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Higher diversity and abundance of ammonia-oxidizing archaea than bacteria detected at the Bayon Temple of Angkor Thom in Cambodia

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ABSTRACT

Ammonia-oxidizing archaea (AOA) and bacteria (AOB) are two groups of the ammonia-oxidizing microorganisms responsible for conversion of ammonia to nitrite, producing acidity and damaging building materials, including valuable cultural heritage stone structures. In this study, the molecular biomarker of *amoA* gene of archaea and bacteria was applied in polymerase chain reaction (PCR) amplification and quantitative real-time (RT) PCR (qPCR) to detect the abundance of AOA and AOB at 4 locations (Pond, Entrance, Gallery and Central Tower) of Bayon temple at Angkor Thom, Cambodia. The results showed that both AOA and AOB were positively detected at all locations and the diversity index suggested that AOA were more diverse than AOB. Sequences of AOA were mainly distributed in Group I.1b and few clustered in Group I.1a and Group I.1a-associated, while AOB were all closely related to β-proteobacteria (*Nitrosospira*). Based on the qPCR results, AOA outnumbered AOB clearly, the gene copy numbers of AOA *amoA* gene ranged from 1.35×10^6 to 2.52×10^8 copies per gram of dry materials, while, in comparison, AOB *amoA* gene copy numbers from 6.54×10^5 to 4.52×10^6 copies per gram of dry materials. In addition, the abundance of *amoA* gene from galleries was the highest than other locations. At gallery section, gene abundance, NH_4^+ and NO_3^- agreed well with the intensity of biofilm development. Based on the community and abundance analysis, nitrogen cycling plays an important role in contributing to deterioration of sandstone monuments at Angkor Thom in Cambodia under tropical climate.

1. Introduction

Microorganisms are the most important primary colonizers of stone and building materials, and cyanobacteria are major biofilm component, weakening historic stone buildings and monuments under different climate conditions (Monte, 1991, 1992; Flores et al., 1997; Urzı and Realini, 1998; Low et al., 2000; Tomaselli et al., 2000; Warscheid and Braams, 2000; Crispim and Gaylarde, 2005; Gaylarde et al., 2006; Adamson et al., 2013; Gu et al., 2013; Mandal and Rath, 2013; Essa and Khallaf, 2014; Gómez-Ortíz et al., 2014; Keshari and Adhikary, 2014). Among the mechanisms involved, sunlight, water availability and porosity of the stone are the key factors contributing to the pioneering colonizers to settle on the substratum and cause damage (Warscheid and Braams, 2000). In addition, fungi are also reported to be involved in attacking of concrete through potentially acid producing process (Gu et al., 1998; Gómez-Alarcón et al., 1994; Resende et al., 1996). Due to the wide spread of air pollution, hydrocarbon-utilizing microorganisms are also found on stone surfaces at high population density at more polluted sites (Mitchell and Gu, 2000). The collected information establishes the common occurrence and also active involvement of microorganisms to biodeterioration of stone.

Ammonia oxidization is the first and rate-limiting step in nitrification, which oxides ammonia to nitrate. This process is carried out by ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) through the ammonia monooxygenase, the key enzyme for ammonia oxidation and the *amoA* gene, which encodes the enzyme's subunit A can be used as molecular biomarker for detection of these microorganisms (Rotthauwe et al., 1997). AOBs have been known and investigated for more than 100 years, but AOAs in comparison are more recent discovery for the last 10 years. AOAs are classified into the phylum of Thaumarchaeota based on 16S rRNA gene and genome (Brochier-Armanet

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et al., 2008) and are able to oxidize ammonia to nitrite under aerobic conditions (Könneke et al., 2005). The phylogeny based on 16S rRNA gene and *amoA* gene showed that sequences of AOA in natural environments distributed into two groups: Group I.1a (mainly from marine environments) and Group I.1b (mainly from soil environments) (Stahl and de la Torre, 2012). Up to date, all known AOB belong to β-proteobacteria including *Nitrosospira* and *Nitrosomonas* lineages and γ-proteobacteria containing the *Nitrosococcus* lineage (Head et al., 1993; Purkhold et al., 2000).

Khmer civilization was at its peak from 9th to the 12th century and many monuments were built during this period with sandstone and laterite by the Angkor Empire in now known as Southeast Asia. Angkor Thom, the large city famous for its bas-relief carving and Khmer smile on the four sides of each tower at Bayon temple (Fig. 1, Fig. S1 and S2), suffers from serious deterioration and damage (Lan et al., 2010; Li et al., 2010; Gu et al., 2013; Hu et al., 2013; Kusumi et al., 2013; Bartoli et al., 2014) similar to many other cultural heritages around the world (Seaward et al., 1989; Danin and Caneva, 1990; Tayler and May 1991; Seaward, 2001; Piñar et al., 2009; Tayler and May 2000; Motti and Stinca, 2011; Adamson et al., 2013; Bertuzzi et al., 2013; Cutler et al., 2013; Zanardini et al., 2016). The large number and some of them massive building structure located in Cambodia, Thailand, Vietnam and Laos under tropical climate with alternating and intense dry and wet seasons show colonization by a wide range of microorganisms and deterioration (Lan et al., 2010; Li et al., 2010; Gu et al., 2013; Hu et al., 2013; Kusumi et al., 2013; Bartoli et al., 2014). Lichen and cyanobacteria are dominant members of the biofilms on sandstone; they are commonly observed on the surface of walls and temples, covering the bas-relief and subsequently damaging the carving and also the more seriously building structure, leading to collapse. Coupling with physical and chemical factors, the biological contribution enhances the deterioration process to the cultural heritage through specific biochemical processes (Monte, 1991; Romão and Rattazzi, 1996; Saiz-Jimenez and Laiz, 2000; Tayler and May 2000; Tretiach et al., 2007; André et al., 2008; Miller et al., 2009; Eyssautier-Chuine et al., 2015). Protection of them is a major effort undertaken by local and also many international organizations to save the world cultural heritage. One critically important information needed for better protection of them is the scientific understanding of the various processes and factors involved so that effective management strategies can be formed in the near future and implemented on sites.

Previous investigations at Bayon temple of Angkor Thom in Cambodia using molecular analysis of 16S and 18S rRNA genes of biofilm on sandstone walls and deteriorated materials showed the presence of both bacteria and fungi and different colors shown on the wall are associated with different microorganisms (Lan et al., 2010; Li et al., 2010; Hu et al., 2013). The presence of these microorganisms on surface may accelerate the damage to the sandstone over time, posing concern over the structure stability of cultural heritage (Lan et al., 2010; Mitchell and Gu, 2000). Sulfur-oxidizing bacteria (SOB) and fungi were responsible for attacking stone (Li et al., 2007, 2010), but the significance of nitrogen-utilizing microbes still remains unknown. Therefore, this study used the biomarker of *amo* gene to analysis the community structure and abundance of ammonia-oxidizing archaea and bacteria at Bayon temple of Angkor Thom in Cambodia and to deduce their effects on heritage deterioration.

2. Materials and methods

2.1. Site description and sampling

Our sampling site is located at Bayon temple of Angkor Thom in Siem Reap of Cambodia, where tropical climate with dry and rainy seasons can be observed distinctively in different time of the year. In our study, a total of 17 samples were collected on August 11, 2014 aseptically at Bayon temple of Angkor Thom, including 6 sediment samples from ponds on either side of the Entrance to Bayon temple, 4 sediment samples from the Entrance of the temple, 5 sediment samples taken from galleries (26-10, 29-8, 31-4 and 39-3) and 2 sediment samples collected from the Central Tower of Bayon temple (Table 1; Fig. S3) and the layout of the Bayon temple is also available elsewhere (Kusumi et al., 2013). The sediment materials were collected from the bottom corner of walls or pillars from deteriorated sections of the monuments; no physical invasive procedure was applied to take any of the samples in this investigation. In August, the average temperature in Siem Reap is 27.8 °C. It is the rainy season and the average monthly precipitation is about 185 mm (http://en.climate-data.org/country/167/). At the sampling locations, bats were commonly found, with approximately 1000 daily at Angkor Thom and 500 daily at the temple in the night (Li et al., 2007). Bat droppings were commonly observed and accumulated on the floor or on the surface of the monument within the sampling area.

2.2. Physical-chemical parameters measurement

Inorganic N in samples was extracted by shaking in 2 M KCl solution with a ratio of 1:5 for 2 h. Ammonium-N and nitrate-N extracted were measured in triplicate with the Lachat QuikChem 8000 Flow Injection Analyzer (Lachat Instruments, Inc. Loveland). Materials' (sediment and sandstone) moisture content was measured through determining the dry weight of sample after drying in oven for 48 h. Total nitrogen (TN) and total carbon (TC) content were determined by elemental analyzer (Euro vector EA3025, UK).

2.3. DNA extraction, PCR amplification and clone library construction

Genomic DNA was extracted using 0.25 g materials (sediment/deteriorated sandstone) with PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc. USA) following the protocol. The quality and concentration of DNA was assessed by NanoDrop™ ND - 2000 (Thermo Scientific, Inc. USA). Sample DNA was stored in −20 °C for further processing.

Primer sets of *amoA*_1F/*amoA*_2R and Arch-*amoA*F/Arch-*amoA*R were selected to amplify fragment of the unique *amo* gene of AOB (491 bp) and AOA (635 bp), respectively (Francis et al., 2005; Rotthauwe et al., 1997). 54 individual PCRs (triplicate for each sample) of 25 μl volume contained 12.5 μl of PCR Master Mixture (Takara, Japan), 10 pmol of each primer, 25 mmol Mg^{2+}, 10 mmol BSA and 10–100 ng DNA template for the PCR reaction.

PCR products were analyzed in 1% agarose gel of TAE buffer via gel electrophoresis. Fragments with correct size were isolated and purified with GFX™ PCR DNA and Gel Band Purification Kit by following the manufacturer's protocol (Amersham Biosciences, UK). About 30–80 ng of PCR products were subsequently cloned into pMD18-T vector (Takara) and transformed into the competent cell - *Escherichia coli* DH5α, then cultured on LB agar plates for 12 h. Finally, the positive clones were picked by blue-white screening for sequencing.

2.4. Phylogenetic analysis

All sequences were aligned by Mafft online (http://mafft.cbrc.jp/alignment/server/) and the low quality sequences were discarded from dataset. The reference sequences for the phylogenetic analysis were obtained from GenBank. All nucleotide sequences translated