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Research article

Report on microbial communities with gene functions and distribution of elements in *Echinomuricea* (Anthozoa: Holaxonia) from Thailand

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Abstract

The genus *Echinomuricea* consists of marine invertebrates that are important to ocean ecosystems. The present study provided the first data on the microsymbiont community with microbial gene profiles and depositions of elements in gorgonian *Echinomuricea* cf. *pulchra* from parts of the western Gulf of Thailand. Among all the microbes identified, the bacterial diversity was the most abundant in gorgonian corals. The Vibrionaceae and Marinilabiliaceae were the predominant identified Gammaproteobacteria and Bacteroidetes, respectively. Of the other microbes, the *Echinomuricea* were dominated by the Mucorales for fungi, the Nitrosopumilales for Archaea and the Herpesvirales for viruses. In the gene prediction of coral microbial communities, replication, recombination, and repair were found mainly among the known function classes as the highest number of matched genes from the reference database of the evolutionary genealogy of genes: Non-Supervised Orthologous Groups. Kyoto Encyclopedia of Genes and Genomes pathway annotation showed a high number of genes related to transport and the catabolism of cellular processes; signaling molecules; and the interactions of environmental information processing, translation, folding, sorting and the degradation of genetic information processing. From the Carbohydrate-Active Enzyme database, glycoside hydrolases, carbohydrate-binding modules and glycosyltransferases predominated in these functional classes. Scanning electron microscopy using an energy-dispersive X-ray identified calcium throughout the sclerites of coenenchyma, but this element was rarely distributed on the axis of corals. Oxygen and nitrogen were observed in all surface fragments, while the magnesium signal was low. Based on these results, this was the first report of a meta-analysis of microbiota with the main elements of *Echinomuricea* surfaces. These data might support the understanding of microbial symbionts associated with *E.* cf. *pulchra*.

Introduction

The Octocorallia are well-known as an anthozoan coral subclass that inhabits a wide range of marine ecosystems worldwide (McFadden et al., 2010; Halász et al., 2014; Breedy et al., 2015; Matsumoto and van Ofwegen, 2016). The gorgonian genus *Echinomuricea* Verrill, 1869, which belongs to the Plexauridae, consists of more than 20 species, including *E. indica*, *E. andamanensis*, *E. philippinensis*, *E. ramosa*, *E. reticulata* and *E. pulchra* (Williams, 1992; Watling and Auster, 2005; Cordeiro et al., 2018). Goh et al. (1997) reported

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on the gorgonian diversity from zonation patterns in the Singaporean Sea, which identified the *Echinomuricea* population in the bottom zone. The genus *Echinomuricea* has been shown to represent the important metazoans in the trophic levels in marine ecosystems (Williams, 1992).

Chung et al. (2012) described a compound isolated from the *Echinomuricea* that exerts cytotoxic activity against tumor cells. Bioactive compounds consisted of echinolabdane A and a sterol, 6-epi-yonarasterol B isolated from the *Echinomuricea*. They were assumed to further exhibit anticancer activity. However, studies on the *Echinomuricea* have rarely been reported, particularly in Thailand, with information lacking on factors such as the symbiont diversity, gene functions and element topography. The aim of this study was to provide knowledge on the metagenomics, the gene profiles of microbes and on the distribution of elements in *Echinomuricea* cf. *pulchra* collected from parts of the western coast of the Gulf of Thailand.

Material and Methods

Coral samples of gorgonian *E.* cf. *pulchra* were collected between Ko Sai and Ko Sadao and Ko Khi Nok in Prachuap Khiri Khan province (Figs. 1A–B) from February to July 2017 (12°28'46.5"N 99°59'46.4"E). The sampling sites were at depths of 2–5 m, located on the western coast of the Gulf of Thailand (Fig. 1B). The coral collection conducted for the current study strictly adhered to the requirements of the Wild Animal Reservation and Protection Act (1992) of Thailand. Eight individuals of *E.* cf. *pulchra* were used in this study (Fig. 1C). The gorgonias were washed with artificial seawater (26 g NaCl, 1 g $CaCl_2$, 0.75 g KCl, 3.95 g $MgSO_4 \cdot 7H_2O$, 6 g $MgCl_2 \cdot 6H_2O$ in 1 L of distilled water) with 10 µg/mL ampicillin and 10 µg/mL paromomycin sulfate (Sigma-Aldrich; Munich, Germany) according to Sweet et al. (2014) for 10 s and subsequently were washed with artificial seawater without antibiotics three times. The gorgonias were acclimatized in artificial seawater without antibiotics containing an automatic air pump, automatic wave maker, chiller and artificial lighting (light emitting diode) under laboratory conditions (27 ± 1°C, pH 7.8, 12 hr light:12 hr dark photoperiod) for 3 d. Fragments (0.5–0.8 cm) of each gorgonian sample were used in the study of genomic extraction.

For microbial genomic analysis, total genomic DNA was extracted from each coral fragment sample using an EZNA®Mollusc DNA isolation kit (Omega Bio-tek; Norcross, GA, USA), according to the manufacturer's protocol. Genomic solutions from each sample were pooled into a single Eppendorf tube and then quantified using a NanoDrop spectrophotometer and a Qubit spectrofluorometer (Thermo Fisher Scientific; Waltham, MA, USA). DNA fragments with a size of approximately 350 bp were prepared using a Covaris S2 AFA sonicator (Covaris; Chicago, IL, USA). Library construction (≈1 µg DNA/sample) was performed using the NEBNext® Ultra™ DNA Library Prep Kit (Illumina Inc.; San Diego, CA, USA). Products were purified with the AMPure XP system (Beckman Coulter; Brea, CA, USA), and the library quality and insert size were determined using an Agilent 2100 bioanalyzer (Agilent Technologies; Santa Clara CA, USA). Cluster generation of the index-coded samples was analyzed using Illumina cBot (Illumina Inc.; San Diego, CA, USA). Library preparations were sequenced on an Illumina HiSeq platform (Illumina Inc.; San Diego, CA, USA), and paired-end sequences were generated.

For metagenome assembly, MEGAHIT (Li et al., 2015) and *Soapdenovo* 2.21 (Luo et al., 2012) were used to clean the data. The interrupted scaffolds were produced at N to obtain the scaftigs. *SoapAligner* 2.21 was preferred for mapping the clean data to scaftigs, unutilized paired-end reads were obtained and all reads were then assembled together with the same assemble arguments. Scaftigs of about 500 bp were filtered for further analysis. For gene prediction and abundance analysis, scaftigs were used for open reading frame (ORF) prediction using MetaGeneMark 2.10 (Zhu et al., 2010), and the ORFs were dereplicated using CD-HIT 4.5.8 (Fu et al., 2012) to create a gene catalogue. The total number of mapped reads and gene length were used to calculate the gene abundance with the computational formula of Oh et al. (2014). In taxonomy annotation and identification, sequences of bacteria, fungi, archaea and viruses were aligned with the 16S/18S/ITS amplicon sequences in the NR database by DIAMOND (Buchfink et al., 2015) and BLASTX (National Center for Biotechnology Information; Bethesda, MD USA). The DIAMOND software was 20,000 times faster than BLASTX for similar sensitivity, especially on short reads with an e-value ≤ $1 \times e^{-5}$. The BLASTX data that resulted in an e-value of less than 10 folds of the minimum e-value were selected for sequential analysis. In sequential analysis, unigenes were assigned using the lowest common ancestor algorithm of MEGAN (Huson et al., 2007) for the classification tool. Blastp was performed using DIAMOND against different databases for each unigene. The relative abundance was ascertained to use each read's k-mer index value sum based on its relative abundance index model, according to the calculation presented in Ai et al. (2018).

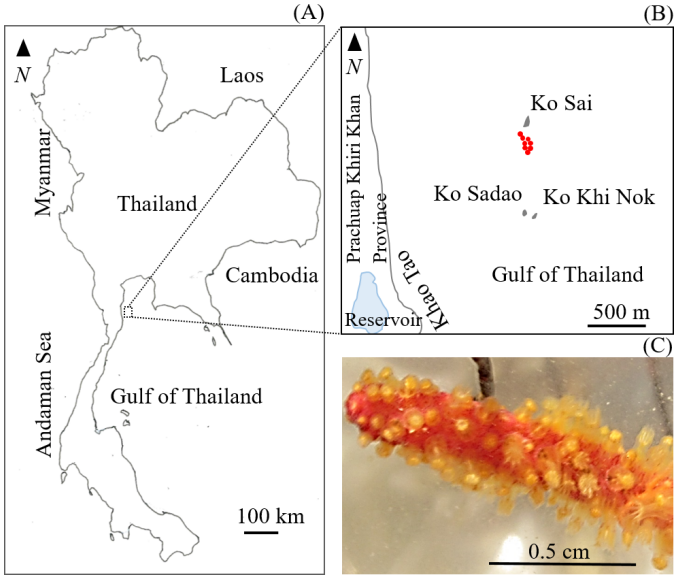

Fig. 1 (A) Locality for collected *E.* cf. *pulchra*; (B) gorgonian sampling (red circles) between Ko Sai and Ko Sadao and Ko Khi Nok in Prachuap Khiri Khan province, Thailand; (C) gorgonian fragment

Orthologous groups and functional annotation followed the database of the evolutionary genealogy of genes: Non-supervised Orthologous Groups with eggNOG version 4.1 (Powell et al., 2014). The evolutionary genealogy of genes: Non-supervised Orthologous Groups was composed of 24 functions. The Kyoto Encyclopedia of Genes and Genomes was used for annotations, associated molecular pathways and gene functions (http://www.genome.ad.jp/kegg/). It exhibited 6 large pathways and 43 seed pathways. The carbohydrate-active enzyme database was applied to annotate the five different enzyme classes using CAZy version: 2014.11.25 (Cantarel et al., 2009). It represented six functions. Experiments and bioinformatics analyses were conducted at Novogene Bioinformatics Technology Co., Ltd., Beijing, China. All molecular data sequences were deposited in the Sequence Read Archive of NCBI with the accession numbers: PRJNA611890, SAMN14351742, SRS6302699 and SRR11287854.

For scanning electron microscopy with energy dispersive X-ray spectroscopy, the fragment samples of corals were moved from 4% glutaraldehyde (Sigma-Aldrich; St. Louis, MO, USA) to 0.1 M phosphate buffer for washing and re-fixed in 1% osmium tetroxide (Merck; Darmstadt, Germany) solution in distilled water at room temperature for 30 min. They were washed with distilled water several times, dehydrated with a grade series of ethanol (50%, 60%, 70%, 80%, 90%, 95% and 99.99% for 15 min at each concentration), and dried using a critical point dryer (Quorum Technologies; Laughton, UK) at 1500 Pa. Specimens on aluminum stubs were coated with Pt/Pd using a sputter-coater operation (SPI Supplies; West Chester, PA, USA) and were viewed using a scanning electron microscope (SEM; SU-8010; Hitachi; Tokyo, Japan) operating at 15 kV. X-ray element analysis was performed using an XFlash 6 detector (Bruker; Billerica, MA, USA) under a high-vacuum mode. The accelerating voltage was applied at 20 kV for energy dispersive X-ray spectroscopy (EDX) images, with 60 s for the counting time.

Results

Based on the taxonomic annotation for the microbial communities, the kingdom Bacteria was more predominant than the Eukaryota, Viruses or Archaea. At the phylum level, high values of relative abundance were identified for the Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Planctomycetes and Candidatus Tectomicrobia. At the class level, the Gammaproteobacteria was the highest among the other Bacteroidia, Alphaproteobacteria, Flavobacteriia, Planctomycetia, Chytridiomycetes, Clostridia and Cytophagia. At the order level, the Vibrionales was the predominant community, followed by the Marinilabiliales, Flavobacteriales, Rickettsiales, Thiotrichales, Clostridiales, Cytophagales and Enterobacterales, respectively. At the family level, the highest relative abundance was in the Vibrionaceae community, followed by the Marinilabiliaceae, Flavobacteriaceae, Anaplasmataceae, Piscirickettsiaceae, Enterobacteriaceae, Cytophagaceae and Enterocytozoonidae respectively. At the genus level, *Vibrio* was dominant with other genera being *Wolbachia*, *Labilibacter*, *Candidatus Entotheonella*, *Flavobacterium*, *Cycloclasticus*, *Epulopiscium*, *Saccharicrinis* and *Anaplasma* (Fig. 2). The bacteria (with a relative abundance value > 0.00035) were identified at the species level represented by: *Labilibacter marinus*, *Saccharicrinis fermentans*, *L. aurantiacus*, *Acinetobacter baumannii*, *Marinifilum fragile*, *Marinilabilia salmonicolor*, *Staphylococcus aureus*, *Geofilum rubicundum*, *Escherichia coli*, *Vibrio nigripulchritudo*, *V. parahaemolyticus*, *Aphanizomenon flos-aquae*, *Photobacterium proteolyticum*, *Sunxiuqinia elliptica*, *Oceanospirillum multiglobuliferum*, *Piscirickettsia salmonis*, *Endozoicomonas atrinae*, *Mariniphaga anaerophila*, *Prolixibacter bellariivorans*, *Pseudomonas aeruginosa*, *V. coralliilyticus*, *Flavobacteriales bacterium*, *S. dokdonensis* and *V. harveyi*.

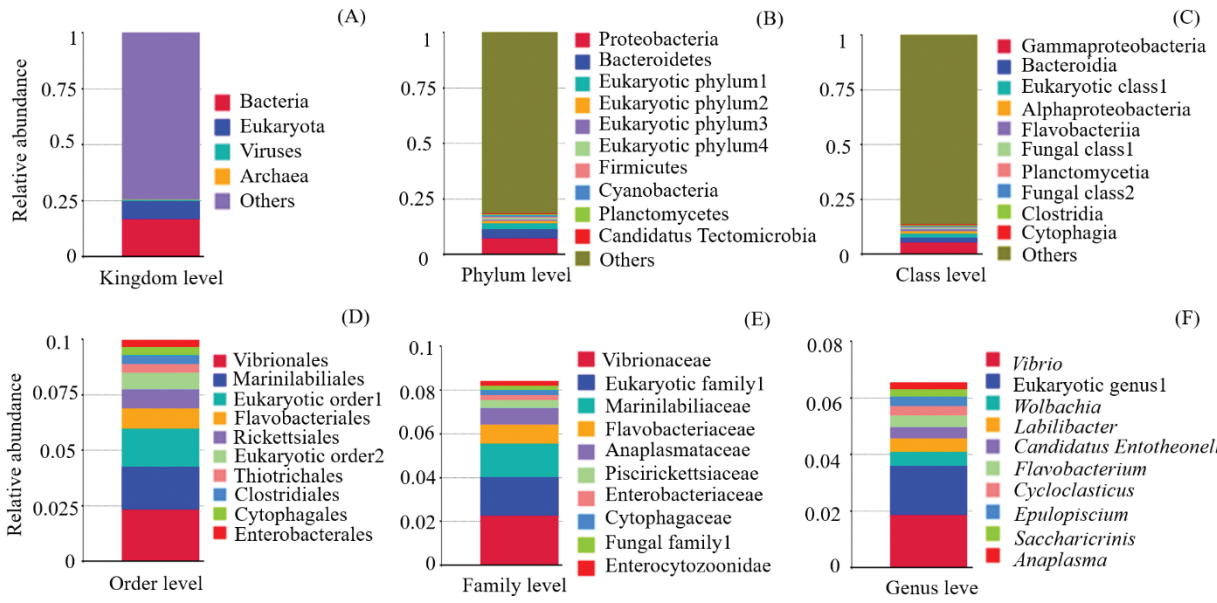

Fig. 2 Relative abundance of microbial communities from *E.* cf. *pulchra* at different levels: (A) kingdom; (B) phylum; (C) class; (D) order; (E) family; (F) genus

In other microbes, *Echinomuricea* were also dominated by fungi from the Mucorales. Nitrosopumilales, Methanosarcinales and Thermoplasmatales were detected for Archaea and Herpesvirales and Caudovirales were detected for viruses (Fig. 3).

For the annotated gene prediction, three databases were used to summarize the evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG), the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Carbohydrate Active EnZyme (CAZy). In eggNOG, replication, recombination and repair were mainly found among the known function classes as the number of matched genes from the annotated number of matched genes. However, the unknown function was the highest and nuclear structure was lowest in all classes (Fig. 4). In KEGG, the pathway annotations showed high gene numbers associated with the transport and catabolism of cellular processes, signalling molecules and interaction of environmental information processing, translation, folding, sorting and degradation of genetic information processing, neurodegenerative diseases and cancer overview of human diseases, carbohydrate metabolism of biochemical metabolism and endocrine system of organismal systems (Fig. 5). In CAZy, glycoside hydrolases, carbohydrate-binding modules and glycosyltransferases were predominant in these functional classes, while in contrast, polysaccharide lyases were the least common (Fig. 6).

Based on scanning electron microscopy with SEM/EDX, the distributions of nitrogen, magnesium, oxygen and calcium based on mean ± SD of the weight % ($n = 3$, error % < 10%) of the coenenchyma were 8.90 ± 1.50%, 6.53 ± 1.17%, 35.67 ± 4.76% and 48.61 ± 2.76%, respectively, of *E.* cf. *pulchra* (Fig. 7A–7D). On the axis of corals, nitrogen, magnesium, oxygen and calcium based on mean ± SD of the weight % (n = 3, error % < 10%) were 15.26 ± 4.94%, 6.71 ± 2.28%, 33.80 ± 3.14% and 44.23 ± 6.67%, respectively. Calcium was present throughout the sclerites of coenenchyma but was rarely distributed on the axis of corals. Oxygen and nitrogen were observed in all surface fragments; however, the magnesium signal was low (Figs. 7E–7H).

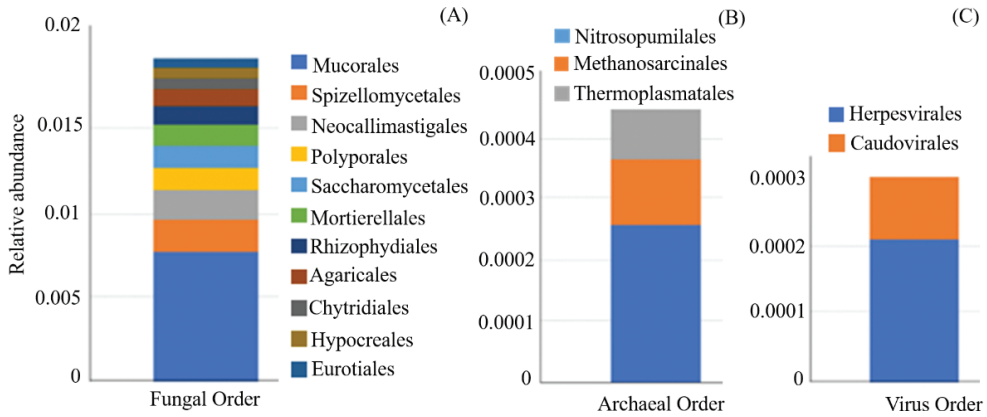

Fig. 3 Relative abundance from *E.* cf. *pulchra* at order level of: (A) fungi; (B) archaea; (C) viruses

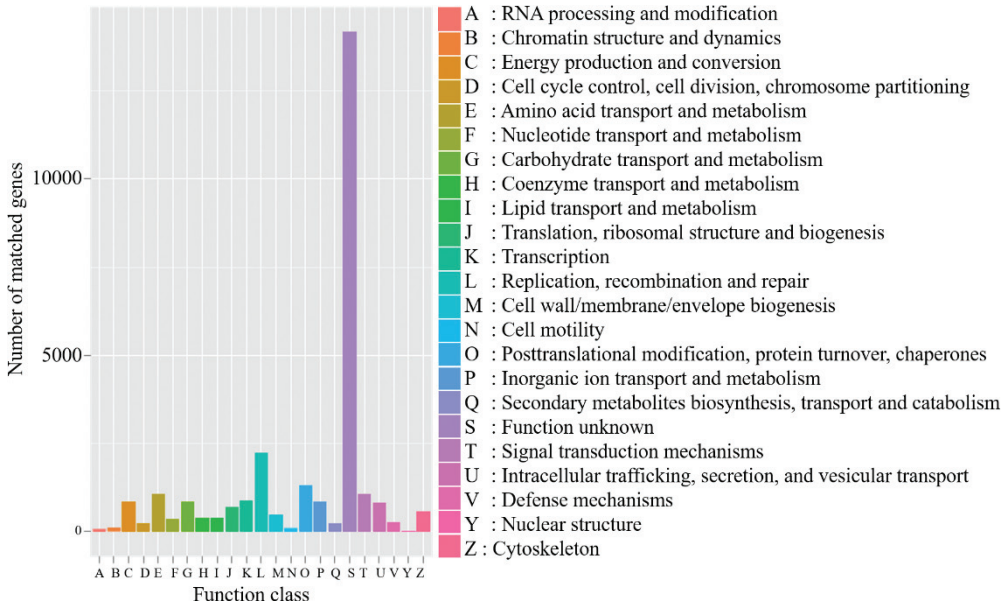

Fig. 4 eggNOG database annotation numbers of unigenes by function class from all microbes associated with *E.* cf. *pulchra*

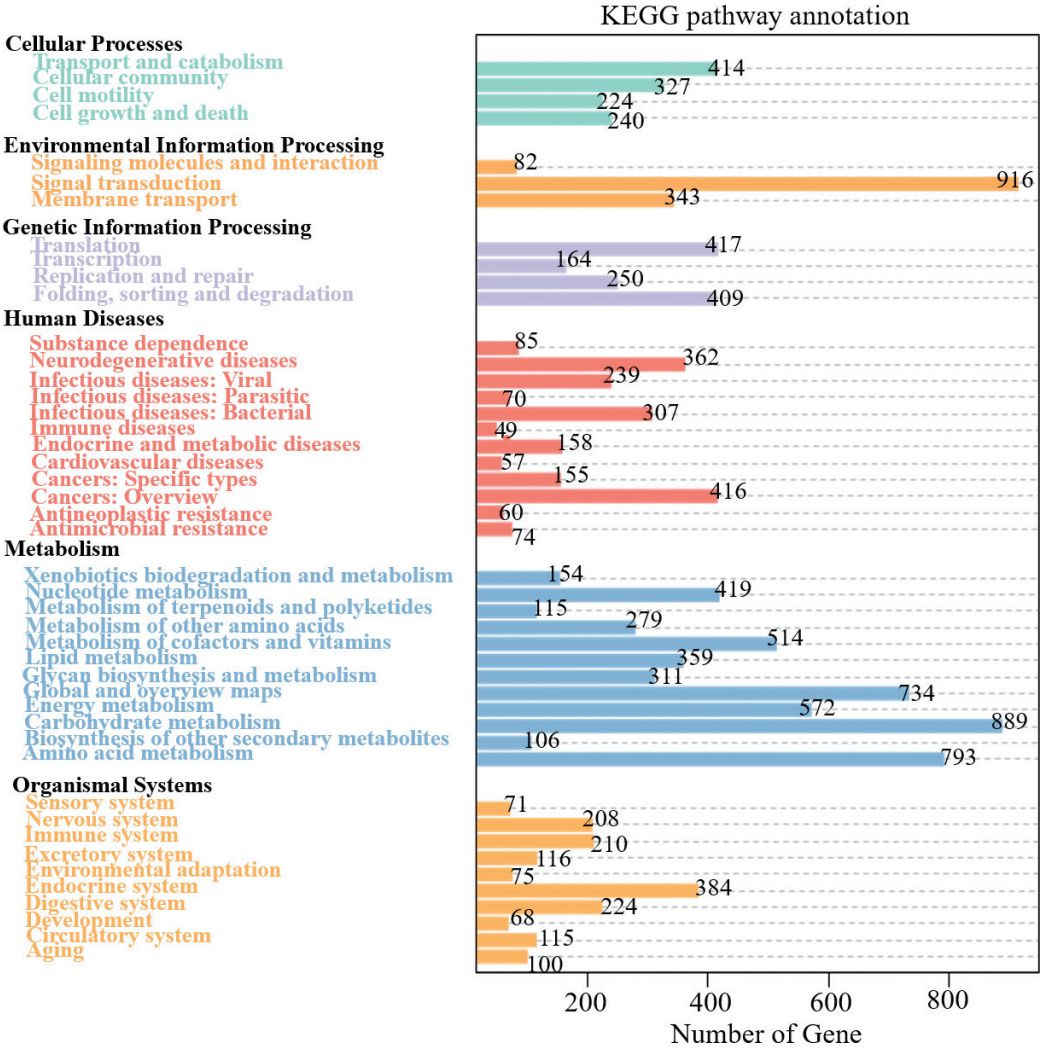

Fig. 5 KEGG pathway-classes of gene number from microbes associated with *E.* cf. *pulchra*

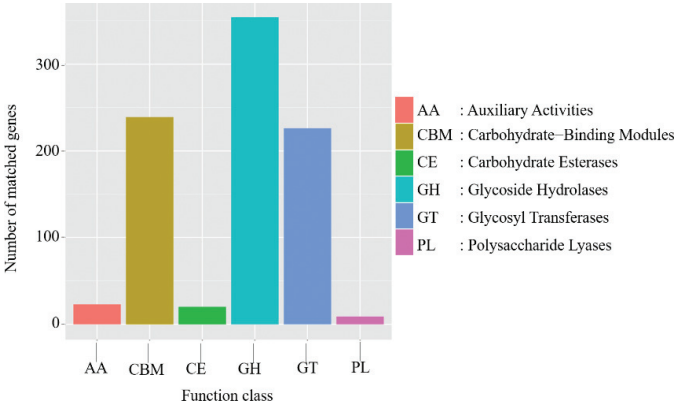

Fig. 6 CAZy database of gene number and function class from microbes associated with *E.* cf. *pulchra*

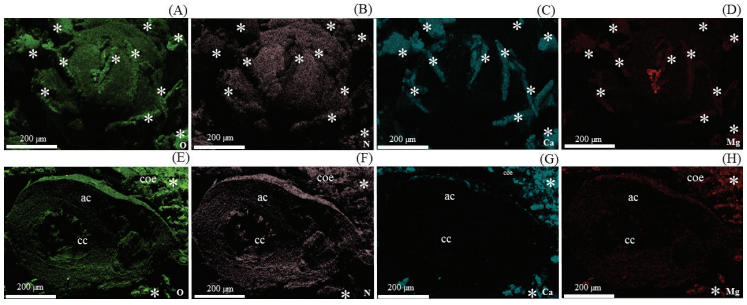

Fig. 7 SEM-EDX images of for oxygen, nitrogen, calcium and magnesium, respectively, in *E.* cf. *pulchra*: (A–D) distribution of elements of coenenchyma; (E–H) distribution of elements on coral axis, where coe = coenenchyma, ac = axis of coral, cc = central chord and asterisk indicates sclerites

Discussion

In this study, the Gammaproteobacteria of the Proteobacteria from coral tissue samples were predominant among the microbial classes. The Gammaproteobacteria class was dominated by the Vibrionaceae, Marinilabiliaceae and Flavobacteriaceae, with high populations of *Vibrio* and *Wolbachia* being identified. Lawler et al. (2016) reported that the percentages of relative abundance of the Proteobacteria and Spirochaetes were high, and the order was dominated by the Oceanospirillales in *Anthothela grandiflorum* from the deep sea of the Atlantic Ocean. Hernandez-Agreda et al. (2018) showed that the Gammaproteobacteria was the dominant class in *Pachyseris speciose* and *Acropora aculeus* from the Great Barrier Reef and the Coral Sea. Vezzulli et al. (2013) indicated that dominant communities of *Endozoicomonas* belonging to the order Oceanospirillales were associated with a healthy purple gorgonian (*Paramuricea clavate*) from pristine locations in the Mediterranean Sea. Bayer et al. (2013) provided data on the microbial communities in the Mediterranean gorgonian *Eunicella cavolini* showing that the genus *Endozoicomonas* within the Gammaproteobacteria was dominant. Robertson et al. (2016) identified a core microbiome of Bahamian *Antillogorgia elisabethae* dominated by the Rhodobacteriales, Rhizobiales, Flavobacteriales and Oceanospiralles. The current results of microbes from *E.* cf. *pulchra* in shallow seawater corresponded to several bacterial communities presented in the above reports. However, these other reports were not rich in the bacterial communities noted in the current study and were dominated by *Vibrio* spp. including *V. coralliilyticus*, which is known as a pathogenic bacterium in several coral genera (Ushijima et al., 2018). In the current study, *V. coralliilyticus* was also detected in all samples of *E.* cf. *pulchra*, although the relative abundance was low. However, this suggested that the vitality or health of these gorgonians may be decreased by an infection with *V. coralliilyticus*.

In corals with fungal symbiosis, *Aspergillus* and *Penicillium* of the Eurotiales were the most common genera in the sea fan *Gorgonia ventalina* (Toledo-Hernández et al., 2007). Soler-Hurtado et al. (2016) reported that aspergillosis in gorgonian octocorals was caused by *A. sydowii*. Hewson et al. (2012) demonstrated that DNA viruses were similar between healthy and diseased tissues of *G. ventalina*. In the coral-associated Archaea, *Nitrosopumilus maritimus* was the dominant Nitrosopumilales in mucus of corals from Israel and Australia (Siboni et al., 2008). In the current study, the Eurotiales dominated in *E.* cf. *pulchra*, which might indicate the health of this coral. However, they do not indicate pathogenic fungi or virus infection since the species level was not identified. In addition, the Nitrosopumilales may be a core archaeal population of *E.* cf. *pulchra*.

In the gene prediction of microbial communities, Carlos et al. (2014) examined the metagenome of the microbial diversity of corals from Buzios Island, Brazil by comparing the reference databases of SEED and Pfam; examples showed that clustering-based subsystems, RNA processing and modification, the plan-prokaryote DOE project, phages and prophages, and protein biosynthesis were enriched in the metagenome of *Madracis decactis*. Furthermore, Badhai et al. (2016) reported on the microbial communities of *Fungia echinata* from the Andaman Sea and found that protein biosynthesis, resistance to antibiotics and toxic compounds, and central carbohydrate metabolism were predominant functional profiles compared with SEED subsystems, and two-component systems (ATP-binding cassette transporters and purine metabolism) were abundant in the KEGG pathway. The predominant metabolism of carbohydrates and amino acids corresponded to microbes associated with *E.* cf. *pulchra* from tropical seawater in the current study, and all functional characterization included the bacteria, fungi, archaea and viruses of these gorgonian tissues.

In terms of the eggNOG database, unknown functions have been detected in several studies of the microbial metagenome in metazoan and mammal hosts (Qin et al., 2015; Thirugnanasambandam et al., 2019; Thomas and Segata, 2019). In the current study, the unknown functions of microbes were also predominantly unigenes in gorgonians. This suggested that the unknown functions might be required for those microbes living in *E.* cf. *pulchra*. Furthermore, they might supply the genomic sources to understand the further evolutionary function of processes in corals associated with microsymbiosis. KEGG annotation might provide insight into microbiome-mediated processes in corals and the ecosystem structure (Hernandez-Agreda et al., 2018; Glasl et al., 2020). Therefore, signal transduction and carbohydrate metabolism might be the main pathway of environmental processes and metabolism, respectively, for the coral microbial communities in *E.* cf. *pulchra*. The Caudovirales and Herpesvirales are viral families detected in corals (Wood-Charlson et al., 2015; Weynberg et al., 2017). In the current study, both viral families were found, corresponding to the results of infectious diseases in KEGG pathway-classes. Thus, the health of *E.* cf. *pulchra* may be determined by using KEGG. In the CAZy database, the families of catalytic and functional domains included in the enzyme source are described (Cong et al., 2017). Carbohydrate-binding modules are present in microbes, such as bacteria, archaea and fungi (Abbott et al., 2008). Glycoside hydrolases are known as a group of enzymes found in almost all organisms (Naumoff, 2011). Glycosyltransferases represent a large group of enzymes that play roles in many crucial biological processes (Ovchinnikova et al., 2016). Carbohydrate-binding modules, glycoside hydrolases and glycosyltransferases were the predominant functions in microbes in gorgonians. These results suggested that carbohydrate-binding modules, glycoside hydrolases and glycosyltransferases might be important functions and enzyme sources for coral microbes co-habiting with *E.* cf. *pulchra*.

Mansur et al. (2005) showed that the microprobe spectra of calcium were high and detected strontium in coral samples from the Brazilian southeast coast. Al-Sawalmih (2016) found that levels of carbon, oxygen and calcium were high among the elements of fragments from *Stylophora pistillata*. Rahman and Oomori (2008) also reported that calcium and magnesium were predominant elements in the sclerite of the soft coral *Sinularia polydactyla*. These findings were consistent with the current calcium results. Therefore, the current study confirmed that calcium and oxygen are the main elements of sclerite in *E.* cf. *pulchra*.

Microbes have been reported in corals as coral-associated

microbes involved in the oxygen and nitrogen cycles and of another trace element (Gregg et al., 2013; Li et al., 2013; Yang et al., 2019). The main microbial communities of *E.* cf. *pulchra* were consistent with those previously reported. This may suggest that they are core microbes for the coral holobionts. In the dinoflagellates, *Symbiodinium* is known as an important diazotroph for the nitrogen cycle in corals such as *Stylophora pistillata*, *Acropora* sp. and *Pocillopora damicormis* (Lema et al., 2012; Sorek et al., 2013; Rädecker et al., 2015; Lesser et al., 2018). However, *Symbiodinium* or members of the Dinophyceae were not found in *Echinomuricea* in the current study perhaps because *E.* cf. *pulchra* might be associated with other symbiotic diazotrophs for nitrogen fixation. Magnesium plays a crucial role in microbial growth in the ocean, including the development of the coral skeleton (Meibom et al., 2004; Heldal et al. 2012). It is well-known that calcium is a major mineral for scleractinian coral skeletons (Goffredo et al., 2011). In prokaryotes, calcium ions also play an important role in the cell biology and physiology of bacteria and archaea (Campbell, 2014). In the current study, the signals of magnesium and calcium were incongruent in sclerites and on the axis, while in addition, the magnesium intensity was low. Therefore, these results suggested that microbial communities may not be endosymbiont and might not be associated with *E.* cf. *pulchra* for magnesium and calcium utilization.

The current study was the first to report on the microbial communities of gorgonian *E.* cf. *pulchra* associated with chemical depositions of this coral from shallow seawater in the western coast of the Gulf of Thailand. The functional profiles of gene prediction were analyzed in the microbiome of gorgonians using comparisons with reference databases.

Ethics Statements

All animal experiments were conducted under the National and Institutional Guidelines for the Animal Care and Use for Vertebrates by the Institute for Animals for Scientific Purpose Development (IAD) National Research Council of Thailand (NRCT) and according to the Thai Wild Animal Reservation and Protection Act, 1992.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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