A gene catalogue of Rhinopithecus gut microbiome provides new insights into dietary adaptation of foregut fermenting animals

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Abstract

Gut microbiome gene catalogues have advanced the knowledge of host-microbiome interactions in many mammals. Snub-nosed monkeys (Rhinopithecus spp) are folivores with foregut fermentation which is similar to ruminants, but their gut microbiota lacks a comprehensive description. In this study, we constructed a comprehensive gene catalogue by performing metagenomic analysis on 143 wild snub-nosed monkeys and compare it to that of ruminants and monogastric animals. Our results demonstrate the classification and functional characteristics of the gut microbiome of snub-nosed monkeys and identified a set of core genera in these mammals. Moreover, we found that the gut of snub-nosed monkeys and other herbivores was enriched with more bacteria and enzymes related to the degradation of structural carbohydrates, indicating the importance of gut microbiota for dietary adaptation. Our study expands resources for gut microbiome studies of nonhuman primates and provide new insights into the evolutionary route of foregut fermenting animals during dietary adaptation.

A gene catalogue of *Rhinopithecus* gut microbiome provides new insights into dietary adaptation of foregut fermenting animals

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Running Title: Gene catalogue of snub-monkey gut microbiome

Abstract

Gut microbiome gene catalogues have advanced the knowledge of host-microbiome interactions in many mammals. Snub-nosed monkeys (*Rhinopithecus* spp) are folivores with foregut fermentation which is similar to ruminants, but their gut microbiota lacks a comprehensive description. In this study, we constructed a comprehensive gene catalogue by performing metagenomic analysis on 143 wild snub-nosed monkeys and compare it to that of ruminants and monogastric animals. Our results demonstrate the classification and functional characteristics of the gut microbiome of snub-nosed monkeys and identified a set of core genera in these mammals. Moreover, we found that the gut of snub-nosed monkeys and other herbivores was enriched with more bacteria and enzymes related to the degradation of structural carbohydrates, indicating the importance of gut microbiota for dietary adaptation. Our study expands resources for gut microbiome studies of nonhuman primates and provide new insights into the evolutionary route of foregut fermenting animals during dietary adaptation.

Keywords: Snub-nosed monkeys, gut microbiome, gene catalogue of gut microbiome, dietary adaptation, gastrointestinal tract

1 | INTRODUCTION

Accumulating evidence indicates that the gut microbiome plays an essential role in modulating the host's whole-body metabolism (Qin et al., 2012; Turnbaugh et al., 2006), immune system (Sylwia Smolinska, 2016; X. Zhang, Chen, Zhao, & Li, 2020) and adaptation to specific diets and extreme environments (Baniel et al., 2021; H. Li et al., 2018; X. Li et al., 2022; Z. Zhang et al., 2016). Therefore, a deeper understanding of host-microbiome interactions is important for understanding host adaptive evolution and health. In recent years, a reference catalogue of gut microbiome genes has been constructed to reduce data redundancy in large-scale gut metagenomic studies to improve the estimation of the diversity and genetic potential of gut microbiome and serve as a basis for metagenome-wide association studies (Xiao et al., 2016; Xiao et al., 2015; Xie et al., 2021). The catalogue of reference genes is a powerful tool to help us gain a comprehensive understanding of the gut microbiome and is also essential for in-depth functional metagenomic analyses such as species/gene profiling, microbial biomarker discovery, and functional annotation (J. Li et al., 2020; Thingholm et al., 2019). To date, many catalogues of reference genes for the gut microbiome have been established in a variety of mammals, including humans (J. Li et al., 2014; Qin et al., 2010), rodents (Degnan et al., 2012) and ruminants (J. Li et al., 2020; Xie et al., 2021). This has greatly promoted knowledge of the interactions between host physiology and their gut microbiome, and is also of great significance for understanding host dietary adaptation and health status monitoring (R. E. Ley et al., 2008; Thingholm et al., 2019).

Nonhuman primates (NHPs) are our closest living relatives and among the most widespread and successful mammals that underwent adaptive radiation. Therefore, studies of the NHP gut microbiome are fundamental in investigations of the ecological and evolutionary adaptation mechanisms of NHPs and even mammals (Amato et al., 2016; Clayton et al., 2018; Goldberg, 2008). Mimicking human dietary patterns in NHP animal models has improved our understanding of the complex interplay between diet, obesity, and the gut microbiome in humans (Newman et al., 2021). Therefore, studies of the NHP gut microbiome may provide important clues for understanding the characteristics of these bacterial communities in the human gut and provide useful guidance and reference for studying the interaction between humans and their gut microbiome (Jianan Sang 2022). In addition, the impact of gut microbiome homeostasis on the conservation of endangered NHPs is of particular concern (Basabose, 2002). Except phylogenetic relationships, the NHP gut microbiome is influenced by multiple other factors, such as diet (Baniel et al., 2021), physiology (Bennett et al., 2016), habitat quality (Barelli et al., 2015), and social interactions (Bennett et al., 2016). For example, the gut microbial structure of geladas (*Theropithecus gelada*) adapted to seasonal dietary changes through rapidly change (Baniel et al., 2021). The richness and diversity of gut microbes in the Udzungwa red colobus (*Procolobus gordonorum*) were reduced due to dietary changes caused by human disturbance and habitat

degradation (Barelli et al., 2015). Therefore, it is very important to study the gut microbiome of NHPs to understand the mechanisms of dietary adaptation and to develop better conservation strategies for these endangered species. However, considering that NHPs occupy a variety of habitats and niches, most previous studies considered a single species or a small number of captive individuals, which is far from being able to completely and accurately represent the general characteristics of intestinal microbiome in NHPs (Degnan et al., 2012; X. Li et al., 2018). The key to detailed analysis of the gut microbiome in NHPs is the availability of comprehensive gene catalogues of wild individuals (Zhu et al., 2020). Therefore, the establishment of a comprehensive reference gene catalogue of the gut microbiome in wild NHPs is of practical significance for further developing the potential resource pool of the gut microbiome in wild NHPs and exploring the relationships of the gut microbiome with ecological adaptation and conservation in NHPs.

Among NHPs, snub-nosed monkeys (*Rhinopithecus* spp) (SNMs) are a group of endangered colobines including five living species of Sichuan SNM (*R. roxellana*), Yunnan SNM (*R. bieti*), Guizhou SNM (*R. brelichi*), Tonkin SNM (*R. avunculus*) and Myanmar SNM (*R. strykeri*), of which Yunnan SNMs, endemic to the Tibetan Plateau, are the NHPs with the highest altitudinal distribution (Quan Gouqiang, 2002). Additionally, the living conditions of SNMs are more severe than those of other primates, with features such as low temperatures and limited oxygen (Li BG, 2002; Long YC, 1994), indicating that SNMs should be suitable for studying the mechanisms adaptation to extreme environments. SNMs have sacculated stomachs and are folivores with foregut fermentation. In recent years, the genomic study of SNMs revealed its genetic mechanism underlying dietary adaptations and found evidence for functional evolution in the colobine RNASE1 gene that enables digestion of the high concentrations of bacterial RNA derived from symbiotic microflora (Zhou et al., 2014). Moreover, the digestion of cellulose by the gut microbiome is also an important part of the dietary adaptation of SNMs (Wang et al., 2021). Studies have shown that the gut microbial structure of SNMs is similar to that of ruminants, but the fermentation efficiency is lower than ruminants (D. J. Chivers, Hladik, C.M, 1980). The sacculated stomachs of SNMs do not exhibit the same strong functional division as the rumens of ruminants (D. Chivers, 1994). It is still not fully understood how the gut microbiome and gastrointestinal tract (GIT) help SNMs extract sufficient energy and nutrients from a high-fibre diet (Ganzhorn, 1992; Wang et al., 2021). Previous studies of single SNM species have limited the description of the overall genetic diversity of their gut microbiome (V. L. Hale et al., 2018; V. L. Hale, Tan, C.L., Niu, K., Yang, Y., Zhang, Q., Knight, R., 2019; Yao et al., 2021). Therefore, a comprehensive exploration of the gut microbiome in a large number of wild SNMs is needed to further elucidate their dietary adaptation mechanism, and the establishment of a reference gene catalogue of the gut microbiome in wild SNMs is a reasonable next step. Such a catalogue will provide an important database and framework for analysing the relationships of the gut microbiome with dietary adaptation and for making conservation plans. It will also provide a data basis and reliable criteria for studies of the gut microbiome as well as health, immunity, and adaptation to extreme environments in SNMs.

Here, we constructed an integrated reference gene catalogue of *Rhinopithecus* gut microbiome (RGC) by processing 143 fecal individual samples from wild populations of SNMs (Yunnan SNM, Sichuan SNM, and Myanmar SNM). The aim of this study was to comprehensively characterize the gut microbiome of SNMs and lay a solid foundation for revealing the relationship between the gut microbiome and host evolutionary adaptation and health. We envisage that the present gut bacterial gene catalogue and functional characterization will serve as a valuable reference and resource for studying the evolution of the gut microbiome in foregut fermenters, also for the study of mammalian evolution and functional adaptation models provides a wide range of perspectives.

2 | METHODS

2.1 | Sample collection and transportation

A total of 143 fecal individual samples with different ages, sexes and altitudes of distribution were collected from different wild populations of Sichuan SNM (81 samples), Yunnan SNM (47 samples) and Myanmar SNM (15 samples) (Table S1). All fresh fecal matter were placed in 15ml of RNAlater immediately after defecation and transported to Institute of Zoology, Chinese Academy of Sciences (CAS) with dry ice, then

stored at -80 until DNA extraction.

2.2 | DNA extraction and sequencing

Fecal DNA extraction was performed using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) according to the standard protocol. The quality and concentration of the DNA were determined using a Nanodrop (ND-1000) spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and the integrity was confirmed by agarose gel electrophoresis. DNA samples were stored at -20°C until use. Shotgun sequencing was performed using an Illumina NovaSeq 6000, with at least 10 Gb per sample.

2.3 | Construction of the gene catalogue

Shotgun sequencing reads for fecal samples from *R.roxellana* , *R. bieti* and *R. strykeri* individuals were independently processed. We filtered the raw data using Trimmomatic (v0.36) (Bolger, Lohse, & Usadel, 2014) to exclude adapter sequences and low-quality reads. Then we used the genomes of *R. strykeri*(assembly ASM2376470v1), *R. bieti* (assembly ASM169854v2),*R. roxellana* (assembly ASM756505v1) and human (assembly GRCh38.p13) to remove contaminants and obtain clean data with Bowtie2 (v2.3.5) (Langmead & Salzberg, 2012). The remaining reads were considered high-quality reads. In total, we obtained 1,448 GB of high-quality reads with an average of 10.13 GB per sample. To construct a comprehensive catalogue of reference genes in the SNM gut microbiome, we individually assembled the high-quality reads from each sample into longer contigs with MEGAHIT (v1.2.6) (Dinghua Li 2015). We obtained 62,306,924 contigs longer than 300 bp. Next, MetaGeneMaker (Wenhan Zhu, 2010) was used to predict open reading frames (ORFs), and we obtained 111,177,047 ORFs from 143 samples with an average of 777,462 ORFs per sample, which were longer than 102 bp, (Table S2). Three non-redundant gene-sets of the *R. roxellana* , *R. bieti* , and *R. strykeri* gut microbiome were independently clustered using CD-HIT (Fu Limin, 2012). We further merged these three non-redundant gene-sets into an integrated catalogue of reference genes in the SNMs by CD-HIT (Fu Limin, 2012), referred to RGC (Figure 1).

2.4 | Taxonomic and functional annotation

The genes of the RGC were translated into amino acid sequences using NCBI Genetic Codes 11 (Kozak, 1983). Taxonomic annotation of amino acid sequences was performed by Kaiju v1.8.0 (Menzel, Ng, & Krogh, 2016) and the NCBI NR database (released on Feb 24th, 2021), providing a detailed overview of the taxonomical composition of SNMs gut microbiome with the parameters '-a greedy -e 5 -E 0.01 -v -z 5'. Functional annotation was performed using the Kyoto Encyclopedia of Genes and Genomes databases (KEGG, Release 100.0, genes from animals or plants were excluded) and Evolutionary genealogy of genes: Non-supervised Orthologous database (eggNOG, v5.0) on the basis of Diamond (v0.9.24) with the parameters '–evalue 1e-5 -k 1' (Benjamin Buchfink, 2015). KEGG and eggNOG annotations were performed using an in-house pipeline, where each protein was assigned to a KEGG orthologous group (KO) or eggNOG orthologous group (OG) when the highest-scoring annotated hits contained at least one alignment with over 60 hits (Qin et al., 2012). Carbohydrate enzyme annotation was carried out through the Carbohydrate-Active enZYmes Database (CAZymes). We mapped protein sequences to entries in the hidden Markov model (HMM) libraries of CAZyme families downloaded from the CAZy database (CAZyDB.07312019) with the hmmscan program in HMMER (3.1b2) (Pattabiraman & Warnow, 2021).

2.5 | Estimation of the relative abundances of genes, taxa and function terms

To calculate the gene relative abundance, high-quality reads from each sample were aligned against the RGC by Bowtie2 (v2.3.5) (Langmead & Salzberg, 2012). Sequenced-based abundance profiling was determined as described in previous studies (Qin et al., 2012). The relative abundances of taxa, KOs, OGs and CAZymes were calculated by summing the abundance of the respective genes belonging to each category per sample, based on the taxonomic assignments and KO, OG and CAZymes annotations, respectively (J. Li et al., 2014).

Published gene relative abundance profiles and gene catalogues of 11 species were downloaded and analysed by the same taxonomic and functional annotation pipeline in our study. These catalogues of 11 species included

human (*Homo sapiens*) gut microbiome (ICG) (J. Li et al., 2014), pig (*Sus scrofa*) gut microbiome (Xiao et al., 2016), mouse (*Mus musculus*) gut microbiome (Xiao et al., 2015), cynomolgus macaque (*Macaca fascicularis*) gut microbiome (X. Li et al., 2018), and gut microbiome of various ruminants (*Bos taurus* ,*B. grunniens* , *Bubalus bubalis* , *Capra aegagrus* ,*Ovis aries* , *Capreolus pygargus* and *Hydropotes inermis*) (Xie et al., 2021) (Table S4).

2.6 | Microbial composition analysis

Alpha diversity of the gut microbiome was calculated using the Shannon index based on the genus and KO profiles by "vegan" packsge in R (Dixon, 2003). The overall differences in the bacterial community structures were evaluated by principal coordinate analysis (PCoA) and hierarchical tree analysis based on the Bray-Curtis dissimilarity matrix, which were performed with "vegan" package in R (Dixon, 2003). To show the special characteristics of the gut microbiome of SNM, the taxonomic (phylum and genus) and function (COGs, KOs and CAZymes) terms present in 90% of individuals in each gene catalog were used for comparison and visualization with UpSet Venn (Xie et al., 2021). Then, we calculated the differences among 12 species by the Wilcoxon rank-sum test or Kruskal–Wallis test, and the p values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) (Yoav Benjamini, 1995).

3 | RESULTS

3.1 | Construction of a reference gene catalogue of the SNM s gut microbiome

We generated a gene catalogue of the gut microbiome in SNMs, comprising 18,169,322 non-redundant genes with an average length of 573 bp and 32.39% of these genes were identified as complete (Figure 1; Table S3). Of these non-redundant genes, only 9,829,597 (54.1%) genes were taxonomically classified. Of these, more than 99.35% could be assigned to bacteria, and the remaining 0.65% being genes from viruses (0.24%) and archaea (0.41%) (Table S5; Figure 2a). At the phylum level, most of the annotated genes belonged to Firmicutes (41.99%), followed by Proteobacteria (3.43%), Bacteroidetes (3.28%) and Actinobacteria (1.17%). Moreover, 6,037,022 (33.2%) and 6,809,541 (37.5%) of the bacterial genes in the RGC could be annotated to the genus and species levels, respectively. At the genus level, most of the annotated genes (5.25%) belonged to *Clostridium* , followed by *Ruminococcus*(2.11%), *Roseburia* (1.38%), *Eubacterium* (1.17%) and*Oscillibacter* (1.05%) (Figure S1; Table S5).

Functional annotation of the gut bacterial genes contained in RGC revealed a total of 8,061 KOs and 44,671 OGs, which represented 33.54% (6,049,083) and 56.30% (10,221,423) of the RGC, respectively (Table S6). These results illustrated that the gut microbiome of SNMs is a highly complex taxonomic assemblage with many unknown genes. Pathways associated with metabolism linked to the degradation of carbohydrate, amino acid, energy, nucleotide, cofactors and vitamins were enriched (Figure S2; Table S9). Further, genetic information processing pathways (replication and repair, and translation), and environmental information processing pathways (membrane transport and signal transduction) were also enriched (Figure S2; Table S9). Additionally, we mapped the RGC to the CAZy database. The results provided us with a complete picture of the carbohydrate enzymes presented in SNMs gut microbiome. We obtained 371 enzyme families from 390,733 annotated genes. These genes accounted for ˜2.15% of the total bacterial genes in the RGC. There is a wide diversity of glycoside hydrolases (GH) catalytic modules in the RGC, including 140 GH families. This indicated that the SNMs have a strong ability to digest structural carbohydrates, which is related to their dietary adaptation. GHs are one of the major enzymes in the gut microbiome and hydrolyse the glycosidic linkage of glycosides (Lee et al., 2014). They play a crucial role in the digestion of complex carbohydrates, such as starch and cellulose.

3.2 | The characteristics of SNMs gut microbes

Based on the taxonomical profiles, Firmicutes and Bacteroidetes were the two main phyla (Figure 2b), and *Clostridium* and *Prevotella*were the dominant genera (Figure 2c) in the RGC. Additionally, we identified a common set of 241 non-redundant genes, 4032 COGs, 624 genera, 45 phyla, 3018 KOs and 191 CAZy families shared by all 143 individuals (Figure 2d; Table S7), suggesting the existence of a core set of genes, microbial

composition and functions in the gut microbes of SNMs.

To assess the extent to which our gene catalogue represents the diversity of the gut microbiome in SNMs, we mapped published data generated from 10 fecal individual samples of wild Sichuan SNM to the RGC. On average, 76.55% of the reads mapped to our gene catalogue (Table S8). The mapping rate was similar to that of SNMs in our study (Table S2), indicating that the RGC should represent the characteristics of the gut microbiome in SNMs. Furthermore, we also mapped 10 fecal individual samples of captive Sichuan SNM to the RGC, and the average mapping rate was 49.83% (Table S 8), indicating that captive environment has a significant effect on the structure of gut microbiome in SNMs.

3.3 | Comparison of the RGC with the gene catalogues of cynomolgus macaque, human, mouse, pig and ruminants

The RGC was compared to the catalogues of monogastric animals (human (IGC) (J. Li et al., 2014), cynomolgus macaque (X. Li et al., 2018), mouse (Xiao et al., 2015) and pig (Xiao et al., 2016)) and ruminants (dairy cattle, water buffalo, yak, goat, sheep, roe deer and water deer) (Xie et al., 2021). Our results showed that the RGC comprised more genes than the human catalogue (9.9 M, 1267 samples), cynomolgus macaque catalogue (1.99 M, 20 samples), mouse catalogue (2.6 M, 184 samples), pig catalogue (7.7 M, 287 samples), roe deer catalogue 13.7 M, 50 samples) and water deer catalogue (7.7 M, 50 samples), but fewer genes than that of the yak (32.9 M, 50 samples), water buffalo (34.4 M, 50 samples), dairy cattle (35.5 M, 60 samples), goat (20.97 M, 60 samples) and sheep catalogues (24.2 M, 50 samples) (Table S6; Figure 3a). Alpha diversity analysis of the 12 species showed that the alpha diversity (Shannon Index) of the RGC was not significantly different from that of most ruminants (goat, sheep, yak and water buffalo) at the genus level (Figure 3b). Since ruminants and SNMs have high levels of structural carbohydrates in their diet, they need to ferment these substances (such as cellulose) for nutrients. At the KO functional level, the alpha diversity results showed that the RGC had no significant difference with water deer (Figure 3c), and monogastric animals generally had higher functional diversity than ruminants and ruminant-like animals (SNMs) (Figure 3c). In conclusion, the gut microbes of SNMs and ruminants are more similar.

We also constructed phylogenetic and hierarchical trees of the 12 species of hosts and their gut microbes at the genus level to further characterize the relationship between SNMs and ruminant gut microbes. The SNMs gut microbes clustered with ruminant gut microbes (Figure 3d). PCoA showed a similar trend at both the functional and taxonomic levels, showing that SNMs gut microbes was more closely related to ruminant gut microbes than to monogastric animal gut microbes (Figure 3e and Figure 3f). SNMs and ruminants have similar dietary characteristics and GIT structures adapted to foregut fermentation (Liu et al., 2022). As the mammalian gut evolved morphologically toward herbivory adaptation, their microbiome reached a compositional configuration similar to that in unrelated hosts that have similar GIT structures and diets (R. E. Ley et al., 2008; Liu et al., 2022).

3.4 | Core genera in mammals

We selected the genera that were shared by all samples of each gene catalogue among the 12 species and termed these core genera. We identified 624 such core genera in the SNMs gut microbiome and 95 in the human gut microbiome (Table S10). Comparing the core genera from these species, we found 74 core genera shared among all 12 species, but we also noted that these genera had different relative abundance between each host (Figure S3). Among these 74 core genera, we further selected the top 30 most abundant genera in each species and displayed them in box plots (Figure S4). Then we found 9 genera shared in all 12 species, including *Clostridium* , *Ruminococcus* , *Prevotella* ,*Bacteroides* , *Eubacterium* , *Parabacteroides* ,*Roseburia* , *Alistipes* , and *Oscillibacter* , which may constitute a core mammalian gut microbiome (Figure S4). These genera belong to the Firmicutes (*Clostridium* , *Ruminococcus* ,*Eubacterium Roseburia* and *Oscillibacter*) and Bacteroides (*Prevotella* , *Bacteroides* ,*Parabacteroides* and *Alistipes*).

3.5 | Effects of physiological structure of the host GIT on the gut microbiome

To investigate the relationship between the physiological structure of the host GIT and their gut microbiome

in host dietary adaptation, we divided the above 12 species into ruminant animals, SNMs (ruminant-like animals) and monogastric animals according to their GIT physiological structures. First, LEfSe test was used to detect the taxonomic and functional (carbohydrate active enzyme families) differences among the three groups. At the genus level, *Ruminococcus* , *Treponema* and *Clostridium* were significantly enriched in SNMs, and *Fibrobacter* ,*Butyrivibrio* and *Prevotella* were significantly enriched in ruminant animals (LDA>4, p <0.05) (Figure 4a, Figure S5). The functions of these genera are all related to the fermentation of complex carbohydrates such as cellulose (Palevich et al., 2019; Ransom-Jones, Jones, McCarthy, & McDonald, 2012; Yang Gu & Jiang, 2010). However, in the monogastric animals, *Bacteroides* ,*Faecalibacterium* , *Roseburia* and *Phocaeicola* were significantly enriched, which were associated with the degradation of fat and protein (LDA>4, p <0.05) (Figure 4a; Figure S5). Carbohydrate active enzyme analysis showed that GHs, which significantly enriched in SNMs (GH78, GH13, GH109) and ruminant animals (GH25, GH5), were related to the degradation of structural polysaccharides. However, GHs that were significantly enriched in monogastric animals (GH43, GH2, GH92, GH97, GH105, GH29, GH28, GH32, GH20) were related to the degradation of oligosaccharides (LDA>3, p <0.05) (Figure 4b; Table S13). These results suggested that the composition and function of the gut microbiome are related to the host's dietary adaptation, and the structure of gut microbes is more similar between SNMs and ruminants.

In addition, we found that the relative abundance of more than 50% (1859) of genera in the SNMs gut microbiome were higher than that in monogastric animals, but lower than that in ruminant animals (Table S14). *Bacillus* (Firmicutes, Bacilli), *Butyrivibrio*(Firmicutes, Clostridia) and *Fibrobacter* (Fibrobacteres, Fibrobacterales) had the highest relative abundance among these genera in the SNMs gut microbiome. Most of these 1859 genera belonged to Firmicutes (129 genera, average relative abundance in SNMs gut microbes was 1.44%), Bacteroidetes (245 genera, average relative abundance in SNMs gut microbes was 0.62%), Proteobacteria (866 genera, average relative abundance in SNMs gut microbes was 0.41%%), Fibrobacteres (3 genera, average relative abundance in SNMs gut microbes was 0.24%), Actinobacteria (264 genera, average relative abundance in SNMs gut microbes was 0.26%), and Tenericutes (4 genera, average relative abundance in SNMs gut microbes was 0.10%). Bacteria in these phyla have the ability to degrade complex carbohydrates, especially in Firmicutes (Yang Gu & Jiang, 2010) and Fibrobacteres (Ransom-Jones et al., 2012). We further found that the relative abundance of 37 GHs in SNMs was higher than that in monogastric animals but lower than that in ruminants. Most of these GHs are associated with the degradation of structural carbohydrates such as cellulose and starch (Figure S6; Table S13). Analysis of the correlations between these genera and these GHs showed that most bacteria correlated positively with these GHs (Table S15). We also found that with the adaptive evolution of the host GIT structure to herbivory, the diversity and abundance of GHs related to the fermentation of structural carbohydrates also increased, and the relationship between these genera and GHs are more complex. (Table S15), because the host needed a stronger fermentation capacity to obtain nutrients.

In summary, the structure of the SNMs gut microbiome was more similar to that of ruminants, both in taxonomy and function. Moreover, the relative abundance of some bacteria and carbohydrate enzymes in SNMs was intermediate to that those of ruminants and monogastric animals, suggesting that the physiological structure of the host GIT plays a regulatory role in the gut microbiome during the adaptation evolution of diet.

4 | DISCUSSION

In this study, we constructed a gene catalogue of the gut microbiome of wild SNMs for the first time by processing 143 fecal individual samples, which contained 18,169,322 non-redundant genes. The RGC also represents the first gene-set of the gut microbiome from wild NHP populations and provides a comprehensive resource for further investigations of the NHP gut microbiome. Compared with previous studies of the SNMs gut microbiome (V. L. Hale et al., 2018; Trevelline & Moeller, 2022; Xu et al., 2015), the RGC comprehensively characterizes the gut microbiome of SNMs and probably contains most of the gut microbial genes prevalent in wild SNM populations. Metagenomic data from 10 wild Sichuan SNMs researched in a previous study showed a mapping rate of more than 75% in the RGC, but there was relatively poor

representation in captive SNMs, with an average mapping rate was 49.83% (Table S8). Previous studies have suggested that the captivity environment has a significant effect on the gut microbiome of SNMs (Hui Zhu, 2018; Mingpu Qi, 2017). Similar results have also been reported in other NHPs (Clayton et al., 2016; Emmanouil Angelakis, 2016; V. L. Hale et al., 2018; Tayte P Campbell, 2020). This proves that the RGC is reliable in describing the gut microbiome of SNMs. Therefore, a comprehensive analysis of the gut microbiome based on the RGC is of practical significance for studying their dietary adaptation, which could further guide the development of better conservation strategies for these endangered species.

We used this catalogue to study the characteristics of the SNMs gut microbiome. In our study, both taxonomic and functional results suggested that the gut microbiome of SNMs was related to structural carbohydrate degradation. Notably, our results were identical to those of previous studies at the phylum level, while the differences were large at the genus level (Wang et al., 2021; Yao et al., 2021). At the phylum level, Firmicutes and Bacteroides were the main groups in the RGC (Figure 2b). This is consistent with the results of previous studies on Sichuan SNMs (Wang et al., 2021; Yao et al., 2021), Yunnan SNMs (Xia et al., 2022) and Guizhou SNMs (V. L. Hale, Tan, C.L., Niu, K., Yang, Y., Zhang, Q., Knight, R., 2019). At the genus level, *Clostridium* ,*Prevotella* , *Ruminococcus* and *Bacteroides* were the groups with the highest relative abundance in the RGC (Figure 2c), which was different from the results of previous single-species studies (V. L. Hale, Tan, C.L., Niu, K., Yang, Y., Zhang, Q., Knight, R., 2019; Wang et al., 2021). This was likely because previous studies on the gut microbiome of SNMs were mainly based on 16S rRNA analysis and small sample sizes (Xia et al., 2022; Yao et al., 2021), preventing the results from describing the general characteristics of the SNMs gut microbiome. The consistency of the main phyla reflected that the gut microbiome of SNMs was related to their dietary adaptation. Many Firmicutes bacteria, including *Clostridium* , are able to utilize xylose, xylan and xyloglucan, which are the major hemicellulose components of plant cell walls (Canfora, Meex, Venema, & Blaak, 2019). These results once again verified the reliability and comprehensiveness of the RGC and preliminarily demonstrated that the gut microbiome composition of SNMs plays an important role in their dietary adaptation.

Alpha and beta diversity analyses also revealed the important role of the gut microbiome in the dietary adaptation of SNMs (Figure 3b, 3c and 3d). The comparative analysis of 12 mammals including SNMs, ruminants and monogastric animals, indicated that the gut microbiome of SNMs was clustered more closely to that of ruminants (Figure 3d), which was similar to previous findings (Zhou et al., 2014). As the morphology of mammalian GIT underwent convergent evolution to adapt to herbivory, their microbiome might have developed similar compositional configurations in unrelated hosts with similar gut structures (R. E. Ley et al., 2008). We further found evidence of coevolution between SNMs and ruminants during dietary adaptation. In SNMs, *Ruminococcus* ,*Treponema* and *Clostridium* were significantly enriched at the genus level, and GH78, GH13 and GH109 were significantly enriched in carbohydrate enzymes (GHs). The genera that were significantly enriched in ruminants were *Fibrobacter* , *Butyrivibrio* and*Prevotella* , and the GHs that were significantly enriched were GH25 and GH5 (Figure 4a and 4b; Figure S5). Although the genera and GHs that were significantly enriched in SNMs and ruminants were different, these genera and GHs were mainly associated with the digestion of structural carbohydrates. For example, *Ruminococcus* ,*Clostridium* and *Butyrivibrio* belong to Firmicutes and are related to the degradation of cellulose (Canfora et al., 2019), and GH5 and GH78 both have cellulase activity (Christiane Liers, 2021). However, the genera and GHs that were significantly enriched in monogastric animals were related to the degradation of fat and protein and oligosaccharides, such as *Bacteroides* (Naofumi Yoshida, 2021) (Figure 4a and 4b). This indicated that the gut microbes of SNMs and ruminants evolved similar digestive strategies in the process of adapting to the plant diet and further suggested that the gut microbiome is an important part of host dietary adaptation.

SNMs and ruminants also display similar physiological adaptation strategies to plant-based diets, and they are both foregut fermentative animals (Liu et al., 2022). The rumen of ruminants and the enlarged saccular stomach of SNMs provide a large space for the bacteria to ferment cellulose and other substances (D., 1998; Karasov & Douglas, 2013; P., 1988), but the fermentation efficiency of SNMs may be lower than that of ruminants, because the sacculated stomach of SNMs does not show as strong functional division as ruminants, and the differentiation is intermediate between those of monogastric animals and ruminants

(D. J. Chivers, Hladik, C.M, 1980). Previous studies have reported that the morphology of the GIT has an important impact on the composition and function of bacterial communities (R. E. Ley et al., 2008). In our study, we found that the relative abundance of 1859 genera (over 50%) and 37 GHs in SNMs was higher than that in monogastric animals but lower than that in ruminants (Table S14). Most of these GHs were associated with degradation of structural polysaccharides, such as GH10, GH12, GH9, GH14 and so on (Table S15). We also found that as the host GIT structure adapted to herbivory, the diversity and abundance of GHs related to the fermentation of structural carbohydrates also increased (Table S14), suggesting that the dual effects of GIT morphology and the gut microbiome promoted the dietary adaptive evolution in foregut fermentative animals, which provides a new perspective for exploring the evolutionary path of the gut microbiome in these animals.

In addition, we defined a set of core gut bacterial genera, including*Clostridium* , *Ruminococcus* , *Prevotella* ,*Bacteroides* , *Eubacterium* , *Parabacteroides* ,*Roseburia* , *Alistipes* and *Oscillibacter* , based on the gut microbiome generated by shotgun sequencing of 12 mammalian species (Figure S4). However, the relative abundance of these genera varied profoundly between each species (Figure S3), which might result from the influence of genetic, dietary and physiological characteristics of the host (R. E. Ley et al., 2008). Our core genera reflected, to some extent, the basic composition of the mammalian gut microbiome. Previous studies reported that most of these genera were important keystone bacteria of the gut microbiome, which are closely related to host food degradation, nutrient absorption, health, and intestinal homeostasis (Ruth E Ley, 2016; Petia Kovatcheva-Datchary, 2015). Therefore, it is highly necessary to focus on these core genera in future analyses of the mammalian gut microbiome.

5 | CONCLUSION

One of the most important features of this study was the large-scale metagenomic analysis of 143 fecal individual samples from three species of SNMs, which generated the first reference gene catalogue covering most of the genes in the gut microbiome of SNMs, enabling us to fully understand the detailed genetic diversity of the gut microbiome of SNMs. The identification of bacteria and carbohydrate enzymes in the gut microbiome of SNMs suggested their adaptation to a plant-based diet. We compared metagenomic diversity and functional potential in SNMs with those in ruminants and monogastric animals, and revealed that the interaction between GIT morphology and the gut microbiome jointly promotes dietary adaptation in foregut fermenting animals. We also identified 9 highly abundant bacterial genera as core genera in these mammals. In conclusion, this study provides a reference and data framework to further reveal the evolutionary adaptation of SNMs and to analyze the evolutionary route of the gut microbiome in animals with foregut fermentation.

Supplementary Material

Supplementary information: figures S1-S6, tables S1–S15 and description.

Author Contributions

M.L. and X.W. conceived this study and designed this project. X.W., J.Z. and H.P. managed this project. Y.C., D.L., Z.X., S.M., M.Z. and Y.S. collected samples. X.W. and J.Z. performed the experiments. X.W., J.Z. and H.P. analyzed the data. J.Z., X.W., H.P. and M.L. wrote the manuscript. D.L., Z.X., S.M., Y.S. and M.Z. provided help with the analysis. All the authors critically reviewed and approved the manuscript. M.L. supervised this study.

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Competing Interests

The authors declare no competing interests.

Ethics Approval

This study was approved by the Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences (CAS).

Availability of data and materials

Raw sequence reads generated from this study have been submitted to the NCBI under BioProject XXXXX. All the gene catalogues, annotation information and relative abundance profiles from this study are available at XXXXXX. All data, code, and materials used in the analyses are available in the main text or the supplementary materials.

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Figure legends

Figure 1. Pipeline for the construction of the gene catalogue of *Rhinopithecus* gut microbiome (RGC).Shotgun sequencing reads for fecal samples from *R. roxellana* ,*R. bieti* and *R. strykeri* individuals were independently processed. We generated three non-redundant gene-sets and merged them into an integrated reference gene catalogue of the SNMs gut microbiome (RGC).

Figure 2. Annotation of the RGC . **A** . Breakdown of taxonomic annotations for the RGC. **B** . The top 11 phyla in the RGC. Firmicutes and Bacteroidetes are the main two phyla in the RGC.**C** . The top 20 genera in the RGC. *Clostridium* is the main genus in the RGC. **D.** Number of shared microbiome features among SNMs at different frequency thresholds for the number of genes (black), COGs (pink), genera (orange), phyla (blue), KOs (purple) and CAZymes (yellow). The percentages of shared items and animals are represented on the y and x axes, respectively. The absolute numbers for each item are indicated at the intercept between the percentages of items and animals at the thresholds of 60% and 100%. Fewer than 2% of genes (207,635 genes) were shared by 60% of individuals, while approximately 36.5% of the KO functions (2,943) were shared by 60% of individuals, suggesting redundancy of genes for similar functions.

Figure 3. Comparisons of the RGC with the catalogues of ruminants and monogastric animal. A. The number of non-redundant genes in 12 mammalian species. **B and C** . Alpha diversity (Shannon index) at the genus, and KO function levels. Data are shown as box plots. The horizontal lines indicate the medians, and the whiskers indicate the lowest and highest points within 1.5× the interquartile ranges into the lower and upper quartiles, respectively. Coloured circles at the bottom indicate significance based on the relative index of each cohort according to the Wilcoxon rank-sum test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. D. Comparison of the host phylogeny tree (right panel; assembled using http://timetree.org/) and their gut microbiome hierarchical tree (upper left panel). **E.** PCoA plot of the gut microbiome composition in the 12 species at the genus level. **F.**PCoA plot of the gut microbiome composition in the 12 species at the KO function level.

Figure 4. Genera and CAZy terms differentially represented among ruminant animals, SNMs

(ruminant-like animals) and monogastric animals (LEfSe). A. LEfSe test at genus level (LDA>0.4, P<0.05). Red circle: enriched in SNMs, green circle: enriched in ruminant animals, yellow circle: enriched in monogastric animals. B. LEfSe test at the CAZy level. Red box: enriched in SNMs, green box: enriched in ruminant animals, yellow box: enriched in monogastric animals. Text in bold: enriched in GH families.

Fig. 1

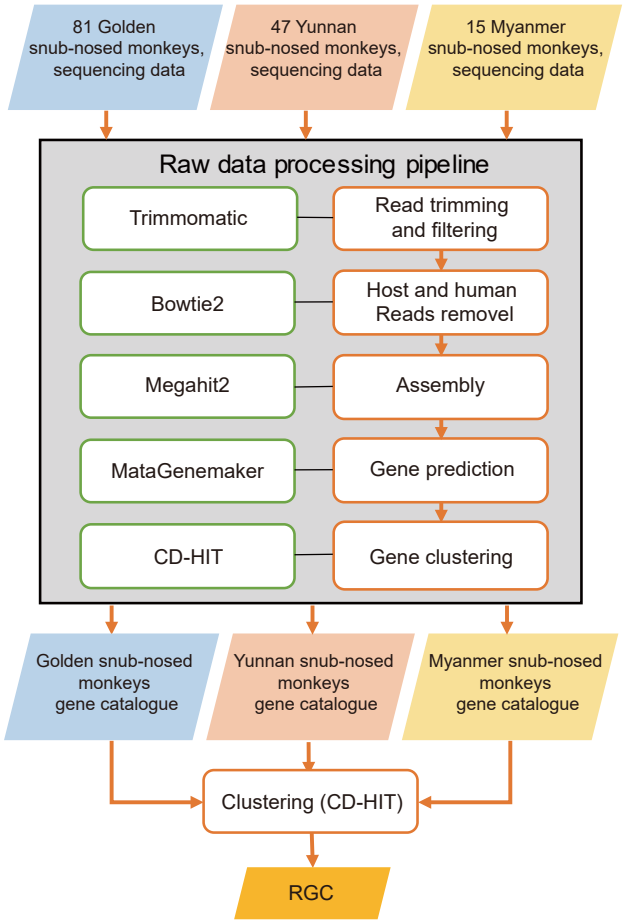

Fig. 2

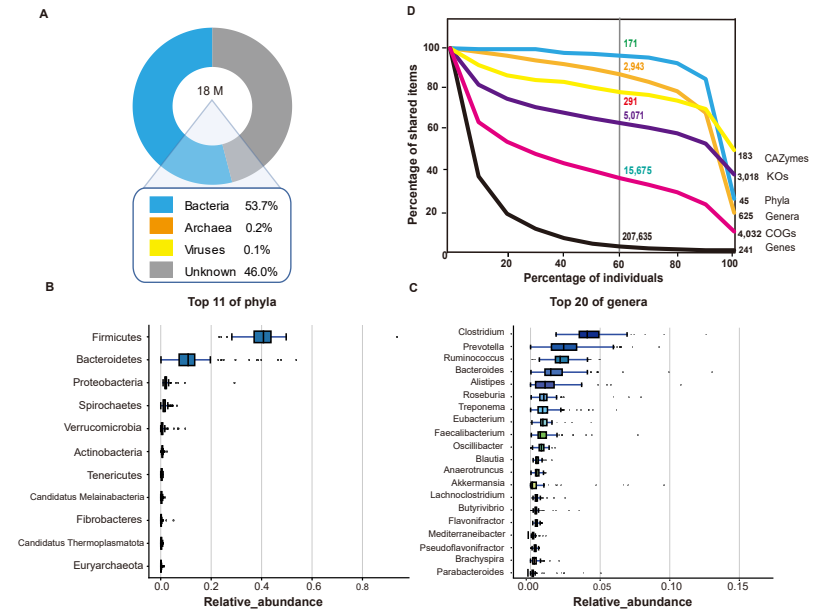

Fig. 3

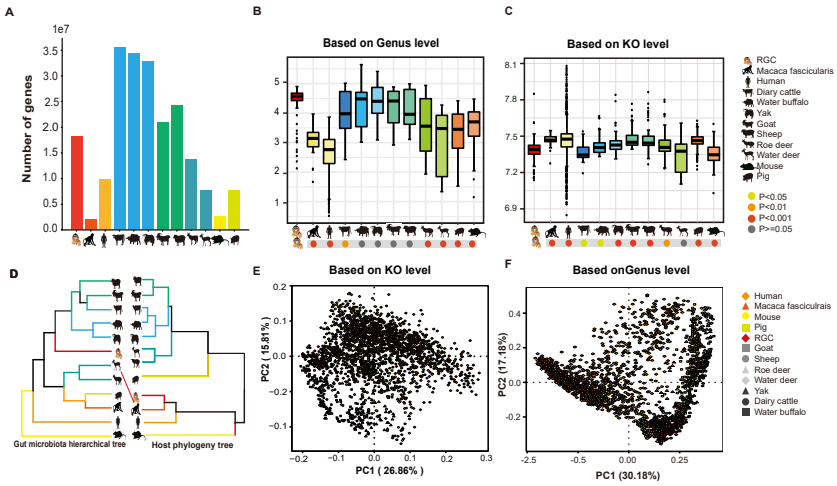

Fig. 4