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DNA Extraction and Library Preparation Optimization for Metagenomic Analysis of Giant Panda in Malaysia

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Abstract

The objective of this study was to determine the expected minimum number of sequence reads needed to achieve full coverage of the microbial species found in the gut of giant panda This was done by first, analyzing five different mammalian metagenomes namely horse, coyote, whitetail deer, humpback whale and the bottlenose dolphin as metagenomic references; based on an approximate of 1,000,000 sequence read estimation. After rarefaction analysis using MG-RAST Version 3.0 analysis pipeline, an average value of 775,075 reads was found to be sufficient for metagenomic analysis. Next, the fecal matter of giant panda was sampled at Zoo Negara, and DNA extraction was performed. DNA extraction was then subjected to DNA qualification and quantification analysis; where the results show that the samples are still viable and sufficient in yield to be used for library preparation. After library preparation, the samples were sequenced using Illumina™ MiSeq® next generation sequencer. The results of this research serve as a foundation for further studies of the giant panda metagenome.

1. Introduction

Ninety-nine percent of the giant panda diet is bamboo, with the remaining percentage of 1% being eggs, fish, fruits, honey, shrub leaves and yams, in contrast to all other ursids (Schaller et al., 1985; Schaller et al., 1989). Nevertheless, its digestive system harbors low-diversity, carnivorous gut microbiota with excessive variation according to seasons; proven by the detection of 13 Clostridian Operating Taxonomic Units (OTUs) of phylum Firmicutes, closely related to *Clostridium* and having cellulose-digesting properties (Collins et al., 1994, Xu et al., 2006). Hence in order to increase the efficiency of cellulose, hemicellulose and lignin digestion, the giant panda has developed morphological-genetic adaptations to compensate digestive inefficiency on bamboo, such as evolution of pseudothumb (Endo et al., 1999), possess dense skulls (Zhang et al., 2007; Wei et al., 2007; Zhan et al., 2006) and large, flat teeth with elaborate crown patterns.

Metagenomics is a field of study that analyzes deoxyribononucleic acid (DNA) extracted directly from mixed microbial communities in a particular environment instead of culturing, due to the fact that not all microbes are culturable under laboratory conditions (Handelsman, 2004). This method serves to determine environmental microbial community diversity and activity, biosynthetic pathways, novel and functional individual genes and this is made possible through next generation sequencing. Hence, phylogenetic diversity and relative abundance of microbial species in the gut of giant panda can be studied, indirectly analyzing and monitoring their activity on cellulose, hemicellulose, and lignin, ultimately providing vital information for the diet management and conservation of the endangered giant panda.

2. Materials and Methods

2.1. Reference Sequence Read - Species Richness Analysis

A metagenome analysis was done on five metagenomes obtained from of five different mammals, namely the coyote (Reference ID: 4526727), horse (Reference ID: 4526729), whitetail deer (Reference ID: 4526730), humpback whale (Reference ID: 4526723) and the bottlenose dolphin (Reference ID: 4526724) to set as a reference for the estimation of the minimal sequence reads needed to attain full gut microbial species richness coverage of the giant panda. The five datasets were obtained and analyzed in MG-RAST Version 3.0 (Meyer et al., 2008), using the best hit classification of the

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organism abundance mode; with maximum e-value cutoff set at 1e-5, minimum percentage identity cutoff at 60%, and minimum alignment length cutoff at 15 units in aa for protein and bp for RNA databases, with Kyoto Encyclopedia of Genes and Genomes (KEGG) as the annotation sources. Then, data visualization in the form of rarefaction plot, organism table, and organism tree was used to visualize and compare the five metagenomes.

2.2. Sampling

Fresh fecal sample of the adult male giant panda was collected and immediately sealed in 2 sterile zip-lock bags kept together with ice packs and stored in a Styrofoam box. During sampling, it is ensured that the fresh feces did not come in contact with foreign environment as much as possible and was handled with disposable gloves to avoid contamination. After sampling, the samples were brought to Malaysia Genome Institute (MGI) to be frozen in the -20°C freezer. Using a sterile spatula, about one third of the 50ml conical centrifuge tube was filled with the fecal sample and topped up with with double distilled water, capped and vortexed to form a fecal suspension. Next, 1ml of the suspension was pipette into 2ml microcentrifuge tube and centrifuged at 14,000 rpm for 10 minutes to form a fecal pellet. The liquid was removed and was repeated for another 12 times to obtain a large cumulative pellet. After the final supernatant removal, 800µl of STE buffer was added and the tube was vortexed for homogenization.

2.3. DNA Extraction, Isolation and Metagenomic Library Preparation

A standard phenol chloroform DNA extraction method was done to extract and isolate DNA from the feces. The results were then quantified for purity and concentration via spectrophotometer at 260nm, 280nm, and 230nm. The extracted DNA was also electrophoresed in a 1% TAE agarose gel electrophoresis. The metagenomic library preparation was carried out in 6 phases, namely DNA fragmentation, End repair and size selection, Addition of adenylate 3' ends, Ligation of adapters, Validation and Normalization and pooling of libraries following the protocol described by Illumina™ and sequenced using Illumina™ MiSeq® system.

3. Results and Discussion

The rarefaction plot (Figure 1) of horse, whitetail deer and humpback whale reference metagenomes showed similar trend; where 920 species count spike is seen in the first 75,250 reads, followed by a gradual rise, leveling off at 1,019 species count for 526,750 reads. Among the three metagenomes, the horse metagenome scored highest alpha diversity of 301.15. The coyote metagenome curve however showed lower increment, with second lowest alpha diversity of 81.04; was leveling off at 1,019 species count (1,279,250 reads). The bottlenose dolphin metagenome scored the lowest alpha diversity of 71.30, with 968 species at a maximum of 1,015,875 reads. The suitable read numbers that was comparable between all reference metagenomes was 775,075 reads. Based on these reference metagenomes, an estimated of 1,000,000 sequence reads was needed to attain whole microbial species coverage in the metagenome of giant panda (Handelsman et al., 2007; Zhu et al., 2011; Xue et al., 2015).

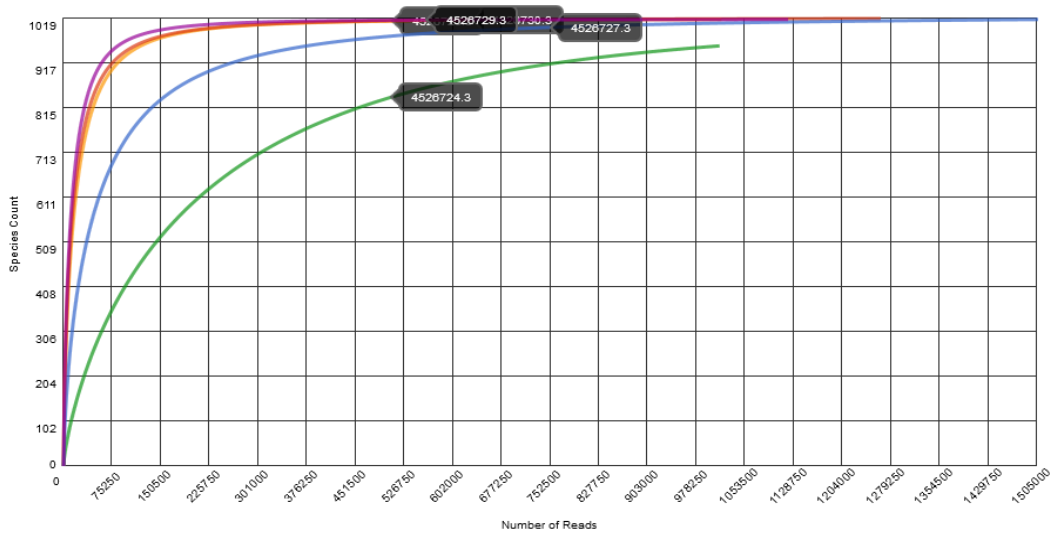

Figure 1: Comparison of species count against number of reads between metagenomes of horse (4,526,729), whitetail deer (4,526,730), humpback whale (4,526,723), coyote (4,526,727), and the bottlenose dolphin (4,526,724) and their respective alpha diversity

The organism tree (Figure 2) showed that 41 phyla was displayed, with only 12 phyla are detectable in all five mammalian metagenomes in this study, with phylum Euryarchaeota scoring the highest number of 9 different classes among all the phyla. The agarose gel electrophoresis (Figure 3) showed thick but faint bands.

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Finally, the spectrophotometer analysis showed a purity of 2.1, signifying slight addition of carrier RNA to the purification procedure. The DNA was sufficient though for library preparation as there was a minimum of 5ug of DNA (Table 1). It is recommended that relatively large-scale sampling and microbial pellet sedimentation process should be done as many times as possible and sample handling should be done at low temperatures while not be exposed to frequent temperature changes.

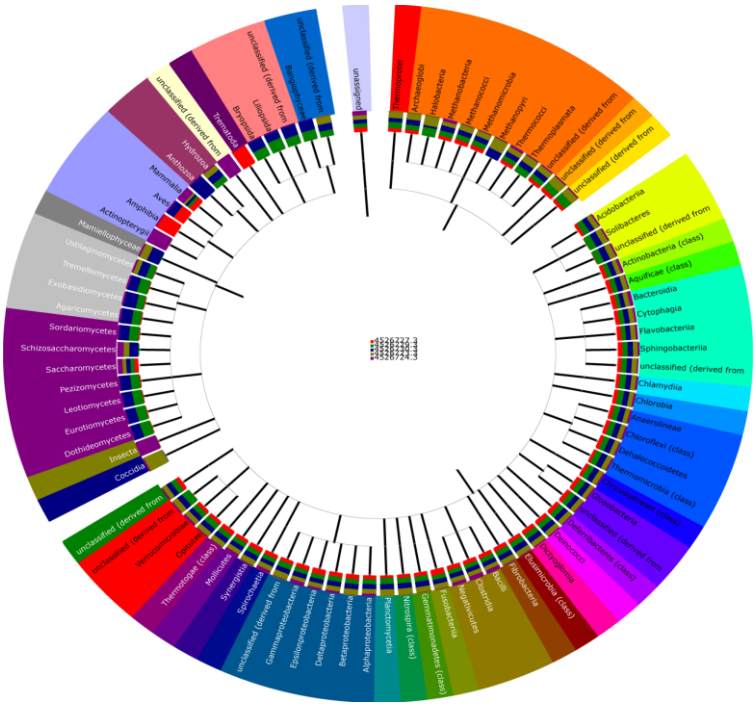

Figure 2: Organism tree of the microbial classes present and their respective relative abundance in the metagenomes of horse (4,526,729.3), whitetail deer (4,526,730.3), humpback whale (4,526,723.3), coyote (4,526,727.3) and the bottlenose dolphin (4,5267,24.3)

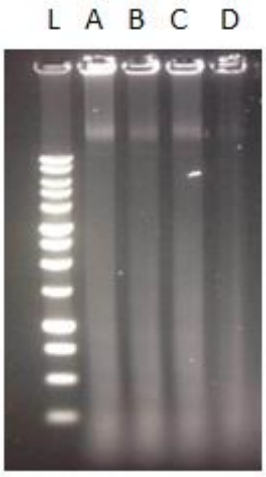

Figure 3: Agarose gel electrophoresis of 4 metagenomic DNA samples. *L = 1Kb ladder, Sample A, B, C and D are all DNA extracted from giant panda feces

Table 1: NanoDrop® ND-1000 spectrophotometer results of four DNA sample purity, concentration, and yield at 230nm, 260nm, and 280nm

Sample	DNA purity (260nm/280nm)	DNA purity (260nm/230nm)	Concentration of DNA (ng/µl)	Volume (µl)	DNA yield (µg)
Panda A	2.06	3.08	217.2	143	31.06
Panda B	2.09	3.19	252.4	143	36.09
Panda C	2.09	3.14	207.4	143	29.66
Panda D	2.06	3.13	249.9	133	33.24

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4. Conclusion

In conclusion, the required amount of reads for a suitable metagenomic analysis for Giant Panda fecal samples are 1,000,000. It is also recomended that the bacterial pellet sedimentation process should be repeated as much as possible to obtain sufficient amounts of bacterial pellet for DNA extraction and isolation.

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