Exploring the diversity and dynamic of bacterial community vertically distributed in Tongguling National Nature Reserve in Hainan Island, China

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Abstract

National nature reserves are important for preserving ecological resources and constructing national ecological security barriers. Tongguling National Nature Reserve (TNNR) is known for its unique tropical island ecosystem and abundant biological resources. This study was conducted to characterize and compare its bacterial community diversity and composition in soils from 10, 20, and 30 cm in depth using high-throughput sequencing of 16S rDNA genes. We found that soils from 20 cm had the highest diversity and might serve as a “middle bridge” to the dynamic distribution between the 10- and 30-cm soil samples. The diversity pattern indicated that the main abundant groups varied distinctly and significantly among soils of different depths. Moreover, Chloroflexi was the most dynamic group in TNNR soils, together with another abundant but rarely reported group, Verrucomicrobia, which greatly enhanced the microbial diversity of TNNR soils. Overall, the results of this study emphasize the urgent need for greater understanding of bacterial community variations in response to human activities and climate change.

Keywords Tropical island · Metagenome · Microbiome · Environment · 16S rDNA gene

Introduction

Microorganisms play key roles in global biogeochemical substance cycling and interact with hosts extensively [1, 2]. Numerous studies using 16S rDNA and metagenomics sequencing have been conducted to investigate the root

microbial diversities of vital crops, and model plants have been reported, including Arabidopsis [3, 4], rice [5], maize [6], barley [7], sugarcane [8], and legumes [9]. Moreover, several studies of these populations have focused on environments that have been disturbed by humans, plants, livestock, or pollutants [10], which has provided insight into plant root microbial community distributions. Simultaneously, human activities as well as biotic and niche-specific abiotic factors have been shown to drive differentiation of these communities and to have significant effects on microbial communities [11]. However, the microbial distributions in soils of unique ecological environment with little human interference, especially those in national nature reserves, have received little attention in microbiome studies. Accordingly, there is a need to understand the bacterial communities of these unique environments.

Recently, the standards for environmental protection have increased to maintain livable areas in China. Tongguling National Nature Reserve (TNNR) is located at the land-sea junction in Wenchang County of Hainan Province, surrounded by sea on three sides, and with rare tropical monsoon forest, coral reefs, mangroves, and other tropical natural landscapes. The *Machrodendron oligophlebium* and *Hydnocarpus hainanensis* are the predominant vegetation. Because of its topography and

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water environment, which consist of marine and terrestrial ecosystems, this area has a unique ecological environment that is home to abundant animals, plants, and coral resources. As a result, the bacterial distribution of soils in this area may differ from that of other farm soils. The TNNR has a good ecological system; its plant and animal resources have been well investigated [12–14]. However, little is known about the microbial community diversity of its soils. Previous studies have confirmed that microorganisms have the capacity to influence climate change and human health [15, 16] and microbial communities have been shown to shift in response to climate and biogeochemistry changes [17–19]. Therefore, consideration of the complex interactions and feedback that occur between microorganism and the environment, human activities, and climate change, with a special focus on the interactions between microorganism and fragile ecological environments, is urgently needed.

This study was conducted to address some of the aforementioned research gaps by conducting a detailed characterization of the TNNR soil microbiome. In addition, the microbial diversity and composition along a vertical distribution of soil samples from the region was characterized, and the interaction relationships among microbial species were investigated. The results presented herein will provide a greater understanding of the microbiome and will facilitate making informed decisions regarding environmental protection in the region.

Materials and methods

Collection and DNA extraction of soil samples

Soil samples were collected from the TNNR. Locus A was located at E: 111° 02' 03", N: 19° 66' 85", with an altitude of 264 m, while the other five loci were 500 m away from locus A as listed in supplemental information figure 1 (SI figure 1). Firstly, the leaves and grass covering the loci were cleared. Then, we used a gasoline engine sampler (diameter 10 cm) to collect soil samples from depths of 10, 20, and 30 cm; at each depth, the soils of three repetitions were mixed for the sequencing soils, after which samples were stored in sterile plastic bags, respectively. Immediately after collection, samples were transported to the laboratory in an ice box, where they were stored at -20°C until analysis.

Aliquots (0.25 g) of the soil samples were processed using a MOBIO PowerSoil® kit. The extracted DNA samples were then analyzed using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA), whereas the DNA quality was checked by 1% agarose gel electrophoresis. The extracted DNA samples were selected and used to conduct microbial community analysis by PCR using the following 16S rDNA primers (V4–V5 hypervariable regions): forward (5'-GTGCCAGCMGCCGCGG-3') and reverse (5'-CCGTCAATTCMTTTRAGTTT-3') [20]. The PCR reactions were as follows: 95 °C, 3 min followed by 27 cycles of 30 s at 95 °C, 30 s

at 55 °C, and 72 °C for 45 s and then final extension at 72 °C for 10 min. The PCR reactions were performed in triplicate in 20 µL mixtures containing 4 µL 5× FastPfu Buffer, 2 µL 2.5 mM dNTPs, 1 µL primer mix (5 µL), 0.4 µL FastPfu Polymerase, and 5 ng extracted DNA as the template. The PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). The products were then quantified using QuantiFluor-ST (Promega, Madison, USA). Purified amplicons were then pooled in equimolar concentrations and paired-end sequenced (2×300) using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to the standard protocols of Shanghai Majorbio Bio-pharm Technology Co., Ltd. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (accession no.: SRP150661).

Diversity analyses of microbial communities

Data were analyzed using the Majorbio I-Sanger cloud platform (<http://www.i-sanger.com/>). The similarity and differences between samples were compared using the shared and unique OTUs of the Venn diagram. The bar and pie analyses were based on the phylum level, and the average abundance of each of the three groups was also calculated. A hierarchical clustering tree based on the OTU level and PCoA analysis of the β -diversity were calculated based on the Bray-Curtis algorithm. To perform significance testing of microbial community variance analysis of the three groups at the phylum level, one-way ANOVA and false discovery rate (fdr) were used with a Scheffe's cutoff value of 0.95. Network analysis was conducted at the OTU level to assess the species-species co-existence relationships of the three groups of samples, after which the species correlation networks were evaluated at the OTU level (based on the 30 most abundant OTUs) to explore the interaction relationships among species. For these analyses, the Kendell correlation coefficient model was used with a cutoff of 0.5.

Results

Bacterial diversity assessment of TNNR soil

Bacterial diversity of soil samples collected from depths of 10, 20, and 30 cm at six different sites was assessed using phylo-type taxonomy. The results revealed that there were 2896 OTUs (SI table 1), and the core OTU number was 1929. Among the 18 soil samples, the number of sequence reads varied from 31,098 to 44,867 with an average of 37,434, and the average reads length of all samples was 435 bp. The results of pan OTU analyses revealed that the total OTU number of soil samples collected at 10, 20, and 30 cm was 2563, 2654, and 2284, respectively, and the unique OTU number was 134, 29, and 57 (Fig. 1A). Furthermore, the result of Student's *t* test indicated

Fig. 1 Venn diagrams showing the number of shared and unique operational taxonomic units (OTUs) between bacterial communities (A). OTUs defined by 97% sequence similarity and Student's *t* test of OTU levels among 10, 20, and 30 cm soil samples (B). *** $P \leq 0.001$

that the Sobds index of the OTU level of 10- and 20-cm soil samples was similar, and they both reached the level of significance when compared with the 30-cm soil samples (Fig. 1B).

Main microbial composition comparison of TNNR soil

The result of each depth soil that represents the average richness of six samples is shown in Fig. 2 (the bacterial relative abundance analyses of 18 soil samples are shown in SI Fig.

2); based on our result, Proteobacteria (42.64%, 32.75%, and 20.16% in 10-, 20-, and 30-cm soil samples, respectively), Acidobacteria (19.74%, 27.98%, and 30.69% in 10-, 20-, and 30-cm soil samples, respectively), Chloroflexi (5.31%, 12.69%, and 25.04% in 10-, 20-, and 30-cm soil samples, respectively), Actinobacteria (10.03%, 8.75%, and 5.26% in 10-, 20-, and 30-cm soil samples, respectively), and Verrucomicrobia (8.07%, 5.71%, and 4.62% in 10-, 20-, and 30-cm soil samples, respectively) were the most abundant groups.

Fig. 2 Average microbial community of soil samples at the phylum level, others means the phylum unknown, y-axis of the column was the mean values of five different loci

Principal coordinates analysis (PCoA) and a dendrogram of the 18 soil samples were also used to compare the samples. The dendrogram (SI figure 3, 4) revealed that the 20- and 30-cm soils were more similar, while the six 10-cm soil samples fluctuated greatly. Furthermore, the vertical axis also showed that the phyla of the 20-cm soil were interpenetrative with the 10- and 30-cm soil samples. The use of PCoA to evaluate the grouping tendency of the OTU level of 10-, 20-, and 30-cm soil samples demonstrated that the soil depth variation could cause the variant of 43.23% microbial communities.

Comparison of microbial communities in different sample groups

To further understand the distinction among microbial communities in 10-, 20-, and 30-cm soil samples of TNNR, the major microbial taxa were listed and compared based on the relative abundance data (Fig. 3). One-way ANOVA showed that up to six phyla (Proteobacteria, Acidobacteria, Chloroflexi, Actinobacteria, Bacteroidetes, and Cyanobacteria) reached a highly significant level (P value < 0.005) among the 10-, 20-, and 30-cm soil sample groups. In addition, the relative abundance distinction of GAL15, Latescibacteria, and Chlamydiae also reached significant levels (P value < 0.05). Based on these results, the OTU relative abundance of Proteobacteria, Actinobacteria, and Bacteroidetes in 10-cm soil samples was significantly higher than that in 20- and 30-cm soil samples, and a similar OTU relative abundance distribution trend was observed for Latescibacteria and Cyanobacteria, while the relative abundance of Acidobacteria and Chloroflexi in 30-cm soil samples was significantly higher than that in 10 and 20-cm soil samples, and the relative abundance of GAL15 in 30-cm soil was the highest and reached the significant level. In the 20-cm soil, the relative abundance of Chlamydiae was the highest.

Top 30 abundant OTUs and network analysis among soil sample groupings

Network analysis is usually used to compare the species abundance between samples; moreover, correlation analysis can provide information regarding the co-existence relationships of species in environmental samples, as well as species interactions and mechanisms of formation of phenotypic differences between samples. Here, network analysis among the 10-, 20-, and 30-cm soil samples revealed the co-existence relationships of species belonging to different soil sample groupings (Fig. 4). The results revealed that there were extensive interactions among the identified species. Notably, a few free OTUs were also found among them, for instance, 29, 10, and 35 free OTUs were found in the 10-, 20-, and 30-cm soil samples, respectively. Moreover, a correlation network of the top 30 abundant OTUs was constructed (Fig. 5), among which

30% of the OTUs (9/30) belonged to Proteobacteria, while another 30% were Acidobacteria, which showed high clustering values with other OTUs. In addition, four OTUs of Chloroflexi (1223, 1427, 2455, 2691), three of Verrucomicrobia (552, 2261, 2266), two of Actinobacteria (1070, 1357), one of Nitrospirae (1022), and one OTU of Firmicutes (2574) were also found. OTU1765 (uncultured soil bacteria) belonging to Proteobacteria, which could interact with up to 21 major OTUs and another four Proteobacteria species (OTU 2495, 1362, 2872, and 2634) also showed extensive interaction with other species and up to 16, 15, 13, and 12 major OTUs, respectively. OTU1370 of Acidobacteria could interact with 18 major OTUs, and two species (OTU1223 and OTU1427) of Chloroflexi could interact with 15 and 14 major OTUs, respectively. Interestingly, OTU880 only interacted with OTU1022, with a positive correlation being observed between them; however, OTU1022 showed a negative correlation with two Acidobacteria species (OTU1714 and OTU2856), two Actinobacteria species (OTU1070 and OTU1357), one Proteobacteria species (OTU1765), and one Firmicutes species (OTU2574).

Discussion

Numerous studies have used high-throughput sequencing methods to identify the microbial populations in food samples, natural environments, and agricultural soils [21, 22], and some studies have provided insights into microbial community distributions in different environments [23, 24]. However, most of these studies have focused on changes in microbiota depending on whether they are associated with host plants, nutrition, genotype, or interference with unique environment factors. Given the high temperature, sunshine, and spatial heterogeneity inherent to soil environments of different depths and the resulting differences in microbial community structure, a spatial sequencing effort across different depths, multiple loci, and varying sequence depths must be analyzed. The present study characterized the bacterial communities of TNNR soil, which harbors a diverse root microbiome that differs quantitatively and qualitatively from that of the soils. The results presented herein characterize the microbial community distribution of a national nature reserve environment that has not been heavily impacted by anthropogenic activity and identifies interaction relationships among different OTUs.

High-throughput sequencing of 16S rDNA genes has been extensively applied to investigations of various complex environments [25, 26]. Previous studies of soil microbiomes have shown that Proteobacteria, Firmicutes, and Actinobacteria were the most common dominant taxa [27, 28], and the root bacterial microbiome typically consists of Proteobacteria, Actinobacteria, and Bacteroidetes [29], which is a general pattern for many soils [30]. However, by

Fig. 3 One-way ANOVA and FDR comparison analysis of microbial communities at the phylum level in 10-, 20-, and 30-cm soil sample groups, the Scheffe's value cutoff was 0.95, *** $P \leq 0.001$, ** $0.001 < P \leq 0.01$, and * $0.01 < P \leq 0.05$

comparing the microbial populations of the three soil groups and conducting network analysis of the 30 most abundant OTUs, we found that Proteobacteria, Acidobacteria, Chloroflexi, and Actinobacteria were the predominant taxa. Additionally, Verrucomicrobia, Planctomycetes, Bacteroidetes, Firmicutes, GAL15, and Nitrospirae were still present at higher relative abundance in TNNR soil than in farm and plant root microbiota [29, 31], which showed a unique microbial diversity in the TNNR soil. To analyze the microbial community variation among the three groups of soil samples, we conducted bacterial distribution characterization analysis and found extensive taxonomic overlap among the

10-, 20-, and 30-cm soil samples, among which the 20-cm soil sample had the maximum number of OTUs, but the least unique OTUs (Fig. 1). By linking to the information of network analysis of the three groups of samples (Fig. 4), we could speculate that the 20-cm soil may serve as a “middle bridge” to undergo the dynamic distribution between the 10- and 30-cm soil samples.

Soil is a complex microcosm replete with inter-organismal interactions. Although cross-talk exists among different species, it is usually difficult to explore. Nevertheless, researchers are paying increased attention to this topic. For example, an investigation of a simplified synthetic bacterial community of

Fig. 4 Network analyses among the 10-, 20-, and 30-cm soil samples. Collinearity relationships among all samples and species are presented in the network diagram. If the species nodes are linked to the sample nodes, the species is included in the representative sample

maize roots revealed that the removal of *Enterobacter cloacae* could lead to the entire community being replaced by *Curtobacterium pusillum* [28]. Thus, *E. cloacae* is a keystone species in that model ecosystem. Based on the correlation network of the 30 most abundant OTUs, we inferred that both OTU1765 and OTU1370 were the keystone species of the TNNR soil because they showed extensive interactions with numerous species. Based on the community distribution (Fig. 2), some microbial taxon distributions associated with soil vertical depth were also shown. Specifically, the abundance of Proteobacteria, Verrucomicrobia, and Bacteroidetes decreased from 10- to 30-cm soil, while the relative abundance

of Acidobacteria, Chloroflexi, and GAL15 increased from 10- to 30-cm soil.

Notably, the relative abundance of Chloroflexi increased as depth increased from 10 to 30 cm and it was the second most abundant organism in the 30-cm soil samples, which expanded the known diversity of the microbial distribution greatly. Moreover, these organisms were present at a higher ratio (22.85–27.51%) than reported in an alpine tundra soil, in which Chloroflexi were the third largest bacterial division sampled ($\approx 13\%$). These findings suggest that Chloroflexi are active at low temperatures, under anoxic environment [32]. Moreover, Chloroflexi have been described as a loose,

Fig. 5 Correlation networks of the top 30 abundant OTUs. The Kendall correlation coefficient model was chosen, and the cutoff of the correlation coefficient was 0.5. Red indicates a positive correlation between species, while green indicates a negative correlation

but coherent group [33], for the uncultivated characterization that few phylogenetic results of this group were known [34, 35], but it was distinct that the Chloroflexi species played important roles among the species-species interaction in TNNR soils (Fig. 5). Thus, the high bacterial community richness reflects the dynamic nature of the TNNR soil chemical and physical environment, together with the fact that was reported in alpine tundra soils, which demonstrated that the Chloroflexi species could utilize geochemical inputs and play a part in material circulation of earth bio-chemical layers. Similarly, Verrucomicrobia is a culture-independent species that has recently been reported. Our result also showed that the Verrucomicrobia was one of the top groups, which expands the microbial diversity distribution characterizations of soil samples. However, in this study, we failed to collect the comprehensive environmental factors; thus, the correlation analysis with microbial diversity was not performed. Future comprehensive studies will help resolve the phylogeny of this diverse and abundant group of TNNR soil bacteria.

Conclusions

Tropical island ecosystems are commonly unique and valuable, and the TNNR contains rich biological resources, providing an ideal environment to study biogeochemistry and predict future responses to global change and human activities. Here, we investigated the highly complex microbial diversity of soils from different depths and conducted a broader description of their microbiome characterizations, along with the interactions among the main taxa groups. Interestingly, changes in soil depth were associated with high diversities and shifts in bacterial community structure. Furthermore, Chloroflexi was one distinct dynamic group, expanding the known diversity of soils. It should be noted that our conclusions regarding TNNR soil microbial diversity are limited by our small sample sizes. Accordingly, it is necessary to conduct more comprehensive studies to investigate the microbial diversity and responses to human activities and climate change.

Author contributions Peng Li, Wenfei Zhang, and Ruiping Wang designed the experiments; Peng Li analyzed the data and wrote the paper; Hongping Wu, Yinghong Jin, Xunyin Huang, Yukai Chen, and Xiaobo Yang helped collect the soil samples; and Wenfei Zhang and Ruiping Wang revised the manuscript.

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