Postprint: High-Throughput Sequencing Analysis of Archaeal Microbiota Diversity in Fecal Samples from Captive Adult Forest Musk Deer

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Abstract

The objective of this study was to investigate the structure and composition of archaea in feces of artificially reared adult forest musk deer using high-throughput sequencing technology, and to compare differences between sexes. Twelve healthy 3-year-old forest musk deer were selected and divided into a male group (M group, n=6) and a female group (F group, n=6) based on sex. Fresh fecal samples were collected, DNA was extracted, the V4-V5 region of archaeal 16S rRNA was amplified by PCR using universal archaeal primers, and the amplicons were subjected to high-throughput sequencing on the MiSeq 300PE platform. The sequencing data were analyzed and statistically processed using QIIME and other software. The results demonstrated that at all taxonomic levels from phylum to genus, differences in relative abundance of archaea between the F and M groups were not statistically significant (P>0.05). The intra-group genetic distances for the M and F groups were $(0.16\$\pm0.03) and (0.27\pm0.06), respectively, while the inter-group distance was (0.24\pm\$0.07)$, indicating high similarity among samples. The archaeal communities in forest musk deer feces could be classified into 3 phyla, with Euryarchaeota being the dominant phylum; at the genus level, they could be divided into 7 known genera, with Methanobrevibacter as the dominant genus, followed by Thermogymnomonas. These findings suggest that there are no significant differences in archaeal structure and composition between male and female forest musk deer feces, and that Methanobrevibacter is the predominant archaeal genus in forest musk deer feces.

Full Text

Fecal Archaeal Diversity of Artificially Bred Adult Forest Musk Deer Analyzed by High-Throughput Sequencing

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Abstract: This study investigated the structure and composition of archaea in the feces of artificially bred adult forest musk deer using high-throughput sequencing technology, and compared differences between sexes. Twelve healthy three-year-old forest musk deer were divided into a male group (M group, n=6) and a female group (F group, n=6). Fresh feces were collected, DNA was extracted, and the V4-V5 region of archaeal 16S rRNA was amplified using universal archaeal primers. The amplicons were sequenced on the MiSeq 300PE platform, and the resulting data were analyzed using QIIME and other software. The results showed that from phylum to genus level, there were no significant differences in archaeal relative abundance between the F and M groups (P>0.05). The intra-group genetic distances were $(0.16\$\pm0.03) for the M group and (0.27\pm0.06) for the F group, while the inter-group distance was (0.24\pm\$0.07)$, indicating high similarity among samples. The archaea in forest musk deer feces could be classified into three phyla, with Euryarchaeota being the dominant phylum. At the genus level, seven known genera were identified, with *Methanobrevibacter* as the dominant genus, followed by *Thermogymnomonas*. These findings suggest that neither the structure nor composition of archaea in forest musk deer feces differs significantly between sexes, and that *Methanobrevibacter* represents the dominant archaeal genus.

Keywords: forest musk deer; archaea; methanogen; diversity

Introduction

The forest musk deer (*Moschus berezovskii*), belonging to the order Artiodactyla, family Moschidae, and genus *Moschus*, is a precious wild animal resource valued for medicinal and fragrance purposes that was once widely distributed across central and southern China. However, due to habitat destruction and overhunting, its population has declined dramatically in recent years. The species is currently listed as endangered by the International Union for Conservation of Nature (IUCN) (http://www.iucnredlist.org/details/13894/0).

China began artificial domestication and breeding of forest musk deer in the 1960s, representing nearly 60 years of history, yet development has been slow. A primary reason for this slow progress is the limited understanding of the digestive physiology of forest musk deer. As herbivorous ruminants, the composition and structure of their gastrointestinal microbial flora remain largely unknown.

Systematic investigation of the gastrointestinal microbiota is essential for effective conservation and artificial breeding efforts. It is well established that the rumen of ruminants harbors a vast microbial community, including bacteria, fungi, protozoa, and archaea. In recent years, rumen methanogenic archaea have attracted increasing attention due to their presumed association with the escalating global greenhouse effect. Methanogenic archaea are widely distributed in the gastrointestinal tracts of herbivores, and numerous studies have identified diverse methanogenic archaea in water buffalo, sheep, kangaroos, dairy cattle, rhinoceroses, beef cattle, camels, yaks, and other animals. Collectively, these studies demonstrate that the diversity of gastrointestinal methanogenic archaea exhibits host specificity. Therefore, we hypothesized that the structure and composition of archaea in forest musk deer might differ substantially from those of other animals. However, no studies have yet reported on methanogenic archaea in forest musk deer. The objective of this experiment was to investigate the structure and composition of archaea in forest musk deer feces using high-throughput sequencing technology and to compare differences between sexes.

1.1 Experimental Animals and Sample Collection

The experimental animals consisted of 12 artificially bred forest musk deer, divided into a male group (M group) and a female group (F group) based on sex, with six animals per group. The six male deer were designated M1-M6, and the six female deer were designated F1-F6. All animals were three years old, healthy, and had not received antibiotics, with an average body weight of 7.58$±$1.596 kg. They were housed at the Sichuan Institute of Musk Deer Breeding in Dujiangyan, Sichuan Province. Deer were individually caged and fed twice daily at 8:00 AM and 5:00 PM with free access to water. The diet composition is presented in . Animals had been fed this diet for at least 28 days prior to sampling. On day 29, fresh feces (20 g per deer) were collected before morning feeding using sterilized disposable gloves and clean bamboo sticks, sampling from the interior of naturally excreted fecal pellets. Samples were stored in liquid nitrogen and transported to the laboratory, where they were stored at -80°C for subsequent DNA extraction.

1.2 DNA Extraction

Total microbial DNA was extracted from forest musk deer fecal samples using the TIANamp Microbial DNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer' s protocol. The extracted DNA was purified using a DNA purification kit, and its concentration and purity were assessed using a spectrophotometer and agarose gel electrophoresis. The purified DNA was stored at -20°C until use.

1.3 16S rRNA Gene Amplification and Sequencing

The V4-V5 region of archaeal 16S rRNA was amplified by PCR using universal archaeal primers 341F (5' -CCTAYGGGRBGCASCAG-3') and 806R (5'

-GGACTACHVGGGTWTCAAT-3') [13]. The 50 L PCR reaction mixture contained 0.5 L each of forward and reverse primers, 5 L of 2.5 mmol/L dNTP mix, 5 L of 10×Ex Taq buffer (containing 20 mmol/L Mg^{2+}, TaKaRa), 0.25 L of Ex Taq DNA polymerase (TaKaRa), 1 L of template DNA, and 37.75 L of milli-Q water. PCR conditions were as follows: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and final extension at 72°C for 5 min. Three replicate PCR reactions were performed for each sample. PCR products from the same sample were pooled and visualized on 2% agarose gels. Archaeal PCR products were excised from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN), followed by further purification with a QIAGEN purification kit. The purified PCR products were sent to Macrogen (South Korea) for high-throughput sequencing on the Illumina MiSeq 300PE platform.

1.4 Data Analysis

Paired-end reads were merged using FLASH software with a minimum overlap of 10 bp and maximum overlap of 220 bp. Successfully merged sequences were assigned to samples based on barcode sequences using QIIME software [14], and low-quality sequences were removed according to the following criteria: reads containing any ambiguous base (N) were removed; reads with three consecutive bases having quality scores < Q20 were removed; reads with mismatched barcode sequences were removed; and reads where the continuous Q20 region comprised less than 75% of the total sequence length were removed. After barcode trimming, high-quality sequences were clustered into operational taxonomic units (OTUs) at $ $97% similarity, and the most abundant sequence in each OTU was selected as the representative sequence. OTU representative sequences were aligned against the RDP database (The Ribosomal Database Project, https://rdp.cme.msu.edu/) using QIIME with default parameters (similarity threshold = 0.9). Non-archaeal OTUs were removed using a Perl script. Chimeras and singletons were filtered out using QIIME, and taxonomic annotation was performed at kingdom, phylum, class, order, family, and genus levels. Alpha diversity indices (Shannon index, Chao index, and observed species index) were calculated for each sample using QIIME default parameters. Genetic distances between samples were calculated based on OTU composition and abundance using unweighted UniFrac distance, and samples were clustered using the unweighted pair group method with arithmetic mean (UPGMA). Shared genera present in all samples were identified using a Perl script, and heatmaps were generated using R software. Differences between the two groups were analyzed using t-tests in SPSS 19.0.

Results

2.1 Sequence and OTU Statistics

Paired-end reads were assembled into single sequences based on overlap relationships. After quality control, a total of 13,130 high-quality archaeal sequences

were obtained, with an average of (1,094$±1, 164)*sequencespersample.ThesesequenceswereclusteredintoOTU* 97±24.5)*OTUspersample*]*and*409*intheFgroup*[(78.8±$27.7) OTUs per sample], with 74 OTUs shared between groups ([Figure 1: see original paper]). The sequence coverage was 80.78% for the M group and 81.91% for the F group.

2.2 Fecal Archaeal Composition Analysis

OTUs were taxonomically annotated from phylum to genus level, with detailed results presented in . Over 90% of OTUs belonged to the phylum Euryarchaeota, while Crenarchaeota and unclassified phyla showed relatively low abundance. No significant differences in relative abundance were observed between groups at any taxonomic level (P>0.05). All Crenarchaeota sequences originated from the class Thermoprotei, which could be further divided into two orders. Only Fervidicoccales could be further classified into one family and one genus. Euryarchaeota comprised four classes: Methanobacteria, Methanomicrobia, Thermoplasmata, and an unclassified class. The unclassified class could not be further resolved, while the other three classes were divided into four orders, six families, and seven genera. Methanobacteria contained one order, one family, and three genera; Methanomicrobia contained two orders, three families, and three genera; and Thermoplasmata contained one order and two families, with only the incertae sedis family Thermoplasmatales incertae sedis resolvable to genus level. At the genus level, *Methanobrevibacter* showed the highest relative abundance, followed by *Thermogymnomonas*. Although no significant differences were observed between groups at the genus level (P>0.05), the relative abundance of *Fervidicoccus* tended to be lower in the F group compared to the M group (P=0.10).

2.3 Alpha Diversity Analysis

Alpha diversity indices (Shannon, Chao , and observed species) for the M and F groups are presented in . These indices primarily evaluate microbial richness and evenness within samples. Although the M group showed numerically higher alpha diversity indices than the F group, the differences were not statistically significant (P>0.05).

2.4 Beta Diversity Analysis

Genetic distances between samples were calculated based on OTU composition and abundance, with smaller values indicating greater similarity in microbial community structure. The intra-group genetic distances were $(0.16\$\pm0.03) for the M group and (0.27\pm0.06) for the F group, while the inter-group distance was (0.24\pm\$0.07)$. Based on these distances, samples were clustered and visualized using PCoA ([Figure 2: see original paper]). Except for samples M4 and M2, the remaining 10 samples showed high clustering density. Although the F group exhibited tighter clustering than the M group, samples did not show clear group-specific clustering patterns.

2.5 Shared Genus Analysis

Since no significant differences were observed in archaeal composition, abundance, or alpha diversity indices between the F and M groups, all 12 samples were analyzed as a whole to identify shared archaeal genera. A heatmap was generated to visualize the abundance of shared genera across all samples ([Figure 3: see original paper]). Shared archaeal genera among the 12 forest musk deer primarily originated from the classes Methanobacteria and Thermoplasmata within Euryarchaeota. Some shared genera exhibited high abundance across samples, such as *Methanobrevibacter* and *Thermogymnomonas*, with average relative abundances of 67% and 11% of total archaeal abundance, respectively. Other sequences were present in all samples but at extremely low abundance, such as the unclassified class within Euryarchaeota. The cumulative relative abundance of shared genera accounted for over 96% of total archaeal abundance in each sample, indicating that sex has minimal impact on the composition of dominant archaeal taxa in forest musk deer feces at the genus level, although the abundance of shared genera varied considerably among individual animals.

Discussion

To our knowledge, this study represents the first report on gastrointestinal archaea in forest musk deer. Previous studies on animal gastrointestinal archaea have predominantly employed clone library sequencing techniques, which are not only time-consuming and labor-intensive but also limited to identifying dominant taxa and fail to comprehensively reflect microbial community structure. High-throughput sequencing technology, adopted in recent years for microbial ecology research, enables more comprehensive identification of microbial diversity. In this study, high-throughput sequencing of fecal samples from 12 forest musk deer yielded 13,130 high-quality archaeal sequences and detected 720 archaeal OTUs. In contrast, previous studies on rumen archaea in ruminants, foregut archaea in kangaroos, and fecal archaea in various animals typically obtained approximately 1,000 sequences and around 100 OTUs per experiment —substantially lower than our results. Taxonomic annotation of our OTUs revealed three phyla and eight known genera. While nearly all previously reported gastrointestinal archaea originated from Euryarchaeota, with very rare reports of Crenarchaeota and a maximum of 4-5 genera detected per study, these discrepancies likely reflect methodological differences. Kim et al. analyzed archaeal sequences from public databases and estimated, based on rarefaction curves, that rumen samples should contain >1,400 archaeal OTUs requiring at least 24,480 sequences for comprehensive coverage. Our sequence and OTU numbers were approximately half these estimates, possibly due to differences in sample type, as previous research has demonstrated significantly lower archaeal diversity in goat feces compared to rumen contents.

Previous studies have identified *Methanobrevibacter* as the dominant archaeal genus in the hindgut of monogastric animals and the rumen of ruminants. For example, Wright et al. reported that 62 of 65 archaeal sequences obtained

from Australian sheep rumen belonged to *Methanobrevibacter*. In our study, *Methanobrevibacter* was not only a shared genus across all 12 samples but also the dominant genus in both male and female forest musk deer feces, with relative abundances of 71.78% and 61.99%, respectively. These results are consistent with numerous previous reports. However, some studies have reported different dominant archaea, such as Sundset et al., who found that 53% of archaea from Svalbard reindeer rumen belonged to unidentified taxa, and Huang et al., who reported that 80.9% of archaea in yak rumen originated from Thermoplasmatales, with only 14.9% belonging to Methanobacteriales, despite identical environmental conditions and diets. These findings demonstrate host-specific differences in gastrointestinal archaeal community structure and composition, emphasizing that the archaeal profile of a particular animal species must be determined empirically rather than extrapolated from other species.

In this study, no significant differences in archaeal relative abundance were observed between male and female forest musk deer at any taxonomic level from phylum to genus. Similarly, alpha diversity indices did not differ significantly between sexes, and genetic distances between samples were small. These results collectively indicate that sex does not significantly influence archaeal community structure and composition in forest musk deer feces. Previous research has identified breed and diet as primary factors influencing gastrointestinal archaea. For instance, Huang et al. reported higher relative abundances of Methanobacteriales (21.5% vs. 12.4%) and Methanomicrobiales (9.8% vs. 1.0%) in cattle compared to yak rumen. Zhou et al. demonstrated differences in rumen archaeal structure between beef cattle with different feed efficiencies, with lower-efficiency animals exhibiting higher archaeal diversity. Min et al. reported that the relative abundance of *Methanobrevibacter* in goat rumen decreased linearly with increasing dietary condensed tannin concentration. To date, no studies have reported sex-related effects on animal gastrointestinal archaea. We speculate that sex itself may have minimal impact on forest musk deer gut archaea, and the lack of significant differences in our study may be further reinforced by the fact that both sexes were fed identical diets.

Conclusion

At all taxonomic levels from phylum to genus, no significant differences in archaeal structure and composition were observed between male and female forest musk deer feces, and *Methanobrevibacter* represents the dominant archaeal genus.

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