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Next-generation sequencing revealed specific microbial symbionts in *Porites lutea* with pigment abnormalities in North Sulawesi, Indonesia

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Abstract

Bacterial diseases affecting corals pose an enormous threat to the health of coral reefs. The relationship between certain bacterial species and coral diseases remain largely unknown. Pigment abnormalities are common in *Porites lutea*. Here we used Illumina 16S rRNA gene sequencing to analyze the bacterial communities associated with healthy *P. lutea* and *P. lutea* with pigment abnormalities. We observed an increase of alpha diversity of the bacterial community of *P. lutea* with pigment abnormalities, relative to healthy corals. We then identified changes in the abundance of individual operational taxonomic units (OTUs) between pigmented and healthy corals. We were able to identify eight OTUs associated with pigment abnormalities, which are possibly the causative agents of pigment abnormalities.

Key words: *Porites lutea*, pigment abnormalities, next-generation sequencing, 16S rRNA, bacterial diversity

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1 Introduction

Microbial symbionts are versatile organisms aiding in the development and health of their host (Neish, 2009; Webster and Taylor, 2012). The interaction of corals and microbes is a fundamental aspect in the dynamics on reefs (Bourne et al., 2009). Corals continuously face changing environmental conditions, both on local and global scales (Bourne et al., 2009; Wegley et al., 2007).

Some bacterial species are reportedly responsible for coral diseases (Ben-Haim et al., 2003; Cervino et al., 2004; Kushmaro et al., 2001). The identification of bacterial pathogens associated with specific coral diseases is not easy, because the causing pathogens should be isolated and confirmed to fulfill Kohl's law (Sussman, 2009). Six pathogens have been identified as causative agents for five kinds of coral diseases.

Culture-independent techniques, based on the characterization of symbiotic microorganisms associated with corals, have been used to gain a better understanding of symbiotic interactions (Morrow et al., 2012), and of the health conditions of coral systems (De Castro et al., 2010; Salipante et al., 2013).

Porites is a stony coral genus that is an accurate recorder of past marine surface conditions (Lough, 2010; Suzuki et al., 2000). *Porites* is thought to be the holobiont of bacterial pathogens, and to be easily affected by environmental stressors (Raymundo et al., 2005; Thurber et al., 2008). Pigment abnormalities are considered an inflammatory response of corals; however, the relationship between pigment abnormalities and bacterial communities remain unclear (Benzoni et al., 2010).

To illustrate the relationship between specific microbial communities and the pigment abnormalities in *P. lutea*, we analyzed the symbiotic microbial community of *P. lutea* with pigment abnormalities, and compared it with the microbial communities of healthy *P. lutea*.

2 Materials and methods

2.1 *Sample collection*

Coral samples were collected during a Sino-Indonesia co-survey in late April to early May in 2013 at the Lembeh Strait. Samples of corals with pigment abnormalities, and of healthy corals, were collected at Site S5 located at the middle of the Lembeh Strait (1°27′13.6″N, 125°14′12.8″E, North Sulawesi, Indonesia) (Fig. 1). Coral samples were cut on site to collect separately pink segments and healthy segments (Fig. 2), and then fixed with 95% ethanol. Three parallels from pink segments and healthy segments respectively were subsampled for DNA analysis.

2.2 *DNA extraction, amplification, and sequencing*

Total genomic DNA was extracted from samples using the MP FastDNA Spin kit for Soil (MP Biomedicals). DNA concentration and purity were monitored on 1% agarose gels. DNA was diluted to 1 ng/μL in sterile water. The regions V3–V4 from the 16S gene were amplified using the specific primer 515F-806R with the barcode designed for the following sequencing. PCR reactions were

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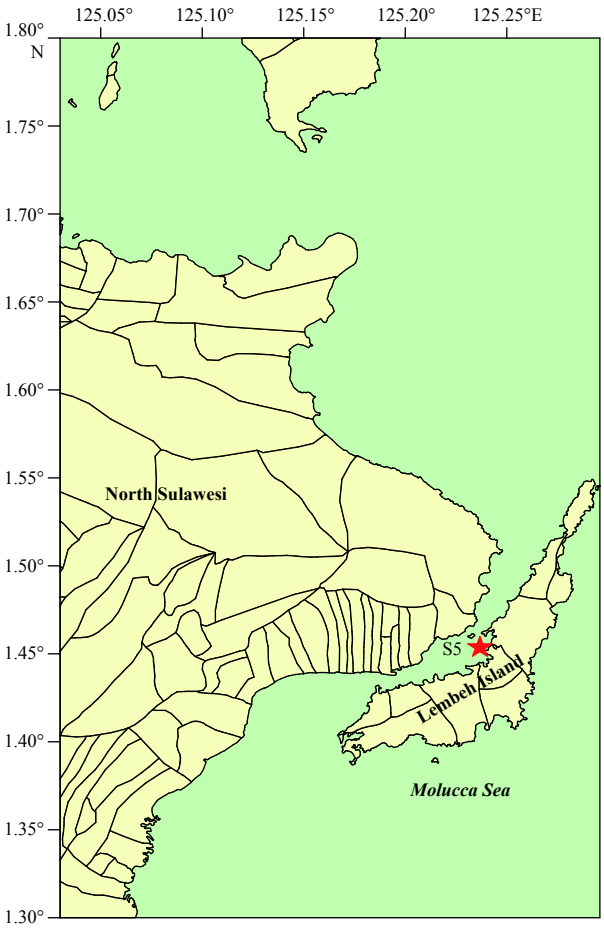

Fig. 1. Location of the sampling Site S5.

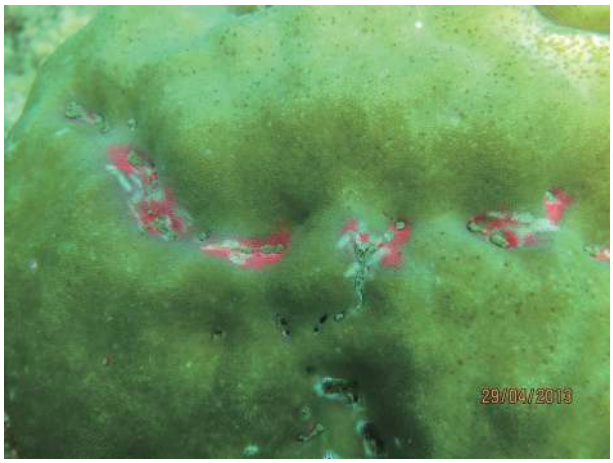

Fig. 2. Pigment abnormalities in *Porites lutea* (photograph by Tri Aryano).

carried out in 30 μL containing 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μmol/L of forward and reverse primers, and approximately 10 ng template DNA. Thermal cycling consisted of an initial denaturation step at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C or for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 60 s, plus a final elongation step at 72°C for 5 min. Mix same volume of 1× loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection, and samples containing a band of 400–450 bp were selected for further experiments. PCR products were mixed in equal ratios. Then, the PCR products were purified using the GeneJET Gel Extraction Kit (Thermo Scientific). Sequencing libraries were generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB), following the manufacturer's recommendations. The library quality was assessed using the Qubit@2.0 Fluorometer (Thermo Scientific) and the Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina MiSeq platform, generating 250–300 bp paired-end reads.

2.3 *Data analysis*

High-throughput sequencing data were processed using the Mothur software, as described in the pipeline of "Costello stool analysis" (Costello et al., 2009). In order to obtain more accurate sequences, sequences that were shorter than 400 bp, or that contained more than six polymers, were eliminated. Sequencing errors corresponding to artifacts of the sequencing process were reduced through the "denoising" algorithm (Reeder and Knight, 2010). After screening, filtering, and pre-clustering, unique sequences were checked for chimeras using Vsearch (Rognes et al., 2016). The qualified sequences were aligned with the reference set in the SILVA SSURef database Release 123 (Pruesse et al., 2007). Sequencing data were subsampled to eliminate the deviation due to the difference of sequence numbers across samples. After achieving an even number of sequences across samples, optimized reads were taxonomically assigned using the RDP-classifier with a bootstrap cut-off of 97% (Wang et al., 2007).

Observed richness, Simpson diversity, and the Shannon–Wiener index were measured based on the frequency of operational taxonomic units (OTUs) and genera in the assigned sequence collections after rare sequences were removed (Hill et al., 2003). Rarefaction curves were computed after discarding singletons. Beta diversity was estimated by computing weighted and unweighted UniFrac distances between samples using QIIME (Caporaso et al., 2010). Correlation analysis between the symbiotic bacterial communities of the samples were carried out using the statistical R package commond cor.test (Liu et al., 2014).

3 Results

3.1 *Diversity and richness of total symbiotic bacteria*

A total of 439 734 sequences from six samples were recovered after Miseq sequencing. After sequence screening, filtering, pre-clustering and removal of chimeras, 425 182 qualified sequences were retained for alignment and OTU classification (these sequences accounted for 96.69% of the total raw sequences). In total 245 333 sequences representing bacterial taxons (16 951 to 98 768 sequences per sample) were recovered from these qualified sequences. As shown in the rarefaction curve based on the bacterial OTU number, the sequencing data can reflect the diversity and richness of the bacterial community of the coral samples, because all the curves reached the near plateau phase (Fig. 3). Additionally, the coverage value shown in Table 1 also indicated that pyro-sequencing data covered 89.39%–96.95% of the bacterial community in the coral samples.

The bacterial community in *P. lutea* with pigment abnormalities had features differing from those of healthy *P. lutea*. The relative diversity of the bacterial community from *P. lutea* with pig-

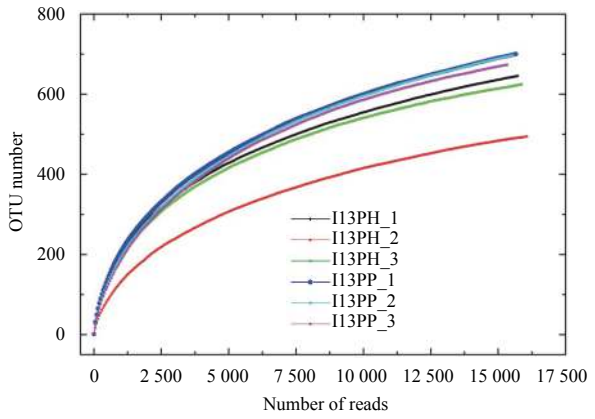

Fig. 3. Rarefaction analysis of bacterial community in three replicates of pigmented (I13PP) and healthy (I13PH) coral samples (OTUs were clustered at 97% similarity level).

Table 1. Mean values[1)] of richness and diversity of symbiotic bacterial communities in pigmented and healthy *P. lutea*

Sample ID	Sobs	Coverage /%	Chao's index	Simpson's index	Shannon's index
I13PH_1	1 854	91.77	10 164.38	0.03	4.92
I13PH_2	1 376	93.83	6 581.10	0.27	3.05
I13PH_3	1 683	92.81	7 911.18	0.05	4.57
Ave. of PH	1 638	92.80	8 218.89	0.11	4.18
I13PP_1	1 967	91.30	10 733.73	0.03	4.87
I13PP_2	2 077	90.66	10 155.41	0.05	4.64
I13PP_3	2 242	89.84	10 661.21	0.05	4.60
Average of PP	2 095	90.60	10 516.78	0.04	4.71

Note: [1)] OTUs were defined at 0.03 level.

ment abnormalities was higher than that from healthy coral samples, as indicated by the Shannon index. The Shannon's index showed different patterns; it increased as the sequence number increased in the samples of *P. lutea* with pigment abnormalities, while in healthy corals the Shannon's index decreased as the sequence number increased. The Simpson's index in the samples of *P. lutea* with pigment abnormalities was higher than that of healthy corals, which also indicates that the bacterial community in *P. lutea* with pigment abnormalities is more diverse than in healthy corals. The community richness represented by Chao's index and sobs showed that the bacterial community in *P. lutea* with pigment abnormalities has higher richness than healthy corals (Table 1).

3.2 *Bacterial community composition*

A total of 16 951 sequences from each sample were clustered into 8 007 OTUs at a similarity of 97%, using the furthest neighbor algorithm by Vsearch. Bray-Curtis distance matrix, based on the correlation analysis of the six samples, indicated that the symbiotic bacterial community structure in *P. lutea* with pigment abnormalities and in healthy corals were different from each other, while those in the three parallels of each group were quite similar (Fig. 4).

From the 8 007 OTUs clustered using Mothur, 7 943 OTUs were classified by blasting against NCBI microbial 16S rRNA database. After removing rare OTUs, 936 qualified OTUs accounted for the 15 335–16 049 sequences that were finally recovered. The most abundant bacteria phyla, as calculated by the size (number of sequences) of the phylum of each sample, were Proteobacteria (58.47% of the total), Actinobacteria (19.49% of the total) and Chlorobi (14.18% of the total). Proteobacteria was the most versatile phylum, accounting for 3 449–10 022 and 10 114–12 767 of the sequences in healthy corals, and in *P. lutea* with pigment abnormalities, respectively.

A total of 399 genera of symbiotic bacteria in healthy *P. lutea* and in *P. lutea* with pigment abnormalities were identified by OTU analysis. Bacteria belonging to the 20 most abundant genera constituted more than 77% of the total identified bacteria in *P. lutea* (77.30% of the total in healthy *P. lutea,* and 78.09% in *P. lutea* with pigment abnormalities). The five most abundant genera, *Vibrio*, *Prosthecochloris*, *Blastococcus*, *Roseibium* and *Pseudoalteromonas* accounted for 16.21%, 14.18%, 10.06%, 6.25% and 3.77% of the total sequences, respectively. Among these genera, the most abundant OTUs of symbiotic bacteria were *Vibrio* (0.97%–8.33% in healthy *P. lutea* and 16.89%–34.57% in *P. lutea* with pigment abnormalities), *Prosthecochloris* (0.20%–61.69% in healthy *P. lutea* and 0.45%–8.67% in *P. lutea* with pigment abnormalities), *Blastococcus* (4.66%–19.69% in healthy *P. lutea* and 4.17%–11.33% in *P. lutea* with pigment abnormalities), *Roseibium* (2.19%–8.77% in healthy *P. lutea* and 5.55%–9.73% in *P. lutea* with pigment abnormalities), and *Pseudoalteromonas* (0.90%–2.32% in healthy *P. lutea* and 4.89%–8.63% in *P. lutea* with pigment abnormalities) (Fig. 5). *Vibrio* species in *P. lutea* with pigment abnormalities (16.21%) were significantly more abundant than in healthy *P. lutea* (4.94%), which suggests that *Vibrio* might play an important role in the pigment development in *P. lutea*.

According to Silva 16S rRNA database, and after removing rare OTUs, a total of 936 bacterial OTUs belonging to 575 species were recovered from subsampled healthy *P. lutea* and *P. lutea* with pigment abnormalities. *Vibrio*, *Photobacterium*, *Acinetobacter*, *Mesorhizobium* and *Desulfovibrio* were most versatile bacteria genus with 24, 9, 7, 6, and 5 species, respectively (Appendix Table A1). After comparing the 22 most abundant bacterial OTUs (with abundance over 1% of the total OTUs) of healthy *P. lutea* and *P. lutea* with pigment abnormalities, we identified 2 OTUs consistently associated with *P. lutea* with pigment abnormalities, and 8 OTUs consistently associated with healthy *P. lutea* (Fig. 6). OTUs associated with healthy *P. lutea* were *Prothecochloris vibrioformis* and *Prothecochloris aestuarii*. OTUs associated with *P. lutea* with pigment abnormalities were *Vibrio hyugaensis*, *Vibrio fortis*, *Roseibium aquae*, *Pseudoalteromonas arabiensis*, *Oscillochloris trichoides*, *Kofleria flava*, *Photobacterium gaetbulicola* and *Vibrio xuii*, with 12.80%, 10.58%, 6.30%, 4.93%, 3.25%, 2.26%, 1.98% and 1.90% of the pigmented group versus 1.95%, 1.85%, 2.05 %, 1.34%, 0.66%, 0.67%, 0.27% and 0.31% of the healthy group (Fig. 6). Among these, six OTUs associated with *P. lutea* with pigment abnormalities were related to bacterial families that include known coral pathogens (Vibrionaceae), to symbiotic bacteria found in diseased marine invertebrates (Rhodobacteraceae), or to algicidal bacteria (Pseudoalteromonadaceae) (Sunagawa et al., 2009).

4 Discussion

The symbiotic bacterial community changed drastically in *P. lutea* with pigment abnormalities, exhibiting an increase in hundreds of OTUs, relative to healthy *P. lutea*. This change was evidenced by increased bacterial diversity and richness, which is consistent with other coral diseases (Pantos et al., 2003; Séré et al., 2013; Sunagawa et al., 2009).

Proteobacteria was the most abundant phylum in the symbiotic bacterial OTUs, which is in keeping with previous 16S rDNA

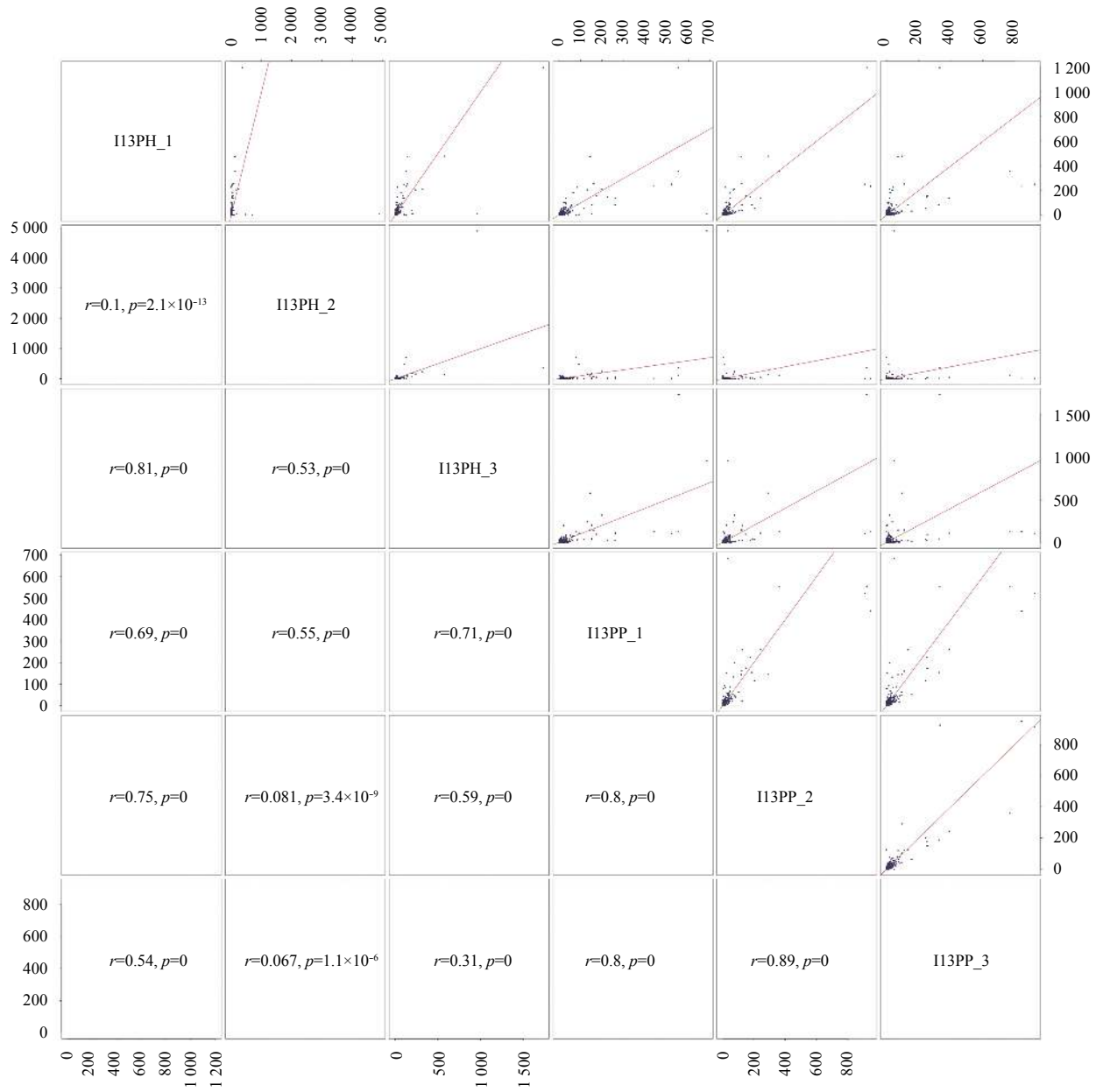

Fig. 4. Correlation analysis between the symbiotic bacterial communities of the six samples. The correlation values were calculated using the "cor. test" function in R.

and metagenomic analysis of bacteria associated with *Porites* (Rohwer et al., 2002; Wegley et al., 2007). The other two most abundant phyla were Actinobacteria and Chlorobi, which were different to those reported in previous studies, in which Firmicutes and Actinobacteria were the second and third most abundant phyla (Rohwer et al., 2002; Wegley et al., 2007).

The five most abundant genera, *Vibrio*, *Prosthecochloris*, *Blastococcus*, *Roseibium* and *Pseudoalteromonas* accounted for more than 50% of the total sequences found in *P. lutea*. These results are similar to previous reports which suggested that *Vibrio* and *Pseudoalteromonas* were the most abundant bacterial genera in *P. lutea* from southern Hainan Island in China. It is thus likely that these genera form a part of the natural microbiota of *P. lutea* (Li et al., 2014). Furthermore, *Vibrio* were more abundant in *P. lutea* with pigment abnormalities than in healthy *P. lutea*, indicating that *Vibrio* might be involved in the etiology of the pigment abnormalities of *P. lutea*.

Several *Vibrio* species are well-known pathogens of marine shrimp, fish, invertebrates and coral (Austin et al., 2005; Ben-Haim and Rosenberg, 2002; Kushmaro et al., 1997). *Vibrio Shiloi*, the first coral pathogen to be identified, is an etiological agent of bleaching of the coral *Oculina patagonica* (Kushmaro et al., 1996; 2001). *Vibrio coralliilyticus* is an etiological agent of bleaching of the coral *Pocillopora damicornis* (Ben-Haim et al., 2003). Four *Vibrio* species are related to yellow blotch/band disease of the coral *Montastrea* spp. (Cervino et al., 2004). In our research, three *Vibrio* species, *Vibrio hyugaensis*, *Vibrio fortis*, and *Vibrio xuii* accounted more than 25% of the total microbial OTUs in *P. lutea* with pigment abnormalities, which suggested these *Vibrio* species may be involved in the development of pigment abnormalities.

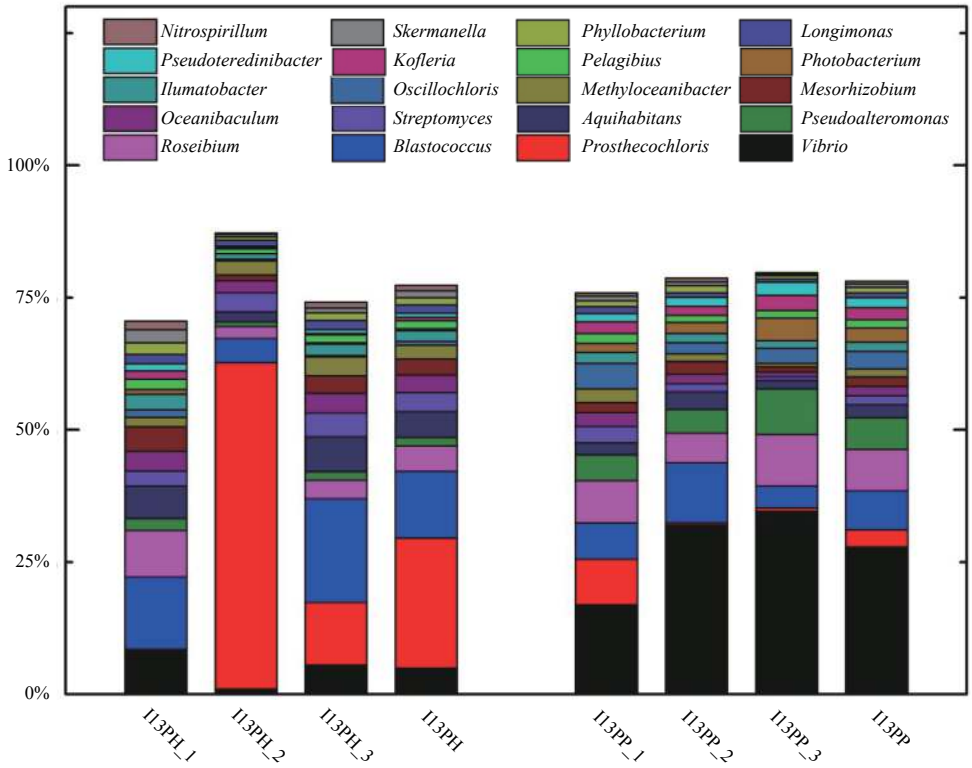

Fig. 5. Percentage of the OTU size of the 20 most abundant symbiotic bacterial genera in healthy *P. lutea* and *P. lutea* with pigment abnormalities.

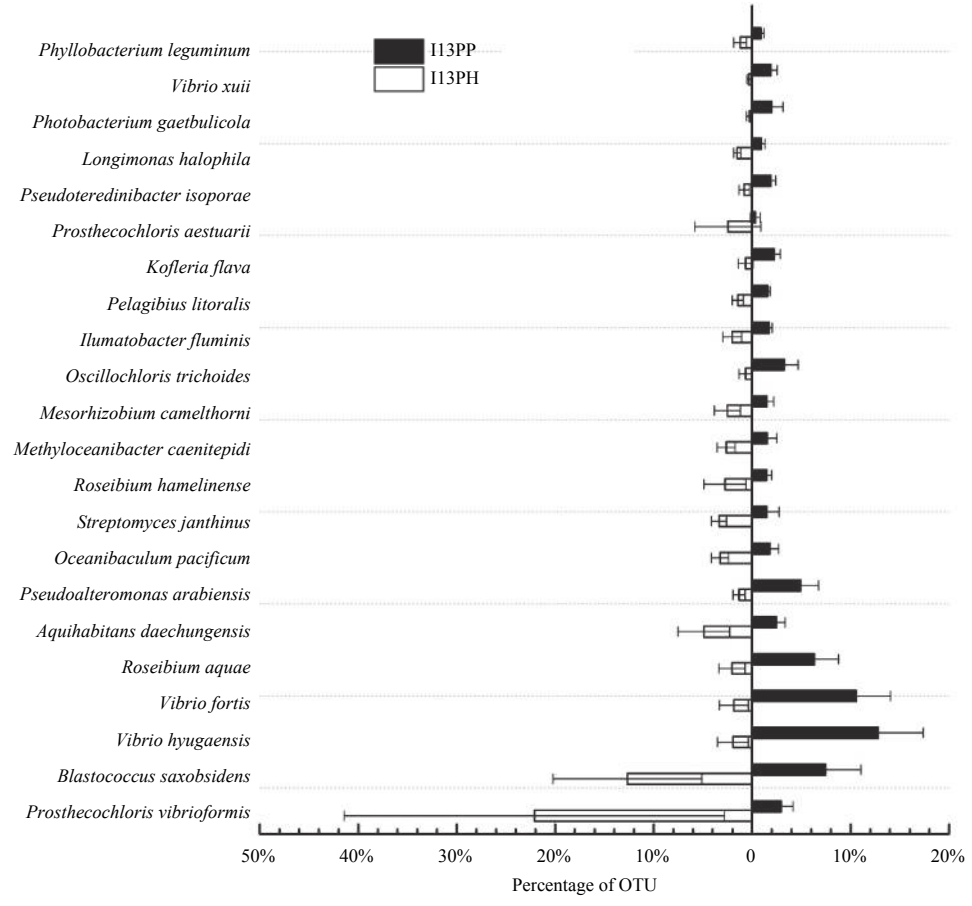

Fig. 6. Most abundant bacterial species associated with healthy *P. lutea* and *P. lutea* with pigment abnormalities.

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Appendix:

Table A1. OTU number of five most versatile symbiont bacterial genera in *Porites lutea* samples

Genus	Species	I13PH	I13PH_1	I13PH_2	I13PH_3	I13PP_1	I13PP_2
Vibrio		1 305	155	869	2 643	4 966	5 301
	V. aestuarianus	78	28	122	44	179	69
	V. alginolyticus	19	2	27	31	101	96
	V. anguillarum	1	2	1	0	1	1
	V. brasiliensis	1	0	0	4	4	14
	V. caribbeanicus	3	0	0	2	5	12
	V. coralliilyticus	5	1	3	27	23	38
	V. europaeus	2	0	2	5	2	2
	V. fluvialis	0	0	1	0	1	1
	V. fortis	502	43	327	1 027	1 972	1 911
	V. gallicus	2	1	0	2	1	0
	V. hangzhouensis	1	0	1	6	11	11
	V. hispanicus	1	0	0	0	1	3
	V. hyugaensis	552	58	310	1 203	2 209	2 524
	V. jasicida	0	0	0	0	3	0
	V. mediterranei	28	2	13	41	67	126
	V. mexicanus	3	0	0	1	5	6
	V. mytili	2	1	0	3	4	6
	V. nigripulchritudo	3	1	0	3	3	16
	V. parahaemolyticus	6	0	1	3	6	9
	V. penaeicida	0	0	0	0	0	3
	V. tubiashii	7	2	6	34	32	43
	V. variabilis	4	2	3	15	14	23
	V. vulnificus	3	0	0	4	6	8
	V. xuii	82	12	52	188	316	379
Photobacterium		140	8	44	271	320	664
	P. aphoticum	0	0	0	0	0	3
	P. damselae	1	0	0	0	1	4
	P. frigidiphilum	28	1	8	60	56	81
	P. gaetbulicola	99	6	23	180	227	509
	P. ganghwense	0	0	5	0	2	1
	P. jeanii	2	0	2	4	3	23
	P. marinum	8	1	5	25	31	38
	P. rosenbergii	1	0	0	1	0	2
	P. swingsii	1	0	1	1	0	3
Acinetobacter		17	6	6	10	18	62
	A. baumannii	3	1	0	2	2	1
	A. indicus	9	3	1	2	8	40
	A. junii	1	0	1	0	3	5
	A. lwoffii	0	1	1	1	0	0
	A. radioresistens	1	0	2	1	1	1
	A. variabilis	0	1	1	1	0	2
	A. venetianus	3	0	0	3	4	13
Mesorhizobium		730	176	527	293	385	155
	M. alhagi	0	0	1	1	0	1
	M. camelthorni	547	162	485	249	334	116
	M. mediterraneum	175	8	32	35	42	31
	M. soli	1	0	0	1	1	0
	M. thiogangeticum	0	1	1	2	2	0
	M. waimense	7	5	8	5	6	7
Desulfovibrio		44	13	41	34	24	17
	D. africanus	2	2	3	4	3	3
	D. aminophilus	24	3	20	17	9	9
	D. indonesiensis	1	2	6	4	1	0
	D. oxamicus	0	2	0	1	0	1
	D. portus	17	4	12	8	11	4