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ENVIRONMENTAL BIOTECHNOLOGY

The response of archaeal species to seasonal variables in a subtropical aerated soil: insight into the low abundant methanogens

Wei Xie[1] · Na Jiao[2] · Cenling Ma[1] · Sa Fang[2] · Tommy J. Phelps[3] · Ruixin Zhu[2] · Chuanlun Zhang[1]

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Abstract Archaea are cosmopolitan in aerated soils around the world. While the dominance of *Thaumarchaeota* has been reported in most soils, the methanogens are recently found to be ubiquitous but with low abundances in the aerated soil globally. However, the seasonal changes of Archaea community in the aerated soils are still in the mist. In this study, we investigated the change of Archaea in the context of environmental variables over a period of 12 months in a subtropical soil on the Chongming Island, China. The results showed that *Nitrososphaera* spp. were the dominant archaeal population while the methanogens were in low proportions but highly diverse (including five genera: *Methanobacterium*, *Methanocella*, *Methanosaeta*, *Methanosarcina*, and *Methanomassiliicoccus*) in the aerated soil samples determined by high throughput sequencing. A total of 126 LSA correlations were found in the dataset including all the 72 archaeal OTUs and 8 environmental factors. A significance index defined as the pagerank score of each OTU divided by its relative abundance was used to evaluate the significance of each OTU. The results showed that five out of 17 methanogen OTUs were significantly positively correlated with temperature, suggesting those methanogens might increase with temperature rather than being dormant in the aerated soils. Given the metabolic response of methanogens to temperature under aerated soil conditions, their contribution to the global methane cycle warrants evaluation.

Keywords Archaea · Aerated soil · Methanogens · Network analysis

Wei Xie and Na Jiao contributed equally.

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✉ Wei Xie
xiewei@tongji.edu.cn

✉ Ruixin Zhu
rxzhu@tongji.edu.cn

[1] State Key Lab of Marine Geology, Tongji University, Shanghai 200092, People's Republic of China

[2] Department of Bioinformatics, School of Life Sciences and Technology, Tongji University, Shanghai 200092, People's Republic of China

[3] Department of Earth and Planetary Sciences, University of Tennessee, Knoxville, TN 37996, USA

Introduction

Archaea, which are firstly thought to be limited to environmental extremes of the earth (Woese et al. 1990; Takai and Horikoshi 1999), now considered as cosmopolitan as Bacteria, adapted to their ecological niches (DeLong 1998; Schleper et al. 2005; Leininger et al. 2006; Auguet et al. 2010). The archaeal communities in soils are majorly composed of a few groups of *Thaumarchaeota* (Auguet et al. 2010; Bates et al. 2011; Cao et al. 2012; Xie et al. 2015) that are possibly key players in soil nitrification (Leininger et al. 2006; Nicol and Schleper 2006). However, methanogens were also found to be ubiquitous but with low abundances (methanogens in some upland aerated soils were below 10^3 copies/g dry weight, while those *Thaumarchaeota* were around 10^8 copies/g dry weight (Angel et al. 2012)) in various well-aerated soils including desert soils (Angel et al. 2011, 2012), pasture soil (Nicol et al. 2003; Prem et al. 2014), and alpine fallow soils (Praeg et al. 2014). Those methanogens were traditionally thought to be restricted to anaerobic environments; however, Peters and Conrad (1995) observed that

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methane could be produced in aerated soils when incubated under anoxic conditions. Subsequent researches also demonstrated that some methanogen strains possess the genetic features to protect themselves against oxidative stress (Brioukhanov et al. 2000; Shima et al. 2000; Erkel et al. 2006). However, comparing with the amount of researches related to the ecological significances of *Thaumarchaeota* in the nitrogen cycles in soils, the significances of those low abundant methanogens in the field are largely unrevealed.

Past studies have addressed the potential methanogenic activities (Peters and Conrad 1995; Angel et al. 2012; Hofmann et al. 2016) including using the stable isotope probing technique (Lee et al. 2012), which does not fully reflect the diversity and dynamics of those low abundant methanogens in the natural environment. With the development of high throughput sequencing, numerous low-abundance microbial taxa recently began to be revealed. For example, Hu et al. (2013b) found the existence of methanogens and their increasing trend with soil pH in the upland soils across southern and northern China. *Methanobacteria* and *Methanomicrobia* were recently detected by high-throughput sequencing in high-altitudinal soils of the Southeast Tibet, China (Wang et al. 2015). However, it remains elusive whether those low abundant methanogens were actively growing as the low abundant *betaproteobacteria* in coastal water became dominant after removing the virus (Bouvier and Del Giorgio 2007; Pedros-Alio 2012).

Network analysis represents an effective approach for exploring the significance of individual species in microbial ecosystems and their response to environment changes. Lupatini et al. (2014) found that only a few genera played a key role as connectors that mainly belonged to phyla *Proteobacteria* and *Actinobacteria* in 36 surface soils in Brazil. Using network analysis of 24 soil samples from Minnesota, Zhou et al. (2011) revealed that soil microbial communities were substantially altered by elevated CO_2. Barberan et al. (2012) further found *Nitrososphaera* in the soils co-occurred with sequences closely related to methane oxidizers, suggesting the potential function of soil *Thaumarchaeota* in methane oxidation. The application of the network analysis might also shed light on uncovering the activity of the low abundant methanogens in aerated soils.

In this study, the grassland surface soils on Chongming Island were monthly sampled over 1 year. qPCR and pyrosequencing targeting the archaeal 16S rRNA gene were conducted to investigate the quantifications and community structures of Archaea in those soils. The local similarity analysis (LSA) was further conducted to investigate the responses of those Archaea to the seasonal variations. Based on those analyses, we sought to understand if those low abundant methanogens were dormant or growing over the sampling periods in nature environments, which might illuminate their ecological function in methane cycle in aerated soils.

Materials and methods

Time-serial sampling, environmental measurements, and amplicon sequencing

The Chongming Island at the mouth of the Yangtze River of China is the largest alluvial island in the world. The island has a subtropical monsoon climate and the annual mean air temperature (MAT) is 15 °C and varies from 3 to 4 °C (the extreme low temperature was −9.8 °C in 1977) in winter to around 27 °C (the extreme high temperature was 37.5 °C in 2004) in summer. Soil mineralogy on Chongming Island is majorly composed of hydromica with minor proportions of kaoline, vermiculite, and chlorite (He 1992).

The sampling grassy land had no record of flooding and stayed aerobic since it was reclaimed from wetlands 40 years ago (Cui et al. 2012). Surface soil (top 2 cm) was collected monthly from within a 4 m^2 open grassy area (31° 31.84′ N and 121° 36.86′ E) on the Chongming Island between March 2012 and March 2013. In detail, the surface grasses were firstly removed. The soils from the four corners of this region were collected and mixed in a pre-sterilized aluminum pan. After removing the visible rocks and grass roots, the samples were divided into two aliquots in the field: one part was kept at 4 °C for soil water content (SWC), TOC, pH, and nutrient analysis; the other part was saved in liquid N_2 and stored at −80 °C for DNA extraction. An iButton (DS-1922, Embedded Data System, US) buried at 2 cm depth in the area recorded the soil temperature every 2 h for the whole 12 months.

SWC was obtained by measuring sample weight before and after freeze-drying. Ca. 5 g of freeze-dried sample was acidified by 10% HCl, washed with deionized water, and dried at 80 °C overnight for analysis of total organic carbon (TOC) and total nitrogen (TN) using an elemental analyzer (CarloErba EA1110). Distilled water extraction from Ca. 4 g of freeze-dried soils were used for pH measurement. 2 M KCl extraction from Ca. 5 g of freeze-dried soils was used for nutrient analysis. NH_4^+ and NO_2^- were measured with an Auto-Analyzer Technicon II (AAII, Bran Luebbe).

DNA was extracted from 0.5 ± 0.1 g of soil using the FastDNA SPIN Kit for Soil (MP Biomedical, OH, USA) with a final elution in 50 μl deionized water. Pyrosequencing was conducted on all 12 samples, targeting the archaeal 16S rRNA gene. The primers were Arch_344F (5′ ACGGGGCG CAGCAGGCGCGA 3′)/Arch_915R (5′ GTGCTCCC CCGCCAATTCCT 3′) for archaeal 16S rRNA gene, which showed higher coverage than other published primer pairs (Gantner et al. 2011). Raw data were extracted from the 454 source data format using mothur (version 1.29.2; Schloss et al. 2009). Sequence reads shorter than 50 bp were removed and the remaining reads were checked with ChimeraSlayer (Haas et al. 2011). The chimeric sequences were excluded from

further analysis. Following the standard pipeline of QIIME software (Caporaso et al. 2010), the remaining sequences were clustered into operational taxonomic units (OTUs) using UCLUST (Edgar et al. 2011) with a 97% sequence identity threshold. Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier 2.2 (minimum confidence 80%) (Cole et al. 2009). The GenBank Sequence Read Run accession numbers are SRR1367259 for the archaeal 16S rRNA gene (Xie et al. 2015).

Defining abundant and rare phylotypes

Rare and abundant OTUs were identified according to their average relative abundance in the community. As the definition by Pedros-Alio (2006) and Fuhrman (2009), OTUs with relative abundance less than 1% were classified as rare, and those with greater than 1% classified as abundant, respectively. The representative sequences of OTUs were compared with reference sequences from the entire SILVA database using BlastN to identify the percentage similarity between the queried sequences and their top hits.

Phylogenetic tree construction

A total of 116 sequences including 72 OTUs representative sequence and 44 archaeal reference sequences downloaded from NCBI were aligned by CLUSTALW. The unrooted tree was based on the results of maximum likelihood analysis of the 116 16S rRNA sequences with 1000 bootstrap and constructed in MEGA6.

Network construction

Local similarity analysis was designed to identify the intervals of correlations between OTUs and environmental parameters (Ruan et al. 2006). OTUs that occurred in no more than 6 sampling months and the average sequences less than 2 were excluded from the analysis; "PercentileZ" was used to normalize the variables and permutation test (n = 5000) was used to calculate the P value for each correlation, which is the probability that a correlation between two nodes is at least as high as the observed value if they are not associated. Q values were calculated to determine a false-discovery rate (Storey 2002). The correlations with $P < 0.01$ and $Q < 0.05$ (false-positive rates) were selected for network construction.

Evaluation of OTU significance

"Significance index" was defined to evaluate the significance of each OTU in the network. Pagerank algorithm (Page et al. 1999) was performed to rank the importance of nodes with R, and the pagerank score as well as relative abundance of OTUi were scaled to (0,1] by dividing the maximum of pagerank score or abundance. Significance index was then calculated as follows:

$$\text{Significance index} = \log_{10}\left(\text{pagerank score of OTUi} \Big/ \text{relative abundance of OTUi} + 1\right) \quad (1)$$

The ratios of pagerank score of OTUi to relative abundance of OTUi were log-transformed to guarantee the normal distribution and 1 were added to the ratios to guarantee the positive values of significance index for further comparison.

Network visualization was performed in Cytoscape3.2.1 (Shannon et al. 2003).

qPCR of the archaeal 16S rRNA gene

The qPCR primers were Arch_334F (5′-ACGG GGCGCAGCA GGCG CGA-3′) (Raskin et al. 1994) and Arch_518R (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer et al. 1993) for the quantification of archaeal 16S rRNA gene. The qPCR analysis of this gene was performed at 95 °C for 30 s and 40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min. Quantification standard comprised a dilution series of purified plasmid DNA from an archaeal 16S rRNA gene clone from the soil collected in March 2013. Triplicate measurements were run for each sample and standard. Melting curve analysis showed that a single melting peak corresponding to the standard DNA was observed for all samples, demonstrating that the signal obtained was consistent with the expected profile for specific PCR products.

Pearson correlation analyses were conducted between abundance of rare methanogens and environmental parameters to determine the environmental factors likely controlling the occurrence and relative abundance of those rare methanogen OTUs.

Statistical analysis

One-way ANOVAs were performed to determine whether the "significance index" was different among any three OTU groups, followed by post hoc Bonferroni's test. Two-tailed t test was performed for Pearson's correlation coefficients. A P value less than 0.05 was considered statistically significant.

Results

The seasonal changes of soil parameters and archaeal abundances on Chongming Island soil

The sampling temperature ranged from 7.85 °C in January 2013 to 29.71 °C in September 2012. The SWCs ranged from 0.8% in June 2012 to 25.8% November 2012. The warmest 4 months corresponded to the driest season (SWC < 7.2%). Nitrite ranged from 0.61 μM in November 2012 to 9.48 μM in December 2012 with an average value of 2.21 ± 2.34 μM. The other five parameters (including TOC, pH, TN, C/N ratio, and NH_4^+) were relatively stable and varied in a narrow range over the sampling seasons (Table S1). Analysis of qPCR showed that the archaeal *16S rRNA* gene copies/g soil (wet wt.) ranged from 6.7×10^6 in April 2012 to 2.9×10^7 in March 2013 with an average value of $2 \pm 0.7 \times 10^7$ over the sampling periods (Table S2).

The diversity of Archaea

Pyrosequencing was used to detect the changes in the archaeal community structure and relative sequence abundance in the Chongming Island soil over the 12 months. A total of 92,049 sequences remained after removal of chimera and low confidence singletons. The archaeal community was dominated by *Nitrososphaera* spp. ranging from 92.9% in March 2012 to 98.8% in February 2013 with minor groups including *Thermoplasmata* (1.4 ± 1.6%) and methanogens (1.1 ± 0.7%) (Xie et al. 2015). Simpson, Shannon, Chao, and ACE indices of the archaeal community varied in narrow ranges from 0.13 to 0.31, 1.9 to 2.6, 60 to 72, and 61 to 73, respectively (Table S1).

Eleven OTUs were abundant (>1% and occurred in more than one sample) and represented 85.4% of all of the archaeal sequences (Fig. S1). All those OTUs were compared with the entire SILVA database to ascertain if they had high or low similarity to reference sequences (defined as globally common and uncommon, respectively) (Hugoni et al. 2013). All 11 abundant OTUs were common, which had higher similarities than 96% to the SILVA database sequences and annotated as *Nitrososphaera* (Fig. S2A). A total of 34 OTUs were common, while the other 27 OTUs were uncommon (Fig. S2B).

The methanogens accounted for a minor part of the archaeal community. A total of 17 methanogen OTUs existed in the soils on the Chongming Island, which represented 5 genera (3 *Methanobacterium*, 3 *Methanocella*, 1 *Methanosaeta*, 1 *Methanosarcina*, and 8 *Methanomassiliicoccus*) and an unclassified *Methanomicrobia* (Figs. 1 and 2). The average percentage of those methanogens was 2.42 ± 1.97% (ranged from 0.7% in May 2012 to 7.9% in March 2012) and their average abundance was $5.0 \pm 4.0 \times 10^5$ copies/g wet soil (ranged from 7.0×10^4 copies/g wet soil in November 2012 to 1.2×10^6 copies/g wet soil in March 2012) over the 12 months (Table S2).

Archaeal OTU-environmental parameter network and significance evaluation

Considering our data were time-serial and LSA could capture unique time-dependent associations by dynamic programming algorithm comparing to traditional methods (Weiss et al. 2016), such as Pearson correlation analysis that generally analyzed the data throughout the whole time interval without considering potential local and time-delayed associations, we constructed the network of archaeal community using LSA from OTU co-occurrence patterns. All 12 samples having 72 OTUs and corresponding eight environmental parameters were included in the analyses. The resulting soil archaeal network consisted of 74 nodes (66 OTUs and the eight environmental parameters OTUs were involved) and 126 edges (Fig. 3 and Table S3).

Based on the structural properties of the network, we calculated the significance level of each OTU involved in this soil archaeal community. Considering that significance of each OTU was determined by both the interactions with other OTUs and its abundance, significance index that comprehensively taken the effects of both pagerank and abundance into account was used to evaluate the significance of an OTU in the network. Briefly, if one specie has more accumulative edges than the other one in the community, it would be supposed to have more significant impact on the community structure (Lupatini et al. 2014). The results showed that the significance indices ranged from 0.09 for a *Nitrososphaera* OTU to 2.7 for a *Methanomicrobia* OTU (Table S3). The significant indices of the 16 methanogen OTUs in the network were significantly higher than those of the 10 abundant *Nitrososphaera* OTUs ($P < 0.01$; Fig. 4), and higher than the 36 rare *Nitrososphaera* OTUs ($P = 0.08$; Fig. 4), suggesting those rare methanogens might significantly participate in ecosystem functioning in those aerated soils.

To further clarify the potential function of archaeal OTUs, representative sequences of 72 OTUs were aligned with 44 reference sequences downloaded from NCBI, and 241 positions were aligned mainly in the V3 region of 16S rRNA gene. The top six significant methanogens were OTU216 (*Methanomicrobia*), OTU692 (*Methanocella*), OTU407 (*Methanosaeta*), OTU207 (*Methanomassiliicoccus*), OTU209 (*Methanobacterium*), and OTU532 (*Methanobacterium*) (Fig. 2, Fig. S3, and Table S3). *Methanosaeta* and *Methanosarcina* are the only two methanogens capable of performing aceticlastic methanogenesis and the predominant methanogens in most natural environments (Liu and Whitman 2008). *Methanocella* is a newly discovered genus, also globally distributed but colonizes rice roots in particular (Lu and Conrad

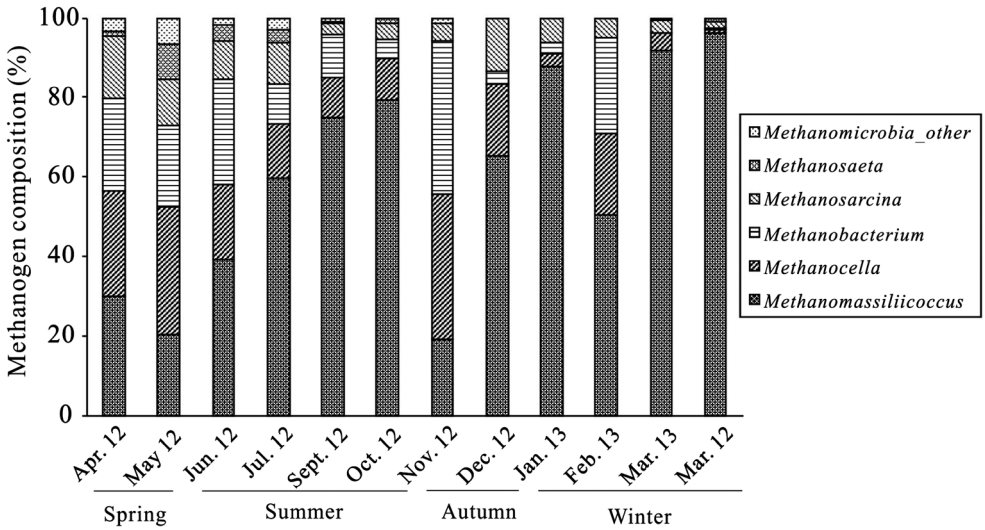

Fig. 1 Monthly changes in methanogen composition in the Chongming Island soil

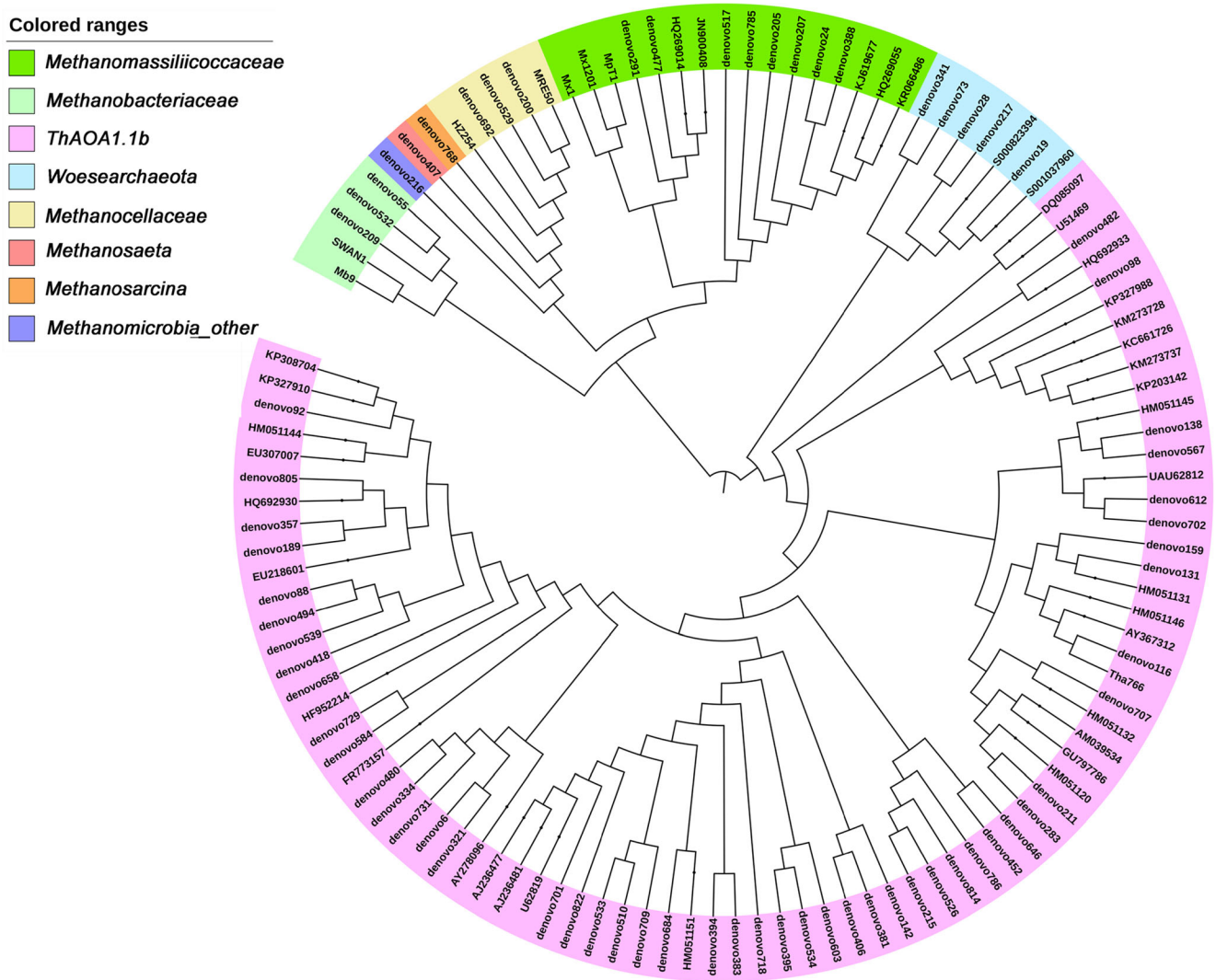

Fig. 2 Maximum likelihood phylogenetic tree of 116 (72 OTUs representative sequence and 44 reference sequences downloaded from NCBI) sequences aligned by CLUSTALW

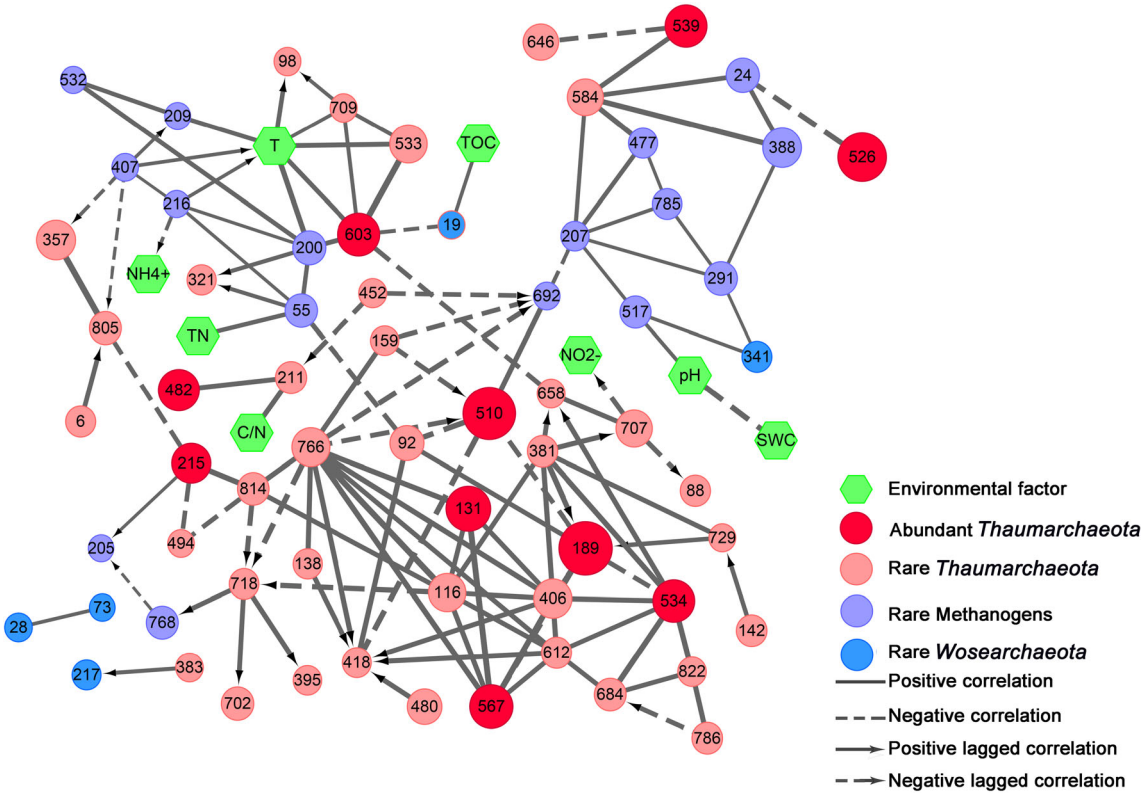

Fig. 3 Overview of the archaeal network. *Circles*, archaeal OTUs; *triangle*, environmental factors. *Solid lines*, positive LS; *dashed lines*, negative LS; *arrow*, 1-month delayed LS correlations that point toward the lagging OTU

2005; Lu et al. 2005; Sakai et al. 2008). Although *Methanocella* were phylogenetically closely related to *Methanosaeta*, they perform different pathways to produce methane. *Methanocella* species can only perform hydrogenotrophic methanogenesis (Sakai et al. 2008; Lü and Lu 2012). Those two genera were frequently found in different aerated soils and could be readily activated by incubating the soils as slurry under anoxic conditions (Angel et al. 2012). The high significance indices of those *Methanosaeta* and *Methanocella* OTUs further indicated their activities in the natural environment. Two of them were phylogenetically clustered with *Methanobacterium*, which have also been found in some aerated soils (Hu et al. 2013b), suggesting it also might be involved in methanogenesis in the aerated soils. OTU207 was phylogenetically clustered with *Methanomassiliicoccus*, which is the seventh order of methanogens and uses methanol and hydrogen as substrates (Iino et al. 2013). All four methanogen OTUs (OTU477, OTU785, OTU291, and OTU517) positively correlating with OTU207 belonged to *Methanomassiliicoccus*, suggesting a close relationship among the *Methanomassiliicoccus* species.

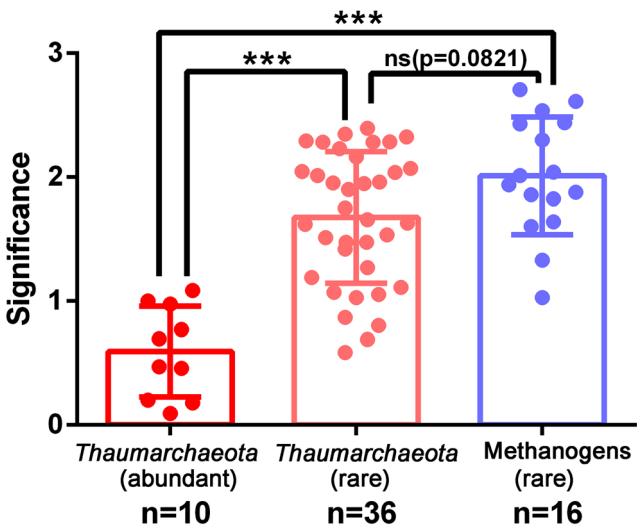

Fig. 4 Comparison of significance index of OTUs in abundant and rare *Thaumarchaeota* and methanogens. Mean ± 95% significance indices of OTUs in abundant *Thaumarchaeota* ($n = 10$, *red*), rare *Thaumarchaeota* ($n = 36$, *pink*), and methanogens ($n = 16$, *purple*) were plotted. *Asterisks* indicate significant differences: one-way ANOVA analysis followed by post hoc Bonferroni's test, $*p < 0.05$, $**p \leq 0.01$, $***p < 0.001$. *ns* not significant

The effects of environmental factors on archaeal community

Pearson correlation analyses showed that none of the alpha diversity indices was significantly correlated with the detected

environmental factors (including temperature, pH, SWC, TOC, TN, C/N, nitrite, and ammonium, Table S1), suggesting the diversity of the archaeal community was not significantly impacted by those environmental factors. However, the ACE index of methanogens was negatively correlated with temperature (Fig. S4), suggesting that increased temperature might reduce the richness of methanogens in the aerated soils on the Chongming Island.

Network analysis also revealed individual OTUs associated with environmental parameters. The temperature had eight edges, while each of the other parameters had just one edge, suggesting the importance of temperature in determining the archaeal community structure. Among those eight OTUs, four were identified as methanogens (a total of 16 methanogen OTUs in the network), while the other four were identified as *Nitrososphaera* (a total of 46 *Nitrososphaera* OTUs in the network; Fig. 3). The proportion of methanogens OTUs (25%) that correlated with temperature was around 3-fold higher than that of *Nitrososphaera* OTUs (8.7%), suggesting temperature had greater effect on methanogens than on *Nitrososphaera* in aerated soil. The four methanogenic OTUs were annotated as *Methanobacterium* (OTU209), *Methanocella* (OTU200), *Methanosaeta* (OTU407), and an unclassified *Methanomicrobia* (OTU216) (Table S3).

To demonstrate the impacts of temperature on methanogens revealed by network analysis, the abundance of methanogens was further estimated by multiplying the abundances of archaeal 16S rRNA gene with the proportion of each OTU. The results showed that the abundances of those methanogens ranged from under detection to 3.1×10^5 copies/g wet soil. Pearson correlation analyses showed that the abundance of *Methanobacterium*_OTU55, *Methanobacterium*_OTU209, *Methanobacterium*_OTU 532, *Methanocella*_OTU200 and *Methanosaeta*_OTU407 were significantly correlated with temperature (Fig. 5a–e, respectively), while the other 11 methanogenic OTUs were not significantly correlated with temperature or other parameters (Table S4), suggesting different responses of methanogens in the aerated soil. It is noteworthy that the SWC was <7.2% during the warmest four sampling months, likely obfuscating its impact on methanogenic abundances (Table S1). The average abundance of *Methanocella*_OTU200, *Methanobacterium*_OTU55, *Methanobacterium*_OTU532, *Methanobacterium* _OTU209, and *Methanosaeta*_OTU407 over the 12 months ranged from $5.84 \pm 5.07 \times 10^3$ to $4.27 \pm 3.84 \times 10^4$ copies/g wet soil. The average abundances of the corresponding five OTUs in high temperature seasons (>20 °C) were 2.7-fold to 5.7-fold higher ($P < 0.05$ for all the five OTUs) than those in lower temperature seasons (<20 °C) (Table S4). Although the individual abundance of five methanogen OTUs was positively correlated with temperature, the total abundances of methanogens were not correlated with temperature, which suggests differential metabolic responses to temperature change at the species level. Based on the results from both network analyses and Pearson correlation analyses, it could be assumed that those methanogens were growing in response to increasing temperature rather than dormant in this aerated soil. Comparing with the high proportion of methanogens OTUs showing positive correlation with temperature, both network analyses and Pearson's correlation analyses showed that low proportion of *Nitrososphaera* OTUs was positively correlated with temperature (4 and 7 out of 46 *Nitrososphaera* OTUs, respectively; Fig. S5).

Discussion

Significance of methanogens in aerated soils

Both *Thaumarchaeota* and methanogens are recently found to be ubiquitous in the aerated soil globally (Auguet et al. 2010; Bates et al. 2011; Angel et al. 2012; Xie et al. 2015). Comparing with *Thaumarchaeota* that have significant impact on the nitrogen cycles in soils, the significances of those rare methanogens in the field are largely unrevealed. In this study, we found the significance index of those rare methanogens were statistically higher than those of *Nitrososphaera*, suggesting methanogens were rare but an integrative component in the structure of the archaeal community in aerated soil. In consistent with the former report verifying the activity of in an oxic soil microcosm by a DNA-SIP (stable isotope probing) experiment in the laboratory (Lee et al. 2012), our results suggested those aerated methanogens in the field may be actively participated in methane cycle.

The diverse methanogens found in aerated soils

Although there are 34 known methanogenic genera according to the List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.net/), only *Methanosarcina* and *Methanocella* were observed by low-resolution molecular fingerprinting tools (such as clone library, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP) and denaturing high-performance liquid chromatography (dHPLC)) in barley field soil (Poplawski et al. 2007), different types of upland soils (Angel et al. 2012), glacier moraine soil (Aschenbach et al. 2013), and subalpine fallow (Praeg et al. 2014). With the development of high-throughput sequencing, more genera are found in the aerated soils. Hu et al. (2013b) found *Methanosaeta*, *Methanosarcina*, *Methanotorris*, and *Methanobacterium* were the key methanogen genera in 59 upland soils covering grasslands, forests, and agricultural soils. Hofmann et al. (2016) recently reported that *Methanomicrobiales* and *Methanocella* were detected in 14 grassland soils and 16 coniferous forest soils, while

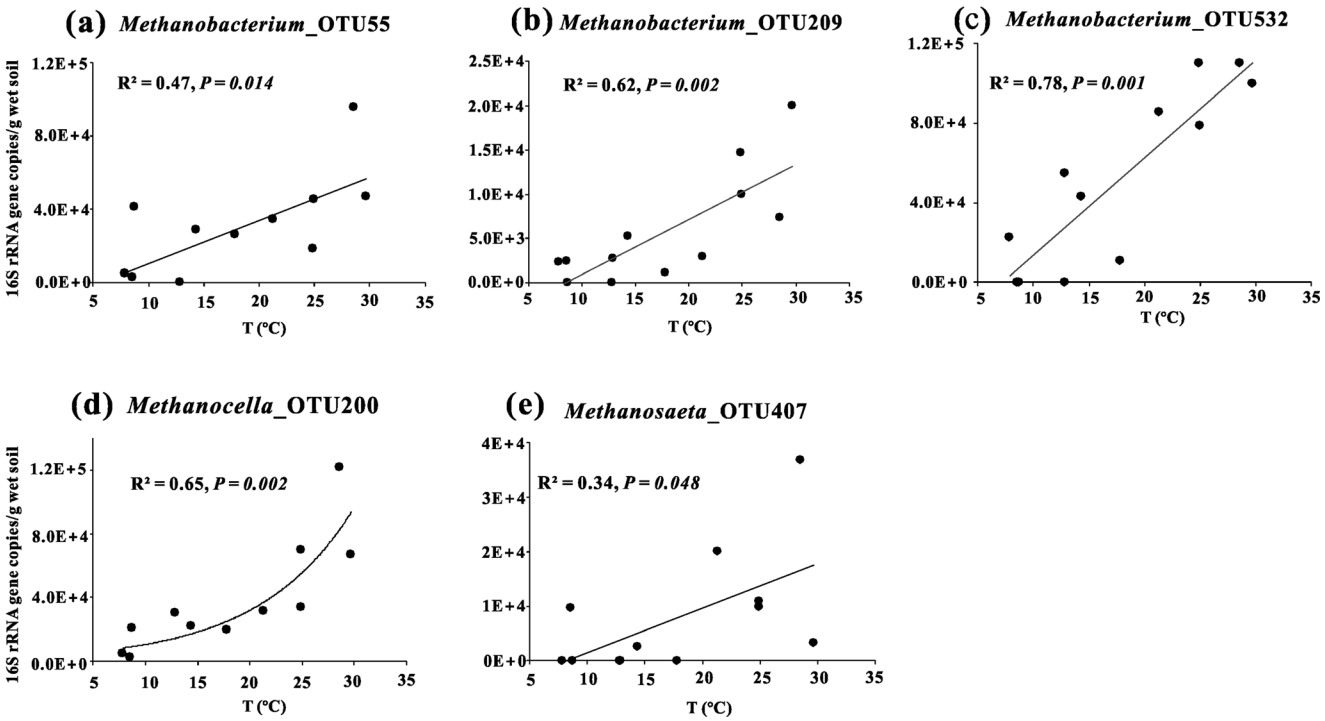

Fig. 5 The simple linear regression plots for relationship between the abundance of *Methanobacterium*_OTU55 (**a**), *Methanobacterium*_OTU209 (**b**), *Methanobacterium*_ OTU532 (**c**), *Methanocella*_OTU200 (**d**), *Methanosaeta*_OTU407 (**e**), and temperature

Methanobacteriales and *Methanosarcinales* were absent in some soils, suggesting a high diversity of methanogens in well-aerated soils. Our study also demonstrated great diversity of methanogens in the aerated soil on Chongming Island. The presences of *Methanobacterium*, *Methanocella*, *Methanosaeta*, and *Methanosarcina* are consistent with previous studies, suggesting their adaptation to the aerated soil environments. *Methanomassiliicoccus*, which represented the seventh order of methanogens and use methanol and hydrogen as substrate, have only recently been found in human guts (Borrel et al. 2012; Borrel et al. 2013), termite guts (Paul et al. 2012), anaerobic sludge (Iino et al. 2013), and wetland (Söllinger et al. 2016). We also found eight *Methanomassiliicoccus* in the aerated soils. A reanalysis of the published archaeal sequences from 59 soil samples across North to South China showed that half of those samples contained *Methanomassiliicoccus* (Hu et al. 2013a), suggesting the wide occurrence of this order. Redundancy analysis (RDA) of the methanogen community and environmental parameters showed that *Methanomassiliicoccus* had a small angle with temperature vector (Fig. S6), suggesting the impact of temperature on the distribution of *Methanomassiliicoccus*. The increasing proportions of *Methanomassiliicoccus* from May to September 2012 (Fig. 1 and Table S1) also supported temperature as a control on this new methanogen order. However, further investigations at larger spatial scales are needed to address the impacts of temperature on the growth of *Methanomassiliicoccus* in the aerated soils.

Dominant influence of temperature on the growth of methanogens

The effect of temperature increase on the function of the methanogenic microbial community in the rice fields has been studied. Both negative (Frenzel and Karofeld 2000; Conrad et al. 2009) and positive effects (Schrope et al. 1999; Cheng et al. 2000; Allen et al. 2003; Watanabe et al. 2005) of temperature have been found on the methanogenic processes. In comparison, the responses of those low abundant methanogens to environmental changes in the aerate soils are largely unexplored. Some former studies have addressed the high potential methanogenic activities of the methanogens from aerated soils in the laboratory (e.g., Angel et al. 2012; Hofmann et al. 2016). However, the dynamics of those low abundant methanogens in aerated soils under nature environments are still in the mist. In this study, although the lack of activity measurements in the laboratory, both Pearson's correlation targeting the qPCR results and LSA analysis targeting the proportions data from Miseq high-throughput sequencing showed that five methanogens were positively correlated with temperature rather than the other seven parameters (soil water content, pH, TOC, TN, C/N, NO_2^-, NH_4^+). Furthermore, after grouping the samples as the local seasons, those five methanogens showed small standard deviations within seasons, but significant different between the winter and summer (Fig. S7). The abundances of those five methanogens in summer were 3- to 5-fold

higher than those in winter, demonstrating their increasing from winter to summer.

The shift of temperature may change not only the rate of CH_4 production but also structure of the methanogenic communities. For example, *Methanosarcinaceae* and *Methanosaetaceae* were more adapted to 15 and 30 °C, respectively, while *Methanobacteriales* and *Methanocella* methanogens were significantly enhanced at 45 °C in the anoxic rice field soil (Peng et al. 2008; Kitamura et al. 2011). In this study, the five methanogens, who increased with temperature ranged from 8.6 to 29.7 °C on Chongming Island, belonged to *Methanobacterium*, *Methanocella*, or *Methanosaeta*, suggesting their similar responses to temperature in the aerated soils as in the anoxic rice field soils.

SWC has previously been shown to control methanogen communities (Mayer and Conrad 1990; Sitaula et al. 1995; Liu et al. 2008; Praeg et al. 2014). In this study, however, it showed no significant correlation with alpha diversity indices of methanogens, the abundance of each methanogen OTUs, or the abundance of the total methanogens. The obfuscated effects of SWC on the methanogens might be due to complication with temperature because the warmest 4 months corresponded to the driest season (SWC < 7.2%) and the wettest 6 months averaged 16 °C cooler (SWC > 19%). However, we admit the limitation of this study on reflecting the changes of those methanogens in large-scale aerated soils. Widely investigations targeting the changes of those methanogens in the field are needed in future.

In conclusion, we found that (i) methanogens were rare but an integrative component in the archaeal community, (ii) the methanogen community was composed of five methanogen genera (*Methanobacterium*, *Methanocella*, *Methanosaeta*, *Methanosarcina*, and *Methanomassiliicoccus*), and (iii) some of those methanogens were positively correlated with temperature in the aerated soil. Considering the cosmopolitan distribution of methanogens in the global aerated soils, the observation of low abundant methanogens sensitive to temperature variation in the Chongming Island soil might illuminate the importance of them in global methane cycle in the terrestrial environment.

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