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ORIGINAL ARTICLE

Metagenomic Analysis of Genes Encoding Nutrient Cycling Pathways in the Microbiota of Deep-Sea and Shallow-Water Sponges

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Abstract Sponges host complex symbiotic communities, but to date, the whole picture of the metabolic potential of sponge microbiota remains unclear, particularly the difference between the shallow-water and deep-sea sponge holobionts. In this study, two completely different sponges, shallow-water sponge *Theonella swinhoei* from the South China Sea and deep-sea sponge *Neamphius huxleyi* from the Indian Ocean, were selected to compare their whole symbiotic communities and metabolic potential, particularly in element transformation. Phylogenetically diverse bacteria, archaea, fungi, and algae were detected in both shallow-water sponge *T. swinhoei* and deep-sea sponge *N. huxleyi*, and different microbial community structures were indicated between these two sponges. Metagenome-based gene abundance analysis indicated that, though the two sponge microbiota have similar core functions, they showed different potential strategies in detailed metabolic processes, e.g., in the transformation and utilization of carbon, nitrogen, phosphorus, and sulfur by corresponding microbial symbionts. This study provides insight into the putative metabolic potentials of the microbiota associated with the shallow-water and deep-sea sponges at the whole community level, extending our knowledge of the sponge microbiota's functions, the association of sponge- microbes, as well as the adaption of sponge microbiota to the marine environment.

Keywords Sponge holobiont · Metagenomics · Metabolic profile · Nutrient element

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Introduction

As the oldest multiple cell animal (i.e., more than 600 million years old), marine sponges form a close association with other organisms including prokaryotes and eukaryotes. Phylogenetically diverse bacteria and archaea have been found to inhabit in sponges (Taylor et al. 2007; Webster, et al. 2010; Lee et al. 2011; Schmitt et al. 2012; Simister et al. 2012; Moitinho-Silva et al. 2014a, b; Reveillaud et al. 2014). Meanwhile fungi (e.g., Ascomycota and Basidiomycota) and algae (e.g., *Chlorella* and dinoflagellate) are also identified as important components of sponge holobionts (Reisser 1984; Garson et al. 1998; Gao et al. 2008; Yu et al. 2013). Particularly, in 2014, we found phylogenetically diverse fungi (e.g., Ascomycota and Basidiomycota), algae (e.g., Chlorophyta, Haptophyta, Streptophyta, Rhodophyta, and Stramenopiles), and protozoa (e.g., Alveolata, Cercozoa, Haplosporidia, and Radiolaria) were associated with 11 species of South China Sea sponges using 454 pyrosequencing, indicating complex eukaryotic community in sponge holobionts (He et al. 2014), and proved

that the fungi, e.g., Pezizomycotina, are active in situ in sponge holobionts (Jin et al. 2014).

The evaluation of functional processes performed by sponge symbionts represents a major challenge in the study of the sponge holobionts. To date, compared with the understanding of microbial diversity of sponge holobionts (Taylor et al. 2007; Schmitt et al. 2012; He et al. 2014), knowledge on the microbiota's functions and the association of sponge- microbes is still lacking (Webster and Taylor 2012; Fan et al. 2013; Li et al. 2014).

Omics provides an efficient approach to understand the metabolic potentials or functions of complex microbial communities including sponge holobionts (Thomas et al. 2010; Liu et al. 2012; Trindade-Silva et al. 2012; Fan et al. 2012; Li et al. 2014; Moitinho-Silva et al. 2014a; Fiore et al. 2015). Thomas et al. (2010) first explored the functional genomic signatures of bacteria associated with the sponge *Cymbastela concentrica* by shotgun sequencing. Fan et al. (2012) demonstrated the existence of functional equivalence in different sponge microbial communities and suggested that the symbiont communities in divergent sponge hosts had evolved with different genomic solutions to perform the same function in different environment or to occupy the same niche. This suggests that the core functions of sponge microbiota mainly depend on the host's selection through its prolonged evolution (Steinert et al. 2000; Fan et al. 2012). Even so, little is known about the total metabolic profiles of microbiota including prokaryotic and eukaryotic symbionts in a single sponge, because nearly all the investigations were focused on bacterial populations of sponge holobionts. Recently, we compared the metabolic profiles of symbiotic prokaryotes and eukaryotes in one sponge holobiont and found different metabolic characteristics between prokaryotic and eukaryotic symbionts in the same sponge host (Li et al. 2014). However, the metabolic differences between the shallow-water and deep-sea sponge holobionts still remain largely unknown.

Symbioses frequently have a nutritional basis, for example nitrogen fixation in *Rhizobium*–legume symbiosis (Lodwig et al. 2003), chemoautotrophy in marine mussels and worms (Woyke et al. 2009), and photosynthesis by the symbionts of corals or ascidians (Schnitzler and Weis 2010; Donia et al. 2011). Kamke et al. (2013) revealed the ability of bacterial heterotrophy to utilize diverse carbon sources and suggested a functional basis interaction of microbial symbionts with sponges. However, nutrient element metabolism of whole sponge microbiota is not very clear, particularly the respective total profiles of microbial communities in different sponge holobionts.

It could be hypothesized that the metabolic potentials of the deep-sea and shallow-water sponge microbiota are different because deep sea has its unique characteristics which are different from that of shallow water (Smedile et al. 2013), e.g., lower light intensity, temperature, and salinity and higher concentrations of inorganic nitrogen and phosphorus. In order to provide evidence for this hypothesis, in this study, a shallow-water sponge *Theonella swinhoei* was selected to compare the whole symbiotic community and metabolic potential in element transformation with that of a deep-sea sponge *Neamphius huxleyi*, using comparative metagenomic approaches.

Materials and Methods

Sponge Sampling

Shallow-water sponge *T. swinhoei* was collected from the South China Sea, Yongxing Island (112° 20′ E, 16° 50′ N), at a depth of ca. 20 m, in July, 2009. Deep-sea sponge *N. huxleyi* was collected at a depth of ca. 1800 m in the Indian Ocean (36° 80′ N, 52° 76′ E), August, 2010, during the scientific investigation on the Ocean No 1. Research Ship, China. Sponges were transferred directly to ziplock bags containing seawater to prevent the contact of sponge tissue with air, stored at −70 °C immediately after collection, and maintained at this temperature until processing. Sponges were identified using the 28S rRNA gene sequence amplified with the primer set NL4F/NL4R (Nichols 2005).

DNA Extraction and Deep Sequencing

Three samples of one species of sponge were used for DNA extraction. Two strategies were used to extract sponge metagenomic DNA: (1) QIAGEN DNeasy® Kit (Qiagen) following the manufacturer's instruction and (2) cetyltrimethyl ammonium bromide (CTAB)-based method according to Taylor et al. (2004). DNA samples extracted by different methods were pooled and mixed thoroughly before deep sequencing (Li et al. 2014).

Deep sequencing was carried out on the Genome Analyzer IIx and HiSeq 2000 systems (Illumina, Inc.) using paired-end technology (2 × 120 and 2 × 100). Total metagenomic DNA was broken up into 300-bp fragments by using the Covaris S2 system and extracted with the QIAquick PCR Purification Kit (Qiagen). Adaptors were ligated to the extracted 300-bp fragments. Then, 300-bp fragments were enriched using Phusion DNA Polymerase through low cycle (8–10 cycles) PCR. Cluster generation and sequencing were performed according to the manufacturer's manual.

The 28S rRNA gene sequences of sponges were deposited in GenBank under accession numbers JF506040 (*T. swinhoei*) and JN162063 (*N. huxleyi*). The metagenome data were deposited in the NCBI Sequence Read Archive (SRA) under accession numbers SRA183005 (*T. swinhoei*) and SRA052801 (*N. huxleyi*).

Phylogeny of Sponge Prokaryotic and Eukaryotic Symbionts

Low quality reads were filtered out by using an in-house developed program. The sequences containing the ambiguous base (*N*) were abandoned using customized perl scripts. Reads with low quality "B" and adapter contamination were trimmed, followed by the trimmed reads shorter than 70 bp were abandoned. The sequences belonging to Metazoa according to NCBI were used as a reference for removing the possible contaminating sequences from metagenome data by MEGABLAST with the threshold at *E* value $<1e^{-3}$, 80 % identity, and 80 % alignment coverage (Altschul et al. 1990). Each sequence was searched against Greengenes, a chimera-checked 16S rRNA gene database (http://greengenes.lbl.gov) and SILVA Eukaryota database (version 104) using MEGABLAST (Altschul et al. 1990; DeSantis et al. 2006; Schloss and Handelsman 2006; Pruesse et al. 2007). The evolutionary distance was calculated separately based on 16S rRNA (V3 and V6) and 18S rRNA (V4) sequences using Mothur (http://www.mothur.org) (Schloss et al. 2009). For prokaryotic symbionts, the online tool RDP classifier was used to assign the sequences to phylogenetic taxonomy at 50 % cutoff (Wang et al. 2007; Cole et al. 2009). In the case of eukaryotic symbionts, BLASTN was used to find the best hit against 18S rRNA genes from SILVA database following parameters: 97 % identity and 90 % coverage (Altschul et al. 1990).

Analyses of Functional Genes and Metabolic Pathways

The qualified reads were assembled using MetaVelvet (an extension of Velvet assembler to de novo metagenome assembly from short sequence reads), with the parameter of hash 61 and minimum length of 500 bp. Open reading frames (ORFs) were identified using two programs, MetaGeneAnnotator and GeneMark (Lomsadze et al. 2005; Noguchi et al. 2006), with ORF length set at a minimum of 60 bp for predicted genes. Based on the deduced amino acid sequences, the annotation was performed through BLASTP against the seed database (Overbeek et al. 2005), with parameter set at *E* value 1e-5. Protein Clusters of Orthologous Groups (COG) assignments were predicted through RPS-BLAST (Marchler-Bauer et al. 2005), using the Conserved Domain Database (CDD) with *E* value 1e-5. The putative metabolic pathway was constructed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database by single-directional best hit (SBH) method (Kanehisa and Goto 2000). For understanding the composition of symbiotic community which contributed to certain function, sequences were compared with non-redundant NCBI nucleotide database using BLASTN and analyzed with MEGAN 4.70 (Huson et al. 2011), which used a last common ancestor algorithm to assign sequences to the NCBI taxonomy.

Gene Enrichment Analysis

The read number of each gene was first calculated using Bowtie and SAMtools (Langmead et al. 2009; Li et al. 2009). Genes were transformed into Reads Per Kilo bases per Million reads (RPKM) (Mortazavi et al. 2008) and identified by DEGseq package using MA-plot-based method with Random Sampling (MARS) model (Wang et al. 2010). The scatterplot was plotted using R with the ggplot2 package. The parameter of differential analysis was set at *P* value <1e-5 and at least fourfold change between two samples. Enrichment of seed subsystem (three hierarchy systems), COG, and KEGG pathways for a given gene list was calculated by a classical hypergeometric distribution statistical comparison. The parameter was set at *P* value <1e-5.

Results

Different Community Structures of the Shallow-Water Sponge *T. swinhoei* and Deep-Sea Sponge *N. huxleyi* Holobionts

Sixty-six and 72 Gb of DNA sequence data were obtained from the metagenomes of *T. swinhoei* and *N. huxleyi*, respectively. After removing the 127 million reads of sequences from sponge hosts, other Metazoa, sequences containing N, or with adapter contamination and shorter than 70 bp, 35.3 and 44.4 Gb high-quality microbiota sequences were obtained for *T. swinhoei* and *N. huxleyi*, respectively. In total, 105,328 and 110,800 contigs (≥500 bp) including 132,863 and 191,923 protein-coding genes were assembled for *T. swinhoei* and *N. huxleyi*, respectively (Supplementary Table S1).

Phylogenetically diverse bacteria, archaea, fungi, and algae were detected in both shallow-water sponge *T. swinhoei* and deep-sea sponge *N. huxleyi* (Supplementary Fig. S1). Even though Proteobacteria was the predominant prokaryotic group of both shallow-water sponge *T. swinhoei* (89.7 %) and deep-sea sponge *N. huxleyi* (80.8 %), different community components were detected between these two distinct sponge species. For example, Deferribacteres, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Thermotogae, BRC1, and SR1 were found only in the deep-sea sponge. Crenarchaeota were the predominant archaea in the shallow-water sponge *T. swinhoei*, while Euryarchaeota dominated in the archaeal community of the deep-sea sponge *N. huxleyi* (Supplementary Fig. S1A).

The community structure of eukaryotic symbionts in the deep-sea sponge was different from that of the shallow-

water sponge. Saccharomycotina (72.6 %), a subphylum of the phylum Ascomycota, was the predominant fungal phylotype in the deep-sea sponge, followed by Ustilaginomycotina (17.9 %), a subphylum within the phylum Basidiomycota which was detected in the deep-sea sponge. In contrast, Pezizomycotina (56.5 %) within the phylum of Ascomycota was detected as the only predominant fungal phylotype in the shallow-water sponge (Supplementary Fig. S1B). Dinophyslales (90.2 %) was the only predominant algae in the shallow-water sponge, whereas Dinophyslales (59.8 %) and Gymnodiniales (7.4 %) were found to be abundant in the deep-sea sponge (Supplementary Fig. S1C).

Overall Metabolism and Nutrient Element Metabolism of the Shallow-Water Sponge *T. swinhoei* and Deep-Sea Sponge *N. huxleyi* Holobionts

According to the protein matches, the major metabolic categories and functional protein categories were similar between these two distinct sponge species (Supplementary Fig. S2), though some statistic differences were observed based on gene abundance analysis, e.g., in cell envelope biogenesis, defense mechanisms, DNA replication, energy production and conversion, posttranslational modification, and secondary metabolite biosynthesis based on COG analysis (Supplementary Fig. S2A). According to seed analysis (Supplementary Fig. S2B), genes related to amino acids and derivatives, carbohydrates, clustering-based subsystems, cofactors, vitamins, prosthetic groups, pigments, fatty acids, lipids and isoprenoids, cell wall and capsule, regulation and cell signaling, and stress response showed statistical difference between these two species of sponges.

In Fig. 1, overall 3696 functional genes were identified based on COG annotation; although the abundance of most annotated genes in *N. huxleyi* was higher than that in *T. swinhoei*, the major metabolic categories were highly similar between these two sponge species. But, there were some genes which only exist in a single species of sponge (locate at the *x*-axis or *y*-axis), and meanwhile, different gene abundance did exist between these two distinct sponge species. For example, nitroreductase (COG0778) and urea transporter (COG4413) in the nitrogen metabolism, fructose-1-phosphate kinase (COG1105) in the carbon metabolism, serine phosphatase (COG2208) in the sulfur metabolism, and phosphotransferase (COG0857) in the phosphate metabolism were relatively enriched in the shallow-water sponge (COG0778 = 62.86 vs 11.74; COG4413 = 51.84 vs 14.84; COG1105 = 24.62 vs 14.73; COG2208 = 95.63 vs 13.06; COG0857 = 30.31 vs 0; RPKM *T.S.* vs *N.H.*), while nitrate reductases (COG5013), nitric oxide reductases (COG3256) in the nitrogen metabolism, pyruvate:ferredoxin oxidoreductase (COG1014) in carbon metabolism, phosphohydrolases (COG1408) in phosphate metabolism, and dissimilatory sulfite reductase (COG2920) in sulfur metabolism were relatively enriched in the deep-sea sponge (COG5013 = 2.04 vs 66.73; COG3256 = 8.02 vs 25.68; COG1014 = 2.79 vs 93.50; COG1408 = 1.75 vs 223.66; COG2920 = 39.19 vs 368.98) (Fig. 1).

In the two metagenomes, nitrogen cycle-related genes such as ammonia monooxygenase (*amo*), glutamate dehydrogenase (GDH2), glutamate synthase (*glt*), hydroxylamine oxidoreductase (*hao*), periplasmic dissimilatory nitrate reductase (*napA*), nitrate reductase (*nar*), nitrogenase (*nif*), copper-containing nitrite reductase (*nirK*), cytochrome cd1-containing nitrite reductase (*nirS*), nitric oxide reductase (*nor*), nitrous oxide reductase (*nosZ*), cytochrome c nitrite reductase (*nrfA*), and nitrite oxidoreductase (*nxr*) were detected in both sponge species. These genes indicated a complex nitrogen cycle including anammox, ammonia oxidation, assimilation, complete denitrification (nitrate, nitrite, nitric oxide, and nitrous oxide reduction), nitrite oxidation, dissimilatory nitrate/nitrite reduction to ammonium (NDRA), and nitrogen fixation in both shallow-water and deep-sea sponges (Table 1). However, *amo*, *nirS*, *glt*, *nxr*, and *nif* genes showed statistical abundance difference between these two species of sponges (Table 1). Besides, gene enrichment analysis indicated that nitrate reductase beta subunit (*narY*, COG1140), nitrate reductase alpha subunit (*narG*, COG5013), *nosZ* (COG4263), and *ntrY* (signal transduction histidine kinase involved in nitrogen fixation, COG5000) genes were enriched in the deep-sea sponge. In contrast, nitrate reductase (*torC*, COG3005), nitroreductase (*nfnB*, COG0778), urea transporter (*utp*, COG4413), nitrate/nitrite transporter (*narK*, COG2223), amino acid permeases (*sdaC*, COG0814), 5-carboxymethyl-2-hydroxymuconate isomerase in amino acid transport and metabolism (*hpaF*, COG3232), nitroreductase (*nfnB*, COG0778), proline hydroxylase (COG3751), nitrate/nitrite transporter (*naarK*, COG2223), and amino acid permeases (*sdaC*, COG0814) genes were enriched in the shallow-water sponge.

Genes related to central carbohydrate metabolism, including one-carbon metabolism, monosaccharides, di- and oligosaccharides, polysaccharides, organic acids, and sugar alcohols, were abundant in these two sponge metagenomes. However, different gene enrichment was observed in some metabolic processes, e.g., 2-keto-3-dehydro-6-phosphogluconate (KDPG) to pyruvate and glyceraldehyde-3-phosphate (GAP), GAP to 1,3-bisphospho-glycerate (BPG), 2-phosphoglycerate (2PG) to oxaloacetate, succinate to succinyl-CoA, isocitrate to oxaloacetate, acetyl-CoA, xylulose 5-phosphate (X5P) to 3-phosphoglycerate (3PG), and ribose 5-phosphate (R5P) to ribulose 5-phosphate (Ru5P) (Table 2). The shallow-water sponge had a relatively higher ratio of CO_2 uptake carboxylase and respiration-related proteins, while in the deep-sea sponge metagenome, a higher number of Calvin-Benson cycle-related proteins as well as a low ratio of CO_2 uptake carboxylase and respiration related proteins

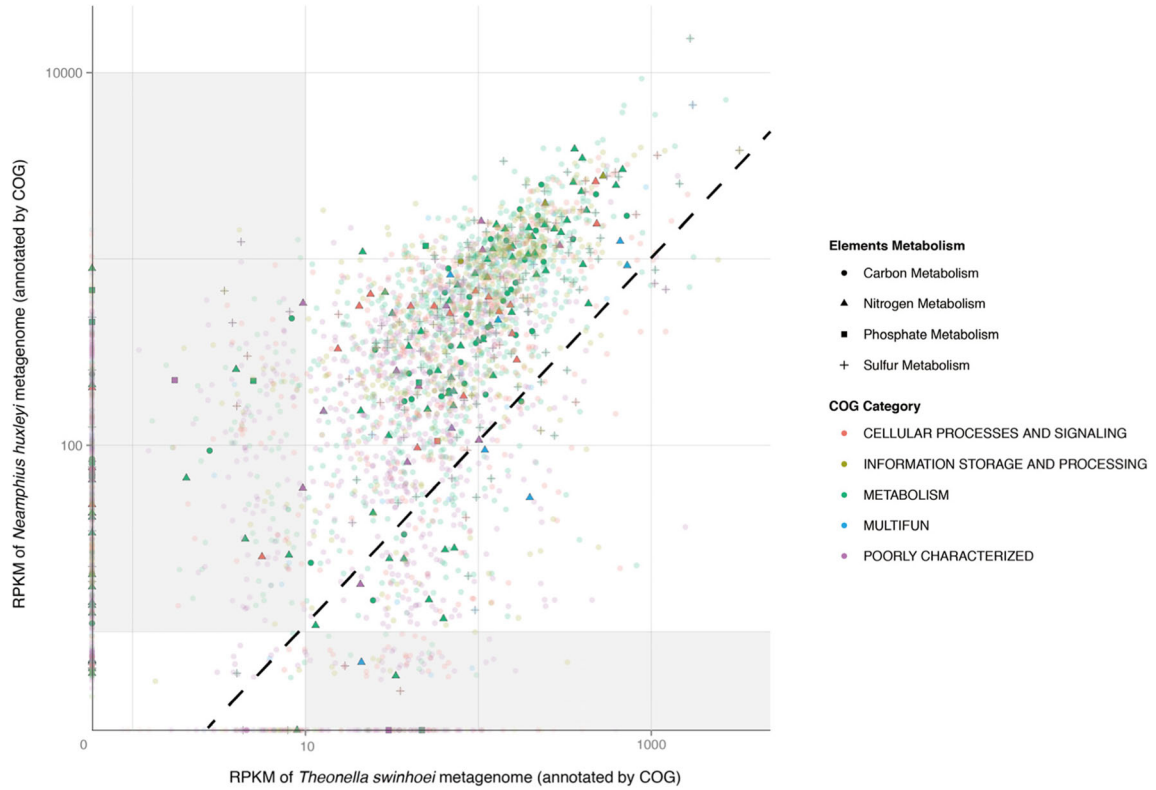

Fig. 1 Enriched COG features in metagenomes of the shallow-water sponge *T. swinhoei* and deep-sea sponge *N. huxleyi*. The map was plotted based on mapping reads to the de novo metagenomic assemblies and calculated by Reads Per Kilo bases per Million reads (*RPKM*). The transparent layer with *filled colored circles* shows the COG categories of all the annotated features (*red*: cellular processes and signaling; *yellow*: information storage and processing; *green*: metabolism; *blue*: gene with multi-function; *purple*: poorly characterized). The top layer with different symbols indicates the carbon, nitrogen, phosphate, and sulfur metabolism-related genes. The *dot line* represents that the log RPKM values between the sponges are equal. The *shadow portions* of the graph represent the genes with relatively high different abundance between the two sponges (using RPKM = 10 as the threshold). The transparent layer with *filled colored circles* shows the COG categories of all the annotated features: *red*: cellular processes and signaling ([D] Cell cycle control, cell division, chromosome partitioning, [Y] Nuclear structure, [V] Defense mechanisms, [T] Signal transduction mechanisms, [M] Cell wall/membrane/envelope biogenesis, [N] Cell motility, [Z] Cytoskeleton, [W] Extracellular structures, [U] Intracellular trafficking, secretion, and vesicular transport, [O] Posttranslational modification, protein turnover, chaperones; *yellow*: information storage and processing ([J] Translation, ribosomal structure, and biogenesis, [A] RNA processing and modification, [K] Transcription, [L] Replication, recombination, and repair, [B] Chromatin structure and dynamics; *green*: metabolism ([C] Energy production and conversion, [G] Carbohydrate transport and metabolism, [E] Amino acid transport and metabolism, [F] Nucleotide transport and metabolism, [H] Coenzyme transport and metabolism, [I] Lipid transport and metabolism, [P] Inorganic ion transport and metabolism, [Q] Secondary metabolite biosynthesis, transport, and catabolism; *blue*: gene with multi-function, *purple*: poorly characterized

were detected. Genes responsible for chemoautotrophic CO_2 fixation via the Calvin-Benson cycle were observed to be enriched in the deep-sea sponge. For lipopolysaccharide biosynthesis, the potential of symbionts in the deep-sea sponge was obviously higher than that in the shallow-water sponge based on gene enrichment analysis. While for other types of O-glycan biosynthesis, the potential of symbionts in the shallow-water sponge was higher than that in the deep-sea sponge. Carboxylesterase type B (*PnbA*, COG2272), spore coat polysaccharide biosynthesis protein F (*spsF*, COG1861), and beta-galactosidase/beta-glucuronidase (*lacZ*, COG3250) genes were enriched in the deep-sea sponge. Glycosyltransferase (COG3306), carbonic anhydrase (*cah*, COG3338), carbohydrate-selective porin (*oprB*, COG3659), exopolysaccharide biosynthesis protein (*epsL*, COG4632), citrate symporter (*citM*, COG 2851), mannito/fructose-specific phosphotransferase system (*mtlA*, COG4668, COG2213), esterase/lipase (COG1647), sugar phosphate permease (*uhpC*, COG2271), and glucose dehydrogenase (COG4993) were enriched in the shallow-water sponge.

In the case of phosphate metabolism, alkylphosphonate utilization represented as an obvious characteristic of phosphorus metabolism in both sponge holobionts; however, the gene number of alkylphosphonase in the deep-sea sponge *N. huxleyi* was higher than that of the shallow-water sponge *T. swinhoei*. In addition, genes encoding phosphate transport regulator (COG1392), *phnD* (COG3221), *phnL* (COG4778) (ABC-type phosphate/phosphonate transport system) and *phnG* (COG3624), *phnH* (COG3625), *phnI* (COG 3626), phospholipid *N*-methyltransferase (COG3963),

Table 1 Gene enrichment analysis of nitrogen-cycling genes

Reaction		Enzyme	Gene no.		Fold change based on RPKM (Ts/Nh)	*P* value
			Ts	Nh		
Anammox		NirS*	3	3	4.08	4.64E-12
Ammonia oxidation		Amo*	12	37	1.55	1.29E-14
		Hao	19	25	0.78	1.38E-8
Assimilation		GDH2	616	1676	1.11	6.02E-76
		Glt*	159	515	0.64	4.4E-184
Denitrification	Nitrate reduction	Nar	160	398	1.12	8.13E-14
		NapA	156	382	1.17	1.45E-25
	Nitrite reduction	NirS*	3	3	4.08	4.64E-12
		NirK	52	88	1.18	1.38E-10
	Nitric oxide reduction	Nor	152	382	0.76	7.00E-69
	Nitrous oxide reduction	NosZ	35	30	0.86	2.52E-4
Nitrite oxidation		Nor	152	382	0.76	7.00E-69
		Nxr*	13	63	0.7	1.95E-9
DNRA		NrfA	26	62	1.19	4.34E-5
Nitrogen fixation		Nif*	22	81	0.57	2.36E-43

Ts T. swinhoei, *Nh N. huxleyi*, *Amo* ammonia monooxygenase, *GDH2* glutamate dehydrogenase, *Glt* glutamate synthase, *Hao* hydroxylamine oxidoreductase, *NapA* periplasmic dissimilatory nitrate reductase, *Nar* nitrate reductase, *nif* nitrogenase, *NirK* copper-containing nitrite reductase, *NirS* cytochrome cd1-containing nitrite reductase, *Nor* nitric oxide reductase, *NosZ* nitrous oxide reductase, *Nrf* A cytochrome c nitrite reductase, *Nxr* nitrite oxidoreductase

*P value <0.05, statistical significance

phosphohydrolases (COG1408), trehalose-6-phosphatase (*otsB*, COG1877), alkaline phosphatase (*phoA*, COG1875), and phosphate/sulfate permease (*pitA*, COG0306) were enriched in the deep-sea sponge. On the other hand, *fruA* (phosphotransferase system, COG1299), *pta* (bioD-like N-terminal domain of phosphotransacetylase, COG0857), and *pstS* (ABC-type phosphate transport system)-related genes were enriched in the shallow-water sponge.

Abundant sulfur metabolism-related genes, e.g., inorganic and organic sulfur assimilation, galactosylceramide and sulfatide metabolism, thioredoxin-disulfide reduction, sulfur oxidation, and reduction, were observed in these two metagenomes using the seed database (Table 3). Overall, the number of genes involved in sulfur metabolism in the deep-sea sponge *N. huxleyi* was higher than that of the shallow-water sponge *T. swinhoei*. Particularly, dimethylsulfoniopropionate (DMSP) mineralization, which was not detected in the shallow-water sponge *T. swinhoei*, was found in the deep-sea sponge *N. huxleyi*. By contrast, much more genes encoding sulfite oxidase were observed in the shallow-water sponge *T. swinhoei* than that in the deep-sea sponge *N. huxleyi* (Table 3). In addition, *cysP* (ABC-type sulfate transport system, COG4150) and disulfide interchange protein (COG4232) were enriched in the deep-sea sponge, while alkyl sulfatase and related hydrolases (COG2015) were enriched in the shallow-water sponge.

The ABC transporter-related genes (Supplementary Fig. S3) showed the metabolic potential of sponge microbiota in nutrient transformation and also indicated different enrichment trends between these two species of sponges. For example, in the deep-sea sponge *N. huxleyi*, phosphate and amino acid transporter and metal-like cation transporter-related genes were enriched, while in the shallow-water sponge, oligosaccharide, polyol, and monosaccharide transporter-related genes were enriched.

The Potentials of Different Microbes in the Nutritional Metabolism of Sponge Holobionts

As shown in Fig. 2, the potential contribution of microbes based on abundance of genes involved in the nitrogen cycle is similar in the two sponges. In total, prokaryotic symbionts' possible contribution to nitrogen metabolism is greater than eukaryotic symbionts based on the gene abundance comparison. Figure 2 also suggests that different microbial species may play similar roles. For instance, Planctomycetes, Bacteroidetes, Actinobacteria, Chloroflexi, Deinococcus-Thermus, Thaumarchaeota, and Euryarchaeota in the deep-sea sponge probably play an important role in the nitrogen cycle. In contrast, Proteobacteria and Poribacteria are probably involved in the nitrogen cycle of the shallow-water sponge *T. swinhoei*. In the case of eukaryotes, the role of eukaryotes associated with the shallow-water sponge *T. swinhoei* was

Table 2 Gene enrichment analysis of carbon metabolism-related genes

Reaction	Enzyme	Gene no.		Fold change based on RPKM (Ts/Nh)	*P* value
		Ts	Nh		
G6P → F6P	5.3.1.9	10	23	1.14	4.45E-2
G6P → glucono-1,5-lactone-6p	1.1.1.49	7	18	1.18	1.81E-2
Glucono-1,5-lactone-6p → 6-P-gluconate	3.1.1.31	6	8	1.59	3.85E-5
6-P-Gluconate → KDPG	4.2.1.12	2	8	0.69	2.48E-4
KDPG → pyruvate + GAP	4.1.2.14*	2	12	0.62	5.63E-6
GAP → BPG	1.2.1.12; 1.2.1.59*	22	60	1.64	5.31E-48
BPG → 3PG	2.7.2.3	16	43	0.83	1.93E-3
3PG → 2PG	5.4.2.1	24	67	0.85	2.17E-4
2PG → PEP	4.2.1.11*	15	45	0.49	1.07E-31
PEP → oxaloacetate	4.1.1.31*	12	23	0.60	5.34E-15
Oxaloacetate → malate	1.1.1.37	16	45	1.21	3.49E-4
Malate → fumarate	4.2.1.2	27	51	1.00	9.85E-1
Fumarate → succinate	1.3.99.1	29	107	0.74	3.76E-17
Succinate → succinyl-CoA	6.2.1.5*	32	107	0.63	1.11E-35
Succinyl-CoA → 2-oxoglutarate	1.2.7.3	31	50	1.30	4.59E-9
2-Oxoglutarate → isocitrate	1.1.1.42	16	38	1.19	1.07E-2
Isocitrate → citrate	4.2.1.3*	43	74	1.67	7.94E-61
Citrate → oxaloacetate + acetyl-CoA	2.3.3.8*	0	2	0.04	6.24E-10
F6P → FBP	2.7.1.11	7	17	0.99	9.61E-1
FBP → F6P	3.1.3.11	12	24	1.00	9.80E-1
FBP → GAP + DHAP DHAP → GAP	4.1.2.13	9	44	0.78	1.00E-4
F6P → X5P + E4P	2.2.1.1	19	55	1.24	3.22E-7
X5P → Ru5P	5.1.3.1*	11	36	0.61	1.65E-14
Ru5P → RuBP	2.7.1.19*	0	5	0.04	9.33E-10
RuBP → 3PG	4.1.1.39*	0	8	0.02	6.97E-17
GAP → R5P	2.2.1.1	19	55	1.24	3.22E-7
R5P → Ru5P	5.3.1.6*	15	32	0.69	4.32E-9
E4P → S7P	2.2.1.2	12	25	1.35	5.80E-7
S7P → R5P	2.2.1.1	19	55	1.24	3.22E-7

Ts T. swinhoei, *Nh N. huxleyi*, *GAP* glyceraldehyde-3-phosphate, *FBP* fructose 1,6-bisphosphate, *DHAP* dihydroxyacetone phosphate, *KDPG* 2-keto-3-dehydro-6-phosphogluconate, *G6P* glucose 6-phosphate, *F6P* fructose 6-phosphate, *FBP* fructose 1,6-bisphosphate, *E4P* erythrose 4-phosphate, *X5P* xylulose 5-phosphate, *GAP* glyceraldehyde-3-phosphate, *Ru5P* ribulose 5-phosphate, *S7P* sedoheptulose 7-phosphate, *R5P* ribose 5-phosphate, *RuBP* ribulose 1,5-bisphosphate, *BPG* 1,3-bisphospho-glycerate, *3PG* 3-phosphoglycerate, *2PG* 2-phosphoglycerate, *PEP* phosphoenolpyruvate

**P* value <0.05, statistical significance

probably greater than that in the deep-sea sponge *N. huxleyi*, e.g., Streptophyta and Oomycetes in the nitrogen cycle of the shallow-water sponge *T. swinhoei*. On the other hand, Ascomycota and Basidiomycota may play an important role in the nitrogen cycle of the deep-sea sponge based on abundance of genes.

According to the microbial carbon metabolism of two distinct sponge species by MEGAN analysis (Fig. 3), prokaryotic symbionts maybe mainly involved in the carbon metabolism than eukaryotic symbionts, for example Bacteroidetes, Actinobacteria, Firmicutes, Cyanobacteria, Acidobacteria, and Thaumarchaeota in the deep-water sponge *N. huxleyi* and Proteobacteria and Crenarchaeota in the shallow-water sponge *T. swinhoei*. The eukaryotic symbionts which are probably involved in the carbon metabolism include Ascomycota, Basidiomycota, Streptophyta, Chlorophyta, and Oomycetes.

The diversity of sulfur metabolism-related prokaryotic and eukaryotic symbionts was lower than that involved in the

Table 3 Gene enrichment analysis of sulfur metabolism-related genes

Reaction	Enzyme	Gene no. Ts	Gene no. Ls	Fold change based on RPKM (Ts/Nh)	*P* value
Sulfate → APS	Sulfate adenylyltransferase subunit 1	0	3	0.04	3.52E-14
APS ↔ sulfite	Adenylylsulfate reductase, subunit A	3	25	0.22	3.34E-35
Sulfite → sulfide	Sulfite reductase (NADPH) Hemoprotein beta-component	8	33	0.29	1.99E-94
PAPS → PAP	aryl sulfotransferase	1	2	0.29	9.68E-16
PAPS → APS	3′(2′),5′-Bisphosphate nucleotidase	6	19	0.55	1.75E-19
Glutathione as a sulfur source	Hydroxyacylglutathione hydrolase	10	27	1.46	5.95E-12
Glutathione as a sulfur source	Glutathione transporter	7	12	1.88	3.75E-14
APS → PAPS	Adenylylsulfate kinase	8	8	2.34	2.07E-34
L-serine → *O*-acetyl-L-serine	Serine *O*-acetyltransferase	7	22	3	1.40E-97
O-acetyl-L-serine → L-cysteine	Cysteine synthase B	8	7	3.14	1.34E-22
Sulfite → sulfate	Sulfite oxidase	1	0	120.61	4.38E-9
Sulfite → sulfide	Sulfite reductase alpha subunit	1	0	–	–
Glutathione as a sulfur source	Glutathione *S*-transferase	48	134	1.01	6.28E-1
Sulfite → sulfide	Sulfite reductase (ferredoxin)	0	1	0.47	3.50E-1
Sulfate → APS	Bifunctional enzyme CysN/CysC	4	11	0.9	2.59E-1
APS → PAPS	3′-Phosphoadenosine 5′-phosphosulfate synthase	1	2	0.65	1.24E-1
Sulfite → sulfide	sulfite reductase (NADPH) flavoprotein alpha-component	5	10	1.26	9.13E-3
APS ↔ sulfite	Adenylylsulfate reductase, subunit B	2	9	0.54	8.23E-3
Sulfate → APS	Sulfate adenylyltransferase subunit 2	3	14	0.76	9.91E-4
Glutathione as a sulfur source	Gamma-glutamyltranspeptidase	24	46	0.85	4.67E-4
Sulfate → APS	Sulfate adenylyltransferase	5	15	0.70	5.45E-5
PAPS → sulfite	Phosphoadenosine phosphosulfate reductase	8	16	1.35	1.24E-5
O-acetyl-L-serine → L-cysteine	Cysteine synthase A	13	36	0.73	2.31E-11

Ts T. swinhoei, *Nh N. huxleyi*

nitrogen and carbon metabolisms (Fig. 4). Thaumarchaeota and Bacteroidetes, Chlorobi, Actinobacteria, Firmicutes, and Cyanobacteria were probably involved in the sulfur metabolism of the deep-sea sponge *N. huxleyi*. By contrast, Proteobacteria, Chloroflexi, and Chlorophyta were probably involved in the sulfur metabolism of the shallow-water sponge *T. swinhoei*.

Discussion

Omics has been successfully used for the functional exploration of sponge microbiota (Thomas et al. 2010; Liu et al. 2012; Trindade-Silva et al. 2012; Fan et al. 2012; Li et al. 2014; Fiore et al. 2015). The extensive non-redundant catalog of functional genes in sponge metagenome provides an opportunity to analyze the metabolic potential of a sponge holobiont. In this study, the whole community's gene sequences except that from sponge and other Metazoa were used for the metabolic potential analysis, i.e., whole symbiotic microbes including prokaryotic and eukaryotic symbionts were included, which is different from the other metagenomic analyses that only focused on the prokaryotic symbionts in sponges (Thomas et al. 2010; Liu et al. 2012; Trindade-Silva et al. 2012; Fan et al. 2012). Consequently, we could gain insights into the whole metabolism profile of microbial symbionts in a sponge holobiont.

Sponges have emerged as significant mediators of biogeochemical fluxes by facilitating both the consumption and release of nutrients since sponges are one of the most abundant benthonic organisms in the coral reef ecosystem and have the ability to filter large amount of seawater (Maldonado et al.

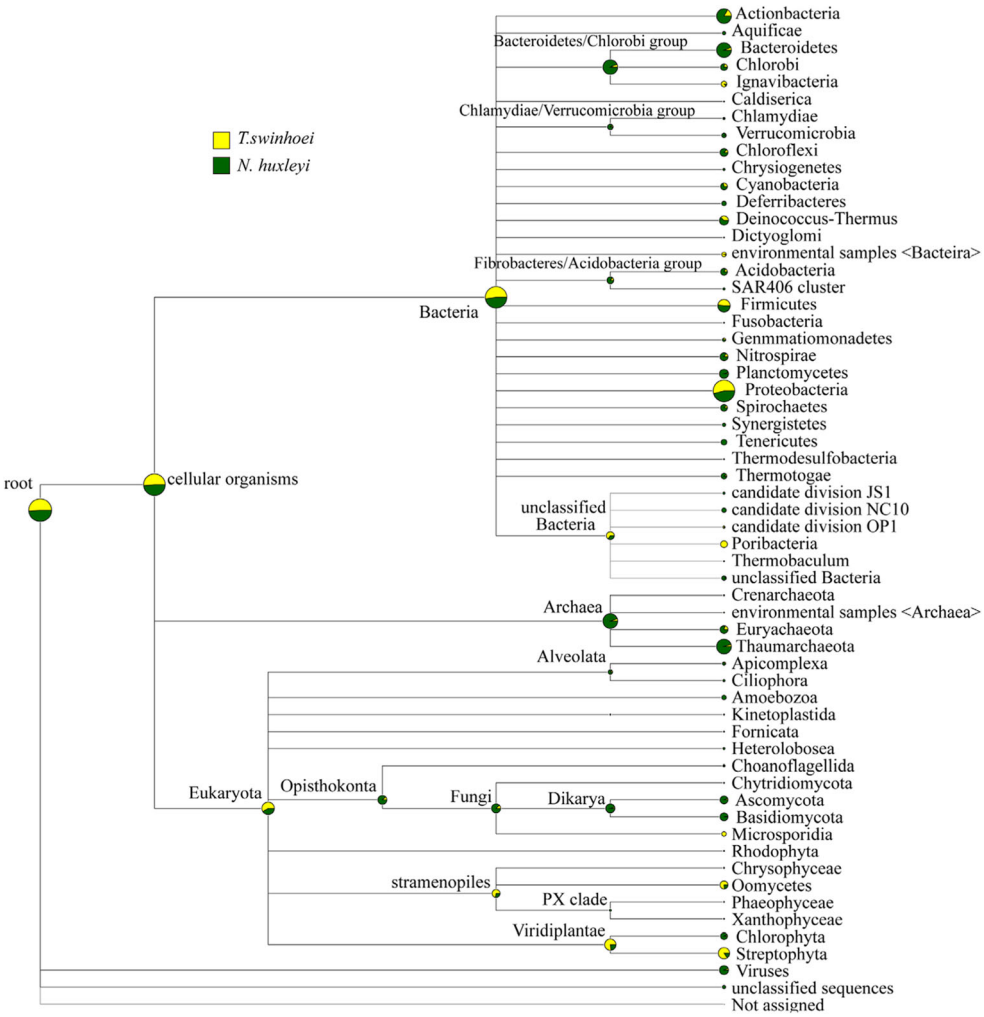

Fig. 2 MEGAN analysis of microbes potentially involved in nitrogen metabolism. Taxonomy classification of genes related to nitrogen metabolism is listed in Table 1. Genes from *T. swinhoei* dataset (*yellow*) and *N. huxleyi* dataset (*green*) were annotated by NCBI nr-database and assigned by MEGAN LCA algorithm using default parameters. The tree is collapsed at phylum level of NCBI taxonomy. The size of the *circles* is relative to the number of genes assigned to each node

2012; Southwell et al. 2008a). The net effect is to release labile nutrient forms (nitrate, nitrite, ammonium, phosphate) from less bioavailable organic molecules (Maldonado et al. 2012; Southwell et al. 2008a, b). It is now known that, in general, sponges host diverse microbial communities that contribute to digestion and nutrient release. For example, Kamke et al. (2013) suggested complex carbohydrate degradation patterns in bacterial symbionts of sponges by single-cell genomic study. DNA-based analysis has revealed the nitrifying potential of the *Nitrosopumilus*, *Nitrosospira*, *Nitrococcus*, *Nitrospira*, and *Nitrospina* lineages in *T. swinhoei* (Bayer et al. 2008). Zhang et al. (2015a) revealed a major and heretofore unknown role regarding marine phosphorus cycle, i.e., polyphosphate (poly-P) production and storage by sponge endosymbionts. Zhang et al. (2015b) found phylogenetically diverse sulfate-reducing *Desulfovibrio* associated with sponges.

The present study shows the total metabolic potential of sponge microbiota in the transformation of nutrient elements such as C, N, P, S, which corroborates some of the findings on the nutrient fluxes through sponges from chemistry investigations (Maldonado et al. 2012; Southwell et al. 2008a, b). Taking nitrogen cycle as an example, both shallow-water and deep-sea sponges have complex nitrogen-cycling network based on the functional gene detection, including anammox, ammonia oxidation, assimilation, denitrification, nitrification, DNRA, and nitrogen fixation (Table 1). Functional genes related to ammonia assimilation dominated the nitrogen metabolism in these two distinct sponge species, by which inorganic NH_4^+ (NH_3) could be transferred to organic nitrogen such as amino acids and proteins. Moreover, the presence of genes responsible for allantoin utilization, urea and nitrite hydratase, and cyanate hydrolysis in the metagenome indicated the microbial symbionts' potentially important role in the removal of metabolic wastes and toxic compounds such as urea, allantoin, and cyanate in the sponge holobionts. Similarly, the sponge microbes may contribute to the C, P, and S transformation in its sponge host according to the metagenomic analysis. For example, genes related to central carbohydrate metabolism were abundant in the two sponge metagenomes.

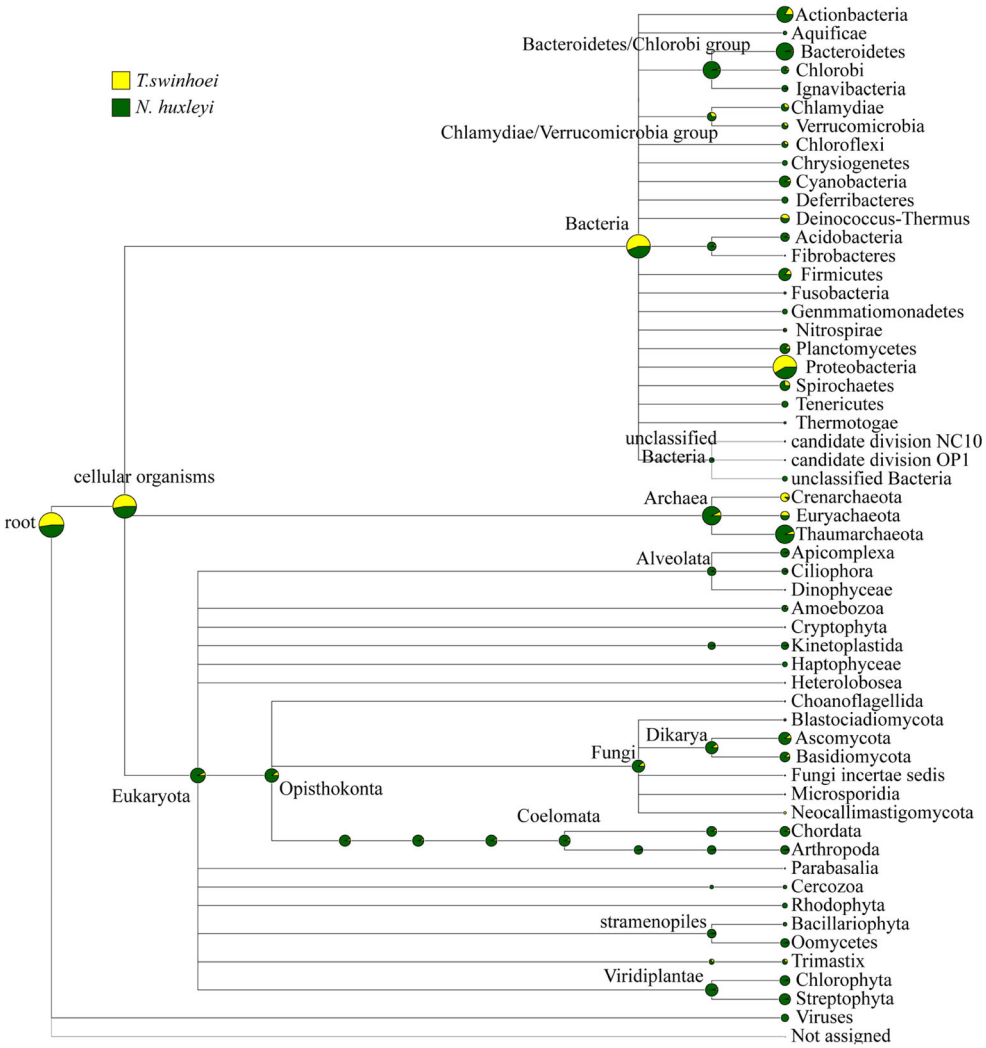

Fig. 3 MEGAN analysis of microbes potentially involved in carbon metabolism. Taxonomy classification of genes related to carbon metabolism is listed in Table 2. Genes from *T. swinhoei* dataset (*yellow*) and *N. huxleyi* dataset (*green*) were annotated by NCBI nr-database and assigned by MEGAN LCA algorithm using default parameters. The tree is collapsed at phylum level of NCBI taxonomy. The size of the *circles* is relative to the number of genes assigned to each node

Interestingly, the two sponge microbiota showed different metabolic characteristics in some carbon metabolic processes based on the gene enrichment analysis, e.g., the shallow-water sponge had a relatively higher ratio of CO_2 uptake carboxylase and respiration-related proteins, while genes responsible for chemoautotrophic CO_2 fixation via the Calvin-Benson

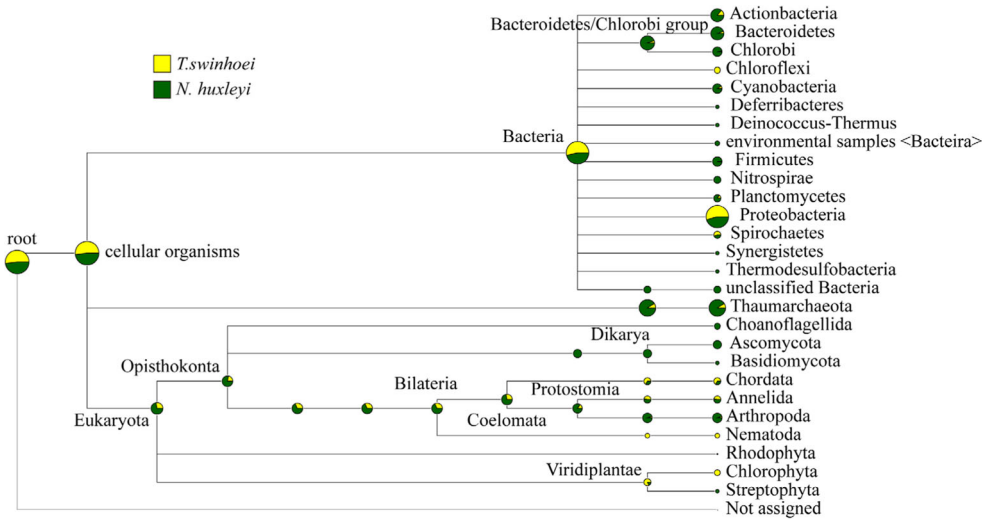

Fig. 4 MEGAN analysis of microbes potentially involved in sulfur metabolism. Taxonomy classification of genes related to sulfur metabolism listed in Table 3. Genes from *T. swinhoei* dataset (*yellow*) and *N. huxleyi* dataset (*green*) were annotated by NCBI nr-database and assigned by MEGAN LCA algorithm using default parameters. The tree is collapsed at phylum level of NCBI taxonomy. The size of the *circles* is relative to the number of genes assigned to each node

cycle were relatively enriched in the deep-sea sponge. For phosphate metabolism, the utilization of alkylphosphonate represented as a characteristic of phosphorus metabolism especially the deep-sea sponge *N. huxleyi*. Abundant sulfur metabolism-related genes, e.g., sulfur assimilation, oxidation, and reduction, were observed in both sponge holobionts. Overall, the number of sulfur metabolism genes in the deep-sea sponge *N. huxleyi* was higher than that in the shallow-water sponge *T. swinhoei*. Consistently with the findings of Fan et al. (2012), these two different sponges had similar core functions in nutrient element transformation. However, the shallow-water sponge *T. swinhoei* and the deep-sea sponge *N. huxleyi* microbiota showed different potentials or strategies in some detailed metabolic processes such as carbon, nitrogen, phosphorus, and sulfur transformation, which maybe probably resulted from the different microbial species and abundance as well as the environment factors.

Marine sponges host highly diverse, specific microbes (Lee et al. 2011; Schmitt et al. 2012; Moitinho-Silva et al. 2014b; Reveillaud et al. 2014). For instance, Schmitt et al. (2012) compared the bacterial communities of 32 sponge species from eight locations around the world's oceans by 16S ribosomal RNA gene amplicon pyrosequencing and found that, consistent with previous studies suggesting at least partially overlapping communities among different sponges, different sponges contained bacterial communities of mainly different bacterial species (species-specific community) and shared very few bacterial species (core community). Similarly in this study, the metagenomic data suggested that the shallow-water and deep-water sponges probably have species-specific microbes, even though Proteobacteria dominated in both of these two distinct sponge species (Supplementary Fig. S1), e.g., Deferribacteres, Fusobacteria, Germmatimonadetes, Lentisphaerae, Thermotogae, BRC1, and SR1 were found only in the deep-sea sponge *N. huxleyi*. The observed metabolic profile differences by gene enrichment analysis might result from the different diversity or abundance of microbial populations in these two different sponge species. Deferribacteres, Fusobacteria, Gemmatimonadetes, and Thermotogae which were observed only in the deep-sea sponge *N. huxleyi* may carry out distinct functions in the N, C, and S transformation according to the MEGAN analysis. Even for the same metabolic process, the contribution of the microbes belonging to the same category may be different between different species of sponges because of their different abundance. For instance, archaea in the deep-sea sponge *N. huxleyi* accounted for about 4.6 % of the total prokaryotes and probably played an important role in the nitrogen, carbon, and sulfur cycles according to gene abundance analysis, while in the shallow-water sponge *T. swinhoei*, archaea accounted for only about 1.0 % of the total prokaryotes and gene abundance suggested only a slight involvement in the nitrogen cycle. A similar phenomenon existed for Actinobacteria and their putative role in the nitrogen cycle of these two sponges. In addition, the putative nutrient transformation roles of eukaryotic symbionts such as fungi and microalgae in the deep-water sponge *N. huxleyi* were suggested to be greater than in the shallow-water sponge *T. swinhoei* according to the MEGAN analysis (Figs. 2, 3, and 4).

Deep sea has its unique characteristics which are different from that of shallow water (Smedile et al. 2013); therefore, it could be supposed that the putative metabolic difference of the sponge microbiota may be related to the marine environmental factors. Regarding nitrogen metabolism, the deep-sea sponge microbiota gene abundance suggested a more vigorous denitrification potential consistent with nitrate and nitrite reduction being higher due to the higher nitrate and nitrite concentration in deep sea (Supplementary Fig. S4), for example in the sampling site of the deep-sea sponge *N. huxleyi*, the concentration of nitrate was increased from 0 mM/L at the surface water to 20 mM at approx. 900 m deep water and maintained nearly unchanged in deep sea water. The higher phosphate (e.g., ca. 1.4 μm/L) content in deep sea water (Supplementary Fig. S4) could favor the more active phosphorus metabolism (indicated by abundant alkylphosphonase) in the deep-sea sponge *N. huxleyi* than that of the shallow-water sponge *T. swinhoei*. Considering carbon fixation as another example, gene abundance in the shallow-water sponge *T. swinhoei* suggests a relatively higher ratio of CO_2 uptake-related carboxylase and respiration-related proteins consistent with the shallow-water sponge microbiota mainly fixing CO_2 by algal or cyanobacterial symbiont photosynthesis in the presence of light. In contrast, the deep-sea sponge microbiota could not use photosynthesis for production of organic carbon molecules; instead, it might utilize CO_2 fixation of chemoautotrophic prokaryotes for this purpose.

Overall, this work has intriguing implications for our understanding of the total metabolic potential of the shallow-water and deep-sea sponge holobionts. As indicated in this study, though the two sponge species with different microbial community structures have similar core metabolic profiles, they might have different strategies in some detailed metabolic processes, e.g., transformation and utilization of carbon, nitrogen, phosphorus, and sulfur, according to functional gene enrichment analysis. The exploration of sponge microbiota's metabolisms or functions using omics is just in its infancy, future comparative omics explorations, particularly metatranscriptomics (Radax et al. 2012; Moitinho-Silva et al. 2014a; Fiore et al. 2015) and metaproteomics (Liu et al. 2012), will give more information on the sponge microbiota's functions and the relationships of sponge microbiota with the sponge hosts and marine environment.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no competing financial interests.

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