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Contributed Paper

16S rRNA Gene Sequencing Assessment of the Prokaryotic Communities in the Southeast Andaman Sea, Thailand and Potential Environmental Alerts

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ABSTRACT

The microbial community in an ecosystem can be a guide to its environmental condition. This research utilized culture-independent 16S rRNA gene sequencing to obtain preliminary profiles of marine prokaryote communities at 15 km (PT) and 52 km (TC) offshore of the coastal Andaman Sea, Phang Nga, Thailand. The more inland, PT site, exhibited lower prokaryote biodiversity and metabolic potentials than the TC site further offshore. The presence of a relatively high level of nitrite oxidizing Nitrosospira at PT (6.34%) raises an alert for potential coastal nitrogen pollution. The presence of selenium-reducing Sedimenticola at TC (8.35%) suggested an unusual presence of selenium that might be caused from underwater seismic activity, as a minimum of four tsunami events have occurred in this area previously. Monitoring these microorganisms may help understand the marine microbial communities around the tsunami-effected area and also the underwater sediment resuspension level. Our sequencing data highlighted the unique hydrography at this oceanographic position comprising freshwater from India and mangrove forest water runoff from Thailand, together with underwater land-slip. Global ocean comparison indicated bacterial diversity around TC and PT, similar to two other sites off Thailand (Tha Wang, Tham Phang and differing to that from the distant GS012, GS110b and GS049 sites. The potentially abundant microbial metabolic subsystems in TC supported its high biodiversity, whereas the missing metabolic subsystems in PT might require vigilant monitoring of the condition of Thai Andaman coast. Compared with the culture-dependent method, more than 26-fold number of species were recorded. Moreover, many cultured isolates were not the major species in the culture-independent profile. The data supported the significance of the culture-independent approach in obtaining the more comprehensive microbial databases.

Keywords: Southeast Andaman Sea, metagenomics, 16S rRNA, pyrosequencing, tsunami

1. INTRODUCTION

The microbial community in an ecosystem can be a guide its environmental condition. Microorganisms are ubiquitous and comprise the vast majority of species on earth. They play major roles in every ecosystem. Studies have reported their complex food network with physical and biological factors, for examples, temperature, nitrogen availability and tsunami occurrence. These factors affected the diversity and composition of the microbial communities [1-3], that might disturb traditional species-species interaction and the metabolic potential of the ecosystems. Studies reported that changes in the microbial community could contribute to shaping of the ecosystem, e.g., in coral ecosystems certain bacteria were reported to help sustain global warming [1]. Nonetheless, the microbiome databases are still missing in most regions of the world.

The Andaman Sea located in the Indian Ocean is biologically rich. It has one of the highest aquatic life abundances, and yet is one of the 25 endangered ecoregions on Earth [4]. Although the Andaman Sea represents just one third of Thailand's coastline, more than half of the country's coral reefs are along this coastline. Despite the 2004 Indian Ocean tsunami, the water, coral reefs and mangrove forests in the southeast Andaman Sea off the west coast of Thailand remain naturally fruitful, with several locations nominated for UNESCO World Heritage sites [5]. The area is home to many fish species, and over 300 species of corals and coral-associated animals were found [6,7]. Further, the Southeast Andaman Sea comprises a unique hydrography, of stratified inflow freshwater from the Irrawaddy River (India) and nutrient-rich water runoff from the mangrove forests of Thailand [6,8,9].

Indeed, in this area four tsunami events have occurred previously. The two most recent ones were in 2004 and the other approximately 600 years ago [10]. These events partly damaged the coral ecosystems while our knowledge about this ecosystem remains limited with no database of the microbial communities and their metabolic potentials. Such information is crucial to help understand and monitor this sensitive ecosystem. Therefore, the present study evaluated, for the first time, the current prokaryotic population structures and the potential metabolic networks at two representative sites that are different in term of their offshore distances within the southeast Andaman Sea, and has been affected by the previous tsunamis. The comparisons between these microbial communities as well as to the rest of the world help reveal the Andaman Sea microbiota and aquatic food networks.

Culture-independent and culture-dependent methods have been utilized to determine biodiversity and the relative abundance of microbes. The present study employed a popular culture-independent, 16S rRNA gene pyrosequencing method to obtain this marine prokaryotic database. This method is more comprehensive for constructing a microbiome database than the traditional culture-dependent approach. The culture-independent method has been used to create databases of various environments, such as freshwater, lakes, oceans, and soil [3,11-13]. Our study also included a culture-dependent method for bacterial diversity in one sample site, allowing a parallel comparison of the results between the two approaches.

2. MATERIALS AND METHODS

2.1 Sample Collection

The two marine sample sites are PT site located 15 km offshore (N8.9334 E98.0976) and TC site located 52 km offshore (N8.8516 E97.5201) of the southeast Andaman Sea coast, Thailand (Figure 1). This research area did not require any ethical approval. Water samples were collected at a depth of ~30 m below the sea surface on the 7th and 10th January 2013 between 14:00-17:00 hrs. For each site, three independent samples of 20 L of seawater were collected. On-site water temperature, conductivity, salinity, dissolved oxygen and pH were measured.

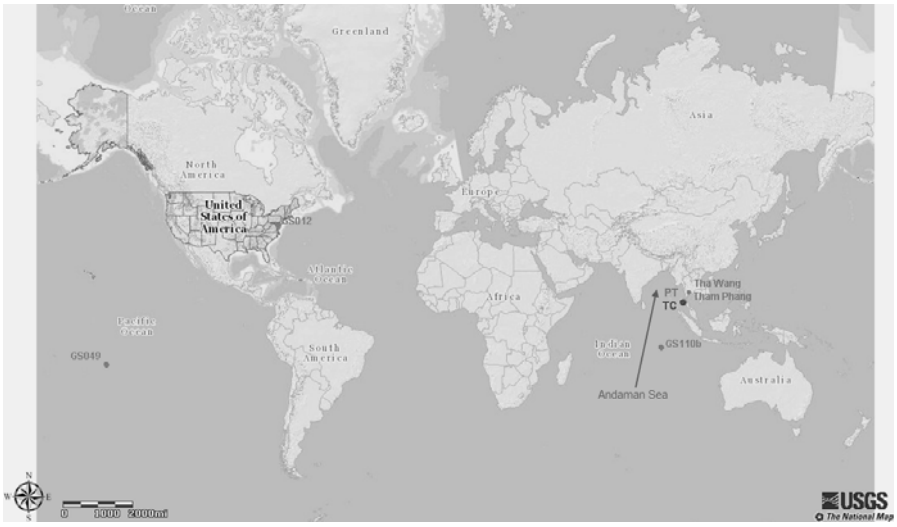


Figure 1. Oceanographic positions of the PT and TC sites in the southeast Andaman Sea, relative to coastal Tha Wang and Tham Phang, GS012, GS110b and GS049 sites. The map is from the USGS National Map Viewer (public domain), retrieved in May 2014.

2.2 Metagenomic DNA Extraction and Quality Examination

Each sample was filtered through a four-layered sterile cheesecloth to remove organisms and debris of ≥ 30 *m*m in diameter sizes. Then the filtrate was filtered through a 0.22 *μ*m filter (Merck Millipore, Massachusetts, USA) to trap microorganisms of ≤ 0.22 *μ*m, and the total DNA was extracted using the Metagenomic DNA Isolation Kit for Water (Epicentre, Wisconsin, USA) [14]. The isolated metagenomes had an apparent size of ~40 kb as recommended by the manufacturer. The DNA concentration and quality were analyzed by agarose gel electrophoresis and absorbance at 260 and 280 nm, respectively.

2.3 Pyrotagged 16S rRNA Gene Library Construction and Pyrosequencing

The universal (conserved across genera) prokaryotic primers 338F and 786R, which target the V3 and V4 regions of the 16S rRNA gene [14-16], with appended 5' *ACATCG AG*-and *TCTACTCG*-tagged barcodes [17], were used to construct the amplicon libraries of the PT and TC samples, respectively. Each 25 *m*l PCR reaction comprised 1 × EmeraldAmp® GT PCR Master Mix (TaKaRa, Shiga, Japan), 0.3 *μ*M of each primer and 100 ng of metagenomic DNA. The thermal cycling profile was 4 min at 95 °C followed by 30 cycles of 94 °C for 45 s, 50 °C for 50 s and 72 °C for 90 s, and then a final extension at 72 °C for 10 min.

For each of the individual triplicate sample, three PCR reactions were performed. The PCR products from the triplicate samples were pooled, purified by PureLink® Quick Gel Extraction Kit (Invitrogen, New York, USA) and pyrosequenced on an eight-lane Roche picotiter plate with the 454 GS FLX system (Roche, Branford, CT) according to the manufacturer's protocols.

2.4 Community Structure Evaluation of PT and TC by Culture-independent 16S rRNA Gene Library Analysis

The 16S rRNA gene sequences obtained were classified as derived from the PT or TC site according to the appended barcode sequence. Sequences of less than 50 nucleotides in length (excluding the primers and the barcode length) and those that did not pass the quality threshold cutoff by the sequencing machine were discarded. An annotated read is defined a read with $\leq 10^{-4}$ E-value for PT and $\leq 10^{-2}$ E-value for TC, by BLASTN against NCBI non-redundant [18], RDP [19] and Greengenes [20] databases. The PT and TC profiles were compared with those from two representative coastal marine sites in Thailand (Tha Wang and Tham Phang) [14] and 73 GOS sites (GS000a to GS149: https://portal.camera.calit2.net/gridsphere/gridsphere) [21,22], using the Yue & Clayton theta similarity coefficient (Thetayc) and Bray-Curtis dissimilarity index (Bray-Curtis) in mothur [23]. The closer the similarity coefficient is to 0.000, the higher the similarity between the compared pair of community structures. The metabolic subsystems and functional groups were predicted based on the available mg-RAST database for metabolic subsystems and functional groups of different bacteria species [24,25].

2.5 Community Structure Evaluation of TC by Culture-dependent Bacteria Plate Culture and Species Identification

Bacteria in the TC samples were cultivated by direct and concentrate plate count methods. For the direct plate count, seawater was 1/10 serially diluted with 0.85% (w/v) NaCl and 0.1 mL of each diluent (10^{0}-10^{-4}) was inoculated in duplicate onto solid salt nutrient agar (SN; 2.7% (w/v) NaCl, 0.3% (w/v) beef extract, 0.5% (w/v) peptone, 1.5% (w/v) agar) [26]. For the concentrate plate count, 500 mL of seawater was poured through a four-layered sterile cheesecloth and a 0.22-μm filter membrane. The microorganisms were washed from the filtered membrane in 0.85% (w/v) NaCl, 1/10 serial diluted (10^{0}-10^{-4}),. and 0.1 mL of each diluent was inoculated in duplicate onto SN agar. Cultures were incubated at 28-30 °C for 1-5 d. Colonies with unique morphologies were streaked to isolate colonies, and species were identified by their morphology, Gram strain and sequence analysis of the V3 to V4 fragment of the 16S rRNA gene, using the primers as outlined above. Each amplified product was commercially Sanger-sequenced using ABI3730XL (Macrogen Inc., Korea) instrument. Species were identified by BLASTN with 10^{-4} E-value cut off and NCBI non-redundant database.

3. RESULTS AND DISCUSSION

3.1 Seawater Characteristics at PT and TC Sites

Despite similar latitude from the equator and water depth, the farther offshore (TC) site had a higher temperature, dissolved oxygen and alkalinity (pH) than the PT (Table 1). The latter two properties might be indicative of a more suitable environment for marine organisms at the TC.

3.2 Culture-independent Analysis of Prokaryotic Diversity at PT and TC Sites

The PT and TC metagenomic DNA concentrations were 23.99 and 14.01 ng per L of seawater, and the metagenomic DNA quality (A260/A280) was 1.86 (PT) and 1.77 (TC), respectively. The 16S rRNA gene fragment libraries were constructed successfully, in terms of amplicons of the appropriate size, and pyrosequencing resulted in a respectable yield and average read length. For the PT site, 13,693 total reads with average read length of 288 bp were retrieved, of which 13,607 reads (99.37%) were annotated by BLASTN with significant E-values. For the TC, 10,808 total reads with 275 bp average sequence length, of which 10,701 reads (99.01%) were annotated.

Table 1. Characteristics of the seawater at PT, TC, Tha Wang, Tham Phang, GS110b, GS049 and GS012 sites.

Station name	Site description	Latitude (N)	Longitude (E)	Sample depth (m)	Temperature (°C)	Salinity (ppt)	Conductivity (mS/cm)	pH	Dissolved oxygen (mg/L)
PT	15 km offshore from Andaman coast,	8.9334	98.0976	30	27.8	32.7	53	7.79	5.02
TC	Phang Nga, Thailand 52 km offshore from	8.8516	97.5201	30	28.4	32.7	53	8.04	6.87
Tha Wang	Andaman coast, Phang Nga, Thailand < 1 km offshore	13.094	100.495	< 1	29.7	32.3	72	7.03	N/A[a]
Tham Phang	from Sichang Island, Chonburi, Thailand < 1 km offshore	13.084	100.483	< 1	30.5	32.8	66	7.39	N/A
GS012[b]	from Sichang Island, Chonburi, Thailand	38.947	-76.417	13.2	1.0	3.5	N/A	N/A	N/A
GS110b[b]	Estuary, Chesapeake Bay, Maryland, USA	-10.446	88.303	1.5	27.0	32.7	N/A	N/A	N/A
GS049[b]	Open ocean, Indian Ocean < 1 km offshore from Cook's Bay, Moorea, French Polynesia, France	-17.453	-149.799	1.2	28.8	32.6	N/A	N/A	N/A

[a]N/A represents data not available.

[b]The data for the latter three marine sites were from https://portal.camera.calit2.net/gridsphere/gridsphere.

PT and TC communities mainly comprised bacteria, particularly Proteobacteria (PT 72.4%, TC 76.87%), meanwhile a minor proportion of archaea (PT 0.04%, TC 0.04%) were present (Table 2). This was consistent with previous reports of 0-0.4% archaea in general marine environments [21,22]. The different species distributions between the two sites and the greater species biodiversity at the TC than at the PT site, was consistent with its higher pH and dissolved oxygen (Table 1). For example, members of the phyla Bacteroidetes, Deferribacteres, Gammatimonadetes and Chlorobi were more common in PT than TC, whereas Nitrospirae and Acidobacteria were more common in TC (Table 2). The PT site contained 275 genera, of which 122 genera (44.4%) were found only at PT. The TC site contained 309 genera, of which 156 genera (50.5%) were found only at TC (Figure 2). The significant abundance of *Sedimenticola*

in the TC site (8.35%), but not present in the PT site and other typical marine microbial communities (Figure 2) might represent an interest. Unique bacteria like *Sedimenticola* can utilize selenium oxyanions from toxic selenium ions, and hence are not generally found since selenium is naturally scarce (less than 0.0001% (w/v) in marine and terrestrial environments) [3,14,21,22]. Accumulated amounts of toxic selenium, which is teratogenic, is found under certain circumstances, such as underwater sediment erosion from the California Coastal Ranges to the San Joaquin Valley, drainage from mines, and combustion of coal and fuel oil [27]. Given that none of mines, coal nor fuel combustion are located around the TC site but the tsunami occurrences, the high proportion of *Sedimentcola* supports the potential accumulation of selenium ions due to tectonic plate movement, associated with the 2014 and 600 yo tsunami events in this area [10]. Monitoring of *Sedimenticola* might help assess tsunami-evolved microbial community and underwater seismic disturbances, and our microbial profiles helped assess the effect of toxic selenium on the marine biodiversity from the microbial and their interacting food networks perspectives. Another interest in our microbial profiles is the significant presence of *Nitrosospira* in phylum Proteobacteria in the PT site (PT 6.34%, TC 2.80%) (Figure 2). Ammonia and its oxidized product, nitrite, are toxic to most organisms and high levels of assimilative nitrogen compounds cause habitat degradation, alteration of food web structures, loss of biodiversity and harmful algal blooms. *Nitrosospira* is a nitrifying bacterium that can oxidize toxic nitrite (derived from the oxidation of ammonia by members of *Nitrosomonas*) to the non-toxic nitrate that many organisms can utilize as a nitrogen source and essential component of macromolecules, such as DNA and proteins. This finding might suggest an early warning of the coastal condition near the PT site for potential nitrogen pollution [28-30], and that *Nitrosospira* play a part in recovering the ecosystem. Indeed, worldwide increase in coastal nitrogen pollution over the past 15 years has been reported, and linked to the increased terrestrial use of synthetic nitrogen fertilizer and meat production activities [1,31].

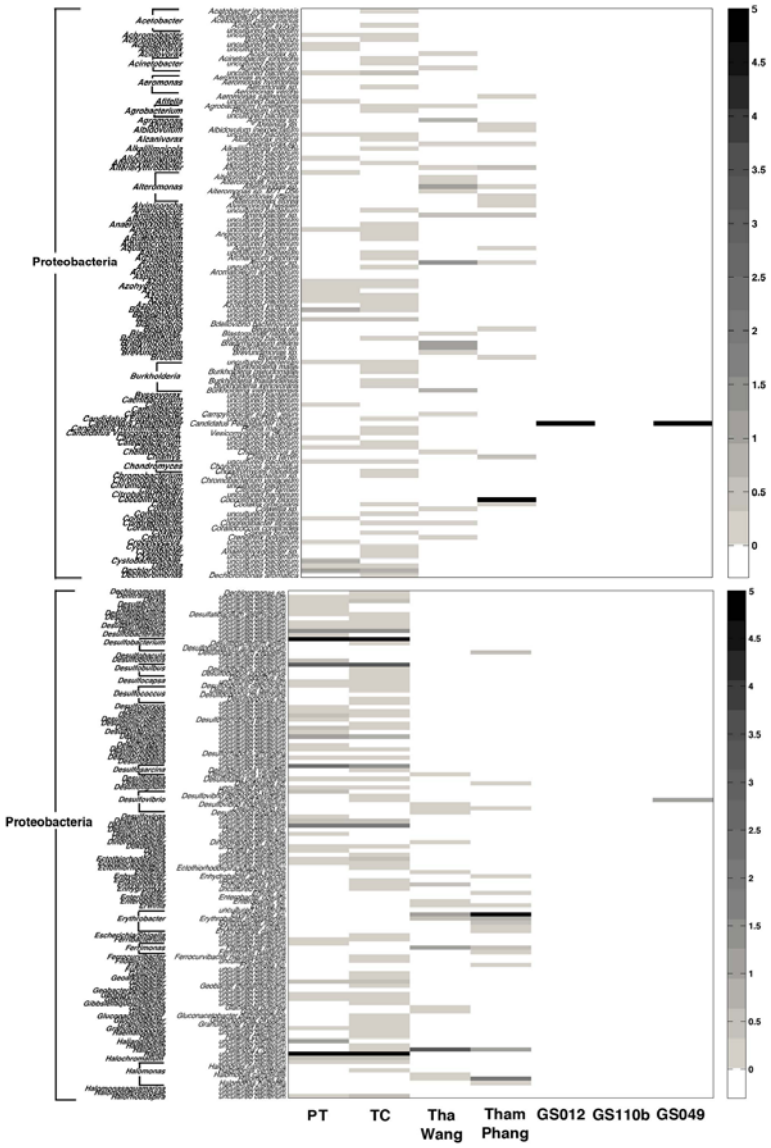
Table 2. Prokaryotic phylum distributions (% prevalence) in PT, TC, GS012, GS110b and GS049 communities.

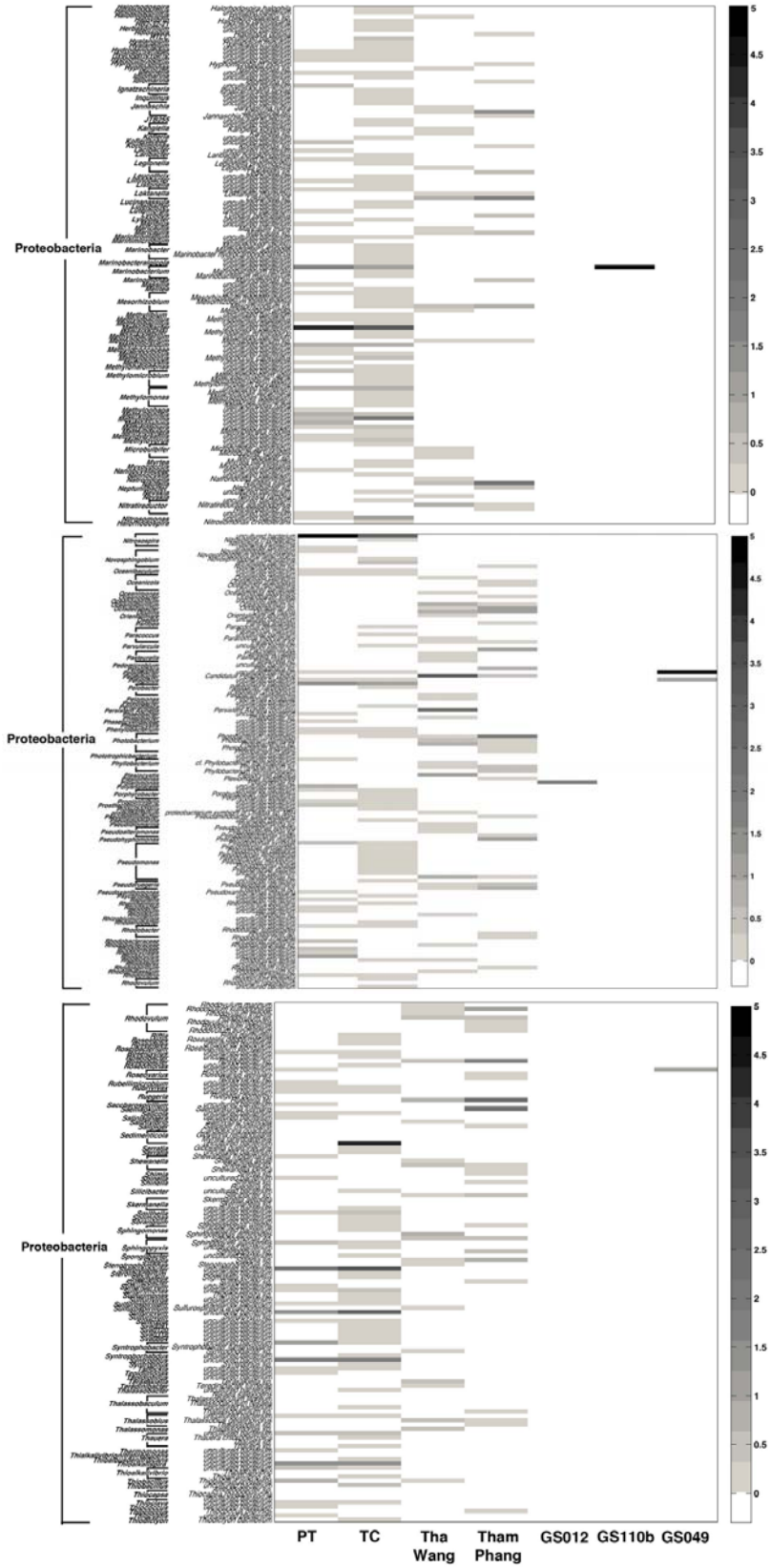
Phylum	PT[a]	TC[a]	Tha Wang[b]	Tham Phang[b]	GS012[c]	GS110b[c]	GS049[c]
Proteobacteria	72.44	76.87	65.30	87.42	35.48	5	55
Bacteroidetes	5.65	2.99	26.84	8.01	3.23	10	0
Deferribacteres	4.42	1.05	0	0	0	0	0
Gemmatimonadetes	4.27	2.38	0	0	0	0	0
Nitrospirae	2.68	8.44	0.16	0	0	0	0
Chlorobi	2.14	1.20	0.33	0	43.55	15	13
Firmicutes	1.81	1.70	2.78	1.63	0	0	0
Spirochaetes	1.67	0.78	0.33	0	0	0	0
Acidobacteria	1.59	2.36	0	0	0	0	0
Chlamydiae	1.20	0.20	0.16	0	0	0	0
Tenericutes	1.09	0.02	0	0	0	0	0
Chloroflexi	0.33	0.22	0	0	0	0	0
Thermotogae	0.25	0.04	0	0	0	0	0
Caldiserica	0.07	0.01	0	0	0	0	0
Fusobacteria	0.07	0.57	2.13	2.45	0	0	0
Lentisphaerae	0.07	0	0.16	0	0	0	0

Table 2. Continued.

Phylum	PT[a]	TC[a]	Tha Wang[b]	Tham Phang[b]	GS012[c]	GS110b[c]	GS049[c]
Verrucomicrobia	0.07	0.70	0.16	0	0	0	2
Cyanobacteria	0.04	0	0.16	0.16	0	65	24
Deinococcus-Thermus	0.04	0.01	0	0.16	0	0	0
Euryarchaeota	0.04	0.04	0.16	0	0	0	0
Planctomycetes	0.04	0	0.16	0.16	0	0	0
SAR407	0.04	0	0	0	0	0	0
Actinobacteria	0	0.30	1.15	0	17.74	0	0
BRC1	0	0.02	0	0	0	0	0
SAR406	0	0.07	0	0	0	5	6
Synergistetes	0	0.01	0	0	0	0	0
Total number of phyla	22	22	14	7	4	5	5

[a]This study
[b]Data from [14]
[c]Data from https://portal.camera.calit2.net/gridsphere/gridsphere




Proteobacteria
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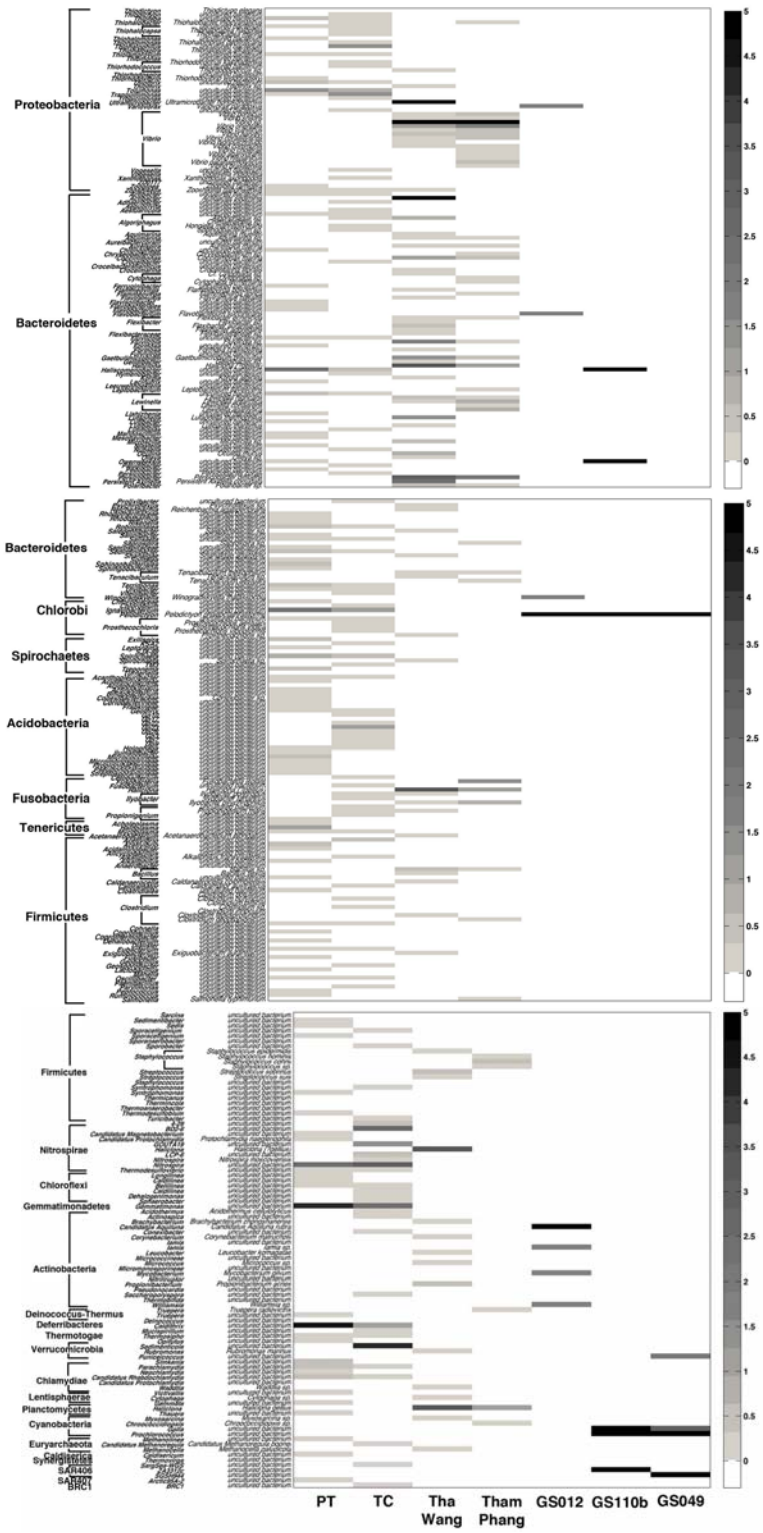

Figure 2. Prokaryotic communities (species level) at PT and TC, compared with those at Tha Wang, Tham Pang, GS012, GS110b and GS049. Percent relative abundance of each species is represented by a grey-scale gradient.

3.3 Comparison of Global Marine Prokaryotic Communities and Their Metabolic Potentials

Global comparison of marine prokaryotic communities indicated PT and TC to be closely related to each other, and to the two marine sites off the Thai coast, Tham Phang (Thetayc 0.72240, Bray-Curtis 0.99191) and Tha Wang (Thetayc 0.58495, Bray-Curtis 0.96466), followed by the more distant GS012 (Thetayc 0.97831, Bray-Curtis 0.99973), GS110b (Thetayc 0.98396, Bray-Curtis 1.00666) and GS049 (Thetayc 0.99756, Bray-Curtis 1.00015), respectively (Figure 2). GS110b and GS049 are within 2-8° latitude of PT and TC, while GS012 is ~30° latitude north where the climate is cold (seawater temperature) (Figure 1). All water samples were of < 30 m depth, supporting the sea level as another considering factor for microbial biodiversity, in addition to the oceanographic location, climate and salinity (Table 1). The low salinity at GS012 could be because it is an estuary and thus the seawater could be diluted by incoming freshwater from the river and a poor water evaporation rate due to the cold climate.

The bacterial diversity around the Thailand maritime zone was relatively large: 22 phyla each were found at PT and TC, 7 and 14 phyla at the other two Thai sites, compared with 4-5 phyla elsewhere (Table 2). While Proteobacteria were common to all sites, Firmicutes and Fusobacteria might represent one microbial diversity characteristic of the Thai maritime zone as these were absent in GS012, GS110b and GS049 (Figure 2), and many other GOS sites [21,22]. Many phyla in the PT and TC sites were thermotolerant, such as *Caldithrix* in Deferribacteres and genera in Thermotogae (Figure 2) [14, 32]. However, the PT and TC sites were considered clean, as species indicative of a polluted environment, such as Actinobacteria (PT 0%, TC 0.3%), were rare (Table 2) [33].

Analyzing potential metabolic networks of prokaryotic communities among these sites, TC and Tha Wang had relatively the most diverse (177 ontology levels from 28 subsystem classifications), followed by Tham Phang, GS012, PT, GS110b and GS049, respectively. PT had 15 fewer functional ontologies and 2 less subsystem classifications than at TC (Supplemental Table 1). Together, the lower bacterial diversity and missing metabolic function in PT compared to TC demonstrated the different marine microbiome across offshore distance from the coastal Andaman Sea of Thailand. These biodiversity data also helped visualize the ecosystem of the coast and the more offshore sites of this area from the microbiological perspective.

3.4 Culture-dependent Analysis of Prokaryote Diversity at TC Site

The traditional culture-dependent approach was performed for TC samples to allow a parallel comparison with the data by the culture-independent. 1.1×10^5 colony forming units (CFU) per mL of seawater were found for aerobic culture, and 2.0×10^3 CFUs/mL for anaerobic culture. The plate count method from direct and concentrated sampling yielded equivalent colony types. A total of 13 clones (12 different species) were isolated (Table 3). This estimate of biodiversity is 25.8-fold less than the 309 species found by the culture-independent method (Figure 2). Additionally, the species found by the culture-dependent analysis, including *Thermosipho*, *Erythrobacter*, *Micrococciniae*, *Staphylococcus* and *Paenibacillus*, were of minor groups in the culture-independent profile (Figure 2). This supported the advancement of this culture-independent technique to obtain more comprehensive microbial databases, as it does not depend on the ability

of culture medium and culture condition, but directly reflects the metagenomic DNA of all microbial species in the sample. Nonetheless, our culture-independent method that relies on independently triplicate PCRs for the 16S rRNA gene library construction may contain some PCR biases against high GC flora.

Table 3. Cultured bacteria diversity from TC sample.

Colony morphology (color, shape, margin)	Cultured condition	Gram stain	BLASTn identification	GenBank accession no.
Dark yellow, circular, entire	Aerobic	Negative cocci	*Erythrobacter* sp. IC114	AB196251.1
No color, circular, entire	Aerobic	Positive cocci	*Stenotrophomonas maltophilia* PISmvs5	KC896401.1
Dark yellow, circular, entire	Aerobic	Positive cocci	Uncultured bacterium	KF500796.1
Yellow, circular, entire	Aerobic	Positive cocci	*Micrococcus luteus* B88	EU240406.1
Cream, circular, entire	Aerobic	Positive cocci	Uncultured bacterium	DQ638472.1
Cream, circular, entire	Aerobic	Negative cocci	*Bacterium* JANV-8	KF453791.1
Very dark yellow, circular, entire	Aerobic	Positive cocci	*Micrococcineae bacterium* ST5-4	AM500680.1
White, circular, entire	Aerobic	Positive cocci	Uncultured *Staphylococcus sp.*	JQ820176.1
No color, circular, entire	Aerobic	Positive cocci	Uncultured *Paenibacillus sp.*	AM489498.1
Beige, circular, entire	Anaerobic	Positive cocci	*Micrococcus luteus* B88	EU240406.1
No color, circular, entire	Anaerobic	Negative cocci	*Paenibacillus* sp. HGH0034	JX520001.1
Yellow, circular, entire	Anaerobic	Negative cocci	*Thermosipho melanesiensis* BI429	CP000716.1
White, circular, entire	Anaerobic	Negative cocci	Uncultured bacterium	GQ467672.1

4. CONCLUSIONS

This study provided marine microbial databases for two different offshore sites (PT and TC) off the Thailand coast in the Andaman Sea to better understand the ecosystem of this area. The data demonstrated the culture-independent 16S rRNA gene sequencing as a powerful method compared with the traditional culture-dependent method, consistent with previous reports [11,21,22]. Comparative global analysis allowed an overview of the southeast Andaman Sea marine microbiota relative to other ocean microbiome, and metabolic potentials.

The necleotide sequences of this 16S

rRNA gene data were deposited in sequence read archive database of NCBI (http://trace.ncbi.nlm.nih.gov/Traces/sra/).

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