Extremophiles (2012) 16:277–283
DOI 10.1007/s00792-011-0428-6

ORIGINAL PAPER

Distribution and phylogenetic diversity of *cbbM* genes encoding RubisCO form II in a deep-sea hydrothermal field revealed by newly designed PCR primers

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Received: 29 September 2011 / Accepted: 13 December 2011 / Published online: 3 January 2012

Abstract To investigate the phylogenetic diversity of putative chemolithoautotrophs possessing the RubisCO form II gene (*cbbM*) in various environments, we designed a new PCR primer set targeting this gene. The primer set was designed to cover more diverse and longer sequences of *cbbM* genes than those reported previously. We analyzed various samples (i.e., benthic sands, basement rocks, sulfide chimneys, vent fluids and overlying bottom seawater) collected in a deep-sea hydrothermal field of the Suiyo Seamount, Izu-Bonin Arc, Western Pacific, by PCR-based analysis using the designed primer set. Most of the *cbbM* phylotypes recovered from the liquid samples were related to those of the SUP05 group that belongs to the *Gammaproteobacteria* and includes putative sulfide-oxidizing chemolithoautotrophs. In contrast, the *cbbM* phylotypes recovered from the solid samples were related to environmental clones with low similarity (74–90%) and not closely related to the SUP05 group (69–74%). The *cbbM* phylotypes recovered from the liquid samples were different from those of the solid samples. Furthermore, the *cbbM* phylotypes recovered from the solid samples were different from each other. Our results expand knowledge of the phylogenetic diversity and distribution of putative chemolithoautotrophs possessing RubisCO form II *cbbM* genes in deep-sea hydrothermal fields.

Keywords RubisCO form II · *cbbM* gene · Carbon fixation · Chemolithoautotroph · Deep-sea hydrothermal field

Communicated by L. Huang.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-011-0428-6) contains supplementary material, which is available to authorized users.

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Introduction

The assimilation of CO_2 into organic compounds by autotrophs is one of the most important biological processes. The reductive pentose phosphate pathway, called the Calvin–Benson–Basham (CBB) cycle, is one of the six known carbon fixation pathways (Hügler and Sievert 2010). Some autotrophic bacteria (*Cyanobacteria*, *Proteobacteria, Firmicutes* and *Chloroflexi*), algae and plants fix CO_2 via the CBB cycle (Tabita et al. 2008; Hügler and Sievert 2010). Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) is a key enzyme in the CBB cycle. RubisCO is categorized into four forms from I to IV based on differences in their sequences and structures (Tabita et al. 2008). Some autotrophs in the *Proteobacteria*, one of the most diverse and widely distributed taxonomic groups on Earth, have RubisCO forms I and II (Badger and Bek 2008). It has been suggested that these forms are used under conditions of different CO_2 and O_2 concentrations (Jordan and Ogren 1981; Tabita 1988; Badger and Bek 2008).

Recent studies of the RubisCO form I and II genes (*cbbL* and *cbbM*, respectively) based on culture-independent PCR-based analysis have shown the phylogenetic diversity of these RubisCO genes in natural environments, such as groundwater (Alfreider et al. 2003, 2009), hypersaline

lakes (Giri et al. 2004; Tourova et al. 2010) and deep-sea hydrothermal vents (Elsaied and Naganuma 2001; Elsaied et al. 2007; Hügler et al. 2010). However, the specific PCR primers used, in particular, for *cbbM*, were designed based on a limited number of gene sequences. Sequences of putative *cbbM* genes can be found in recently deposited whole-genome sequences and metagenomic sequences in public databases. For a better understanding of the diversity and distribution of putative autotrophs possessing *cbbM* genes, it is advisable to design new primers capable of detecting more diverse *cbbM* genes and to apply the designed primers for PCR-based analysis.

A variety of chemolithoautotrophs thrives in deep-sea hydrothermal fields, and some of these microorganisms fix CO_2 via the CBB cycle (Nakagawa and Takai 2008; Hügler and Sievert 2010). The Suiyo Seamount is an active submarine volcano that is located along the volcanic front of the Izu-Bonin Arc, Western Pacific. Hydrothermal vents were found on the caldera floor of the seamount (Watanabe and Kajimura 1993). Microbiological investigations with PCR-based analysis targeting 16S rRNA genes have been conducted in this field, indicating the presence of novel as-yet-uncultivated prokaryotes (Higashi et al. 2004; Nakagawa et al. 2004; Sunamura et al. 2004; Hara et al. 2005; Kato et al. 2009b). Although the diversity of *cbbM* genes has been investigated in this field previously (Elsaied et al. 2007), the actual diversity seemed to be considerably underestimated because the primers used were designed based on only a few *cbbM* genes (Elsaied and Naganuma 2001). Here, we report the phylogenetic diversity of *cbbM* genes in representative habitats of the Suiyo Seamount hydrothermal field (i.e., benthic sands, basement rocks, sulfide chimneys, vent fluids and overlying bottom seawater) using the newly designed PCR primers.

Materials and methods

Site description and sample collection

Fluid samples from a natural vent and overlying seawater, and solid samples of benthic sands, a sulfide chimney and a rock were collected in the deep-sea hydrothermal field of the Suiyo Seamount during the NT05-16 cruise (22 September to 7 October 2005) of R/V *Natsushima* (JAMSTEC, Japan) with the remotely operated vehicle *Hyper-Dolphin* (JAMSTEC, Japan). The locations of the sampling points are shown in Fig. S1. Detail information about the boreholes APSK08 and APSK10 has been described previously (Kato et al. 2009b). The overlying bottom seawater (2–3°C) was collected at three points next to each borehole or the vent using Niskin bottles at approximately 1 m above the seafloor. The vent fluids (27°C) were collected in the point AP04 using a BAG sampler as described previously (Kato et al. 2009c). The rock was collected approximately 10 m away from AP04 using a manipulator on the vehicle. The sands were collected using a closable sediment sampler. The sulfide chimney discharging hydrothermal fluids (45°C) was collected by a manipulator on the vehicle. To trap particles in the collected liquid samples, 1 l of the overlying seawater and 0.3 l of the vent fluid were filtered through 0.2-μm pore-size polycarbonate membranes (Advantec, Tokyo, Japan) using a vacuum pump on board. The rock and chimney samples were crushed into pieces using an autoclaved hammer and chisel in a clean box. The filters and solid samples were stored in DNA/RNA-free plastic tubes at −80°C until DNA extraction.

Primer design for *cbbM* genes

To design a new PCR primer set targeting the *cbbM* gene, twenty-seven *cbbM* gene sequences were extracted from whole-genome sequences of cultured species and environmental metagenomic sequences deposited in the public databases (Figs. 1, S2). These *cbbM* sequences were aligned using MUSCLE (Edgar 2004). Based on the alignment, we designed the forward and reverse PCR primers, cbbM343F (5′-GGYAAYAACCARGGYATGGG-3′) and cbbM1226R (5′-CGYARBGCRTTCATRCCRCC-3′), respectively (Fig. S2). The expected amplicon size is approximately 800–900 bp. The primer set of cbbM343F and cbbM1226R covers 21 of the total 27 sequences (77.8%) in the alignment (Fig. 1).

PCR clone library analysis

The PCR clone libraries were constructed as previously described (Kato et al. 2009a, c) with minor modifications. In brief, genomic DNA was extracted from the filtrated filters and the solid samples (approximately 0.5 g) using a Fast DNA kit for soil (Qbiogene, Carlsbad, CA, USA). For all solid samples, the seawater-exposed portions (0–1 cm depth from the surface) were used for analysis. A portion of the *cbbM* genes in the genomic DNA extracts was amplified by PCR using the primer set cbbM343F–cbbM1226R. PCR was performed with 30 cycles of the following program: 95°C for 1 min, 50°C for 2 min, and 72°C for 3 min. The optimal annealing temperature was determined by multiple tests with 40–65°C at intervals of 5°C. The PCR products were cloned and the nucleotide sequences of randomly selected clones were determined on an ABI PRISM 3130xl Genetic analyzer (Applied Biosystems, CA, USA). The nucleotide sequences of *cbbM* clones were aligned using MUSCLE. Maximum-likelihood (ML) trees were constructed by PHYML (Guindon and Gascuel 2003) with non-gap homologous positions in the alignment

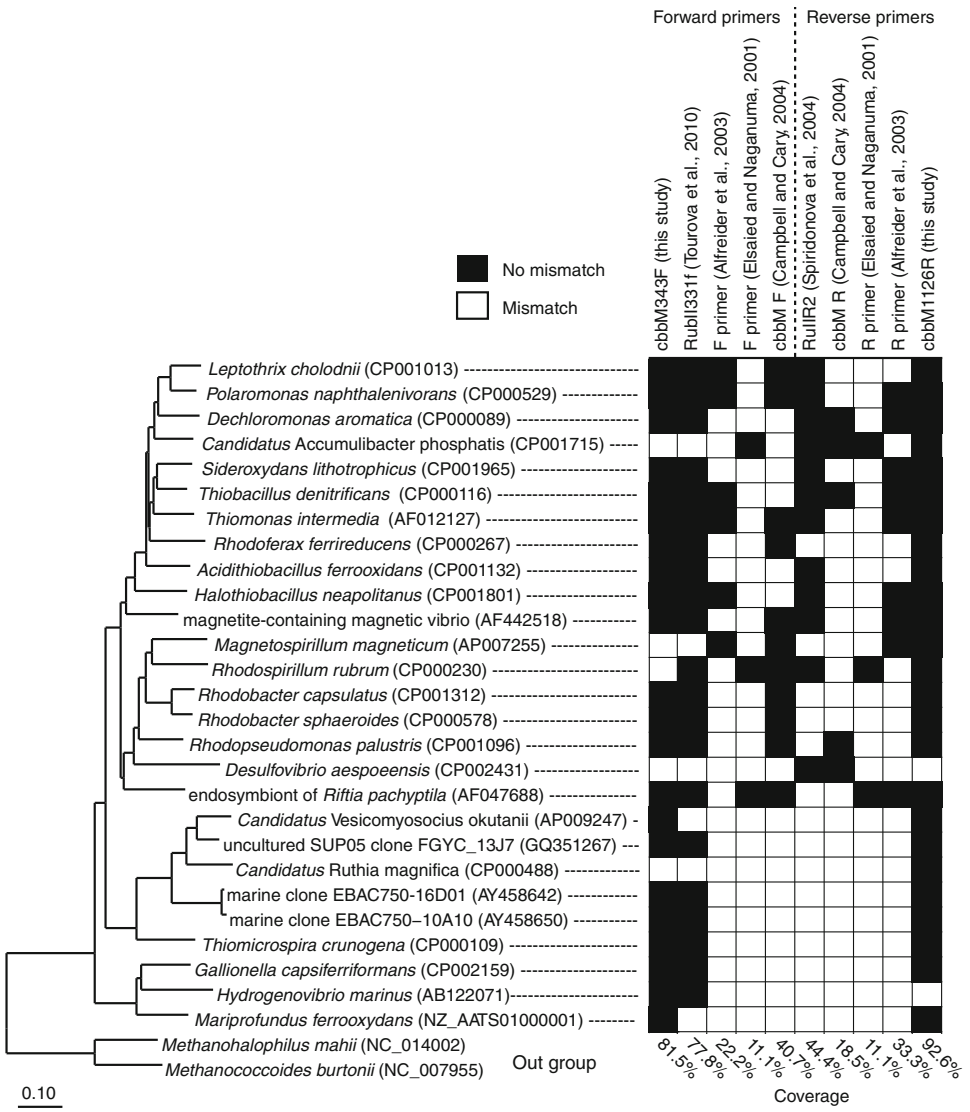

Fig. 1 Comparison of the coverage of the *cbbM* primers against the known *cbbM* genes. A neighbor-joining tree of nucleotide sequences for the known *cbbM* genes is shown. The *cbbM* gene sequences of *Methanohalophilus mahii* (NC_014002) and *Methanococcoides burtonii* (NC_007955) were used as outgroups. The *cbbM* genes in the tree were collected from whole-genome sequences of cultured species and environmental metagenomic sequences deposited in the public databases

dataset with the nucleotide substitution model GTR + I + G. The nucleotide sequences reported in this paper have been deposited in the DDBJ database under accession numbers: AB629624 to AB629696. Rarefaction curves, Good's coverage, Shannon diversity index and Chao1 richness estimators for each clone library were calculated using mothur (Schloss et al. 2009).

Results and discussion

Numerous studies using 16S rRNA gene analysis have been performed to understand the microbial ecosystems in natural environments. However, the physiology of the microorganisms present could not be determined but only inferred from the phylogeny of the 16S rRNA genes. The PCR-based analyses targeting RubisCO genes have been done to determine the potential autotrophs using the CBB cycle in microbial communities (Pichard et al. 1997; Elsaied and Naganuma 2001; Alfreider et al. 2003, 2009; Giri et al. 2004; Selesi et al. 2005; Tourova et al. 2005, 2010; Elsaied et al. 2007; Lisa and Gary 2007; Hügler et al. 2010; Kovaleva et al. 2011). In the present study, we showed the diversity of the *cbbM* gene phylotypes in various habitats (i.e., sand, rock, chimney, vent fluids and overlying bottom seawater) of the Suiyo Seamount hydrothermal field by PCR-based analysis with the newly designed primers for *cbbM*. A total of 301 *cbbM* clones were recovered from the samples. We revealed that there were novel and diverse *cbbM* phylotypes in this field. Our results expand knowledge of the phylogenetic diversity and distribution of putative chemolithoautotrophs possessing RubisCO form II encoded by the *cbbM* gene in deep-sea hydrothermal fields.

Phylotypes of cbbM genes recovered from fluid samples

Most of the *cbbM* gene phylotypes (>95% of the total clone numbers) recovered from the seawater and vent fluid samples were affiliated with the SUP05 group (Figs. 2a, S4). The SUP05 group was firstly reported in the hydrothermal plume of the Suiyo Seamount, and the 16S rRNA genes of this group belong to the *Gammaproteobacteria* (Sunamura et al. 2004). Our *cbbM* phylotypes from the liquid samples were related to the *cbbM* genes in metagenomic sequences, the clone FGYC_13J7 (Walsh et al. 2009) and *Candidatus* Vesicomyosocius okutanii (Kuwahara et al. 2007) (87 and 83% similarities, respectively), which are affiliated with the SUP05 group. The other phylotypes obtained from the liquid samples were related to the *cbbM* genes of members of *Thiomicrospira*, the deep-sea fosmid clone EBAC750-10A10, and environmental *cbbM* clones recovered from deep-sea hydrothermal fields. In the present study, the cluster including the sequences in the SUP05 group, the deep-sea fosmid clones (EBAC750-10A10 and EBAC750-16D01), *Candidatus* Ruthia magnifica str. Cm (Newton et al. 2007) and several clones detected in marine environments is defined as the Marine *cbbM* cluster I(MCMCI) (Figs. 2a, S4).

The *cbbM* gene phylotypes recovered from the vent fluid and seawater samples may be derived from putative sulfide-oxidizing chemolithoautotrophs belonging to *Thiomicrospira* and the SUP05 group in the *Gammaproteobacteria*. The genus *Thiomicrospira* includes sulfide-oxidizing chemolithoautotrophs possessing RubisCO form II (*cbbM*) in addition to form I (*cbbL*) (Tourova et al. 2006). The metagenome sequence of FGYC_13J7 in the SUP05 group has a complete gene set for the sulfide oxidation pathway in addition to a *cbbM* gene (Walsh et al. 2009). 16S rRNA genes related to *Thiomicrospira* and the SUP05 group have been previously detected in the Suiyo Seamount hydrothermal field (Higashi et al. 2004; Nakagawa et al. 2004; Kato et al. 2009b) and were also detected in the same DNA extracts used in the present study (Kato et al., in preparation: AB629103, AB629108, AB629110, AB629111, AB629113, AB629260, AB629268, AB629272, AB629298, AB629324, AB629332, AB629367 and AB629321 for the representative 16S rRNA gene phylotypes related to the SUP05 group and *Thiomicrospira*). It has been shown that the SUP05 group was predominant in the hydrothermal plume in the Suiyo Seamount hydrothermal field (Sunamura et al. 2004); however, their metabolic functions have not been determined. Our results imply that the SUP05 group plays a role in primary production in the microbial ecosystem in the overlying seawater including hydrothermal plumes on the seafloor of the Suiyo Seamount hydrothermal field.

Phylotypes of cbbM genes recovered from solid samples

A number of *cbbM* gene phylotypes affiliated with MCMCI were recovered from the rock and sand samples, although these phylotypes were out of the SUP05 group (Fig. 2a). The *cbbM* phylotypes related to *Rhodobacter* and *Rhodopseudomonas* in the *Alphaproteobacteria* (79% similarity or lower) and to environmental clones detected in deep-sea hydrothermal fields (75–90%) were also recovered from the rock and sand samples (Fig. 2).

One phylotype (the representative clone, ChcbbM06) recovered from the sulfide chimney sample was related to the *cbbM* genes of *Mariprofundus ferrooxydans* in the *Zetaproteobacteria* (80% similarity) and *Gallionella capsiferriformans* in the *Betaproteobacteria* (74%), which are microaerobic iron-oxidizing bacteria. This phylotype was also related to the *cbbM* genes of *Hydrogenovibrio marinus* and members of *Thiomicrospira* in the *Gammaproteobacteria* (76–81%), which include hydrogen- and sulfide-oxidizing microaerobes and facultative anaerobes. The cluster including these *cbbM* genes related to ChcbbM06 is defined as a Deep-branching *cbbM* cluster, which is strongly supported by the high bootstrap value (Fig. 2a). The other phylotypes recovered from the sulfide chimney were relatively close to the *cbbM* genes of *Acidithiobacillus*, *Halothiobacillus* and *Thiohalomonas* in the *Gammaproteobacteria*, *Magnetospirillum* in the *Alphaproteobacteria* and members of the *Betaproteobacteria* (88% similarity or lower).

Our results indicate that diverse putative chemolithoautotrophs possessing *cbbM* genes are likely to be present in the rocks, sands and sulfide chimneys on the seafloor of the Suiyo Seamount hydrothermal field, which have been unrecognized previously (Elsaied et al. 2007). Although novel and diverse 16S rRNA genes including yet-uncultivated members of the *Alpha-*, *Beta-* and *Gammaproteobacteria* have also been detected in these samples (Kato et al., in preparation: the bacterial 16S rRNA gene sequences are available in the DDBJ database, AB629096–AB629489), it is difficult to identify the taxonomic affiliations of these putative chemolithoautotrophs with *cbbM* genes including MCMCI, except the SUP05 group, because the phylogeny of the *cbbM* phylotypes cannot be directly linked to the 16S rRNA gene phylotypes using parallel PCR analysis of each gene. Further cultivation efforts and whole-genome sequencing analysis are needed to identify the taxonomic affiliation and physiological characteristics of these putative chemolithoautotrophs.

Diversity of the *cbbM* gene phylotypes

For the overlying seawater samples, the coverage values were 97% or higher and the rarefaction curves reached

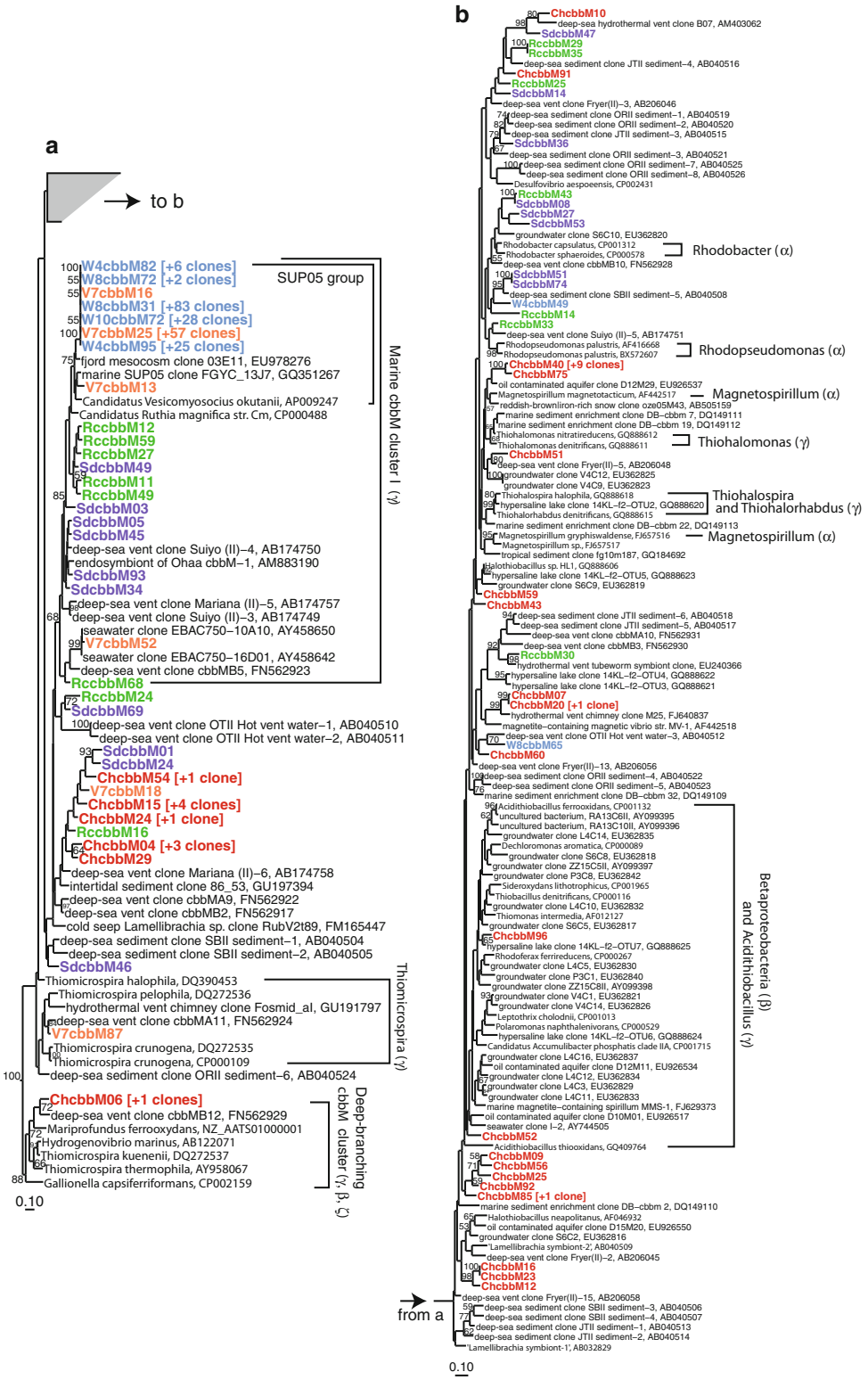

Fig. 2 Phylogenetic tree of nucleotide sequences for the *cbbM* genes detected. **a** The whole topology of the tree and **b** a part of the tree are shown. The ML tree was constructed using 246 homologous positions in the alignment dataset. The *cbbM* gene sequences of *Methanohalophilus mahii* (NC_014002) and *Methanococcoides burtonii* (NC_007955) were used as outgroups. Bootstrap values (higher than 50%) based on 100 replicates are shown at the branch points. The *scale bar* represents 0.1 nucleotide substitutions per sequence position. *Bold letters* indicate our clones detected in this study. The *numbers* following the detected clones indicate the number of clones

nearly plateau (Table 1; Fig. S3). Most *cbbM* phylotypes detected in the seawater samples were related to the SUP05 group as described above (Fig. 2). These results suggest that there are only a few members of autotrophs possessing *cbbM* genes (i.e., the SUP05 group) in overlying seawater on the caldera floor of the Suiyo Seamount. These seawater environments, which are likely to be influenced by hydrothermal activity, may be suitable to the SUP05 group and not to other autotrophs possessing *cbbM* genes. This is consistent with the previous report that the SUP05 group

Table 1 Diversity of 16S rRNA gene clone libraries

Sample type	Sample ID	Sampling site	Total clone number	Total phylotype number	Coverage (%)	Chao1 species richness[a]	Shannon score[a]
Ambient seawater	Sm8sw	APSK08	88	2	98.9	1 (1–1)	0
	Sm10sw	APSK10	32	1	100.0	1 (1–1)	0
	Sm4sw	AP04	34	2	97.1	1 (1–1)	0
Natural vent fluid	Sm4hw	AP04	63	5	93.7	1 (1–1)	0
Rock	Sm4rk	Near AP04	15	15	0.0	120 (54–296)	2.71 (2.36–3.05)
Benthic sand	Smhsd	Marker H475-2	21	21	0.0	231 (112–506)	3.04 (2.75–3.34)
Active chimney	Smmcs	Marker #387-1	48	29	52.1	9 (6–29)	1.68 (1.21–2.15)

[a] Values were normalized to the smallest total clone number (15 for Sm4rk) using mothur. Numbers in parentheses indicate the 95% confidence interval

predominated in the hydrothermal plume of the Suiyo Seamount (Sunamura et al. 2004).

The diversity analysis results (Table 1; Fig. S3) clearly indicate that the putative chemolithoautotrophs possessing *cbbM* genes in the solid samples have higher diversity than those in the liquid samples, which can be confirmed in the phylogenetic tree of the *cbbM* genes (Fig. 2). An extremely steep environmental gradient (e.g., temperature, pH, reductants and oxidants) is likely to occur in the chimney wall by mixing of hydrothermal fluids with seawater (Tivey 1995). There may also be an environmental gradient (e.g., oxygen concentrations) within the sands and rocks on the seafloor by microbiological/chemical consumption. The high diversity of the *cbbM* genes recovered from the bulk of the solid samples may be originated from the broad environments with various geochemical characteristics. In any case, the low coverage values (Table 1) and the non-plateau rarefaction curves (Fig. S3) for the solid samples suggest that more diverse *cbbM* phylotypes are expected to be recovered from these samples by additional sequencing efforts using the same primer set.

Conclusions and perspectives

In the present study, we showed the presence of novel and diverse *cbbM* gene phylotypes and their distribution in the various habitats in the deep-sea hydrothermal field of the Suiyo Seamount by PCR-based analysis using the newly designed primer set targeting *cbbM* genes. Chemolitho-autotrophs possessing *cbbM* genes are mainly anaerobes/microaerobes (Badger and Bek 2008). Therefore, the studies related to *cbbM* genes will provide insights into biomass production and carbon cycling in suboxic to anoxic environments. Although PCR-based analyses of *cbbM* genes have been done in variable environments using *cbbM* gene-specific primers (Pichard et al. 1997; Elsaied and Naganuma 2001; Alfreider et al. 2003, 2009; Giri et al. 2004; Selesi et al. 2005; Tourova et al. 2005; Elsaied et al. 2007; Lisa and Gary 2007; Hügler et al. 2010; Tourova et al. 2010; Kovaleva et al. 2011), these primers may have a significant limitation to detect *cbbM* genes (Fig. 1). Considering this limitation, there is a possibility that these previous studies considerably overlooked the members of microaerobic/anaerobic autotrophs in these environments. Our primer set designed in the present study is one of the choices for further investigation of the diversity and distribution of chemolithoautotrophs possessing *cbbM* genes in suboxic to anoxic environments.

Acknowledgments We would like to thank the crew of the R/V *Natsushima* and the operation team of the ROV *Hyper-Dolphin* for their cooperation in sample collection. We are also grateful to Mr. Hironori Oba, Ms. Seiko Ono and the other scientists that joined the NT05-16 cruise for providing valuable samples and for helpful discussions. We would like to thank two anonymous reviewers for their helpful comments. This research was funded by the Ministry of Education, Culture, Science and Technology (MEXT), Japan, through a special coordination fund (Archaean Park Project: International Research Project on Interaction between Sub-Vent Biosphere and Geo-Environments) and a special coordination fund (Project TAIGA: Trans-crustal Advection and In situ biogeochemical processes of Global sub-seafloor Aquifer), and partly by the RIKEN Special Postdoctoral Researchers Program.

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