

Diversity of Epiphytic Bacterial Communities on Male and Female *Porphyra haitanensis*

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Abstract

Purpose

Epiphytic bacteria play an important role in macroalgae growth, development, and morphogenesis. However, epiphytic bacterial communities on male and female macroalgae have not been reported. *Porphyra haitanensis* is one of the main economic macroalgae. In order to explore the similarities and differences of epiphytic bacterial community structure between male and female macroalgae of *Porphyra haitanensis*.

Methods

We investigated the composition, diversity of epiphytic bacterial communities between male and female *Porphyra haitanensis* by 16S rDNA high-throughput sequencing.

Results

The divergences of bacterial community compositions occurred between males and females. Both males and females had their unique bacterial microbiota, such as, Armatimonadetes and Rokubacteria are the unique phyla of male *Porphyra haitanensis*, Chlamydiae is a unique phylum of female *Porphyra haitanensis*. The epiphytic bacteria on both male and female *Porphyra haitanensis* have the similar predictive functions, but they also have their own specific functions, respectively. The specific functions of epiphytic bacteria on female *Porphyra haitanensis* were sulfite_respiration, nitrogen_fixation, nitrate_ammonification, chlorate_reducers and anoxygenic_photoautotrophy_S_oxidizing.

Conclusions

This study provides a basis for exploring the mechanism of epiphytic bacterial communities on dioecious algae and are of great significance for further understanding the relationships between epiphytic microbial communities and the sex of algae.

1 Introduction

Macroalgae is an important natural resource, and its products are various, such as food, alginate, agar, carrageenan, fertilizer and animal feed additives. Most of the macroalgae for these products are produced by aquaculture. The macroalgae farming industry has developed rapidly in many countries around the world, with China accounting for a large portion of the global annual production (Roesijadi et al., 2010). A rich and diverse group of microbes lives on macroalgae, which play important roles in the lives of their hosts (Hollants et al., 2013), and the interactions between macroalgae and their epiphytic bacteria are intricate. Macroalgae release many organic substances during growth (Croft et al., 2005), which are absorbed and utilized by the surrounding epiphytic bacteria. Some of these organic substances are metabolized by the bacteria and released back to the ocean in mineral or other forms, providing nutrients and essential growth factors for algal growth (Florez et al., 2017). Meanwhile, macroalgae could

also produce chemical defense agents (antibacterial compounds such as peroxides) to prevent some bacteria from adhering (Campbell et al., 2015). Nevertheless, bacteria have developed metabolic pathways to combat these chemical defenses (Oliveira et al., 2012), and they can even inhibit algal growth or disrupt algal cells. The epiphytic bacteria of macroalgae can be both mutually beneficial and antagonistic, and they can be selectively enriched through specific interactions with their host macroalgae.

Epiphytic bacteria play a vital role in the normal growth and development of algae. Macroalgae have metabolic products on their surfaces that nourish epiphytic bacteria. Algae can regulate epiphytic bacteria on their surfaces in a variety of ways. In addition, seasonal changes, spatial differences and environmental factors can also affect the composition of epiphytic bacteria on the surface of algae.

The community structure of epiphytic bacteria in macroalgae is host-specific, and the community structure of epiphytic bacteria is different in different macroalgae. Selvarajan (Selvarajan R et al., 20) studied the structure of epiphytic bacterial communities of eight common seaweeds growing in the intertidal zone of Cape Verde, South Africa. The results showed that the bacterial communities of brown algae were very similar to those of green algae, while that of red algae formed an independent branch. WU Hongqing (WU Hongqing et al.) used PCR technology to conduct phylogenetic analysis on the community structure of epiphytic bacteria of four large red algae. The results showed that *Gammaproteobacteria* accounted for the absolute dominant position of epiphytic bacteria in *Gracilaria lemaneiformis*. The dominant flora of epiphytic bacteria in *Porphyra yezoensis* was *Bacteroides spp.* All these have fully illustrated that epiphytic bacteria of macroalgae have host specificity.

Seasonal changes and spatial differences are important factors affecting the structure of epiphytic bacterial communities in macroalgae. Natalia (Natalia et al.) studied the epiphytic bacterial communities of *Ulva lactuca* growing in Santa Marta La Punta de La Loma, Colombia, using the high-throughput sequencing technique and found that the changes in abundance of Rhodobacteraceae, Hyphomonadaceae and Flavobacteriaceae might be due to the increase in sea water temperature. Mensch B (Mensch B et al.) tested the epiphytic bacteria of *Fucus vesiculosus* in the Baltic Sea continuously every three weeks for one year using high-throughput sequencing technology. The results showed that the composition of bacterial community changed with time, and with the increase of temperature, the number of beneficial bacteria in epiphytic bacteria of *Porphyra haitanensis* was decreased, while the number of harmful bacteria was increased. Particularly, in spring and summer, the number of potential pathogenic bacteria and bacteria related to intensified micro-pollution was increased. Alexandra (Alexandra et al.) studied the community structure of epiphytic bacteria on *Ulva lactuca* collected from Spain and Australia. The results showed that the community structures of epiphytic bacteria on *Ulva lactuca* collected from Spain and Australia were different, and there was a small correlation between them (similarity of 18%), reflecting a relationship of attenuation with distance, the decrease in similarity between microbial communities was related to the increase in geographical distance.

The difference in epiphytic bacteria on dioecious higher plants was confirmed. Epiphytic bacterial communities in the rhizosphere of *Populus euphratica* are affected by gender (Liu et al., 2020). However, there are few studies on epiphytic bacterial communities of dioecious macroalgae. Understanding the differences in the diversity of epiphytic bacteria of macroalgae will help to understand the role of male and female differentiation in the construction of epiphytic bacterial communities.

2 Material And Methods

2.1 Sampling site and sampling male and female *Porphyra haitanensis*

The sampling site was in the rocky intertidal zone of Niushan Island (25°N, 119°E) in Fujian, on January 19, 2020. Three samples from male *Porphyra haitanensis*, three from female and three samples from seawater were collected from same site.

2.2 Samples of epiphytic bacteria of male and female *Porphyra haitanensis*

Sample collection of epiphytic bacteria of *Porphyra haitanensis*: take the sample of *Porphyra haitanensis*, wipe a sample evenly with 10 swabs, put swabs into sample tube after wiping, quickly put them into dry ice, take them back to the laboratory and store them in the refrigerator at -80 °C until DNA is extracted.

Environmental water sample treatment: the seawater around *Porphyra haitanensis* was collected by aseptic water bottle, 500ml seawater was collected for each sample, and the diameter of each sample was 47 mm and the pore diameter was 0.22 mm μm polycarbonate filter membrane to collect individual microorganisms. The polycarbonate filter membrane rich in microorganisms was stored in dry ice, frozen and taken back to the laboratory, and stored in the refrigerator at -80 °C until DNA extraction.

2.3 16S rDNA high-throughput sequencing

DNA was extracted from 0.22- μm membranes using an E.Z.N.A. Stool DNA Kit (Omega Biotek, USA) according to the manufacturer's instructions. The V3-V4 region of the 16S rDNA gene was amplified with universal primers. High-throughput sequencing was performed on an Illumina NovaSeq platform by LC-BIO Technologies Co., Ltd. (Hangzhou, China). Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (v.1.2.7). Quality filtering on the raw reads were performed under specific filtering conditions to obtain the high-quality clean tags according to the fqtrim (v0.94). Chimeric sequences were filtered using Vsearch software (v2.3.4). Regarding the high-throughput sequencing analysis, the optimized sequences were clustered according to similarity and operational taxonomic units (OTUs) were defined based on 97% similarity.

2.4 Data analysis

Data analysis was based on methods described according to previous research (Mathai et al., 2018). Quality control including chimeric sequence removal from the dataset was completed, along with subsequent removal of *Porphyra haitanensis* DNA and exclusion of chloroplast and mitochondrial sequences. Chao1 and Shannon indices were used for comparison of α -diversity by using the Wilcoxon rank sum test. While, β -diversity was calculated using unweighted UniFrac distances and represented in principal coordinate analyses (Principal coordinates analysis, PCoA), revealed by ANOSIM test and NMDS analyses. The Bray-Curtis dissimilarity index was used to assess beta diversity (between sample diversity). All of them were performed using the QIIME2 scripts and R software (v 3.6.1). The sequences were classified using BLAST and the SILVA (release 132) databases. SparCC method was used to analyze correlations among the bacterial genera. The correlation between quantitative variables was estimated by the Spearman coefficient correlation (ρ). R package igraph (1.2.6) was used to make the network plots. Using updated PICRUSt2 software (Douglas et al., 2019), the unigenes were annotated with Kyoto Encyclopedia of Genes and Genomes (KEGG) level 2 and 3 categories by using the t-test. Univariate and multivariate statistical analyses were adopted to analyze the results by SPSS 18.0 software.

3 Results

3.1 Sexual differences in richness and diversity of microbial communities

In total, 1119154 high-quality bacterial sequences were obtained from 9 samples (Table S1). The rarefaction analyses indicated that the diversity of all samples was well represented by the number of sequences analyzed (Fig. S1). The bacterial sequences in the female and male microbial communities were clustered into 1417 and 1706 OTUs at a 97% sequence similarity, respectively. The bacterial sequences in the microbial community in the surrounding seawater were clustered into 1608 OTUs with 97% sequence similarity. Both females, males and seawater had their shared and unique bacteria OTUs, (Fig. S2). As to the alpha-diversity indexes, there was significant difference between male and female algae epiphytic bacterial communities in Chao1 and ACE index ($P < 0.05$), but there was no significant difference between male and female bacterial communities in Shannon and Simpson index ($P > 0.05$) (Fig. 1). Similarities of the phyllosphere bacterial communities between sexes were compared by NMDS and Adonis test based on Bray-Curtis. NMDS analysis showed that bacterial communities of males, females and seawater were distributed in different quadrants (Fig. 2). To verify the dominant role of sex selection on microbial communities, the variations between the individual samples and males vs females vs seawater were determined using Adonis test (P values) (Table 1). As shown in the Table 1, the inter-plant (males vs females vs seawater) variation was greater than that of intra-plant (among individuals) in microbial communities.

Table 1
Permutational multivariate (Adonis) analysis of variance of the bacterial composition

diffs	Df	SumsOfSqs	MeanSqs	Fvalue	R ²	Pvalue	significant
Male-vs-Female-vs-Seawater	2	2.0999	1.05	18.5187	0.8606	0.002	**

3.2 Sexual differences in the composition of the bacterial community

The dominant bacterial phyla were Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria and Planctomycetes (Fig. 3A). The phylum Proteobacteria accounted for 40.28% and 28.74% of the total sequences in the male and female bacterial communities. The dominant bacterial genera were *Tenacibaculum*, *Maribacter*, *Granulosicoccus*, and *Hellea*, representing 36.72% and 31.4% of the total sequences in males and females, respectively (Fig. 3B). At the genus level, 82 bacterial genera were unique in male (Fig. 3A). Such as *Rubriphilellula* (0.079%), *Lysinibacillus* (0.053%), *Oceanicella* (0.05%) etc. 71 bacterial genera were unique in the female, such as *Candidatus_Udaeobacter* (0.19%), and some rare genera (relative abundance \leq 0.1%) *Pontibacter*, *Leucobacter*, *Hydrogenoanaerobacterium* etc. (Fig. S3).

3.3 Analysis of indicative species

There were 11 and 3 indicative epiphytic bacteria taxa for the male and female *Porphyra haitanensis*, respectively (Fig. 4) and among them, bacteria from the *Maribacter* (male: 14.67%, female: 1.48%) genus and the *Tenacibaculum* (male: 1.40%, female: 22.38%) genus were the most indicative for male and female *Porphyra haitanensis* (Fig. S4), respectively, according to the negative linear discriminant analysis (LDA) score.

3.4 Predicted functional analysis for microbial communities through PICRUSt2 and Tax4Fun

The function of the bacterial community was predicted using the Tax4Fun tool. Figure 5 shows the heat map of the 20 KEGG class 2 functional pathways with relatively high abundance.

Malaria, Neomycin, kanamycin and gentamicin biosynthesis, Novobiocin biosynthesis were unique function in male. Ethylbenzene degradation, Complement and coagulation cascades and Dorso-ventral axis formation were unique function in female. (Fig. S5) Moreover, some functions of bacteria attached to *Porphyra haitanensis* surface were significantly different between male and female algae (Fig. S6). For example, male algae have more advantages in Fatty acid biosynthesis, Lipopolysaccharide biosynthesis and D-Arginine and D-ornithine metabolism etc. Female algae have more advantages in Ethylbenzene degradation, Polyketide sugar unit biosynthesis and Photosynthesis etc.

4 Discussion

Our results indicated that the influence of male and female *Porphyra haitanensis* on epiphytic bacterial community was statistically not significant, which is consistent with previous studies on male and female epiphytic bacteria in higher plants. Liu (2020) found that there was no significant difference between female and male phyllosphere bacterial communities, but there was significant difference in relative abundance of phyllosphere bacterial and fungal at genus level between female and male *Populus cathayana* by using 16S rRNA/ITS1 gene-based MiSeq sequencing. Our results are similar to those above, and support the view that sex has little effect on epiphytic bacterial diversity. It is speculated that sex differentiation of *Porphyra haitanensis* has minor impact on the epiphytic bacterial communities. At the genus level, there were bacteria with significant differences in relative abundance between male and female algae, and both male and female algae had their own unique flora. These results will help us to understand the differences of epiphytic bacteria in dioecious plants.

There were no significant differences in the alpha diversity indexes between males and females, which was consistent with the findings of a previous study showing that microbial community structure of the same species showed little variability (Yang et al. 2001). Previous study had also shown that there was far more variability in microbial community structure across agle species than within species in the same habitat (Redford et al. 2010). The samples in this study were collected from the same hillside with similar elevation, temperature, rainfall, or solar, which might explain the undifferentiated diversity indexes between males and females. However, the differences in element contents, stomatal distribution, gas exchange, and net photosynthetic rates (Liu 2003; Wang et al. 2010; Xu et al. 2008a) between males and females might lead to the divergence of the microbial communities (Yadav et al. 2005).

In the results of this high-throughput sequencing, Proteobacteria and Bacteroidetes constitute the most abundant bacterial phylum of *Porphyra haitanensis*, which is consistent with previous studies on other algae (Bengtsson mm et al., 2010; Lachnit T et al.,2011; Mancuso FP et al.,2016; Serebryakova Alexandra et al.,2018; Mancuso FP et al.,2016). In previous studies, at the class level, Alphaproteobacteria flora showed functions such as nitrogen fixation and phenol degradation (Cernava t et al., 2017), Gammaproteobacteria flora has the functions of phosphorus solubilization, combined nitrogen fixation and xylan degradation (Kimy et al., 2015), while Bacteroides have the ability to produce heat shock proteins; Iron carrier, sulfur and potassium solubilization. At the genus level, *Loktanella* have high adaptability and are famous for their ability to utilize and rapidly metabolize organic carbon sources in seaweed secretions (Bengtsson MM et al.,2011; Wahlm et al., 2012), most of the algae related functions reported by these bacteria are related to the growth and development of algae. Therefore, the community construction of *Porphyra haitanensis* attached bacteria is not only species-specific, but also closely related to the growth and development of algae itself.

Maribacter (male: 14.67%, female: 1.48%) was the most significant difference among the male strains of *Porphyra haitanensis*, which was reported to be the dominant genus of epiphytic bacteria of *Porphyra yezoensis*. There are also significant differences in the relative abundance of *Myxococcales* between

male and female algae of *Porphyra haitanensis*, which shows that the relative abundance of *Myxococcales* of male plant is significantly higher than that of female plant. The research shows that *Myxococcales* is a drug-derived microorganism and can produce many metabolites with antibacterial activity. The most significant difference in female *Porphyra haitanensis* was *Tenacibaculum* (male: 1.40%, female: 22.38%). *Wenyingzhuangia*, another female algae whose relative abundance is significantly higher than that of male algae, has also been reported to produce agarase and have algae dissolving ability. The relative abundance of *Sphingomonas* belonging to Alphaproteobacteria on female algae was higher than that on male algae. *Sphingomonas* can prevent ultraviolet radiation. It has also been reported in the study of foliar bacterial communities of many plants such as *Arabidopsis*, rice and *Magnolia*. *Sphingomonas phyllosphaerae* strain isolated from plant leaf boundary was found to significantly reduce the growth of pathogens and enhance the resistance to leaf pathogens. At the same time, *Sphingomonas paucimobilis* strain can also reduce the impact of pollutants on plant growth by degrading organic pollutants. In addition, Delmotte also detected that *Sphingomonas* has regulatory factors related to environmental stress response, such as phyr and ecfg. Therefore, we infer that males may have stronger survival ability under adversity. The metabolic ability of bacteria to grow and divide very rapidly may lead to faster response of bacteria to external pressure than their host (Mancuso Francesco p.et al., 2016). Therefore, the difference between male and female algae may affect the selection of algae by bacteria. For example, unique bacteria appear on male and female algae.

Since different bacteria may have the same function, and the emergence of "generalist" bacteria also has more impact on the algal environment, the study of bacterial function is helpful to identify host specificity and speculate the difference of algal function (Burke et al., 2011). In the attached bacterial community of *Porphyra haitanensis*, there are key biosynthetic pathways of male and female algae, and most of the common functions are related to the growth and metabolism of bacteria.

There are some functional differences between male and female algal attachment bacterial communities of *Porphyra haitanensis*. The functions of these bacteria are compatible with the functions carried by algae. For example, the functions of male algal bacteria are mostly concentrated in amino acid metabolism, lipid metabolism, carbohydrate metabolism, etc. for example, the bacteria on male algal bodies are stronger in the metabolic pathway of essential amino acids such as valine and leucine. Male algal bodies also have some unique functions, mainly concentrated in malaria, neomycin, kanamycin and gentamicin biosynthesis, Neomycin biosynthesis. It is worth mentioning that male algal bacteria are also positively related to the function of peroxyredoxin, which can protect plants from harmful reactive oxygen species and play a role in signal transduction by regulating the concentration of H₂O₂ in cells.

The functions of female bacteria are mainly related to membrane transport and energy metabolism. Such as ABC transporters, photosynthesis, etc. Female algal bacteria also have some unique functions, including ethelbenzene degradation, completion and coagulation, cascades and dorso ventral axis formation. It should be noted that in the unique functional expression of female algal bacteria, there are many proteins related to spore germination and maturation, which may promote the growth of young sporophytes of *Porphyra haitanensis*.

5 Conclusion

Taken together, the results of this study underscore the role of sex selection in driving variations in the male and female microbial communities of *Porphyra haitanensis*. Though there were no significant differences between the females and males in the alpha diversity indexes, the microbial composition results clearly demonstrated that some microbes discriminately inhabited on females or males. These bacterial genera appeared to have special ecological function, which could be reflected in the different physiological response to environmental changes in dioecious plants. Our results will improve our understanding of the sex discrimination of microorganism in the dioecious *Porphyra haitanensis*.

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Figures

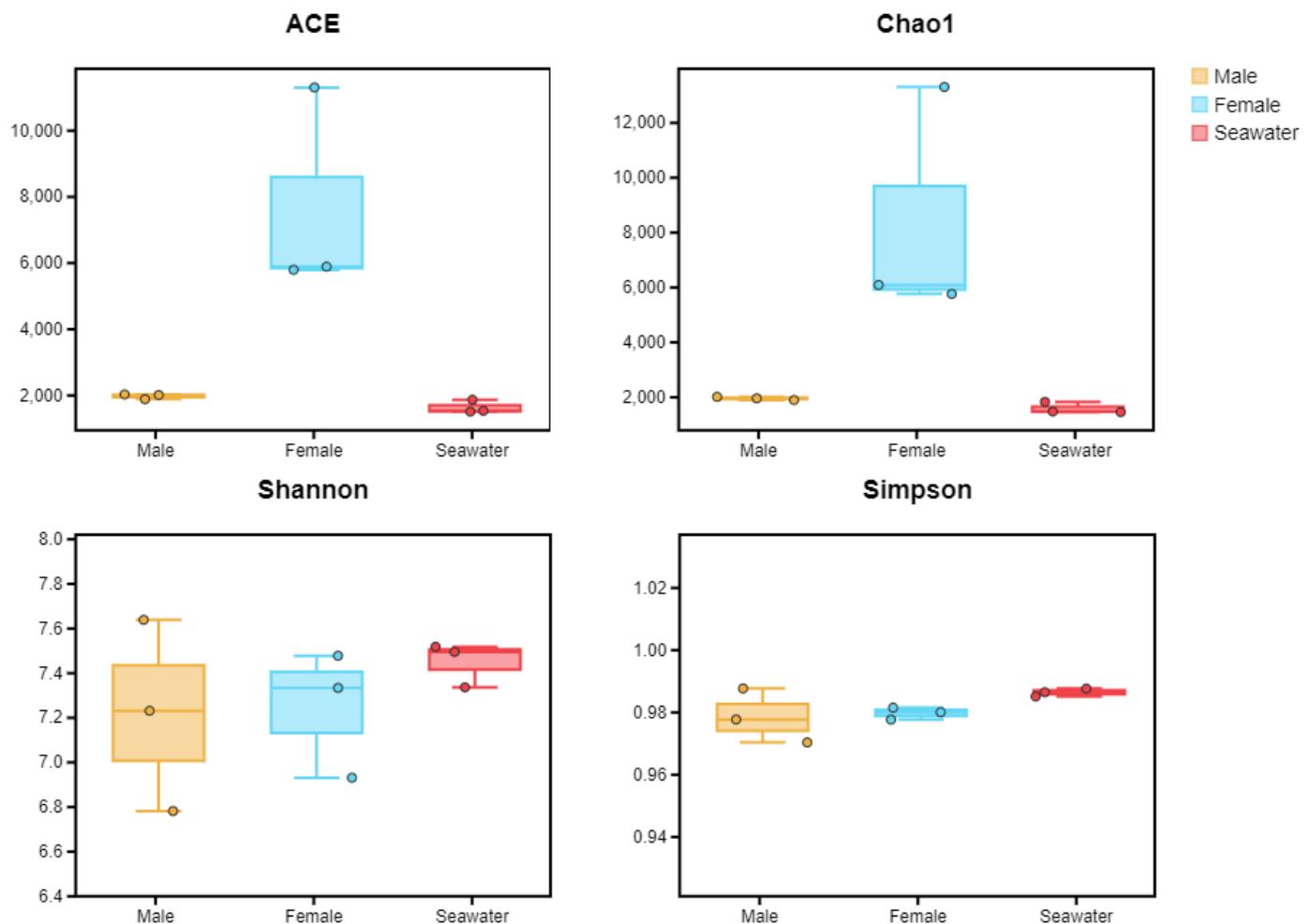


Figure 1

Alpha diversity indexes of bacteria communities in the phyllosphere of male, female *Porphyra haitanensis* and seawater

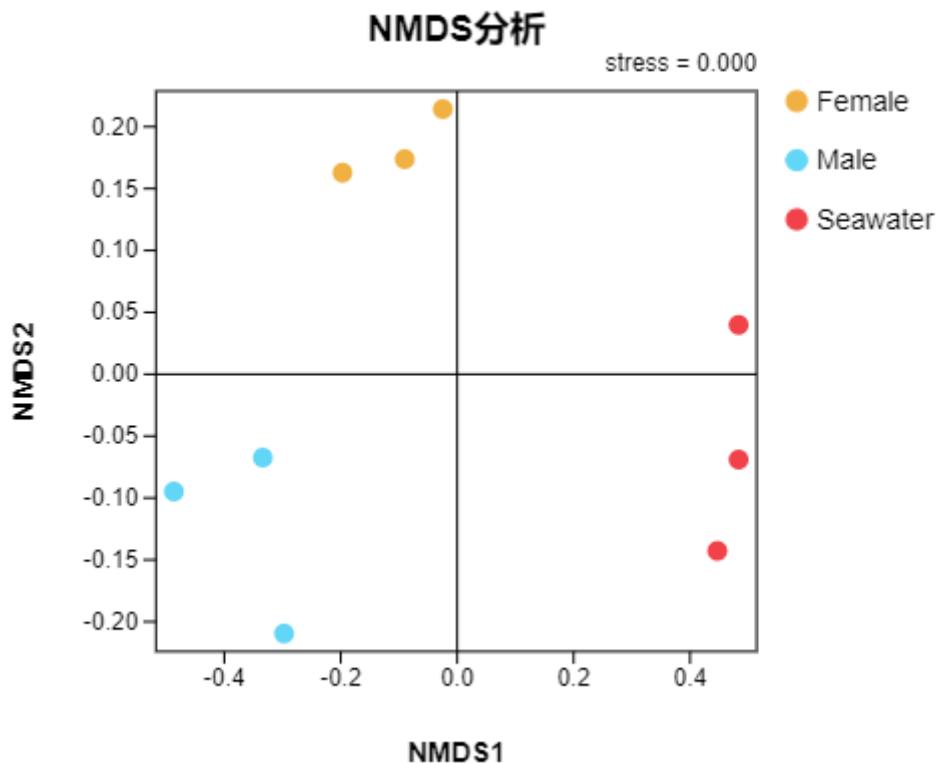


Figure 2

NMDS analysis based on Bray-Curtis distance showing microbial community composition differences between males, females and seawater

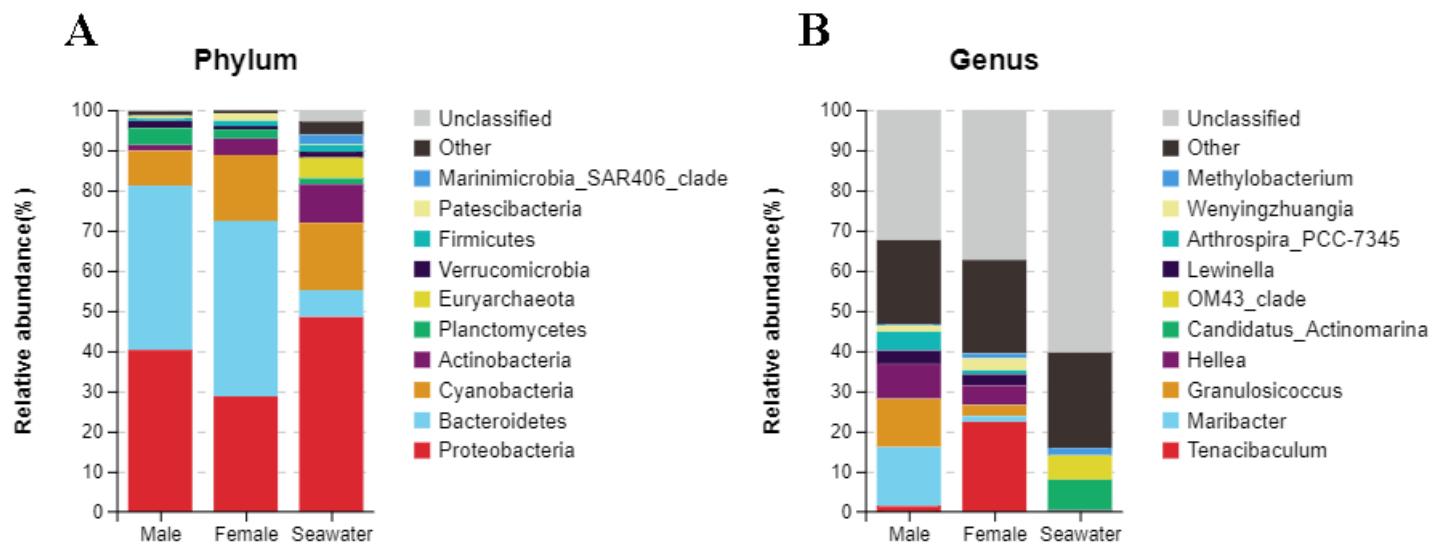


Figure 3

(A) Phylum-level taxonomic composition of the bacterial community.

(B) Genus-level taxonomic composition of the bacterial community.

Figure 4

Histogram of linear discriminant analysis Score distribution regarding epiphytic bacteria on male and female *S. thunbergii*. (less_strict=2 more_strict=4 LDAS CORE>4)

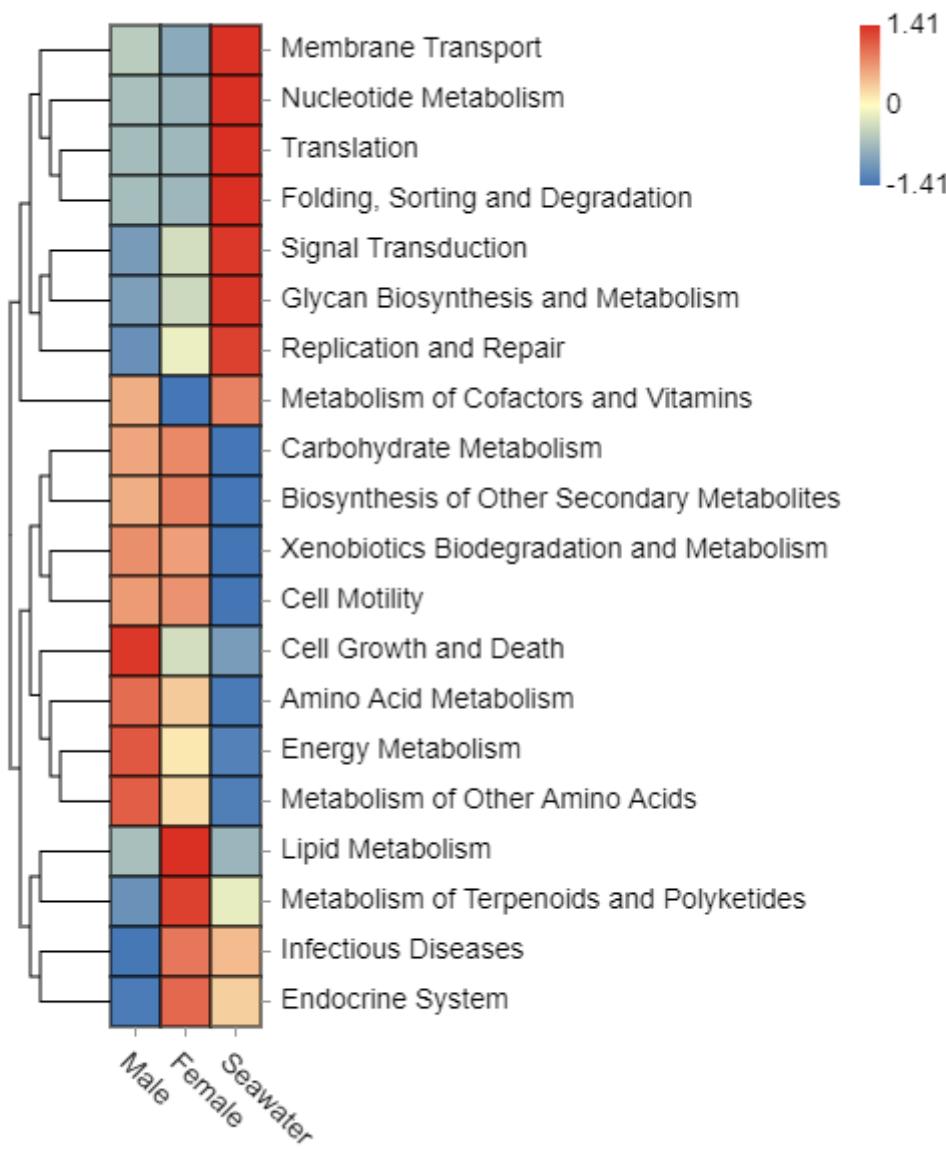


Figure 5

Heat map of the 20 KEGG level-2 functional pathways with relatively high abundance for prokaryotic communities of all samples.

The normalized relative abundance of each KEGG pathway is indicated by a gradient of color from blue (low abundance) to red (high abundance).

Supplementary Files

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