Postprint: DNA Metabarcoding Study of Symbiotic Fungal Diversity in the Locust Gut

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Date: 2017-11-01T00:00:00+00:00

Abstract

Using DNA composite barcode technology, we investigated the diversity of gut symbiotic fungi in 11 locust samples. The results demonstrated that ITS identified 5 phyla, 16 classes, 29 orders, 40 genera, and 2786 fungal OTUs across the studied species. Analysis of gut fungal community composition revealed that Auriculariales and Tremellales were the most abundant fungal taxa in the gut of all species, with Oedipodidae exhibiting the highest relative fungal taxonomic diversity and Catantopidae the lowest, indicating significant variation in microbial diversity among locust guts. Alpha diversity analysis showed that Oedipodidae had the highest richness and diversity of symbiotic fungal communities, whereas Catantopidae had the lowest. Beta diversity analysis indicated that: (1) the gut fungal community structures of species within the same family showed smaller differences, while those of species from different families exhibited larger differences; (2) the gut fungal community structure of Acrididae showed relatively low similarity with other species, and the fungal community structure of Acrida cinerea collected from two different sampling sites also showed relatively low similarity. Cluster analysis revealed that: (1) gut fungi from locusts of the same family clustered together first, with relatively high community similarity; (2) three genera—Bullera, Endocarpon, and Exophiala—were dominant in the gut fungi of locusts.

Full Text

Study of the Biodiversity in Intestinal Symbiotic Fungi in Grasshopper Species by Using DNA Meta-Barcoding

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Abstract

DNA meta-barcoding technology, which combines DNA barcoding with high-throughput sequencing, is a highly efficient method for monitoring microbial diversity. In this study, we investigated symbiotic fungi in the gut of eight grasshopper species collected from Qinling Mountain and the campus of Shaanxi Normal University, with respect to alpha diversity, beta diversity, and cluster analyses, and evaluated the differences in microbial diversity of samples collected from the two locations.

A total of eight individuals from eight species collected from Qinling Mountain (namely, *Trilophidia annulata*, *Pedopodisma tsinlingensis*, *Atractomorpha sinensis*, *Acrida cinerea*, *Chorthippus chinensis*, *Fruhstorferiola omei*, *Euchorthippus unicolor*, and *Xenocatantops brachycerus*) and three individuals from three species collected from the campus of Shaanxi Normal University (namely, *Trilophidia annulata*, *Atractomorpha sinensis*, *Acrida cinerea*) were sampled. The grasshoppers were starved overnight and dissected, and the intestinal gut was fixed in 100% ethanol for DNA extraction. Internal transcribed spacers (ITS) were selected as barcoding sequences. After DNA extraction and PCR amplification using fungus ITS universal primers, the amplicons were sequenced using the 454 FLX+ platform. Two software packages, QIIME and Mothur, were used to analyze the raw data and obtain an operational taxonomic unit (OTU) list. Ecological analysis was subsequently performed using Excel, R, and QIIME software.

Analysis of the fungal species composition revealed a total of 2,786 OTUs, 40 genera, 29 orders, 16 classes, and 5 phyla. With respect to community composition, analysis of the ITS sequences revealed that the orders Tremellales and Auriculariales contained the highest number of species, and fungus diversity was the highest in Oedipodidae and lowest in Catantopidae. These findings indicated that the diversity of fungi in the gut of the grasshoppers was significantly different. Moreover, the alpha diversity analysis showed that Oedipodidae had relatively high values of community richness and diversity when compared with the other families. The beta diversity analysis demonstrated that the intestinal fungal community structure of the grasshoppers showed no significant difference within a family; in contrast, it exhibited more differences among species of different families. However, the gut fungal community structure of *Acrida cinerea* in different environments showed a relatively low similarity. Finally, the cluster analysis showed that grasshoppers from the same family primarily clustered together and their community similarity was relatively high. *Bullera*, *Ochrolechia*, and *Exophiala* were the dominant genera among the grasshoppers' intestinal fungi. This also showed that the fungal populations in the intestines of the grasshoppers were highly diverse in terms of the number of species and their community structure composition, although the differences in the fungal communities varied with the grasshopper host species and surrounding environment.

Keywords: DNA meta-barcoding; grasshoppers; intestinal symbiotic fungus; fungus diversity

Introduction

DNA meta-barcoding is an identification method that utilizes high-throughput sequencing technology to simultaneously obtain barcode sequences from many species for community taxonomic unit composition analysis [1]. This technology enables rapid species identification from large mixed samples, avoiding the low efficiency of single-species barcoding identification. With its high efficiency and other advantages, more researchers and project managers can easily conduct biodiversity assessments. Since its inception, meta-barcoding has been widely applied in microbial community diversity research. Schmidt et al. [2] analyzed the internal transcribed spacer (ITS) region of fungi from soil samples in Flörsheim, Germany, using high-throughput second-generation sequencing, and identified 1,200 operational taxonomic units (OTUs), while demonstrating that community similarity gradually decreased with geographical distance. Siddique and Unterseher [3] used meta-barcoding technology to analyze the fungal community diversity of European birch leaves across different regions with gradient altitudes. In the study of symbiotic fungal diversity, Christian et al. [4] developed an accurate and effective data processing pipeline for full-length fungal sequencing through rDNA-ITS sequence analysis. Sui et al. [5] optimized the ITS-PCR system using the Tiangen DP330 soil extraction kit to extract total DNA from soil and establish the best ITS-PCR system. Meta-barcoding has also been widely applied in plant diversity [7], animal diet analysis, and fecal studies [8].

Grasshoppers are worldwide agricultural pests with wide distribution and large populations in China. Due to abnormal climate and ecological environment deterioration, the occurrence of grasshopper disasters in China has increased year by year, seriously affecting the sustainable development of agriculture and animal husbandry and the quality of farmland and grassland ecological environments [9]. Understanding the symbiotic fungi in grasshopper intestines, mastering the relationship between symbionts and hosts, and the composition of intestinal fungal communities has potential application value for using parasitic fungi or fungal metabolites to control grasshoppers. Currently, research on insect intestinal symbiotic fungi is limited. Domestic literature has reported on the gut fungi of tea geometrid larvae feeding on different tea varieties [10] and the isolation of a laccase-producing endophytic fungus from the gut of *Odontotermes formosanus* [11]. International literature has reported on bacteria and fungi in the guts of wood-feeding beetles in tropical rainforests of Costa Rica, with *Trichoderma* being the most abundant fungal genus [12]. However, research on the diversity of gut symbiotic fungi in grasshoppers is relatively scarce. This study selected the internal transcribed spacer (ITS) region of the fungal ribosomal gene cluster as a barcode marker and used DNA meta-barcoding technology to conduct a preliminary investigation of the composition and community struc-

ture of gut symbiotic fungi in grasshoppers, comparing the differences in fungal diversity composition among different grasshoppers and preliminarily revealing the diversity of gut fungi in grasshoppers.

Materials and Methods

Sample Collection and Processing Grasshopper specimens used in this study were collected in September from Nanwutai in the Qinling Mountains and the campus of Shaanxi Normal University (Table 1). The grasshoppers were starved overnight to empty their gut contents. Using sterilized forceps, the intestinal tract was gently removed from the dorsal membrane between the head and thorax of each grasshopper and immediately immersed in a 50 mL centrifuge tube containing anhydrous ethanol. The insect body was also placed in the tube for later identification. Samples were stored at -20°C for subsequent analysis.

DNA Extraction The grasshopper intestines preserved in anhydrous ethanol were removed with sterilized forceps, gently placed on absorbent paper, and allowed to air dry in a sterile workstation. After the ethanol had evaporated completely, total DNA was extracted using the chloroform method [13].

PCR Amplification and Sequencing Following White et al. [14], we used universal fungal ITS primers for amplification: forward primer ITS5 (5 -GGAAGTAAAAGTCGTAACAAGG-3) and reverse primer ITS4 (5 -TCCTCCGCTTATTGATATGC-3). To enable simultaneous sequencing of multiple samples and to distinguish each sample' s sequences in subsequent analyses, unique tag sequences were added to the primers for each sample. The amplification length was approximately 750 bp. The PCR reaction system (20 L) contained 10 L 2×Taq PCR StarMix with Loading Dye, 1.0 L of each primer (10 M), 1 L template DNA, and 7 L ddH O. The amplification program was: 98°C for 5 min; 35 cycles of 98°C for 30 s, 47.6°C for 45 s, 72°C for 60 s; and a final extension at 72°C for 5 min. The PCR products were sent to Shanghai Personal Biotechnology Co., Ltd. for high-throughput sequencing on the 454 FLX+ platform.

Data Processing and Analysis

Raw Sequence Processing The raw sequencing data were initially processed to extract FASTA files and quality information. The sffinfo command in Mothur software [15] was used to extract sequence files and quality information from the binary SFF files, which already involved preliminary quality filtering of the raw data. The tag sequences used to distinguish samples were then used to extract effective sequences for each sample. Since PCR amplification may produce chimeric sequences and sequencing errors such as point mutations may occur

during the sequencing process, further filtering and chimera removal were necessary to ensure result accuracy. The QIIME software [16] was used for sequence filtering, and Mothur software was used for chimera removal, classification, and annotation.

Community Diversity Analysis High-quality sequences were clustered at 97% similarity using the uclust method in QIIME [17]. The longest sequence in each cluster was selected as the representative sequence. Representative sequences were compared against the UNITE database for taxonomic annotation. Based on the annotation results, Mothur software was used to generate community percentage stacked bar charts at the order level and between samples. According to species abundance tables, alpha diversity indices were calculated for each sample using the summary.single command in Mothur, including Chao1, ACE, Shannon, and Simpson indices. Chao1 and ACE are community richness indices; higher values indicate greater community richness. Shannon and Simpson are community diversity indices; higher Shannon values indicate greater diversity, while lower Simpson values indicate greater diversity.

Beta diversity analysis was performed based on the evolutionary and abundance information of species from each sample to obtain a distance matrix between samples, followed by non-metric multidimensional scaling (NMDS) analysis [18-19]. The heatmap program package in R software was used for cluster analysis at the genus level and to generate heatmaps [20-21].

Results

Sequencing Quality Evaluation After initial quality filtering and assembly, 99.54% of the fungal sequences were retained as high-quality sequences. The number and proportion of sequences for each sample are shown in Table 2. According to sequence quality control standards, after optimization, the proportion of high-quality sequences reached 99%. The proportion of high-quality sequences for each sample ranged from 97.71% to 99.97%.

Taxonomic Composition and Community Structure Comparison of high-quality sequences with the database revealed a total of 2,786 OTUs across all samples. The taxonomic composition at the total level included 5 phyla, 16 classes, 29 orders, and 40 genera. The composition of each sample is shown in Table 1, and the classification at the total level is illustrated in Figure 1. The Venn diagram shows that there were obvious changes in microbial diversity among samples, which can be reflected by the species composition proportions at different taxonomic levels.

Comparative analysis of fungal community composition among different samples revealed that the order Tremellales had the highest proportion in samples N-A, N-B, N-G, N-H, and X-A, while the order Auriculariales had the highest proportion in samples N-E and N-F, reaching up to 54.4%. Other orders accounted for relatively small proportions in each sample. Tremellales and Auriculariales were

the most abundant fungal orders in the grasshopper gut microbial community structure [Figure 2: see original paper].

Alpha Diversity Analysis Alpha diversity analysis showed that sample N-D had the highest community richness, while N-E and N-F had the lowest. Samples N-D and N-E had the highest community diversity, while N-C had the lowest. Overall, Oedipodidae had the highest community richness and diversity, while Catantopidae had the lowest. The diversity indices for each sample are shown in Table 3.

Beta Diversity Analysis Using species evolutionary and abundance information between samples, a distance matrix was obtained and used for NMDS analysis. In the NMDS diagram, closer distances between two points indicate smaller differences and greater similarity between microbial communities. Samples with smaller community differences were N-F (-0.76), N-D (-0.24), and N-G (-0.06), while samples with larger differences were N-A (-1.04) and X-A (-1.05). The NMDS plot based on the two most important factors affecting community structure is shown in [Figure 3: see original paper].

Cluster Analysis Cluster analysis at the genus level showed that *Atractomorpha sinensis* from two different environments clustered together, indicating high community similarity of gut symbiotic fungi at the genus level. *Chorthippus chinensis* and *Fruhstorferiola omei* clustered into another group, indicating high similarity between their gut fungal communities. From the perspective of richness distribution, *Bullera*, *Tilletiopsis*, *Ochrolechia*, *Lipomyces*, and *Exophiala* had relatively high proportions and were dominant genera across the 11 samples. In contrast, *Penicillium* and *Fusarium* were minor genera. The heatmap of gut fungi at the genus level for each sample is shown in [Figure 4: see original paper].

Discussion

Relationship Between Gut Microbes and Grasshopper Habitat From the perspective of taxonomic composition and community structure, grasshoppers from the campus environment (*Trilophidia annulata*, *Atractomorpha sinensis*, and *Acrida cinerea*) had more diverse fungal groups than their counterparts from Nanshantai in the Qinling Mountains. Additionally, Oedipodidae had more fungal groups than Pyrgomorphidae and Acrididae. This indicates that environmental changes can affect the fungal diversity of the same grasshopper species. Previous research has shown that habitat diversity is positively correlated with insect gut microbial diversity and insect diversity itself [25], suggesting that the diversity and function of microorganisms in insect guts are closely related to the habitats where insects live. These results have important implications for understanding how reduced habitat biodiversity may affect the ecological functions of species living there.

Relationship Between Gut Microbes and Grasshopper Diet Alpha diversity refers to diversity within a specific area or ecosystem, commonly measured by microbial community richness (the estimated number of species in the identified microbial groups) and evenness of each sample. In this study, *Trilophidia annulata* from Nanshantai had the highest gut microbial community richness and diversity, while *Pedopodisma tsinlingensis* and *Fruhstorferiola omei* had the lowest. These differences may be caused by dietary variations, as the impact of diet on insect gut microorganisms is complex [26]. Different grasshopper species have their own feeding characteristics, and these dietary differences lead to variations in gut fungal community richness and diversity.

Relationship Between Microbial Composition and Taxonomic System Beta diversity compares diversity between different ecosystems and represents changes in species composition across different environments or communities. It is an important component of biodiversity and is closely related to many ecological and evolutionary biology questions, commonly measured by similarity. In this study, beta diversity was used to explain differences in gut microbial community composition among grasshoppers from different families and sampling locations. Smaller community differences indicate higher similarity and lower beta diversity, while larger differences indicate lower similarity and higher beta diversity.

The analysis showed that *Trilophidia annulata* and *Atractomorpha sinensis* from different environments clustered together, indicating that their gut symbiotic fungal community structure did not change with sampling location. *Chorthippus chinensis* and *Euchorthippus unicolor* had similar gut symbiotic fungal community structures, as did *Fruhstorferiola omei* and *Xenocatantops brachycerus*. However, *Acrida cinerea* from different environments showed low similarity in fungal community composition, and its community composition also had low similarity with other species. Grasshoppers from the same family had similar gut fungal community structures, while those from different families were dissimilar (except for *Acrida cinerea*). This conclusion may be explained by feeding habits: grasshoppers from the same family may have similar feeding habits, resulting in similar gut fungal community composition, while those from different families have different feeding habits.

Meta-barcoding technology has expanded the scope of microbial diversity research, opening a door for studying microbial diversity in aquatic and soil environments, and can be used more rapidly and efficiently for investigating animal gut microbial diversity. Christian et al. [27] detected bifidobacteria and other bacterial communities in the human gut by amplifying 16S rDNA using Ion Torrent PGM, demonstrating that meta-barcoding technology is a rapid method suitable for investigating human gut microbial community composition. Shelomi et al. [28] first combined high-throughput sequencing with barcoding to study the microbial diversity of fat bodies, foregut, and hindgut in two stick insect species, finding that most bacteria belonged to the genus *Spiroplasma* and

were primarily located in the hindgut of parthenogenetic species. Zhu et al. [29] analyzed 16S rRNA gene sequences from fresh panda feces and found that 13 of 17 bacterial groups were unique to the panda gut, with metagenomic analysis further confirming that *Clostridium* can degrade cellulose and hemicellulose. These studies demonstrate the feasibility and effectiveness of meta-barcoding technology for gut microbial identification. We hope this method will be more widely applied to research on grasshopper gut microorganisms in the future, helping us understand differences in fungal communities, the functional roles of symbiotic fungi, and their effects on grasshopper growth, development, and metabolism, thereby finding new methods for biological control of grasshoppers.

Conclusion

1. A total of 2,786 OTUs were identified from the class composition analysis. At the order level, Tremellales and Auriculariales were the two main fungal orders infecting grasshoppers in both Nanshantai and campus environments.
2. Alpha diversity analysis revealed that Oedipodidae had the highest fungal community richness and diversity, while Catantopidae had the lowest.
3. Beta diversity analysis showed that grasshoppers from the same family had similar gut fungal community structures, while those from different families were dissimilar (except for *Acrida cinerea*).
4. Cluster analysis showed that *Atractomorpha sinensis* from both locations clustered together, while *Chorthippus chinensis* (Acrididae) and *Fruhstorferiola omei* (Catantopidae) clustered together, indicating high pairwise similarity in their fungal communities.
5. At the genus level, *Bullera*, *Ochrolechia*, and *Exophiala* were dominant genera, while *Penicillium* and *Fusarium* were minor genera.

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** Table 1 Information of studied samples and the results of Fungi identification**

** Table 2 The results of the sequence and proportion of each sample**

** Table 3 Diversity index of Fungi in each sample**

[Figure 1: see original paper] Fig. 1 The Venn diagram of interblock for ITS in the administrative level

[Figure 2: see original paper] Fig. 2 The community composition of Fungi at the Order level among different grasshopper species

[Figure 3: see original paper] Fig. 3 The NMDS diagram for each sample in the two most important factors that affect the community structure

[Figure 4: see original paper] Fig. 4 The heatmap diagram for Fungi at the Genera level

Note: Figure translations are in progress. See original paper for figures.