The cultivation of *Pyropia haitanensis* has important impacts on the seawater microbial community

Wenlei Wang [1,2,3] · Lei Wu [1,2,3] · Kai Xu [1,2,3] · Yan Xu [1,2,3] · Dehua Ji [1,2,3] · Changsheng Chen [1,2,3] · Chaotian Xie [1,2,3] (ORCID)

Abstract

Microorganisms play important roles in the growth and development of macroalgae. Still, the biodiversity of the epiphytic microbial community associated with the economically important red alga *Pyropia haitanensis* during the cultivation period remains uncharacterized, especially the effects of *P. haitanensis* cultivation on the microbial community of surrounding seawater. Here, we isolated epiphytic microbes from *P. haitanensis* during the thallus stage during oceanic cultivation and the conchocelis stage during industrial cultivation. The dynamic diversity patterns, as determined by 16S and 18S rRNA gene sequencing of the bacterial and fungal communities, respectively, associated with *P. haitanensis* and seawater in the presence and absence of algal cultivation were investigated. A notable distinction was observed between the microbial communities of seawater with and without *P. haitanensis* cultivation. Additionally, the alpha-diversity of seawater with *P. haitanensis* cultivation was significantly greater than without *P. haitanensis* cultivation. Cyanobacteria were the dominant species in the latter, while Rhodobacteraceae was enriched in the former. Furthermore, there were significant differences in the microbial community of *P. haitanensis* at the thallus and conchocelis stages. Seawater properties had significant direct effects on the microbial diversity of *P. haitanensis* and cultivation seawater, but not on non-cultivation seawater. The enriched microbial presence might promote thallus morphogenesis and be beneficial for the growth and development of both the thallus and conchocelis stages. These findings expand our knowledge of the bacteria and fungi that are beneficial for *Pyropia* nursery seeding and cultivation, as well as the effects of *P. haitanensis* cultivation on the seawater microbial community.

Keywords *Pyropia haitanensis* · Microbial communities · Conchocelis stage · Thallus stage · Oceanic and industrial cultivation

Introduction

Microbes are widely distributed in seawater and selectively enrich the phycosphere (Bell and Mitchell 1972). These microbes can form communities with unique functions and structures, which may play important roles in marine food webs and biogeochemical cycles (Cole 1982; Armstrong et al. 2001; Farooq and Francesca 2007). Additionally, host

microbe interactions play crucial roles in marine ecosystems (Egan et al. 2013; Dittami et al. 2019); van der Loos et al. 2019. The interactions of bacteria with algae have evolved as the result of the mutual exploitation of signals in the environment, such that the *Roseovarius* sp. *MS2* and *Maribacter* sp. *MS6* strains release morphogenetic compounds and ensure proper thallus morphogenesis of *Ulva mutabilis* (Kessler et al. 2018). Moreover, the surfaces of seaweeds also provide a habitat for microbial communities (Beleneva and Zhukova 2006; De Mesquita et al. 2019). The cultivation area of economically important seaweeds in China comprised about 145,000 ha in 2017 (China Fishery Bureau 2018). Thus, the microbes of seaweeds are an important component of marine ecosystems on the Chinese coastline, and interactions between cultivated seaweed and seawater may shape the marine microbial community.

The red alga *Pyropia haitanensis*, which naturally inhabits the coastal waters of southern China, has been extensively cultured in the Fujian, Zhejiang, and Guangdong provinces

Wenlei Wang and Lei Wu contributed equally to this work.

✉ Chaotian Xie
   ctxie@jmu.edu.cn

1   Fisheries College, Jimei University, Xiamen 361021, China

2   Key Laboratory of Healthy Mariculture for the East China Sea,
    Ministry of Agriculture, Xiamen 361021, China

3   Fujian Engineering Research Center of Aquatic Breeding and
    Healthy Aquaculture, Xiamen 361021, China

for more than 50 years. Its cultivation accounts for more than 70% of all *Pyropia* production in China (China Fishery Bureau 2018). The total annual harvest and cultivation area of *P. haitanensis* were about 116,000 t (dry weight) and 29,000 ha in the aforementioned three provinces in 2017. The annual life cycle of *P. haitanensis* consists of a thallus stage, which is subject to marine aquaculture in the cool season (from September to December), and a shell-living conchocelis stage, which is cultivated in industrial seedling nurseries in the hot season (from January to August) (Zeng 1985; Blouin et al. 2011). During the conchocelis stage, the filamentous conchocelis develops into sporangial branchlets from which conchospores are released. Subsequently, during conchospore germination, meiotic division gives rise to a four-celled thallus to initiate the thallus stage for oceanic cultivation. The thallus and conchocelis stages are entirely different in morphology, longevity, ploidy, and living environment between thallus stage and conchocelis stage. Therefore, microbes may play different roles during different cultivation stages of *P. haitanensis*.

Recently, attempts have been made to elucidate the relationship between *Pyropia* and the epiphytic microbial community. For example, an analysis of the epiphytic microbial community of *P. haitanensis* thalli using the denaturing gradient gel electrophoresis method revealed that the microbial community varied based on growth form, culture time, and environment (Shen et al. 2013). Microbial communities on *Pyropia yezoensis* thalli and in seawater were significantly different when infected with the oomycetic genus *Pythium* (Yan et al. 2019). In addition, industrial cultivation of the conchocelis stage is also an important component of the *Pyropia* industry. Gai and Zhang (2016) observed significant differences between the microbial community compositions of healthy and diseased shell-living conchocelis of *P. yezoensis* using next-generation sequencing. However, these studies focused on the impacts of pathogenic bacteria on the microbial community of *Pyropia*. Whether the epiphytic microbial community associated with *P. haitanensis* differs between different stages of its life cycle is unknown.

In this study, we used high-throughput sequencing based on 16S rRNA gene and 18S rRNA gene to explore the dynamics of the microbial community on *P. haitanensis* during the entire cultivation period and the effects of *P. haitanensis* cultivation on the seawater microbial community. The samples were collected from all the important stages of the life cycle, including both the conchocelis stage (filamentous conchocelis, sporangial branchlets, and conchospores) and the thallus stage (young, adult, mature, and advanced mature thalli). Our main objectives were as follows: (i) to explore the dynamics of the microbial community on *P. haitanensis* during different life stages, (ii) to explore the effects of *Pyropia* cultivation on the microbial community in surrounding seawater, and (iii) to investigate the interactions between the

seaweed and epiphytic microbes during the cultivation of *P. haitanensis*. Taken together, these findings will enhance our understanding of the interactions between seawater and *P. haitanensis*, and the dynamics diversity changes in the microbial community during the cultivation of *P. haitanensis*.

Materials and methods

Sample collection

Conchocelis-stage samples were collected from a nursery farm at Ningde City, Fujian Province, China. The filamentous conchocelis and sporangial branchlets growing inside calcium carbonate shells were scraped from the shell surface with a sterilized trowel. Each sample was collected from 30 to 50 shells. The shells with mature conchocelis were stimulated with flowing seawater by hanging from a boat overnight for about 12 h and then moved to the laboratory and placed upside-down on sterilized Petri dishes to encourage the release of conchospores. Thallus-stage samples were obtained from the coast at Quanzhou City, Fujian province, China. Young thalli (1–3 cm), adult thalli (20–30 cm), mature thalli bearing antheridia or carpogonia, and advanced mature thalli (decaying thalli after the formation and release of carpospores) were collected from culture ropes. Three biological replicates from each sample were used for subsequent experiments. All the samples were stored at − 20 °C until analysis.

Three cultivation seawater samples, three non-cultivation seawater samples, and three algal samples were collected for each of the afore-mentioned seven life cycle stages. These samples were collected concurrently. For the conchocelis stage, cultivation seawater was sampled from the culture ponds of the nursery farm, whereas non-cultivation seawater was collected from a reservoir that was used as the seawater source for the culture pond. For the thallus stage, cultivation seawater was collected from around the thalli, whereas non-cultivation seawater was collected ~ 100 m from the culture area. Two liters of each sample were passed through 0.22-μm microporous filter paper and stored at − 20 °C until analysis.

DNA extraction and PCR amplification

The filter papers used for the seawater samples were cut into small pieces and placed in a 50-mL centrifuge tube. Extraction buffer (4 g CTAB, 16.4 g NaCl, 20 mL of 1 M Tris-HCl, 8 mL of 0.5 M EDTA, and 200 mL distilled water) was added to the tube together with 500-mg glass beads. The tube was vortexed at maximum speed until the sample was thoroughly homogenized. Then, the sample tubes were incubated at 90 °C for 10 min to ensure that all unicellular organisms were lysed. Subsequently, the tubes were centrifuged at 4000×*g* for 10 min at 4 °C. The clear supernatant was transferred to a

new tube, and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added. The liquid phase was removed and extracted with an equal volume of chloroform/isoamyl alcohol (24:1). Next, 50 mL of 3 M sodium acetate was added together with a 60% volume of isopropanol to the solvent phase. The solution was then incubated at −20 °C overnight. The DNA was pelleted (13,000×g, 15 min) and washed with 70% ethanol. An identical extraction procedure was used for the conchocelis, thallus, and seawater samples.

The hypervariable V4-V5 regions of the 16S and 18S rRNA genes were amplified using the primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGAC TACHVGGGTWTCTAAT-3′) (Zhu et al. 2016) and the primers SSU0817F (5′-TTAGCATGGAATAA TRRAATAGGA-3′) and 1196R (5′-TCTGGACCTGGTGA GTTTCC-3′) (Rousk et al. 2010), respectively. The PCR reactions were performed in a 50-μL volume containing 1× PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4), 5.0 mM $MgCl_2$, 0.5 μM of each primer, 200 μM of each dNTP, 4 μL DNA templates, and 2.5 U Taq polymerase. The PCR reactions for amplifying 16S and 18S rRNA genes were conducted using the following program: 3 min of denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30 s for annealing at 55 °C, and 45 s for elongation at 72 °C, followed by a final extension at 72 °C for 10 min.

Illumina MiSeq sequencing

The PCR products were extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA) in accordance with the manufacturer's instructions and then quantified using a QuantiFluor ST fluorometer (Promega, USA). Sequencing was performed using the Illumina MiSeq platform at the Majorbio BioPharm Technology Co., Ltd., Shanghai, China.

Processing of sequencing data

Data from Illumina sequencing were quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) the reads were truncated at any site receiving an average quality score < 20 over a 50 bp sliding window; (ii) sequences whose overlap was longer than 10 bp were merged according to their overlap with a mismatch of no more than 2 bp; (iii) sequences of each sample were separated according to barcodes (exactly matching) and primers (allowing 2 nucleotide mismatching), and reads containing ambiguous bases were removed. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) with a novel "greedy" algorithm that performs chimera filtering and OTU clustering simultaneously. The taxonomy of each 16S rRNA gene sequence was analyzed by the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using a confidence threshold of 70%, and each 18S rRNA gene sequence was analyzed against the silva128/ 18S_eukaryota database using a confidence threshold of 70% (Vilo and Dong 2012). The alpha-diversity level of microbial community was described for each sample using the metrics Chao1 and Shannon (Hartmann and Widmer 2006).

Structural equation models (SEMs) were used to evaluate direct and indirect effects of seawater properties, microbial diversity of *Pyropia haitanensis* and seawater without Pyropia cultivation on the microbial diversity of cultivation seawater (Eisenhauer et al. 2015). All the data used in the SEMs was standardized using Z scores. A covariance matrix was calculated by pairwise correlations using SPSS statistical software (version 20, SPSS Inc.,, USA) and was then imported into AMOS 21 (SPSS Inc.) for SEMs construction using maximum likelihood estimation. Goodness-of-fit index (N0.90), χ^2 test (P N0.05), and root mean square errors of approximation (RMSEA b 0.05) were used to test the overall goodness of the model fit.

A non-metric multidimensional scaling (NMDS) analysis of Bray–Curtis distance (Kent 2011) and the alpha-diversity (diversity, richness and evenness) (Hartmann and Widmer 2006) were calculated using the "vegan" package in the R 3.4.0 software for investigating the dissimilarities among samples and visualized with Orginlab 2019. Adonis tests (Su et al. 2015) were used to determine significant differences among communities. An analysis of differences between samples was performed on the I-Sanger online platform (https://www.i-sanger.com). All statistical tests using one-way ANOVA test were conducted by SPSS Software V20.0 (IBM, USA).

Results

Characterization of prokaryotic and eukaryotic microbial communities of seawater with or without *Pyropia haitanensis* cultivation

A total of 4498 OTUs, with 3,085,384 high-quality prokaryotic sequences, were obtained, in which, 982,608 sequences of epiphytic microbes on *P. haitanensis* were distributed among 3824 OTUs, 1,925,622 cultivation seawater sequences were distributed among 3633 OTUs, and 2,278,090 non-cultivation seawater sequences were distributed among 1921 OTUs (Table 1). For the eukaryotic community, 1139 OTUs, with 3,590,560 high-quality sequences, were obtained, among which, 1,223,131 sequences of epiphytic microbes on *P. haitanensis* were distributed among 751 OTUs, 2,044,299 cultivation seawater sequences were distributed among 790 OTUs, and 2,398,942 non-cultivation seawater sequences were distributed among 617 OTUs (Table 1).

Table 1 Detailed description of 16S rRNA gene data set and 18S rRNA gene data set during *Pyropia haitanensis* cultivation

	All OUT number	All sequence number	Stage	Category	*P. haitanensis*		Cultivation seawater		Non-cultivation seawater	
					OUT number	Sequence number	OUT number	Sequence number	OUT number	Sequence number
16S rRNA gene			Conchocelis stage	Filamentous conchocelis	1205	155,215	1099	146,288	625	149,216
				Sporangial branchlets	752	188,050	1059	144,633	836	160,968
				Conchospores	373	179,056	1311	142,280	1508	147,116
	4498	3,085,384		All	1600	522,705	2138	433,919	2012	457,987
			Thallus stage	Young thallus	459	132,048	1310	154,446	1156	122,138
				Adult thallus	167	176,127	1866	154,898	1702	134,923
				Mature thallus	238	152,565	1351	117,351	1318	127,986
				Later mature	115	156,641	864	132,464	805	119,334
				All	624	617,567	2456	560,548	2284	505,624
				In total	3824	982,608	3633	1,925,622	1921	2,278,090
18S rRNA gene			Conchocelis stage	Filamentous conchocelis	331	159,126	349	154,821	341	187,188
	1139	3,590,560		Sporangial branchlets	151	185,064	239	185,861	150	164,003
				Conchospores	322	166,755	399	167,634	196	165,840
				All	528	511,037	617	508,407	486	517,062
			Thallus stage	Young thallus	196	179,419	204	172,671	194	160,233
				Adult thallus	301	202,782	247	165,261	60	179,361
				Mature thallus	210	146,721	207	175,605	51	168,621
				Later mature	181	187,692	183	151,393	69	173,193
				All	403	716,769	384	665,089	256	681,424
				In total	751	1,223,131	790	2,044,299	617	2,398,942

The alpha-diversity of diversity, evenness, and richness between the seawater with and without *Pyropia* cultivation samples showed that the biodiversity of cultivation seawater was significantly greater ($P < 0.01$) than that of non-cultivation seawater, but had no distinction from *Pyropia* samples base on the 16S rRNA gene analysis (Fig. 1). For the eukaryotic microbial diversity, the richness of cultivation seawater was also significantly higher ($P < 0.01$) than that of non-cultivation seawater (Fig. 1).

An NMDS analysis based on a Bray–Curtis dissimilarity matrix revealed significant ($P < 0.001$) differences between the seawater with and without *Pyropia* cultivation samples at prokaryotic and eukaryotic microbial level (Fig. 2). Additionally, the thallus and conchocelis stages among cultivation seawater samples were also significantly ($P < 0.001$) distinctive and clustered with the thallus and conchocelis stages of *Pyropia* samples, respectively (Fig. 2).

The differences in the bacterial and eukaryotic community compositions between the seawater with and without *Pyropia* cultivation were further demonstrated in detail at phylum and family levels. For the bacterial community composition at the

phylum level, Proteobacteria and Cyanobacteria were the dominant species among seawater samples. Cyanobacteria were present at a greater level in the non-cultivation seawater compared with in the cultivation seawater during the thallus stage, while Bacteroidetes, Actinobacteria, and Proteobacteria were present at greater levels in the cultivation seawater compared with in the non-cultivation seawater (Fig. 3). For the eukaryotic community composition at the phylum level, Ciliophora, SAI-3C06, and Cryptophyta were present higher levels in *Pyropia* cultivation seawater than in the seawater without thallus cultivation, while Ascomycota were higher in seawater without conchocelis cultivation than in cultivation seawater (Fig. 3).

The compositions of bacterial and eukaryotic communities in the seawater with and without Pyropia cultivation during the thallus and conchocelis stages at family level (with > 1% relative abundance) are shown in Fig. 4. For the bacterial community, during the conchocelis stage, Rhodobacteraceae, Flavobacteriaceae, Oceanospirillaceae, Litoricolaceae, Saprospiraceae, Rhodospirillaceae, Hyphomonadaceae, and Alteromonadaceae were significantly higher ($P < 0.05$) in cultivation seawater than in non-cultivation seawater (Fig. 4).
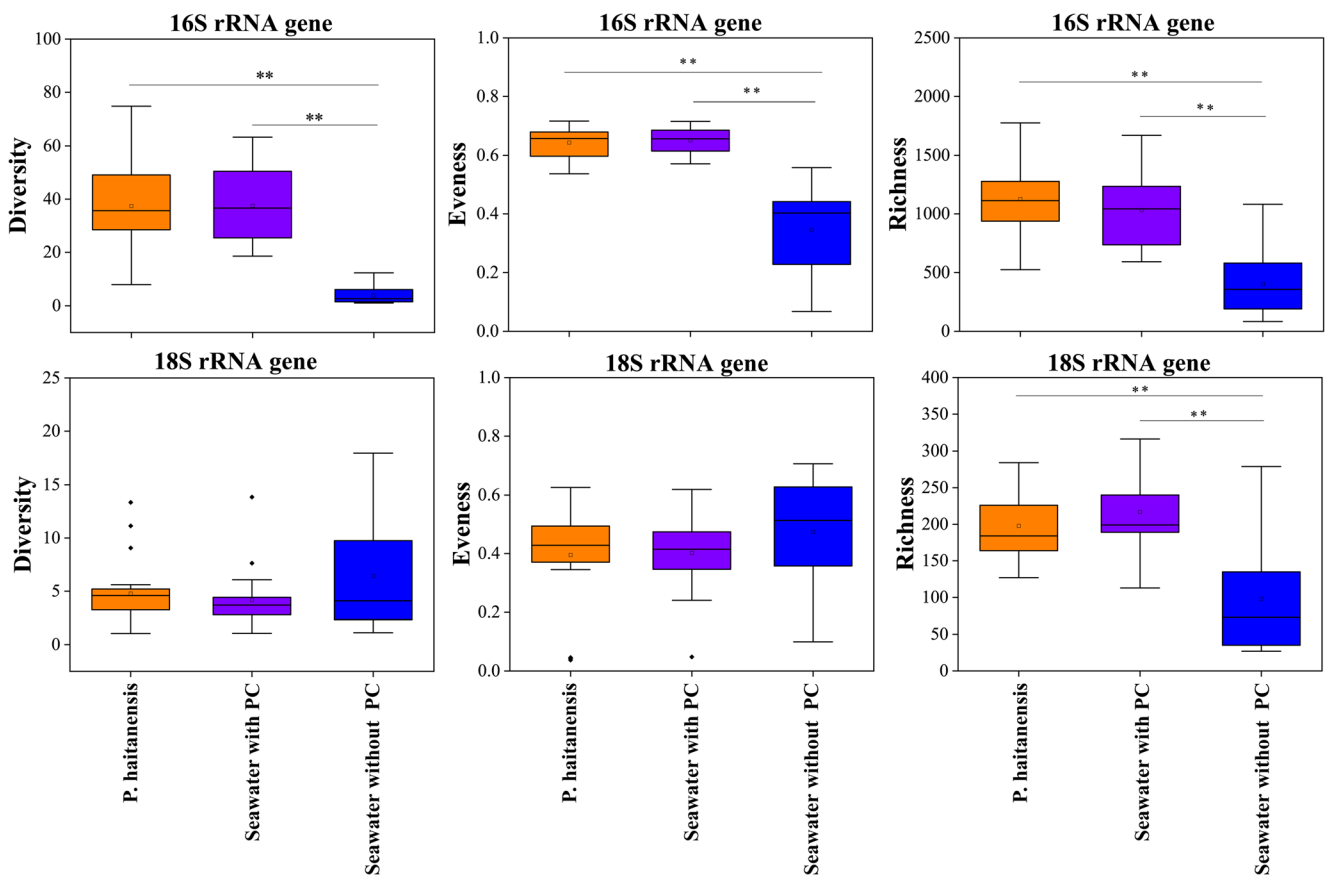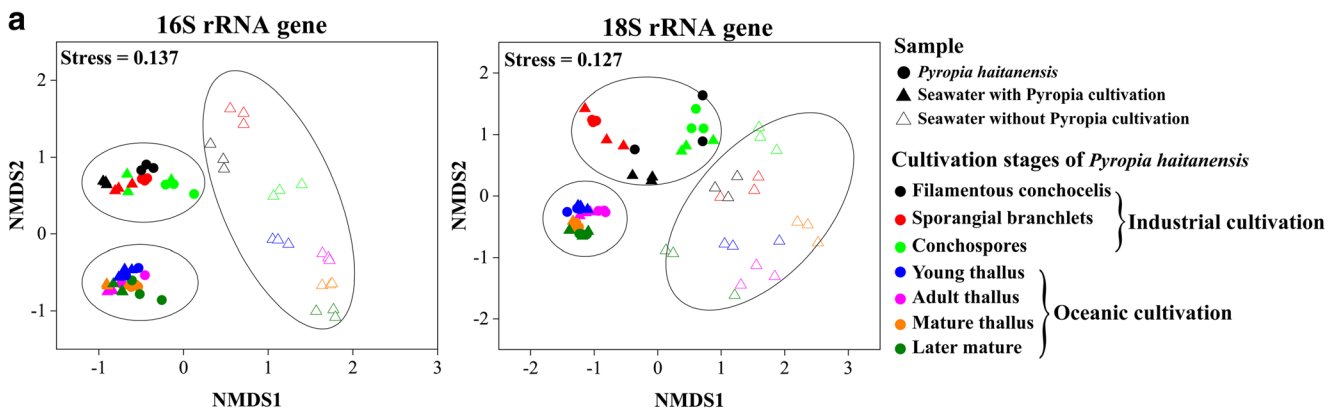
Fig. 1 Alpha-diversity (diversity, evenness, and richness) of bacterial and eukaryotic communities among samples from *Pyropia haitanensis* and seawater with and without *Pyropia* cultivation. PC *Pyropia* cultivation

Fig. 2 Microbial community structures based on Bray–Curtis distances during the industrial and oceanic cultivation stages of *Pyropia haitanensis*. **a** NMDS results based on 16S and 18S rRNA genes of all the OTUs. **b** Statistical analysis of 16S and 18S rRNA genes based on groupings
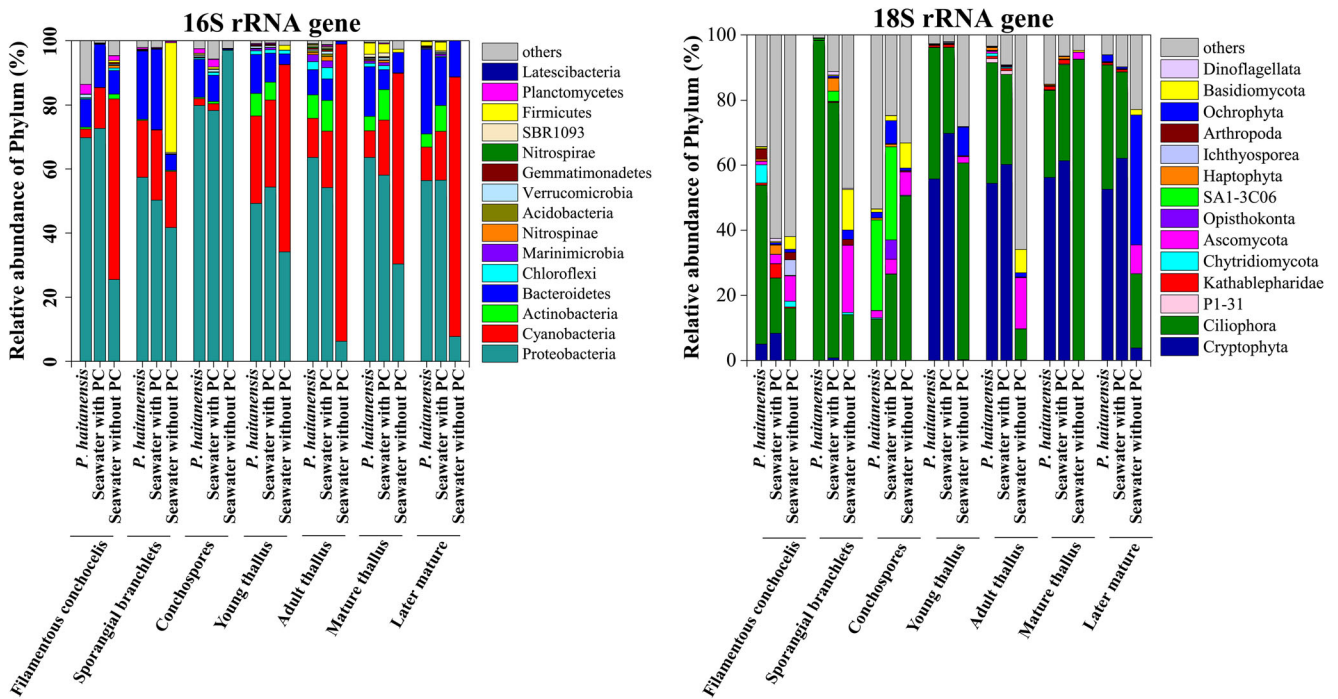
Fig. 3 Relative abundances of bacterial and eukaryotic community members among samples from *Pyropia haitanensis*, cultivation seawater, and non-cultivation seawater at the phylum level. PC *Pyropia* cultivation
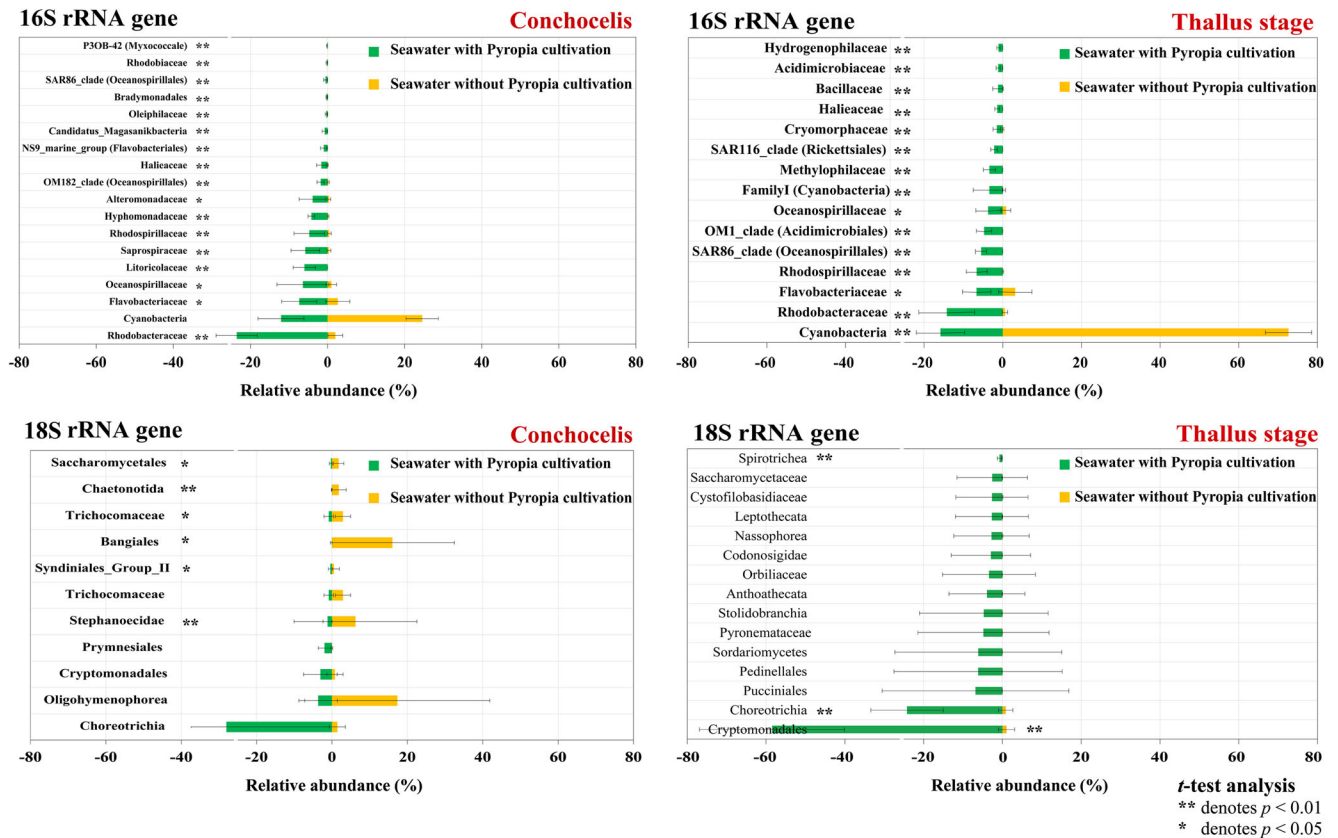
Fig. 4 Significant differences in the bacterial and eukaryotic families present in the seawater with and without *Pyropia* cultivation were observed. Families with > 1% relative abundances in the cultivation and non-cultivation seawaters are presented

During thallus stage, Rhodobacteraceae, Flavobacteriaceae, Rhodospirillaceae, Oceanospirillaceae, Methylophilaceae, and Cryomorphaceae were significantly higher ($P < 0.05$) in cultivation seawater than in non-cultivation seawater (Fig. 4). For the eukaryotic community, during conchocelis stage, Stephanoecidae and Trichocomaceae were significantly higher ($P < 0.05$) in non-cultivation seawater than in cultivation seawater (Fig. 4).

The effects of *Pyropia haitanensis* cultivation on the microbial community in surrounding seawater

One important capability of SEM is to partition direct and indirect effects that one variable may have on another. Our models explained 18 and 60% of the variances found in patterns of bacterial diversity on *P. haitanensis* and in seawater without *Pyropia* cultivation, respectively (Fig. 5a). However, for the bacterial diversity in the cultivation seawater, 22% of the variance was explained by our model. Seawater properties had a direct positive effect on *P. haitanensis* bacterial diversity ($\lambda = 0.42*$, $P < 0.05$), which strongly positively affected the cultivation seawater bacterial diversity ($\lambda = 0.46*$, $P < 0.05$; Fig. 5a). In addition, seawater properties had a direct negative effect on bacterial diversity in non-cultivation seawater ($\lambda = -0.78***$, $P < 0.05$), but this had no significantly direct effect on cultivation seawater bacterial diversity (Fig. 5a). For the eukaryotic community, our models explained 43 and 7% of the variances found in patterns of eukaryotic diversity on *P. haitanensis* and in the seawater without *Pyropia* cultivation, respectively (Fig. 5b). However, for the eukaryotic diversity of cultivation seawater, 81% of the variance was explained by our model. Seawater properties had a direct positive effect on *P. haitanensis* eukaryotic diversity ($\lambda = 0.66**$, $P < 0.01$), which strongly positively affected cultivation seawater eukaryotic diversity ($\lambda = 0.75***$, $P < 0.001$; Fig. 5b). However, seawater properties had no significantly direct effects on the eukaryotic diversity of the seawater with and without *Pyropia* cultivation. The eukaryotic diversity in non-cultivation seawater also had no significantly direct effect on the eukaryotic diversity in cultivation seawater (Fig. 5b).

Characterization of prokaryotic and eukaryotic microbial communities of *Pyropia haitanensis* during different cultivation stages

To evaluate the biodiversity at the same sequencing depth, 34,200 bacterial sequences and 45,602 eukaryotic sequences per sample were randomly selected to generate rarefaction curves. The alpha-diversity (Fig. 6) showed a distinct level of epiphytic microbes on *P. haitanensis* among different cultivation stages based on diversity, richness and evenness. For the bacterial community, adult thallus showed the highest diversity and richness levels compared with other cultivation
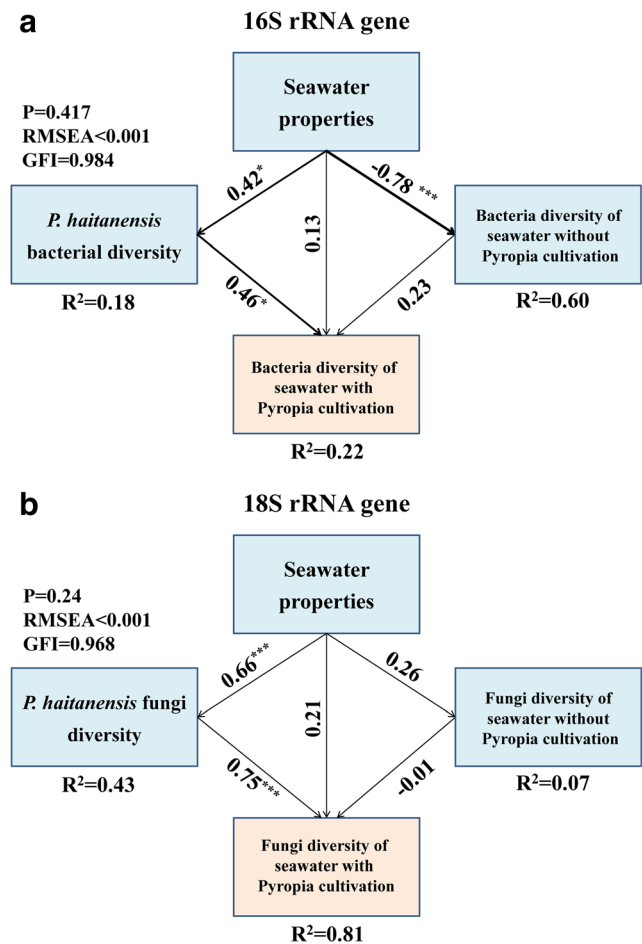

Fig. 5 Structural equation models show the direct and indirect effects of seawater properties, bacterial (**a**) and eukaryotic (**b**) diversity of *Pyropia haitanensis* and seawater without *Pyropia* cultivation on the microbial bacterial (**a**) and eukaryotic (**b**) diversity of seawater with *Pyropia* cultivation. Solid and dashed arrows indicate significant and non-significant relationships, respectively. Numbers adjacent to the arrows are path coefficients, and the width of the arrows is proportional to the strength of the path coefficients. R^2 denotes the proportion of variance explained. Significance levels are indicated: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Standardized effects (total, direct, and indirect effects) are derived from the structural equation models. The hypothetical models fit our data well: $\chi^2 = 0.658$, $P = 0.417$, CFI = 1, GFI = 0.984, and RMSEA < 0.001 (**a**); $\chi^2 = 1.381$, $P = 0.240$, CFI = 0.991, GFI = 0.968, and RMSEA < 0.001 (**b**)

stages. For the eukaryotic community, cochospores exhibited the highest diversity and evenness levels compared with other cultivation stages (Fig. 6).

Variations in the bacterial community composition, visualized by the NMDS analysis based on a Bray–Curtis dissimilarity matrix, revealed a significant difference ($P < 0.001$) between the thallus and conchocelis stages of epiphytic bacterial and eukaryotic community on *P. haitanensis* (Fig. 7). Additionally, different developmental periods among the thallus or conchocelis stages of epiphytic bacterial on *P. haitanensis* were also separated, as were those of the eukaryotic community (Fig. 7).
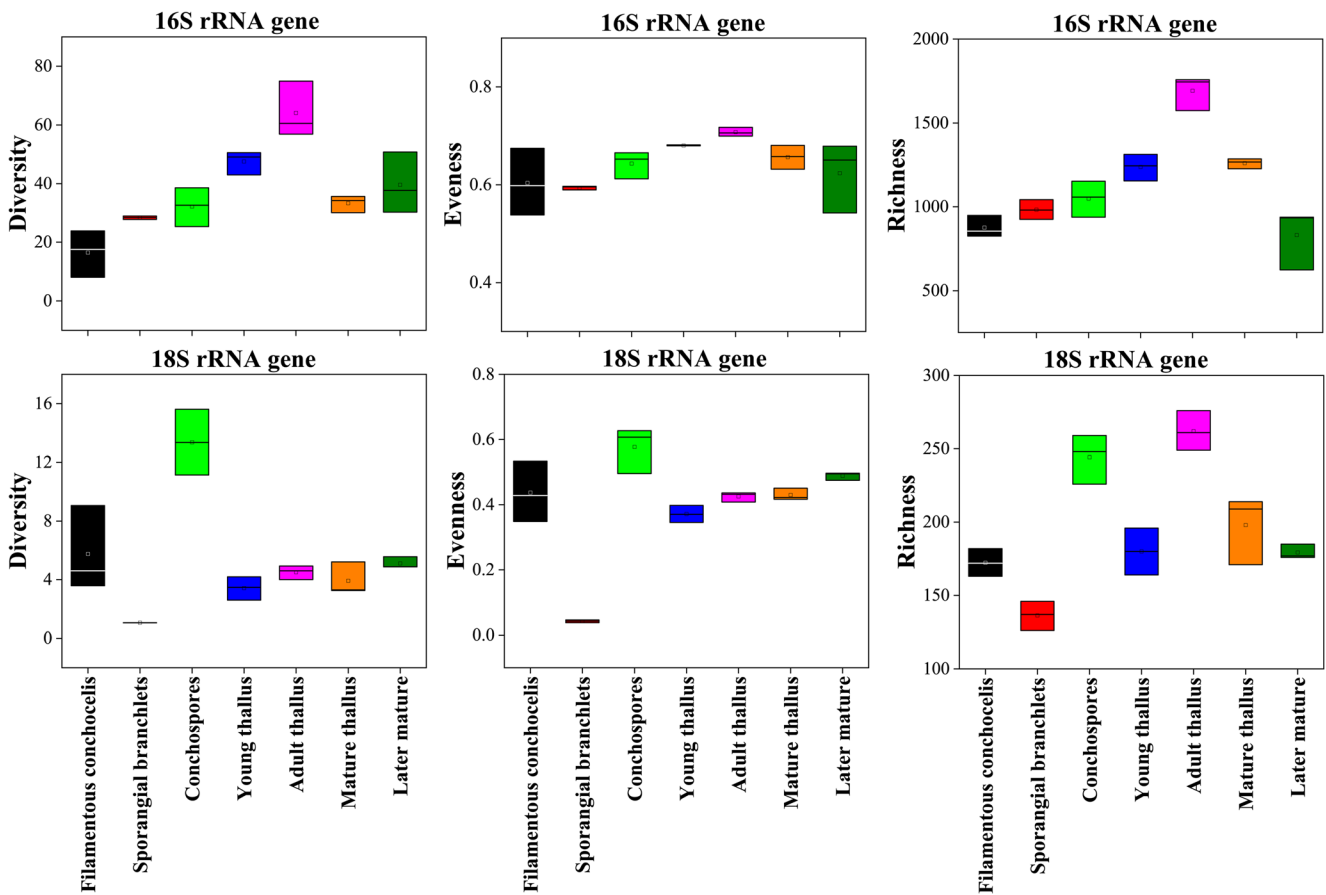
Fig. 6 Alpha-diversity (diversity, evenness, and richness) of bacterial and eukaryotic communities among epiphytic microbe samples from different stages of *Pyropia haitanensis* cultivation

Shifts in the bacterial and eukaryotic community composition were demonstrated in detail by examining community compositions at phylum and family levels. At the phylum level, Proteobacteria, Cyanobacteria, and Bacteroidetes were dominant among the epiphytic bacterial community on *P. haitanensis* during the entire cultivation period. Compared with the conchocelis stage, Proteobacteria decreased during

the thallus stage, while Actinobacteria and Cyanobacteria increased (Fig. 8). Ciliophora and Cryptophyta were the dominant eukaryotic microbes of *P. haitanensis* during the entire cultivation stages at phylum level. Compared with the conchocelis stage, Ciliophora decreased during the thallus stage, while Cryptophyta increased (Fig. 8). The composition of the epiphytic bacterial and eukaryotic community on
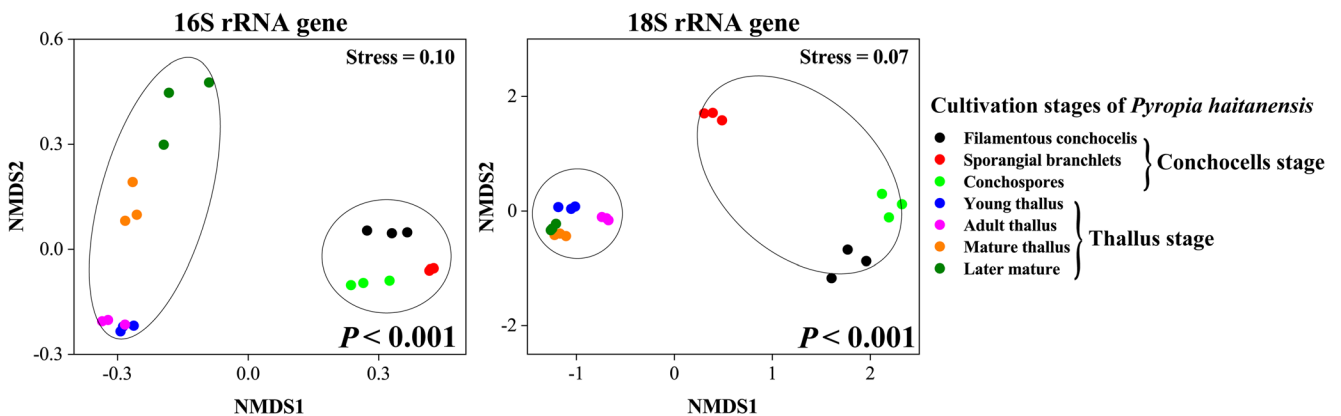

Fig. 7 Community structures of microbes based on Bray–Curtis distances during the thallus and conchochelis stages of epiphytic microbes on *Pyropia haitanensis*
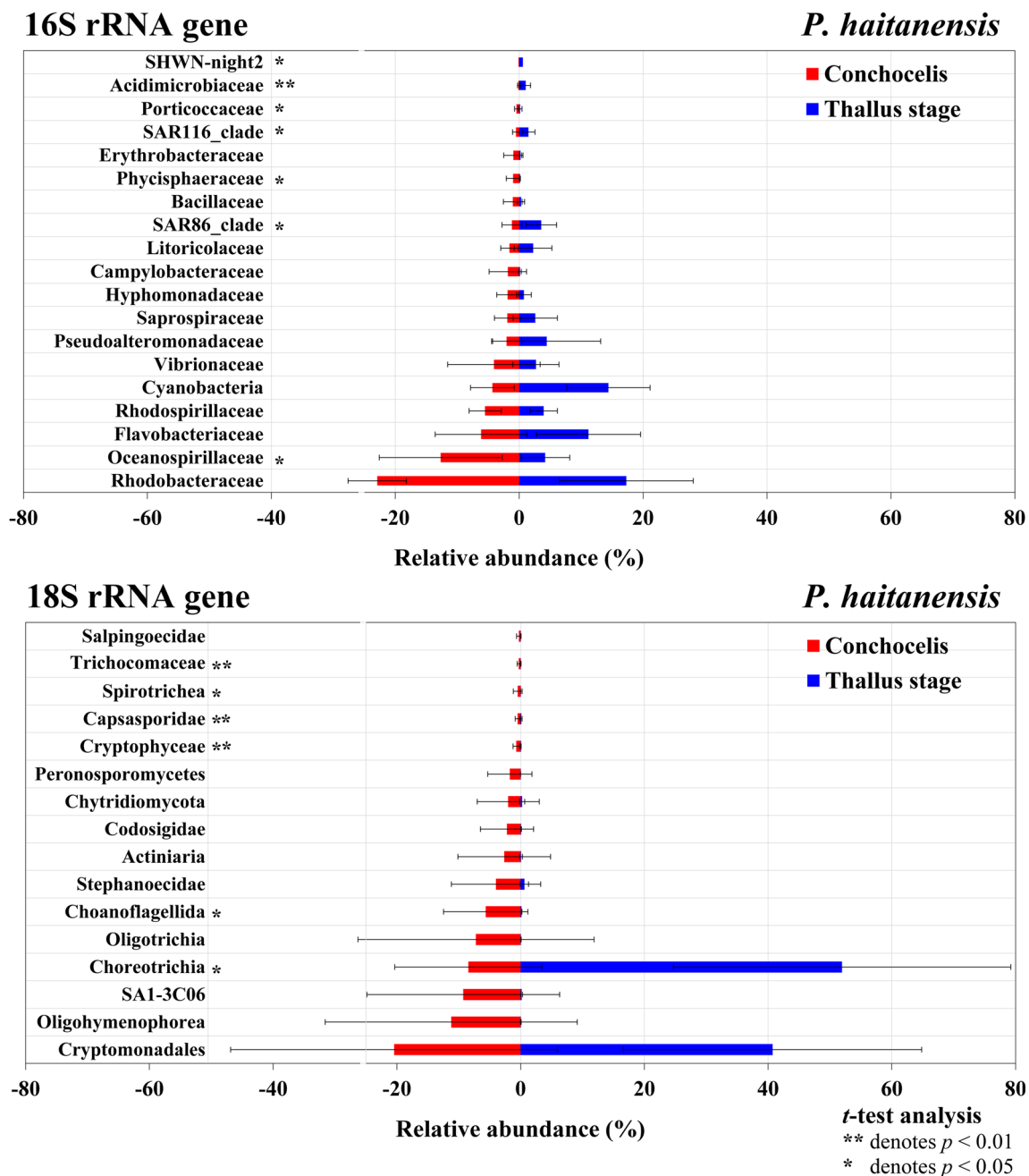
Fig. 8 Significant differences in the bacterial and eukaryotic families between the thallus and conchocelis stages of epiphytic microbes on *Pyropia haitanensis* were observed. Families with > 1% relative abundances in the thallus and conchocelis stages are presented

P. haitanensis between the thallus and conchocelis stages at the family level (with > 1% relative abundance) is shown in Fig. 8. Oceanospirillaceae was present at a significantly higher level ($P < 0.05$) during the conchocelis stage than the thallus stage, while Acidimicrobiaceae, SAR116_clade and SAR86_clade were present at significantly higher levels ($P < 0.05$) during the thallus stage than the conchocelis stage. Among the fungal community members on *P. haitanensis*, the level of Choreotrichia was significantly higher ($P < 0.05$) during the thallus stage than the conchocelis stage (Fig. 8).

Conversely, Choanoflagellida, Cryptophyceae, Capsasporidae, and Trichocomaceae were present at significantly higher levels ($P < 0.05$) during the conchocelis stage than the thallus stage (Fig. 8).

Discussion

Macroalgae are living hosts, performing essential and defining roles in coastal ecosystems (Burke et al. 2011). Microbial

biofilm communities in the marine environment positively affect the health, growth, and development of their hosts (Marshall et al. 2006; Burke et al. 2011). However, studies concerning the effects of the mariculture of economic red algae *Pyropia* on microbial communities are scarce. Additionally, the *Pyropia* life cycle contains two clearly different stages: the conchocelis and thallus stages (Blouin et al. 2011). However, the degree of microbial variation between the two stages is unknown. In the present study, therefore, deep sequencing was applied to investigate the microorganisms associated with *P. haitanensis* during the entire cultivation period.

The effects of *P. haitanensis* cultivation on the microbial community of the surrounding seawater The present data showed that *P. haitanensis* cultivation had obvious impact on the microbial community of the surrounding seawater (Figs. 1, 2, 3, 4, and 5), such that *P. haitanensis* bacterial diversity strongly positively affected cultivation seawater bacterial diversity and eukaryotic diversity (Fig. 5). Many bacteria were enriched remarkably in *P. haitanensis* cultivation seawater. This result is consistent with the previous study that *Gracilaria lemaneiformis* cultivation altered the microbial community composition and structure in cultivation seawater (Xie et al. 2017). The surfaces of marine macroalgae support diverse microbial biofilms (Lemay et al. 2018). The seaweed can also secrete various organic substances that can be utilized by bacteria (Singh and Reddy 2014). The algal polysaccharides (e.g., glucose, mannose and galactose) are potential sources of carbon and energy for numerous marine bacteria (Goecke et al. 2010; Hehemann et al. 2012). For example, Proteobacteria are known to digest galactan sulfates in red algal cell walls (Miranda et al. 2013). Roseovarius can also use glycerol provided by the marine macroalga *Ulva mutabilis* as a carbon source (Kessler et al. 2018). Here we found the most dominant phylum in seawater was Rhodobacteraceae in response to *P. haitanensis* cultivation (Fig. 3). Rhodobacteraceae, belonging to Alphaproteobacteria, are key players of biogeochemical cycling and account for up to 30% of bacterial communities in pelagic environments (Simon et al. 2017). Marine Rhodobacteraceae generally function in fucoidan desulfonation and the synthesis of the plant hormones and compatible solutes (e.g., ectoin and carnitine) (Simon et al. 2017). Additionally, Flavobacteriaceae exhibited higher abundance in seawater with *Pyropia* cultivation compared with non-cultivation seawater (Fig. 4), and they have the capacity to use brown seaweed fucoidan, a mixture of sulfated fucose-containing polysaccharides (Sakai et al. 2002). Moreover, Jiao et al. (2010) showed that dissolved organic matter released by algae can be utilized directly by microbes, constituting a microbial loop in aquatic ecosystems. Therefore, *P. haitanensis* may provide a habitat and nutrients for microbes able to degrade algal polysaccharides. The

seaweeds have the capacity to exude organic carbon and nutrients, whereas the seawater without seaweed cultivation contains relatively low nutrient concentrations. This might explain the strong differences in microbial communities between seawater with and without seaweed cultivation (Pregnall 1983).

In addition to eukaryotes, a dominated fungal species is Choreotrichia, which provides a key trophic link between the microbial and macroscopic components of the pelagic food web (Doherty et al. 2010). This finding indicated the important role of *P. haitanensis* cultivation in shaping the microbial diversity of cultivation seawater.

Differences in the microbial communities during various life stages of *P. haitanensis* The annual life cycle of *P. haitanensis* consists of a thallus stage and a shell-living conchocelis stage (Zeng 1985; Blouin et al. 2011). These stages are remarkably distinct in morphology, longevity, ploidy, and living environment. The conchospore develops into the thallus after undergoing meiosis. Meiosis in *P. haitanensis* occurs during the first two divisions of the germinating conchospore. The initial four cells of a developing conchosporeling constitute a linear genetic tetrad, resulting in the formation of chimeric blades of *P. haitanensis* (Yan et al. 2005). In the present study, the diversity, evenness and richness of the bacteria were highest in the adult thallus stage (Fig. 6). Additionally, the microbial community was obviously different between conchocelis and thallus stages (Figs. 6, 7, and 8). Firmicutes and the SAR86 group that belong to uncultured marine γ-proteobacterial were enriched in the thallus stage (Figs. 3 and 8). Interestingly, although the relative abundance of Firmicutes is very high in seawater without sporangial cultivation, it was not detected in sporangia (Fig. 3). This indicated that Firmicutes play important roles in thallus growth and development. Firmicutes and Proteobacteria (Alphaproteobacteria and Gammaproteobacteria) are also abundant on *Porphyra umbilicalis* blades collected in different seasons (Miranda et al. 2013). Proteobacteria and Firmicutes are involved in the morphogenesis of the green alga *Ulva* by synthesizing the differentiation inducer thallusin (Matsuo et al. 2005). *Roseovarius* sp. MS2 is able to sense dimethylsulfoniopropionate released by *Ulva* and promote the subsequent *Ulva* development and morphogenesis (Kessler et al. 2018). Matsuo and colleagues (2003) also found that the normal morphology of *Monostroma oxyspermum* depends on the presence of particular bacteria and not on bacteria in general. Therefore, the present data suggested that the enriched bacteria might promote *Pyropia* morphogenesis.

The fungal enrichment results showed that Cryptophyta was dominant during the thallus stage, while Choanoflagellida, Cryptophyceae, Capsasporidae, and Trichocomaceae were enriched during the conchocelis stage. We also found that seawater properties had a direct positive

effect on *P. haitanensis* eukaryotic diversity ($\lambda = 0.66^{**}$, $P < 0.01$; Fig. 5b). This difference, therefore, may result from the higher water temperature in the culture ponds during industrial cultivation of conchocelis compared with the seawater temperature during oceanic cultivation of thalli. High temperature is known to improve the growth rates of marine fungi. In addition, the water exchange rate in the conchocelis culture ponds was lower than that in the ocean, and the slow flow rate in the ponds may facilitate the accumulation of fungi. *Pyropia* disease outbreaks always occur during conchocelis cultivation, especially in hot summers, and results in serious economic losses (Guan et al. 2013). Thus, excessive temperature changes should be prevented and the seawater should be refreshed in a timely fashion in the culture ponds to protect conchocelis from disease induced by fungi. Furthermore, the diversity, evenness, and richness of eukaryotes were the greatest in the conchospore stage, while they were very low in the sporangial stage (Fig. 6), even though conchospore are released after sporangial mature. This might occur because conchospores are single-celled algae that lack a cell wall (Fan et al. 2008), which allows epiphytic eukaryotic to easily parasitize or invade algae and even induce disease (Egan et al. 2013). For example, *Olpidiopsis porphyrae* utilizes encysted zoospores to infect *Pyropia* (Sekimoto et al. 2008). In addition, some epiphytic and endophytic fungi secrete defensive compounds to resist invasion from other microorganisms, such as terpenoids, alkaloids, and lipids, which exhibit antibacterial and antifungal activities (Rateb and Ebel 2011; Egan et al. 2013). However, the functions of eukaryotes in the alga–fungus symbiosis require further investigation.

The advantages of microbes for *P. haitanensis* growth and development One topic in the macroalgal holobiont is the advantages of the presence of microbes on macroalgae. For example, the microbes produce plant growth regulators (e.g., indole-3-acetic acid and cytokinins) (Maruyama et al. 1990; Singh et al. 2011), quorum sensing molecules (Twigg et al. 2014; Singh et al. 2015), and bioactive compounds (e.g., vitamin B_{12}) (Singh et al. 2011; Singh and Reddy 2014). Here, Bacillaceae were enriched in the seawater with *Pyropia* cultivation (Fig. 4). Isolates of *Bacillus* species from the rhizosphere of soybean (*Glycine max*) produce indole-3-acetic acid and stimulate increased root lengths, shoot lengths, and numbers of lateral roots in soybean seedlings (Wahyudi et al. 2011). Furthermore, *Bacillus pumilus* and *B. licheniformis*, isolated from the rhizosphere of alder (*Alnus glutinosa*), produce high concentrations of gibberellins to promote host plant growth (Gutiérrez-Mañero et al. 2001). A *Bacillus megaterium* strain promoted the growth of *Arabidopsis thaliana* and *Phaseolus vulgaris* seedlings by regulating cytokinin signal transduction (Ortíz-Castro et al. 2008). *Pyropia haitanensis* co-cultured with *Bacillus* sp. WPySW2 grew better than wild type at 20 °C (Xiong et al. 2018). The present data suggested that Bacillaceae may be applicable as plant growth and development promoters for *Pyropia*. Moreover, Tait et al. (2005) demonstrated that the disruption of quorum sensing (N-acylhomoserine lactone) in seawater prevents the attraction of *Ulva* zoospores to bacterial biofilms. Additionally, many macroalgae are rich in vitamin B_{12}, including *Pyropia* (Nakamura et al. 2013), but vitamin B_{12} can only be synthesized by prokaryotes. Thus, a mutualistic relationship has been proposed to exist between vitamin B_{12}-dependent algae and heterotrophic bacteria. In the present study, Firmicutes were a key factor in the interactions among different bacteria in the thallus stage (Fig. 3). Firmicutes are generally one of the most abundant bacteria on seaweed surfaces and are potential candidates as producers of vitamin B_{12} (Singh and Reddy 2014). *Pyropia/Porphyra* may obtain vitamin B_{12} from its abundant bacterial epiphytes (Brawley et al. 2017). Thus, the enriched microbial community in the cultivation seawater might be beneficial for the development and growth of the host seaweed (Singh et al. 2011; Singh and Reddy 2014).

Conclusion

We observed that *P. haitanensis* harbored phylogenetically diverse bacteria and eukaryotic organisms during the entire cultivation period. A distinct shift in the microbial community composition of *P. haitanensis* was observed between seawater with and without *Pyropia* cultivation as well as between the oceanic cultivation of thalli and industrial cultivation of conchocelis. The detected potential candidate bacteria might promote *Pyropia* morphogenesis and produce vitamin B_{12} and various plant hormones that promote the growth and development of *Pyropia*. The present results enhance our knowledge of the important role of seaweed cultivation in shaping the microbial diversity of cultivation seawater and the beneficial bacterial and fungal associations with seaweed.

Compliance with ethical standards

References

Armstrong E, Yan L, Boyd KG, Wright PC, Burgess JG (2001) The symbiotic role of marine microbes on living surfaces. Hydrobiologia 461:37–40

Beleneva IA, Zhukova NV (2006) Bacterial communities of some brown and red algae from Peter the Great Bay, the Sea of Japan. Microbiology 75:348–357

Bell W, Mitchell R (1972) Chemotactic and growth responses of marine bacteria to algal extracellular products. Biol Bull 143:265–277

Blouin NA, Brodie JA, Grossman AC, Xu P, Brawley SH (2011) *Porphyra*: a marine crop shaped by stress. Trends Plant Sci 16:29–37

Brawley SH, Blouin NA, Ficko-Blean E, Wheeler GL, Lohr M, Goodson HV, Jenkins JW, Blaby-Haas CE, Helliwell KE, Chan CX, Marriage TN, Bhattacharya D, Klein AS, Badis Y, Brodie J, Cao Y, Collén J, Dittami SM, Gachon CMM, Green BR, Karpowicz SJ, Kim JW, Kudahl UJ, Lin S, Michel G, Mittag M, Olson BJSC, Pangilinan JL, Peng Y, Qiu H, Shu S, Singer JT, Smith AG, Sprecher BN, Wagner V, Wang W, Wang ZY, Yan J, Yarish C, Zäuner-Riek S, Zhuang Y, Zou Y, Lindquist EA, Grimwood J, Barry KW, Rokhsar DS, Schmutz J, Stiller JW, Grossman AR, Prochnik SE (2017) Insights into the red algae and eukaryotic evolution from the genome of *Porphyra umbilicalis* (Bangiophyceae, Rhodophyta). Proc Natl Acad Sci U S A 114:E6361–E6370

Burke C, Thomas T, Lewis M, Steinberg P, Kjelleberg S (2011) Composition, uniqueness and variability of the epiphytic bacterial community of the green alga *Ulva australis*. ISME J 5:590–600

China Fishery Bureau, Fishery production (2018) China fishery statistical yearbook (in Chinese). Chinese Agriculture Express

Cole JJ (1982) Interactions between bacteria and algae in aquatic ecosystems. Annu Rev Ecol Syst 13:291–314

de Mesquita MMF, Crapez MAC, Teixeira VL, Cavalcanti DN (2019) Potential interactions bacteria-brown algae. J Appl Phycol 31:867–883

Dittami S, Arboleda E, Auguet JC, Bigalke A, Briand E, Cárdenas P, Cardini U, Decelle J, Engelen A, Eveillard D, Gachon CMM, Griffiths S, Harder T, Kayal E, Kazamia E, Lallier FH, Medina M, Marzinelli EM, Morganti T, Pons LN, Pardo S, Valverde J, Saha M, Selosse M, Skillings D, Stock W, Sunagawa S, Toulza E, Vorobev A, Leblanc C, Not F (2019) A community perspective on the concept of marine holobionts: state-of-the-art, challenges, and future directions. PeerJ arXiv: 1907.05017

Doherty M, Tamura M, Vriezen JA, McManus GB, Katz LA (2010) Diversity of Oligotrichia and Choreotrichia ciliates in coastal marine sediments and in overlying plankton. Appl Environ Microbiol 76:3924–3935

Egan S, Harder T, Burke C, Steinberg P, Kjelleberg S, Thomas T (2013) The seaweed holobiont: understanding seaweed–bacteria interactions. FEMS Microbiol Rev 37:462–476

Eisenhauer N, Bowker MA, Grace JB, Powell JR (2015) From patterns to causal understanding: structural equation modeling (SEM) in soil ecology. Pedobiologia 58:65–72

Fan XL, Wang GC, Li DM, Xu P, Shen SD (2008) Study on early-stage development of conchospore in *Porphyra yezoensis* Ueda. Aquaculture 278:143–149

Farooq A, Francesca M (2007) Microbial structuring of marine ecosystems. Nat Rev Microbiol 5:782

Gai S, Zhang W (2016) Microbiota shell-boring conchocelis of *Pyropia yezoensis* determined by the next-generation sequencing. Oceanol Limnol Sin 47:990–996 (in Chinese)

Goecke F, Labes A, Wiese J, Imhoff JF (2010) Chemical interactions between marine macroalgae and bacteria. Mar Ecol Prog Ser 409:267–299

Guan X, Li J, Zhang Z, Li F, Yang R (2013) Characterizing the microbial culprit of white spot disease of the conchocelis stage of *Porphyra yezoensis* (Bangiales, Rhodophyta). J Appl Phycol 25:1341–1348

Gutiérrez-Mañero FJ, Ramos-Solano B, Probanza AN, Mehouachi J, Tadeo FR, Talon M (2001) The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. Physiol Plant 111:206–211

Hartmann M, Widmer F (2006) Community structure analyses are more sensitive to differences in soil bacterial communities than anonymous diversity indices. Appl Environ Microbiol 72:7804–7812

Hehemann JH, Correc G, Thomas F, Bernard T, Barbeyron T, Jam M, Helbert W, Michel G, Czjzek M (2012) Biochemical and structural characterization of the complex agarolytic enzyme system from the marine bacterium *Zobellia galactanivorans*. J Biol Chem 287:30571–30584

Jiao N, Herndl GJ, Hansell DA, Brenner R, Kattner G, Wilhelm SW, Kirchman DL, Weinbauer MG, Luo T, Chen F, Azam F (2010) Microbial production of recalcitrant dissolved organic matter: long-term carbon storage in the global ocean. Nat Rev Microbiol 8:593–599

Kent M (2011) Vegetation description and data analysis: a practical approach. Wiley, Hokoben

Kessler RW, Weiss A, Kuegler S, Hermes C, Wichard T (2018) Macroalgal–bacterial interactions: role of dimethylsulfoniopropionate in microbial gardening by *Ulva* (Chlorophyta). Mol Ecol 27:1808–1819

Lemay MA, Martone PT, Keeling PJ, Burt JM, Krumhansl KA, Sanders RD, Parfreyet LW. (2018) Sympatric kelp species share a large portion of their surface bacterial communities. Environ Microbiol 20(2):658–670

Marshall K, Joint I, Callow ME, Callow JA (2006) Effect of marine bacterial isolates on the growth and morphology of axenic plantlets of the green alga *Ulva linza*. Microb Ecol 52:302–310

Maruyama A, Maeda M, Simidu U (1990) Distribution and classification of marine bacteria with the ability of cytokinin and auxin production. Bull Jap Soc Microb Ecol 5:1–8

Matsuo Y, Imagawa H, Nishizawa M, Shizuri Y (2005) Isolation of an algal morphogenesis inducer from a marine bacterium. Science 307:1598

Miranda LN, Hutchison K, Grossman AR, Brawley SH (2013) Diversity and abundance of the bacterial community of the red macroalga *Porphyra umbilicalis*: did bacterial farmers produce macroalgae? PLoS One 8:e58269

Nakamura Y, Sasaki N, Kobayashi M, Ojima N, Yasuike M, Shigenobu Y, Satomi M, Fukuma Y, Shiwaku K, Tsujimoto A, Kobayashi T, Nakayama I, Ito F, Nakajima K, Sano M, Wada T, Kuhara S, Inouye K, Gojobori T, Ikeo K (2013) The first symbiont-free genome sequence of marine red alga, Susabi-nori (*Pyropia yezoensis*). PLoS One 8:e57122

Ortíz-Castro R, Valencia-Cantero E, López-Bucio J (2008) Plant growth promotion by *Bacillus megaterium* involves cytokinin signaling. Plant Signal Behav 3:263–265

Pregnall AM (1983) Release of dissolved organic carbon from the estuarine intertidal macroalga *Enteromorpha prolifera*. Mar Biol 73:37–42

Rateb ME, Ebel R (2011) Secondary metabolites of fungi from marine habitats. Nat Prod Rep 28:290–344

Rousk J, Bååth E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R, Fierer N (2010) Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME J 4:1340–1351

Sakai T, Kimura H, Kato I (2002) A marine strain of Flavobacteriaceae utilizes brown seaweed fucoidan. Mar Biotechnol 4:399–405

Sekimoto S, Yokoo K, Kawamura Y, Honda D (2008) Taxonomy, molecular phylogeny, and ultrastructural morphology of *Olpidiopsis porphyrae* sp. nov. (Oomycetes, straminipiles), a unicellular obligate endoparasite of *Bangia* and *Porphyra* spp. (Bangiales, Rhodophyta). Mycol Res 112:361–374

Shen M, Yang R, Luo Q, Wang S, Ren J (2013) Microbial diversity of *Pyropia haitanensis* phycosphere during cultivation. Acta Microbiol Sin 53:1087–1102 (in Chinese)

Simon M, Scheuner C, Meier-Kolthoff JP, Brinkhoff T, Wagner-Döbler I, Ulbrich M, Klenk HP, Schomburg D, Petersen J, Göker M (2017) Phylogenomics of Rhodobacteraceae reveals evolutionary adaptation to marine and non-marine habitats. ISME J 11:1483–1499

Singh RP, Reddy CRK (2014) Seaweed–microbial interactions: key functions of seaweed-associated bacteria. FEMS Microbiol Ecol 88:213–230

Singh RP, Bijo AJ, Baghel RS, Reddy CRK, Jha B (2011) Role of bacterial isolates in enhancing the bud induction in the industrially important red alga *Gracilaria dura*. FEMS Microbiol Ecol 76:381–392

Singh RP, Baghel RS, Reddy CRK, Jha B (2015) Effect of quorum sensing signals produced by seaweed-associated bacteria on carpospore liberation from *Gracilaria dura*. Front Plant Sci 6:117

Su JQ, Wei B, Ou-Yang WY, Huang F-Y, Zhao Y, Xu H-J, Zhu Y-G (2015) Antibiotic resistome and its association with bacterial communities during sewage sludge composting. Environ Sci Technol 49:7356–7363

Tait K, Joint I, Daykin M, Milton DL, Williams P, Camara M (2005) Disruption of quorum sensing in seawater abolishes attraction of zoospores of the green alga *Ulva* to bacterial biofilms. Environ Microbiol 7:229–240

Twigg MS, Tait K, Williams P, Atkinson S, Cámara M (2014) Interference with the germination and growth of *Ulva* zoospores by quorum-sensing molecules from *Ulva*-associated epiphytic bacteria. Environ Microbiol 16:445–453

van der Loos LM, Eriksson BK, Salles JF (2019) The macroalgal holobiont in a changing sea. Trends Microbiol 7:635–650

Vilo C, Dong Q (2012) Evaluation of the RDP classifier accuracy using 16S rRNA gene variable regions. Metagenomics 1:1–5

Wahyudi AT, Astuti RP, Widyawati A, Mery A, Nawangsih AA (2011) Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria. J Microbiol Antimicrob 3:34–40

Xie X, He Z, Hu X, Yin H, Liu X, Yang Y (2017) Large-scale seaweed cultivation diverges water and sediment microbial communities in the coast of Nan'ao Island, South China Sea. Sci Total Environ 598:97–108

Xiong Y, Yang R, Sun X, Yang H, Chen H (2018) Effect of the epiphytic bacterium *Bacillus* sp. WPySW2 on the metabolism of *Pyropia haitanensis*. J Appl Phycol 30:1225–1237

Yan XH, Li L, Aruga Y (2005) Genetic analysis of the position of meiosis in *Porphyra haitanensis* Chang et Zheng (Bangiales, Rhodophyta). J Appl Phycol 17:467–473

Yan Y, Yang H, Tang L, Li J, Mao Y, Mo Z (2019) Compositional shifts of bacterial communities associated with *Pyropia yezoensis* and surrounding seawater co-occurring with red rot disease. Front Microbiol 10:1666

Zeng C (1985) Seaweed aquaculture. Shanghai Scientific & Technical Publishers, Shanghai

Zhu Y, Wu M, Gao N, Chu W, Wang S (2016) Impacts of nitrate and electron donor on perchlorate reduction and microbial community composition in a biologically activated carbon reactor. Chemosphere 2016:134–143