *et al.*, 2001; Huber *et al.*, 2002; Kvist *et al.*, 2007; Mirete *et al.*, 2011), Kamchatka in Russia (Perevalova *et al.*, 2008; Kublanov *et al.*, 2009; Reigstad *et al.*, 2010;

Kochetkova *et al.*, 2011) and north-eastern Australia (Kimura *et al.*, 2005; Weidler *et al.*, 2007; 2008).

The north-eastern edge of the Yunnan–Tibet geothermal zone is one of the most active geothermal areas in the world and hosts thousands of hot springs. These hot springs possess a variety of hydrothermal features, such as hydrothermal explosion craters, geysers, fumaroles and boiling springs (Kearey and Wei, 1993). Previously, several cultivation-dependent and -independent studies were performed in Yunnan hot springs. For example, some thermopiles were isolated and their practical applications were evaluated (Xue *et al.*, 2001; Lin *et al.*, 2002; Xiang *et al.*, 2003). Diversities of *Actinobacteria*, *Crenarchaeota* and ammonia-oxidizing *Archaea* were also studied (Pearson *et al.*, 2008; Zhang *et al.*, 2008; Song *et al.*, 2009; Jiang *et al.*, 2010; Song *et al.*, 2010). These studies suggested that the Yunnan hot springs host many new taxonomic and functional lineages. However, a comprehensive investigation on the community diversity and composition in Yunnan hot springs is still lacking. In addition, microbial communities in several Tibetan hot springs were studied by using clone library and denaturing gradient gel electrophoresis (DGGE) (Lau *et al.*, 2006; 2009; Huang *et al.*, 2011). However, these two conventional techniques only produced limited sequencing information in comparison with high-throughput sequencing techniques (e.g. 454 pyrosequencing). The objectives of this study were: (i) to fully understand the bacterial and archaeal diversity in the selected hot springs (with a range of pH and temperature conditions) of Yunnan Province and Tibet, and (ii) to examine microbial difference between hot springs of Yunnan Province and Tibet and Yellowstone. To fulfil these two purposes, the 16S rRNA gene phylogenetic analysis was conducted by using high-throughput 454 pyrosequencing in conjunction with detailed geochemical analyses.

Results

Environmental characteristics

A total of 16 hot spring sites from five thermal fields (Tengchong, Longling and Eryuan in Yunnan Province, elevations at ~ 1800 m; Gulu and Qucai in Tibet, elevations at ~ 4700 m) were selected for field measurements and sample collections. Hot springs investigated in this study possess a range of temperature (47–96°C) and pH (3.2–8.6) conditions (Table 1). Generally speaking, Yunnan springs possess higher concentrations of nitrate and nitrite than Tibetan springs. The four acidic springs Sx1, Drty14, Drty4 and Zzq from Yunnan Province had higher concentration of ferrous iron than non-acidic springs (Table 2). Chemistry-based principal component analysis (PCA) showed that Tibetan hot springs were grouped together and were separated from those of Yunnan Province, indicating that chemistry of Tibetan hot springs is more similar to each other than to the ones of Yunnan Province (Fig. 1). The acidic springs (Drty4, Zzq and Drty14; pH: 3.2–4.5) fell into the same sector, indicating that their chemistry is more similar to each other than to other hot springs (Fig. 1).

Pyrosequencing data

A total of 41141 bacterial and 30651 archaeal sequences were identified. The coverage calculation (Tables 3 and 4) and rarefaction analysis (data not shown) indicated that the analysed sequences covered the diversity of bacterial

Table 1. Description of hot spring samples investigated in this study.

Sample code	Pinyin name – English name	Sample description	Temperature (°C)	pH	GPS location (N/E)
Yunnan Province					
Zzq	Zhenzhuquan – Pearl Spring	Brown sandy sediment	96	4.3	24°57′03″/98°26′09.5″
Dgg	Dagunguo – Great Boiling Pot	Ashen geyserite	94	8.1	24°57′12.7″/98°26′17.4″
Hmz2	Hamazui 2 – Frog Mouth Spring 2	Black sediment	82	7.8	24°57′12.6″/98°26′17.5″
Eynj2	Eryuanniujie 2 – Eryuan Niujie Spring 2	Black mat	73	7.3	26°15′01.2″/99°59′22.2″
Hmz1	Hamazui 1 – Frog Mouth Spring 1	Grey mat	77	7.8	24°57′12.6″/98°26′17.5″
Eynj3	Eryuanniujie 3 – Eryuan Niujie Spring 3	Black sandy sediment	78	7.4	26°15′01.2″/99°59′22.2″
Drty4	Diretiyanqu 4 – Experimental Site 4	Brown sediment	67	3.2	24°57′12.7″/98°26′17.4″
Sx4	Shangxiao 4 – Shangxiao 4	Black sandy sediment	66	8.0	24°39′23.3″/98°40′03.4″
Sx1	Shangxiao 1 – Shangxiao 1	Black and green sandy sediment	53	6.0	24°39′23.3″/98°40′03.4″
Drty14	Diretiyanqu 14 – Experimental Site 14	Brown sediment	47	4.5	24°57′12.7″/98°26′17.4″
Tibet					
Gl15	Gulu 15 – Gulu 15	Ashen geyserite	84	8.6	30°52′34.1″/91°36′38.8″
Gl16	Gulu 16 – Gulu 16	Grey sandy sediment	78	7.0	30°52′34.8″/91°36′40.6″
Qc4	Quca i4 – Qucai 4	White sandy sediment	76	7.0	30°39′59.2″/91°35′28.2″
Qc8	Quca i8 – Qucai 8	Brown sandy sediment	74	7.0	30°39′57.7″/91°35′29.3″
Qc1	Quca i1– Qucai 1	Grey sandy sediment	71	7.0	30°40′0.4″/91°35′27.9″
Qc3	Quca i3 – Qucai 3	Brown and green mat	57	6.7	30°39′59.4″/91°35′28.4″

Table 2. Water chemistry of the investigated hot springs.[a]

Sample	PO_4^{3-}	NH_4^+	NO_2^-	NO_3^-	Fe^{2+}	SO_4^{2-}	S^{2-}	SiO_4^{2-}	Al	Ca	K	Mg	Mn	Na	P	TDS
Zzq	1.96	164.27	2.21	5.49	0.201	ND	ND	645.68	0.5373	3.901	25.32	0.5995	0.521	59.64	0.0155	ND
Dgg	1.74	BD	0.36	3.35	BD	ND	ND	677.39	0.134	1.75	116.4	0.1197	BD	387.4	0.1272	ND
Hmz2 & 1[b]	4.99	0.56	0.67	3.9	BD	ND	ND	679.22	0.0537	1.975	69.32	0.1429	BD	299.3	0.034	ND
Eynj2	1.06	BD	0.48	1.88	BD	ND	ND	643.7	0.0253	37.06	38.51	14.04	0.034	206.3	0.0356	ND
Eynj3	19.65	441.85	BD	2.34	BD	ND	ND	677.57	0.0324	39.61	47.12	18.07	0.1135	235.2	0.0511	ND
Drty4	1.94	BD	1.22	1.82	3.688	ND	ND	680.59	7.301	23.95	18.58	0.8083	0.0409	20.49	0.4202	ND
Sx4	8.2	382.08	0.02	2.34	BD	ND	ND	629.74	0.3423	28.09	8.213	1.371	0.0524	73.94	0.0467	ND
Sx1	3.71	0.76	0.67	3.88	1.017	ND	ND	172.33	2.05	31.03	20.3	2.318	0.1603	176.5	0.0923	ND
Drty14	5.83	BD	0.49	27.88	1.913	ND	ND	681.15	0.6235	84.4	24.51	9.223	0.9865	47.05	0.0685	ND
Gl15	0.91	0.13	0.008	0.03	0.01	4	0.8	ND	ND	ND	ND	ND	ND	ND	ND	2600
Gl16	2.11	0.23	0.016	0.06	BD	80	0.25	ND	ND	ND	ND	ND	ND	ND	ND	1337
Qc4	1.49	0.29	0.052	0.04	0.01	10	0.02	ND	ND	ND	ND	ND	ND	ND	ND	1085
Qc8	1.13	0.31	0.067	0.01	0.01	12	0.02	ND	ND	ND	ND	ND	ND	ND	ND	1620
Qc1	2.57	0.29	0.114	0.04	0.03	6	0.05	ND	ND	ND	ND	ND	ND	ND	ND	1037
Qc3	1.63	0.03	0.055	0.04	0.02	11	BD	ND	ND	ND	ND	ND	ND	ND	ND	1111

a. Values are reported as milligrams per litre.
b. The Hmz2 and Hmz1 are connected with each other and thus have same water.
TDS, total dissolved solids) (mg l^{-1}); BD, below the detection limit (0.001 mg l^{-1}); ND, not determined.

and archaeal populations in the investigated hot springs. Both bacterial and archaeal richness is markedly different among the investigated hot springs. For example, the numbers of bacterial and archaeal operation taxonomic units (OTUs) (cut-off: 0.03) ranged from 180 to 637 and from 109 to 420 respectively (Tables 3 and 4). *Bacteria* were more diverse than *Archaea* in each spring (Tables 3 and 4). UniFrac analysis clearly showed a significant (*P*-value < 0.0001) difference in the archaeal and bacterial compositions among the investigated springs. Interestingly, more than a half of the springs showed a high relative abundance (> 20%) of unique OTUs to total OTUs (Table 5).

Bacterial diversity

All the obtained bacterial sequences fell into the following phyla: *Acidobacteria*, *Actinobacteria*, *Aquificae*, *Bacteroidetes*, *Chlamydiae*, *Chlorobi*, *Chloroflexi*, *Cyanobacteria*, *Deferribacteres*, *Deinococcus-Thermus*, *Dictyoglomi*, *Firmicutes*, *Fusobacteria*, *Gemmatimonadetes*, *Nitrospira*, Candidate division OD1, Candidate division OP10, *Planctomycetes*, *Proteobacteria*, *Spirochaetes*, Candidate division SR1, *Thermodesulfobacteria*, *Thermomicrobia*, *Thermotogae*, Candidate division TM7, *Verrucomicrobia* and unclassified bacteria. Sequences affiliated with *Proteobacteria*, *Firmicutes*, *Aquificae*, *Deinococcus-Thermus* and *Thermotogae* dominated the springs (Fig. 2), and these major groups varied in relative abundance among them. UniFrac analysis showed significant (*P*-value < 0.0001) differences in the archaeal and bacterial compositions among the springs.

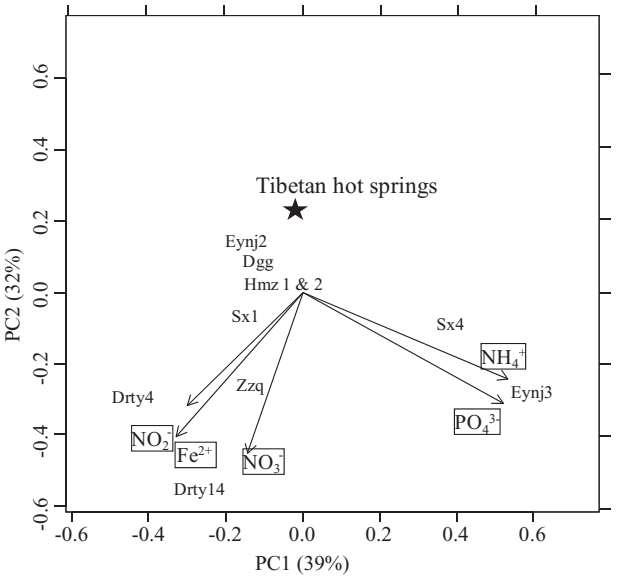

Fig. 1. The first two principal coordinate axes (P1 and P2) for PCA and the distributions of sampling locations in response to these axes. Rectangles represent chemical factors.

Table 3. Observed bacterial richness and diversity estimates based on 97% and 95% OTU clusters respectively.

Community	No. sequences	No. OTUs		Coverage (%)		Richness (ACE)		Shannon's index (Chao & Shen)		Simpson index (MLE)	
		97%	95%	97%	95%	97%	95%	97%	95%	93%	95%
Zzq	1 723	431	324	87	90	851	625	5.34	4.87	0.014	0.020
Dgg	10 628	396	269	99	99	658	502	3.83	3.09	0.058	0.108
Hmz2	1 817	196	131	95	97	367	253	4.07	3.31	0.038	0.095
Eynj2	2 078	435	330	89	92	882	639	5.31	4.82	0.011	0.019
Hmz1	2 055	416	297	89	93	832	593	5.16	4.64	0.015	0.024
Eynj3	1 835	325	237	91	94	633	432	4.58	4.02	0.045	0.067
Drty4	2 263	180	102	97	98	290	176	4.04	3.31	0.034	0.062
Sx4	2 498	337	223	94	96	613	396	4.72	4.11	0.023	0.037
Sx1	1 533	257	189	92	94	486	384	4.69	4.20	0.020	0.032
Drty14	1 272	185	110	93	97	318	177	4.21	3.61	0.035	0.049
Gl15	1 942	339	254	91	94	656	475	4.69	4.12	0.037	0.060
Gl16	1 861	224	146	95	96	388	271	4.26	3.68	0.032	0.049
Qc4	2 593	364	271	93	95	715	542	4.73	4.23	0.021	0.032
Qc8	1 964	327	228	92	94	625	445	4.94	4.27	0.016	0.030
Qc1	3 152	637	454	90	93	1223	864	5.52	4.92	0.012	0.022
Qc3	1 927	428	332	88	91	837	661	5.24	4.80	0.015	0.021
Total	41 141	3582	2302	–	–	–	–	–	–	–	–

Proteobacteria, Aquificae, Deinococcus-Thermus, Bacteroidetes and *Firmicutes* dominated bacterial communities in the four acidic samples (Zzq, Drty4, Drty14 and Sx1). Within the *Proteobacteria*, *Alphaproteobacteria* dominated the acidic springs Zzq and Drty14 (34% and 75% of proteobacterial reads respectively). Most of these sequencing reads fell into the genus *Acidicaldus*, which had been detected in acid-sulfate-chloride and acid-sulfate hot springs in Joseph's Coat and Rainbow Springs in Yellowstone National Park (Kozubal *et al*., 2012). A large number of betaproteobacterial sequences (14% and 20% of proteobacterial sequencing reads respectively) were detected in the Drty14 and Sx1 hot springs, and most of them belonged to the *Burkholderiales*, specifically the *Thermothrix* and *Thiomonas*. The Sx1 hot spring had the highest abundance of deltaproteobacterial sequences (70% of proteobacterial sequencing reads), which fell into several subgroups: *Desulfobacca*, *Desulfomonile*, *Desulfurella*, *Desulfatibacillum* and *Geobacter*. The Drty14 and Drty4 hot springs also harboured a certain amount of deltaproteobacterial sequences (36% and 19% of proteobacterial sequencing reads respectively) and most of these sequences were affiliated with the *Desulfurella*. The Zzq, Drty4 and Drty14 samples contained high abundance of *Aquificae* sequences, which accounted for 24–52% of the total sequences in each sample. Above 99% of these sequences were affiliated with *Hydrogenobaculum*, the only acidophilic genus within the *Aquificae*.

Table 4. Observed archaeal richness and diversity estimates based on 97% and 95% OTU clusters respectively.

Community	No. sequence	No. OTUs		Coverage (%)		Richness (Chao1)		Shannon's index (Chao & Shen)		Simpson index (MLE)	
		97%	95%	97%	95%	97%	95%	97%	95%	97%	95%
Zzq	3 939	293	107	0.98	0.99	416	156	4.60	3.17	0.020	0.073
Dgg	3 605	288	148	0.97	0.98	466	247	4.26	3.24	0.034	0.088
Hmz2	1 690	285	155	0.92	0.96	481	252	4.77	3.92	0.022	0.041
Eynj2	1 016	229	152	0.88	0.92	448	297	4.54	3.67	0.035	0.082
Hmz1	1 781	228	138	0.94	0.96	442	243	4.29	3.50	0.036	0.066
Eynj3	1 079	301	191	0.84	0.90	660	380	5.14	4.34	0.014	0.033
Drty4	2 389	259	105	0.96	0.98	360	169	4.57	3.30	0.025	0.066
Sx4	2 015	178	107	0.95	0.97	386	252	3.54	2.47	0.076	0.176
Sx1	716	130	91	0.90	0.93	267	184	3.75	3.01	0.088	0.181
Drty14	2 091	113	62	0.98	0.98	212	187	3.05	2.36	0.094	0.154
Gl15	1 387	109	61	0.97	0.98	164	106	3.25	2.08	0.097	0.289
Gl16	2 165	172	97	0.97	0.98	290	198	3.71	3.08	0.059	0.088
Qc4	1 735	217	134	0.94	0.96	433	265	3.96	3.11	0.062	0.121
Qc8	1 004	284	175	0.85	0.92	574	319	5.12	4.37	0.017	0.030
Qc1	2 253	374	214	0.92	0.96	637	327	5.01	4.19	0.018	0.036
Qc3	1 786	420	259	0.88	0.94	825	409	5.36	4.69	0.013	0.020
Total	30 651	2587	1245	–	–	–	–	–	–	–	–

Table 5. The relative abundance of unique (present in a single hot spring) OTUs to total OTUs and the relative abundance of sequences represented by unique OTUs to total sequences of each investigated sample.

	Archaea				*Bacteria*			
	Relative abundance of unique OTUs to total OTUs (%)		Relative abundance of sequences represented by unique OTUs to total sequences (%)		Relative abundance of unique OTUs to total OTUs (%)		Relative abundance of sequences represented by unique OTUs to total sequences (%)	
Sample	97%	95%	97%	95%	97%	95%	97%	95%
Zzq	48	32	20	6	27	23	18	13
Dgg	48	33	13	4	66	54	47	23
Hmz2	43	33	16	9	39	28	12	6
Eynj2	47	32	20	11	48	41	27	17
Hmz1	36	30	9	4	44	43	21	16
Eynj3	46	34	17	9	33	27	10	6
Drty4	37	31	16	4	36	30	5	3
Sx4	43	26	24	3	49	37	21	10
Sx1	54	42	33	20	55	44	22	13
Drty14	65	50	38	25	50	48	24	18
Gl15	65	46	20	7	60	48	34	18
Gl16	32	26	7	2	31	27	6	3
Qc4	34	26	9	5	39	34	10	6
Qc8	24	15	9	4	23	21	7	4
Qc1	49	35	20	8	48	42	18	12
Qc3	75	65	62	45	71	63	55	42

Some other phyla were also recovered from acidic hot springs such as *Firmicutes*, *Deinococcus-Thermus*, *Bacteroidetes* and *Actinobacteria* (Fig. 2).

In comparison, non-acidic hot springs in Yunnan province harboured more diverse bacterial population than acidic hot springs (Fig. 2), and they were dominated by *Proteobacteria*, *Aquificae*, *Thermotogae*, *Bacteroidetes*, *Firmicutes*, *Deinococcus-Thermus* and *Thermodesulfobacteria*. *Proteobacteria* comprised 3–49% of the total sequences in each non-acidic spring. Alpha-, bata-, delta- and gamaprotebacterial sequences were detected in all non-acidic samples but with variable abundances, respectively, and the related subgroups included: *Rhodobacteraceae*, *Caulobacteraceae*, *Beijerinckiaceae*, *Syntrophaceae*, *Moraxellaceae*, *Thiotrichaceae* and *Xanthomonadaceae* The *Firmicutes* comprised 0.2–5.5% of the total sequences in each non-acidic spring, and can be classified into two classes: *Clostridia* and *Bacilli* among which *Clostridia* sequences were predominant (67%). In addition, 16% of the *Firmicutes* sequences could not be assigned to any descried classes. The *Bacteroidetes* and *Deinococcus-Thermus* were also recovered from all non-acidic samples, with the former being mainly represented by the class *Sphingobacteria* and the later being mainly represented by the genus *Thermus*. The *Thermotogae* sequences were detected from Sx4, Eynj2, Eynj3 Hmz2

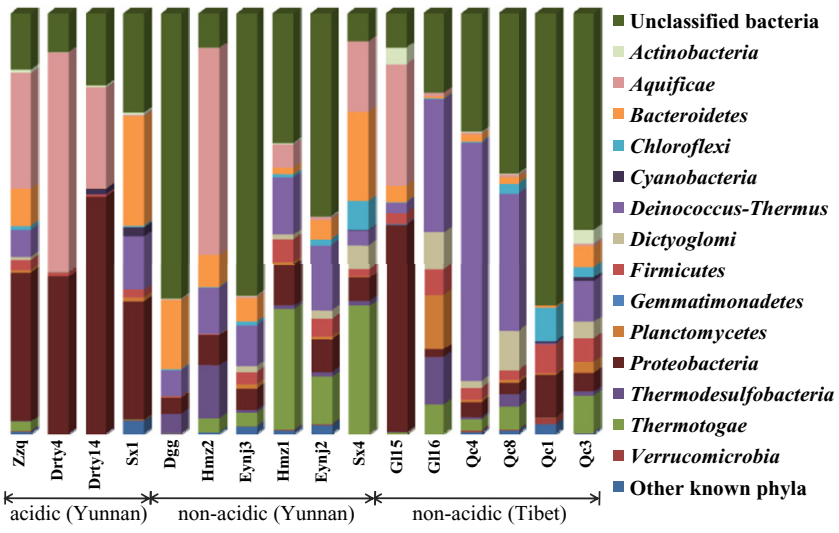

Fig. 2. Bacterial diversity in the different hot springs. Stacked bar graph represents the relative distribution of major phyla in the different samples.

and Hmz1 with abundances ranging from 3% to 30%, and the *Fervidobacterium* represented the predominant genus of this phylum. Some other phyla (e.g. *Actinobacteria*, *Dictyoglomi*, *Acidobacteria* and *Planctomycetes*) were also detected from non-acidic hot springs in Yunnan but with low abundances (Fig. 2).

All the investigated Tibetan hot springs were non-acidic. The major phyla of these Tibetan hot springs were similar to the Yunnan non-acidic samples, but showed different relative abundances (Fig. 2). For example, *Bacteroidetes* constituted 0.4–5% of the total sequences obtained from each of the Tibetan hot springs, versus 2–21% of the total sequences in each Yunnan non-acidic spring; *Deinococcus-Thermus* sequences constituted 30–57% of the total sequences obtained from three Tibetan hot springs (Gl15, Qc4 and Qc8), versus 4–15% of the total sequences obtained from each Yunnan non-acidic hot spring.

Phylogenetic analysis of Aquificae *and* Proteobacteria

All the obtained *Aquificae*-related sequences were distributed among 15 hot springs with the relative abundances of 0.2–52% of the total sequences, and can be clustered into 176 OTUs. The majority (154 out of 176) of the *Aquificae* OTUs were clustered into five known genera of *Thermocrinis*, *Sulfurihydrogenibium*, *Hydrogenobacter*, *Hydrogenobaculum* and *Persephonella* (23, 3, 34, 78 and 16 OTUs respectively). The phylogentic tree showed that the majority of the representative *Aquificae*-related OTUs from this study formed four groups (Fig. 3). Group 1 was composed of the *Persephonella*-related sequences (BLAST similarities ranging from 90% to 97%) and were present in springs Hmz2, Hmz1, Eynj2, Eynj3 and Sx4. Group 2 was composed of *Hydrogenobaculum*-related sequences and dominated the *Aquificae* taxa (94–97% of *Aquificae*-related sequences and 24–52% of total bacterial sequences) in three acidic hot springs (Zzq, Drty4 and Drty14; pH ranged from 3.2 to 4.5). Groups 3 and 4 had close relationship, comparing with other two clades. Group 3 was discovered in eight neutral and/or alkaline hot springs and was mainly related to *Hydrogenobacter* and *Thermocrinis*, which were dominant in Gl15 and Hmz2 respectively. Group 4 was composed of OTUs that could not be assigned to any described classes (BLAST similarities ranging from 91% to 94%).

The representative alpha-, bata-, delta- and gamaprotebacterial OTUs, on the other hand, did not cluster together into some tight clades in the proteobacterial phylogentic tree (Fig. 4). Many of them were similar to those detected from Yellowstone especially for betaproteobacterial sequences, or clustered with the sequences detected from thermophilic microbial mats of hot spring in the central Tibet (Lau *et al.*, 2009).

Archaeal diversity

All the obtained archaeal sequences can be clustered into 2587 OTUs (cut-off: 97%). These archaeal OTUs were affiliated with the following taxa: *Crenarchaeota* (including *Sulfolobales*, *Thermoproteales*, *Desulfurococcales* and *Caldisphaerales*), *Euryarchaeota* (including *Thermoplasmata*, *Methanobacteria*, *Halobacteria*, *Archaeoglobi*, *Methanomicrobia* and *Methanococci*), *Nanoarchaeota* and *Korarchaeota*. The *Nanoarchaeota* were only detected in three springs and *Korarchaeota* from seven hot springs with low relative abundances (< 2.3%). The *Euryarchaeota* and *Crenarchaeota* were the dominant phyla in the investigated hot springs.

The three acidic hot springs (Zzq, Drty4 and Drty14) had simpler communities than non-acidic ones. The majority (98%) of total archaeal sequences recovered from the high temperature spring Zzq fell into the single genus *Sulfolobus*, belonging to the class *Sulfolobales* of the *Crenarchaeota*. The archaeal community of low-temperature spring Drty14 was dominated by class *Thermoplasmata* of the *Euryarchaeota* (97% of archaeal sequences from this sample), and 84% of the *Thermoplasmata* sequences belonged to the genus *Picrophilus*. It appeared that the Drty4 hot spring had an archaeal community structure somewhere between Drty14 and Zzq: 20% of the archaeal sequences of Drty4 fell into *Thermoplasmata* (Fig. 5), and 79% were affiliated with *Sulfolobales*. Different from Zzq, Drty4 harboured a small portion (6%) of sequences belonging to genus *Metallosphaera*. In contrast to the above acidic hot springs, the slightly acid hot spring Sx1 had more diverse and different phylogenetic groups: *Sulfolobales*, *Thermoproteales*, *Desulfurococcales*, unclassified *Crenarchaeota* and *Euryarchaeota* (Fig. 5).

Class *Desulfurococcales* and unclassified *Crenarchaeota* were the two dominant (50–90%) groups in non-acidic hot springs in Yunnan (Fig. 5). *Thermoproteales* was also detected in almost all these non-acidic springs but with a low relative abundance (except Eynj3 and Hmz2). For *Euryarchaeota*, most of the obtained sequences could not be assigned to any known subgroups. The unclassified *Euryarchaeota* were recovered from all non-acidic samples, and were especially dominant in the Sx4 hot spring that constituted 47% of total archaeal species. In addition, a large number of methanobacterial sequences (29% of the total sequences) were recovered from one sample (Hmz1). Similar to those from non-acidic Yunnan hot springs, *Desulfurococcales* and unclassified *Crenarchaeota* were the predominant (representing 16–89% and 0.4–48% of the obtained total archaeal sequences respectively) lineages in Tibetan hot springs. In addition, some other groups were abundantly distributed in certain Tibetan springs. For example, the

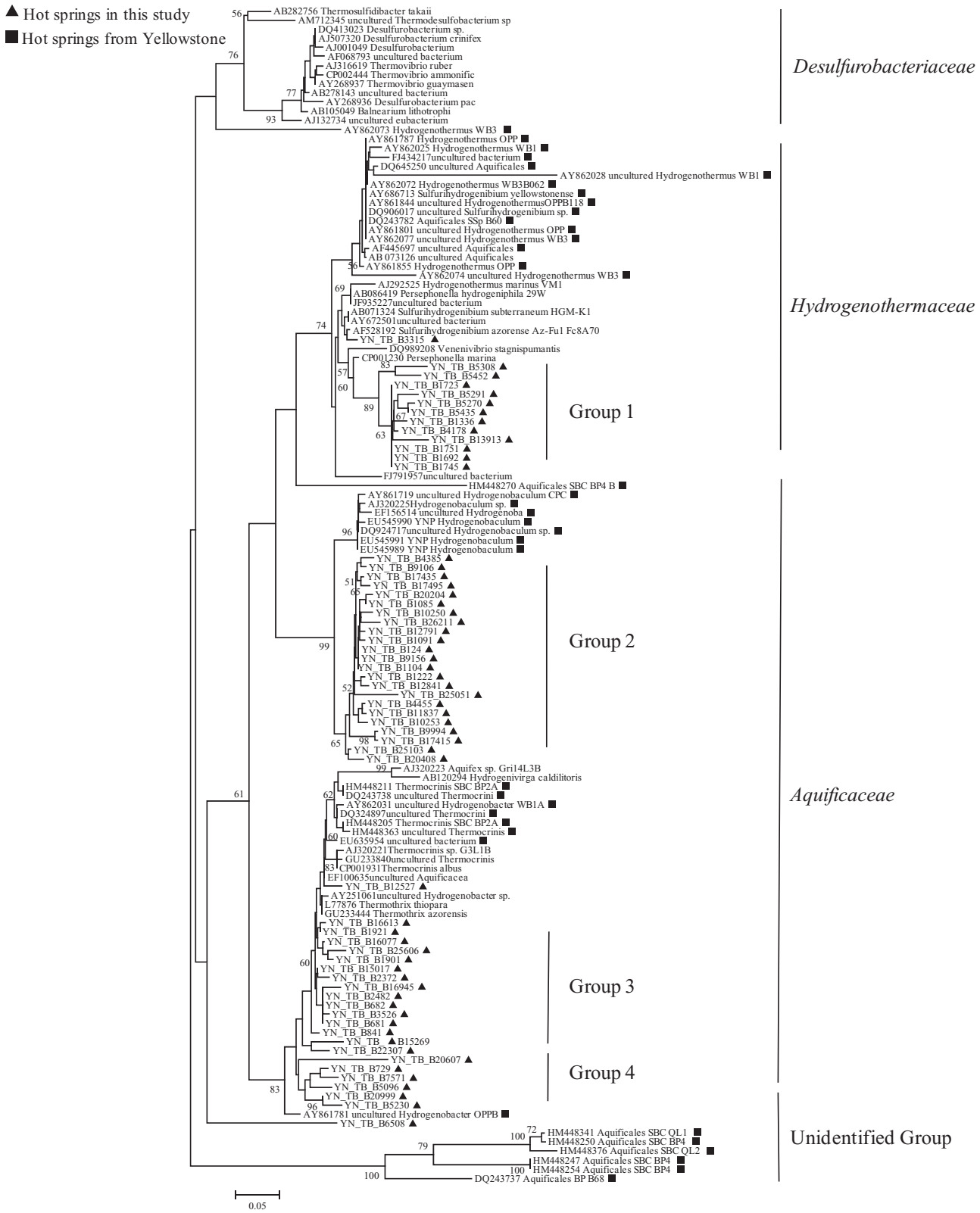

Fig. 3. Phylogenetic relationships between the *Aquificae*-related 16S rRNA gene sequences obtained in this study and those from Yellowstone hot springs and other geothermal features.

Fig. 4. Phylogenetic relationships between the *Proteobacteria*-related 16S rRNA gene sequences obtained in this study and those from Yellowstone hot springs and other geothermal features.

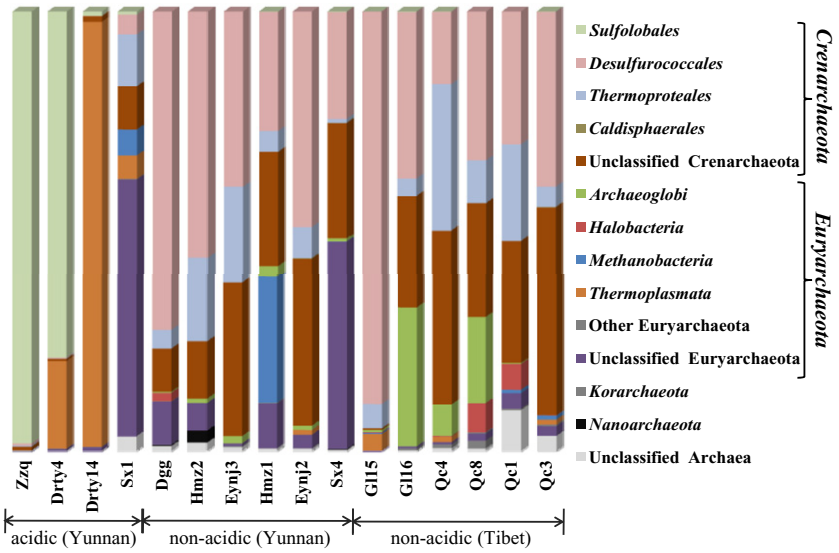

Fig. 5. Archaeal diversity in the different hot springs. Stacked bar graph represents the relative distribution of the mainly phyla in the different stations.

class *Archaeoglobi* of *Euryarchaeota* represented 31% and 20% of archaeal population in Gl16 and Qc8 hot springs respectively. *Halobacteria* were also detected in Qc1 and Qc8 with 6% and 7% of total archaeal species respectively.

Phylogenetic analysis of Desulfurococcale *and unclassified* Crenarchaeota

The *Desulfurococcales* and unclassified *Crenarchaeota* represented a large portion of archaeal community in all non-acidic hot springs (50–90% of total archaeal species in each sample) in this study. Previous studies have recovered many new and representative uncultured phylogenetic clades belonging to these two groups (Barns *et al.*, 1994; 1996; Meyer-Dombard *et al.*, 2005; 2011); however, the automatic classifiers at the RDP level lack the fine classification of these subgroups. Thus, to better define the affiliation of *Desulfurococcales* and unclassified *Crenarchaeota* taxa in this study, we selected some representative OTUs and reference sequences to perform the phylogenetic analysis. The nomenclatures of partial clades were referred to Meyer-Dombard's work at Yellowstone (Meyer-Dombard *et al.*, 2005; 2011). Our sequences were distributed in a broad spectrum of archaeal phylogenetic lineages in Fig. 6. Few sequences were similar to those detected from Yellowstone, and the most clusters were in tight clades within each known group, especially for the class *Desulfurococcales* and 'uncultured *Crenarchaeota*' (UC) group II and IV. The latter group harboured more OTUs than the other groups and some of them formed a large subgroup (character A denoted) (Fig. 6). This subgroup, however, was only found in five Tibetan hot springs (Qc3, Gl16, Qc1 Qc4 and Qc8).

Discussion

High-throughput 454 pyrosequencing is a robust tool in analysing the microbial communities from diverse environments and can detect rare members of the microbial community while providing several thousand sequences of sufficient length to accurately identify consortia members and to estimate microbial diversity and richness (Youssef *et al.*, 2009). With the use of the high-throughput 454 pyrosequencing technique, we recovered community structures from Yunnan and Tibetan hot springs with greater detail than previous studies using conventional clone library and DGGE approaches. For example, we previously studied the crenarchaeotal population in the Zzq and Dgg hot springs by using the clone library technique and only four to six OTUs were obtained (Song *et al.*, 2010). In comparison, more than 200 creanarchaeotal OTUs were recovered from the Zzq and Dgg hot springs in this study. Huang and colleagues (2011) detected 82 bacterial and 41 archaeal OTUs (cut-off: 97%) from 10 Tibetan hot springs using the clone library method, and the bacterial OTUs were affiliated with 10 bacterial phyla (*Firmicutes, Proteobacteria*, *Cyanobacteria*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, *Nitrospirae*, *Planctomycetes*, *Thermodesulfobacteria*, *Aquificae* and unclassified *Bacteria*) with *Proteobacteria*, *Cyanobacteria* and *Chloroflexi* being the dominant groups. In comparison, 1711 bacterial and 1161 archaeal OTUs (cut-off: 0.03) were obtained from six Tibetan hot springs in this study, and in addition to the 10 bacterial phyla mentioned above, 13 other bacterial phyla (with low relative abundances) were also detected in this study. The dominant phylogenetic groups were *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Deinococcus-Thermus* and unclassified *Bacteria* for the Tibetan hot springs in this study.

Fig. 6. Phylogenetic relationships between the crenarchaeotal 16S rRNA gene sequences obtained in this study and those from Yellowstone hot springs and other geothermal features.

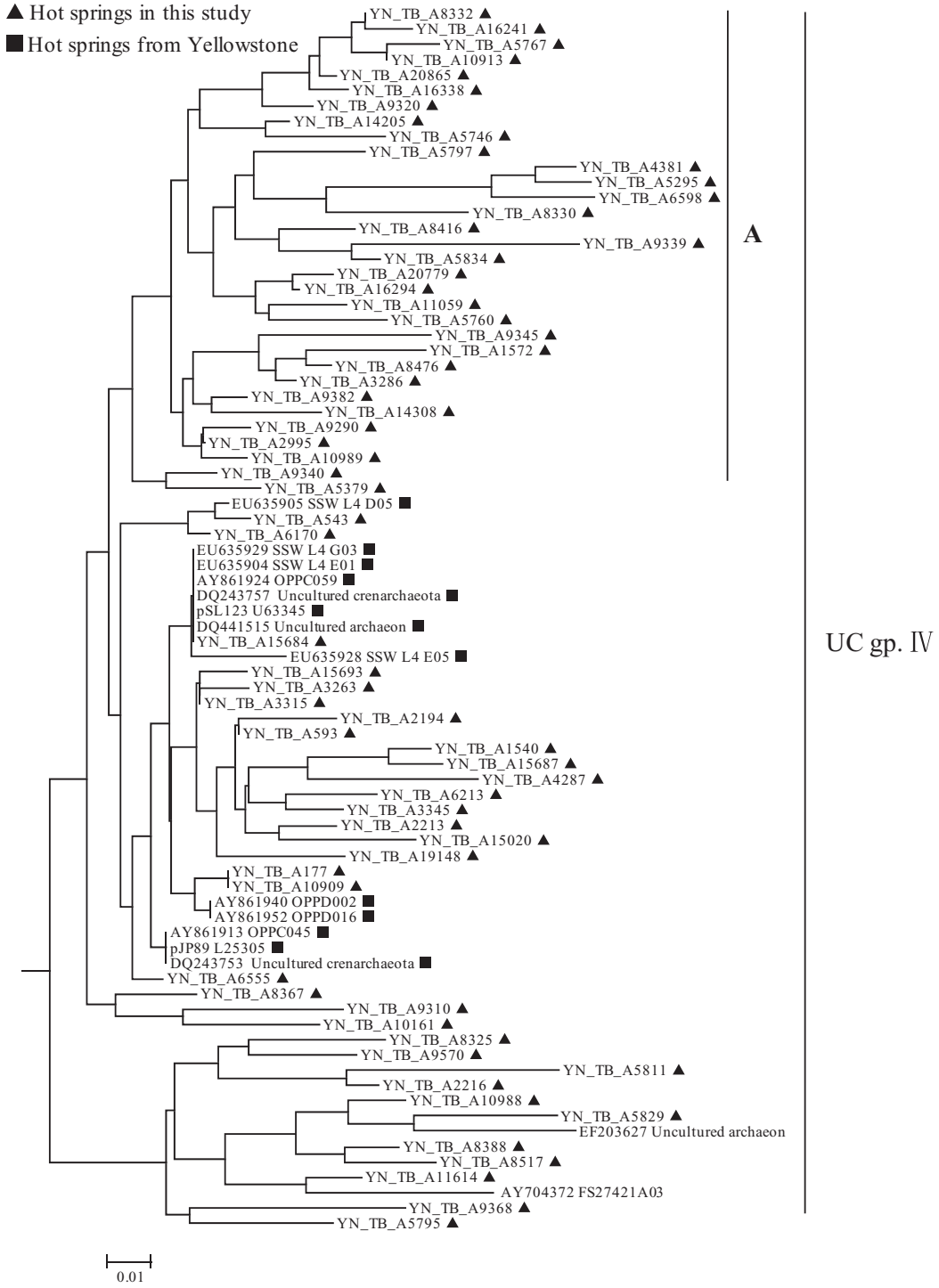

Fig. 6. *Continued*

A recent study revealed the bacterial and archaeal diversities in 11 Fe-rich acidic hot springs (temperature: 53–88°C; pH 2.4–3.6) located in Joseph's Coat and Rainbow Springs in Yellowstone National Park (Kozubal *et al.*, 2012), which were similar to acidic hot springs Zzq, Drty4 and Drty14 (temperature: 47–96°C; pH: 3.2–4.5) in this study. The acidic Yunnan hot springs (Zzq, Drty4 and Drty14) possess similar preponderant bacterial taxonomic groups (e.g. *Hydrogenobaculum*, *Acidicaldus*) to the acidic hot springs in Joseph's Coat and Rainbow Springs

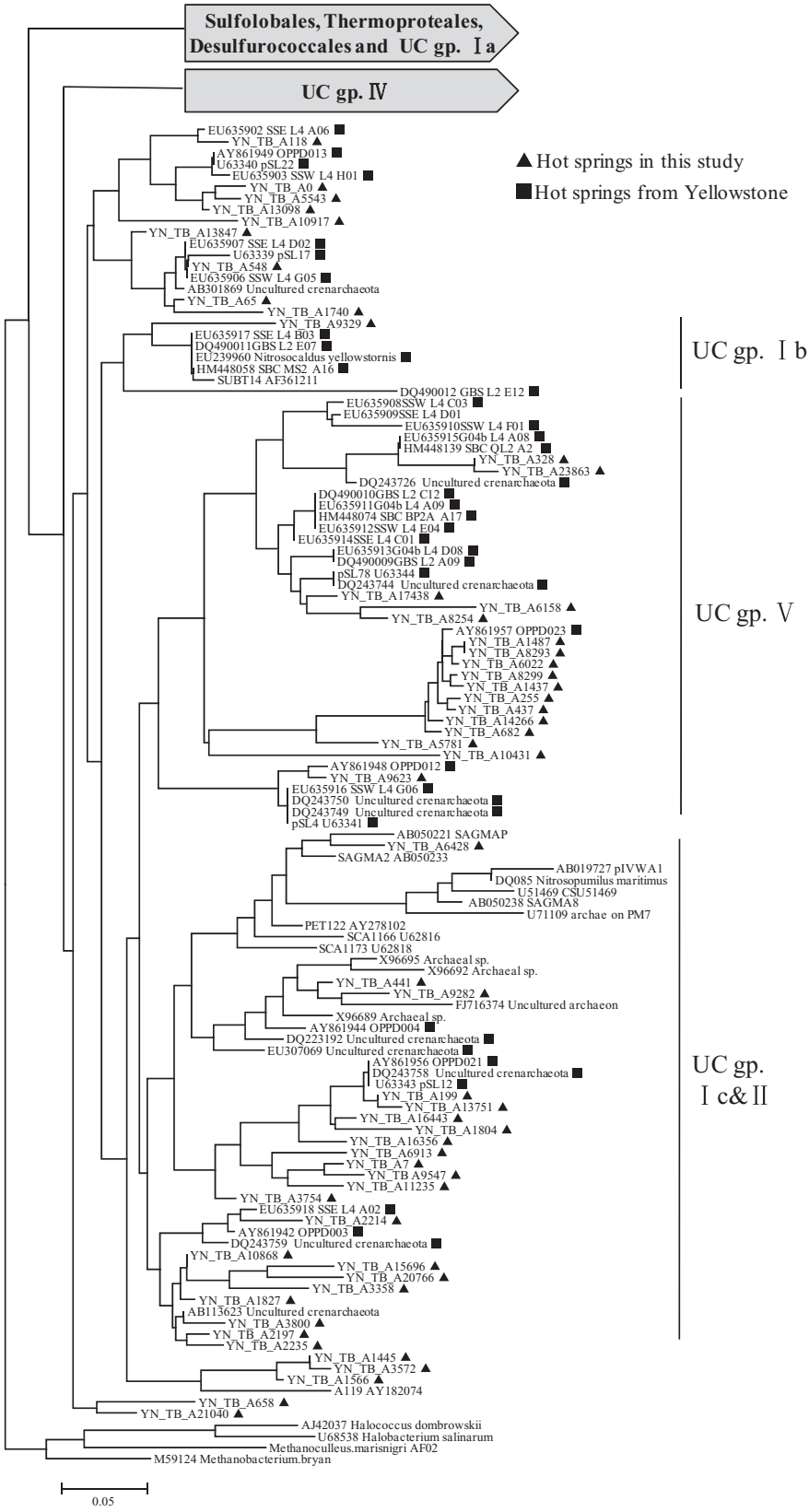

Fig. 6. *Continued*

in Yellowstone (Kozubal *et al*., 2012). However, some bacterial taxonomic groups differ between Yunnan and Yellowstone hot springs. For example, *Thermus*, *Thermothrix*, *Desulfurella* and *Thiomonas* were only detected with relative high abundance from Yunnan hot springs. In contrast, *Acidimicrobium*, *Methylacidiphilum* and *Meiothermus* were only recovered from Yellowstone springs with high abundance (Kozubal *et al*., 2012).

For *Archaea*, the above Yellowstone hot springs were dominated by *Crenarchaeota*. Generally speaking, *Crenarchaeota* always dominate hot springs of Yellowstone and other regions, such as hot springs in Iceland, Kamchatka (Russia), Thailand (Purcell *et al*., 2007; Wilson *et al*., 2008; Costa *et al*., 2009; Kublanov *et al*., 2009). However, in this study the majority (97.6%) of total sequences in Drty14 (temperature: 47°C; pH: 4.5) fell into the class *Thermoplasmata* of *Euryarchaeota*. The Sx1 and Sx4 hot springs were also dominated by unclassified euryarchaeotal sequences. It is unclear whether geography plays a role in the disparate distribution of archaeal species from acidic environments between China and other regions mentioned above.

Desulfurococcales and unclassified *Crenarchaeota* were the dominant groups in most of the investigated non-acidic hot springs from Yunnan and Tibet, which is consistent with previous studies in Yellowstone hot springs (Meyer-Dombard *et al*., 2005; 2011; Spear *et al*., 2005). However, the hot springs of Yunnan Province and Tibet harboured more diverse unclassified crenarchaeotal (UC) group IV lineages than Yellowstone. Furthermore, the UC group IV contained a large subgroup (character A assigned), which has never been revealed from Yellowstone (Fig. 6). Interestingly, sequences affiliated with this subgroup were only from the Tibetan hot springs, indicating this group of *Crenarchaeota* may not be ubiquitous in terrestrial hot springs. Indeed, sequences of this subgroup have also been detected from a hot spring (64°C) located in another thermal field in Tibet. Temperature might be a limiting environmental factor since the group denoted by character A dominates the low-temperature springs and has low abundance in high-temperature springs. In addition, GenBank sequences closely related to our sequences within the A group are mainly from moderate-temperature hot springs in Uzon Caldera, Kamchatka, Russia and Thailand (Kanokratana *et al*., 2004; Burgess *et al*., 2012). Taken together it appears that the A group of *Crenarchaeota* may prefer low–moderate temperature habitats.

In summary, we investigated the prokaryotic diversity in hot springs of Yunnan Province and Tibet using 454 pyrosequencing technologies. Our study shows that the 454 pyrosequencing technique is more robust than the clone library and DGGE methodologies in characterizing microbial diversity in hot springs. This study shows more diverse prokaryotic diversity in the investigated hot springs than our previous studies (using clone library and DGGE techniques) in the same region. The phylogenetic analysis shows that Yunnan and Tibetan hot springs possess high abundance of sequences affiliated with *Aquificae* and *Crenarchaeota*, which is consistent with previous studies in Yellowstone hot springs. However, the detailed community composition of *Aquificae* and *Crenarchaeota* differed between hot springs of Yellowstone and Yunnan/Tibet: many *Aquificae*- and *Crenarchaeota*-sequences obtained in the investigated hot springs are phylogenetically separated from those from Yellowstone hot springs.

Experimental procedures

Field measurement and sample collection

At each hot spring, water temperature and pH were determined using a Hach pH meter equipped with a pH probe and a temperature probe. Nitrite, nitrate, ammonium, phosphate and ferrous iron were measured using Hach kits according to the manufacturer's instructions. After chemical measurements, mat-containing sinter or sediments were collected into 50 ml Falcon tubes and immediately were stored in liquid nitrogen (at the sites in Yunnan Province) or dry ice (at the Tibetan sites). The samples were kept in liquid nitrogen or dry ice in the field and during transportation, and then were stored at −80°C in the laboratory until further analysis.

Nucleic acid extraction and 454 pyrosequencing

One to two grams of sinter or sediments were subjected to DNA extraction with the use of an E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, USA) according to the manufacturer's instructions. Quadruplicates were performed for each sample. The resulting DNA extracts from each sample were mixed and were used for downstream PCR experiments. The bacterial and archaeal hypervariable V4 region of the 16S rRNA genes were amplified using primer sets of B-forward (5′-AYTGGGYDTAAAGNG-3′)/B-reverse (5′-TACNVGGGTATCTAATCC-3′) and A-forward (5′-YMGCCRCGGKAAHACC-3′)/A-reverse (5′-CTACNSGGGTMTCTAAT-3′) respectively (http://pyro.cme.msu.edu/pyro/help.jsp). The employed primer sets targeted regions with high classification accuracy using the RDP's naive Bayesian rRNA classifier. To pool multiple samples for one run of 454 sequencing, a sample tagging approach was used (Meyer *et al*., 2008). Each tag was added to the 5′ end of forward primer (http://pyro.cme.msu.edu/pyro/help.jsp). PCR amplification consisted of an initial denaturation at 95°C for 10 min, 25 cycles of denaturation at 95°C for 45 s, annealing at 42°C and 55°C for bacteria and archaea respectively) for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. Individual reagents and their concentrations were as follows: 1× PCR buffer with 1.5 mM Mg_2Cl, dNTPs (100 M each), 0.25 M each primer, 2.5 U of DNA polymerase (Ex-Taq) (TaKaRa, Dalian, China) and ~ 50 ng of total DNA. PCR products were purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek,

USA) according to the manufacturer's instructions. The purified PCR products were annealed to oligonucleotides that are complementary to an adaptor sequence that are tethered onto μm-size beads. Subsequently, nucleic acid was pyrosequenced using Roche Diagnostics GmbH/454 Life Sciences Corporation (Branford, CT, USA). The raw sequence reads were deposited into the NCBI sequencing read archive under Accession No. SRA053606.

Pyrosequencing data processing

According to Sogin's description (Sogin *et al*., 2006), low-quality reads were removed. All available bacterial and archaeal sequences were longer than 200 bp. All sequence reads were compared with a reference database of known 16S rRNA genes [obtained from SILVA and Ribosomal Database Project (RDP) databases] and taxonomically assigned with rdp_classifier-2.0 at 50% of confidence threshold (Wang *et al*., 2007), then the invalid reads, which cannot be classified in any *Archaea* or *Bacteria*, were removed. Subsequently alignment and OTUs cluster of the available sequences were performed through RDP pipeline (http://pyro.cme.msu.edu/). Based on the results of OTUs cluster and assigned taxonomy of the available sequences, the comprehensive statistics including the distribution and abundance of each OTU in the investigated hot springs were obtained by using a Python script.

One sequence was selected from each OTU (cut-off value: 0.03) and submitted to GenBank and compared with the NCBI (National Center for Biotechnology Information) database using BLAST. On the other hand, according the accession numbers, which have been published, the sequences isolated from Yellowstone hot springs were downloaded and clustered into OTUs (cut-off value: 0.02) by using DOTUR 1.53 (Schloss and Handelsman, 2005). The sequences of OTUs of this study and Yellowstone hot springs were combined and aligned using CLUSTALX1.83. Neighbour-joining phylogenetic trees were constructed from dissimilar distance and pairwise comparisons with the Jukes-Cantor distance model using the MEGA (molecular evolutionary genetics analysis) program, version 5 (Tamura *et al*., 2011) Bootstrap replications of 500 were assessed. The sequences performed in phylogenetic analyses were deposited in the EMBL database under Accession Nos HE980022 to HE980080 (*Aquificae*), HE980081 to HE980160 (*Proteobacteria*) and HE980161 to HE980324 (*Archaea*).

Statistical analysis

A rarefaction analysis was performed with the use of Rarefaction version 1.3 (http://www.uga.edu/strata/software/Software.html). Statistical analysis of OTU richness via rarefaction, Chao1 and ACE (abundance-based coverage) estimates were performed using the SPADE (Species Prediction and Diversity Estimation) software (Chao, 2010). The bacterial and archaeal community classification was determined separately with unweighted Fast UniFrac environmental clustering. The online Fast UniFrac program (http://bmf2.colorado.edu/fastunifrac/) takes molecular evolutionary distances of the sequences and their environmental occurrences for microbial community similarity analyses, particularly suitable for very large sequence data sets such as pyrosequencing data (Hamady *et al*., 2010). PCA of the sulfate, nitrite, nitrate, ammonium, ferrous iron were performed using R software (version 2.6.0).

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