Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis Analysis of Gastrointestinal Microbiota Diversity in a Deceased Wild Sichuan Golden Snub-Nosed Monkey Postprint

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Abstract

This study aimed to analyze the gastrointestinal microbiota diversity and construct a phylogenetic tree based on clone sequencing bands from a deceased wild golden snub-nosed monkey. Gastrointestinal contents were collected from the deceased wild golden snub-nosed monkey and subjected to polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) analysis. Combined with clone sequencing of bands, cluster analysis, and principal component analysis (PCA), microbiota diversity was examined and a phylogenetic tree was constructed. The results showed: 1) The entire gastrointestinal tract of the wild golden snub-nosed monkey harbored a large number of bacteria, and samples from the stomach and small intestine clustered into one major group, samples from the large intestine clustered into another group, while fecal samples formed a separate cluster. 2) A total of 18 bands were recovered from the DGGE profile. Bacterial identification revealed five major phyla—Proteobacteria (38.89%), Firmicutes (22.22%), Bacteroidetes (5.56%), Actinobacteria (5.56%), and Verrucomicrobia (5.56%)—as well as uncultured bacterium (22.22%). Among these, Proteobacteria and Firmicutes were distributed throughout the entire gastrointestinal tract. 3) Phylogenetic tree analysis of the microbiota indicated that only one type of uncultured bacterium showed evolutionary classification similarity to the identified Enterococcus faecalis, while other uncultured bacteria differed significantly from known bacterial species in their evolutionary branches, suggesting that a substantial amount of microbiota information in the gastrointestinal tract of wild golden snub-nosed monkeys remains unidentified. The results suggest that the dominant microbiota in the gastrointestinal tract of the deceased wild golden snub-nosed monkey examined in this study was Proteobacteria, and that microbiota diversity exhibited a high-low-high trend along the

anterior-to-posterior axis of the gastrointestinal tract.

Full Text

Analysis of Gastrointestinal Microbiota Diversity in a Dead Wild Golden Snub-Nosed Monkey Using PCR-DGGE Technology

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Abstract: This study aimed to analyze the gastrointestinal microbiota diversity of a dead wild golden snub-nosed monkey (*Rhinopithecus roxellana*) and construct a phylogenetic tree based on cloned sequencing bands. Gastrointestinal contents were collected from the deceased wild individual and analyzed using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), combined with band cloning, sequencing, cluster analysis, and principal component analysis (PCA) to assess microbiota diversity and construct a phylogenetic tree. The results showed that: 1) The entire gastrointestinal tract harbored abundant bacteria, with samples from the stomach and small intestine clustering into one group, large intestine samples forming another cluster, and fecal samples separating independently. 2) A total of 18 bands were recovered from the DGGE profiles, with identified bacterial taxa belonging primarily to five phyla: *Proteobacteria* (38.89%), *Firmicutes* (22.22%), *Bacteroidetes* (5.56%), *Actinobacteria* (5.56%), *Verrucomicrobia* (5.56%), and uncultured bacteria (22.22%). *Proteobacteria* and *Firmicutes* were distributed throughout the entire gastrointestinal tract. 3) Phylogenetic tree analysis revealed that only one uncultured bacterium showed evolutionary similarity to the identified *Enterococcus faecalis*, while other uncultured bacteria differed substantially from known taxonomic branches, indicating that numerous microbial species in the wild golden snub-nosed monkey gastrointestinal tract remain unidentified. These findings suggest that *Proteobacteria* represents the dominant microbiota in the gastrointestinal tract of this deceased wild golden snub-nosed monkey, with microbiota diversity exhibiting a high-low-high trend along the anterior-posterior axis of the gastrointestinal tract.

Keywords: *Rhinopithecus roxellana*; gastrointestinal microbiota; PCR-DGGE; cloning sequencing; phylogenetic tree

Introduction

The golden snub-nosed monkey (*Rhinopithecus roxellana*), also known as the Sichuan snub-nosed monkey, belongs to Mammalia, Primates, and the genus *Rhinopithecus* [1]. As a nationally protected first-class species endemic to China, it is primarily distributed across Sichuan, Gansu, Shaanxi, and Hubei provinces [2]. With elegant appearance and small population size, this species is a typical forest arboreal animal that lives in groups in alpine dense forests and maintains a complex herbivorous diet consisting mainly of plant materials [3]. However, increasing human activities have severely degraded its habitat, and combined with environmental factors and limited natural predators, its population continues to decline. The establishment of three national nature reserves—Sichuan Nanping Baihe, Shaanxi Zhouzhi, and Hubei Shennongjia [4]—along with artificial breeding programs have gradually restored its reproduction and population numbers. Research on gastrointestinal microorganisms has become an important measure for wildlife reproduction and population conservation [5], as gut microbial composition and function are closely associated with host digestion, immune response, and physiological functions [6-7].

Recent studies have preliminarily reported the structure and composition of microbiota in certain intestinal segments and feces of golden snub-nosed monkeys [3,8-9], but information on the entire gastrointestinal tract microbiome remains unknown. Therefore, investigating the structure and composition of the complete gastrointestinal microbiota is crucial for the breeding and conservation of this species. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) is a widely used molecular biological technique for studying microbial diversity, providing powerful support for investigating microbial community structure and composition [10]. This study employed PCR-DGGE technology to analyze the microbiota diversity throughout the gastrointestinal tract (stomach, duodenum, jejunum, ileum, cecum, colon, rectum, and feces) of a deceased wild golden snub-nosed monkey, with subsequent cloning and sequencing of dominant bands to construct a phylogenetic tree, aiming to enrich the knowledge base of golden snub-nosed monkey gastrointestinal microbiota and provide scientific guidance for its husbandry, health, and population conservation.

Materials and Methods

1.1 Sample Collection

In March 2016, an emaciated, moribund wild aged female golden snub-nosed monkey (18 years old) was discovered in Pingwu County, Mianyang City, Sichuan Province, and transported to Chengdu Zoo for emergency treatment. The animal received nutritional supplementation and fluid therapy (5% glucose, saline, and sodium bicarbonate) but consumed no food during this period. Fecal samples were collected during treatment. The monkey died despite intervention, and necropsy revealed only chronic myocardial infarction with fibrosis and calcification, suggesting death from heart disease. Gastrointestinal

content samples (stomach, duodenum, jejunum, ileum, cecum, colon, and rectum) were collected into sterile 2 mL centrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

1.2 Total DNA Extraction

Bacterial total DNA was extracted from samples using the QIAamp® DNA Stool Mini Kit and dissolved in 200 L Buffer AE solution, then stored at -20°C until use.

1.3 16S rDNA V3 Region PCR Amplification

Total bacterial DNA was used as template for PCR amplification of the 16S rDNA V3 region using universal bacterial primers [10]. The forward primer was 5 -CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3 (with the underlined portion representing the GC-clamp), and the reverse primer was 5 -ATTACCGCGGCTGCTGG-3 .

The PCR reaction mixture (50 L) contained: 2 L template DNA, 2 L each of forward and reverse primers (10 mol/L), 25 L 2×Taq Master Mix, and double-distilled water (ddH_2O) to a final volume of 50 L. PCR conditions were: initial denaturation at 94°C for 4 min; 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min; final extension at 72°C for 10 min. PCR products were verified by 1.0% agarose gel electrophoresis.

1.4 PCR-DGGE Fingerprint Analysis and Band Cloning/Sequencing

PCR products (10 L) were analyzed using the Bio-Rad Dcode system with a 35%-65% gradient gel, electrophoresed at 100 V and 60°C for 16 h in 1×TAE buffer. Gels were stained with silver nitrate and scanned using a Bio-Rad® GS800 Calibrated Densitometer. Dominant and specific bands were excised from DGGE profiles, purified using a Gel Extraction Kit (Omega, USA), cloned into pMD19-T vector, and transformed into *E. coli* DH5α competent cells. Positive clones were sequenced by Shanghai Bioengineering Co., Ltd. Sequences were compared against the GenBank database, and phylogenetic analysis was performed using MEGA software.

1.5 Data Analysis

DGGE fingerprint diversity was statistically analyzed using Excel 2013. PCA was performed using SPSS 19.0, cluster analysis using NTSYS 2.1, and phylogenetic tree construction using MEGA software. Diversity indices were calculated as follows:

- Shannon-Wiener index (diversity index): $H = -\sum(p_i)(\ln p_i)$
- Evenness: $E = H/H_{max}$
- Richness: R (number of bands in DGGE profile)

where p_i represents the relative abundance proportion of species i.

Results

2.1 Cluster Analysis of Gastrointestinal Microbiota PCR-DGGE Fingerprints

DGGE analysis of 16S rDNA PCR products from gastrointestinal samples is shown in [Figure 1: see original paper]. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis revealed that stomach, duodenum, and jejunum samples clustered together with a similarity coefficient of 0.97; ileum, cecum, colon, and rectum samples formed another cluster with a similarity coefficient of 0.60; while fecal samples separated independently with a similarity coefficient of only 0.45 compared to other gastrointestinal samples. DGGE band patterns demonstrated that the wild golden snub-nosed monkey gastrointestinal tract harbored abundant bacteria, with variations in species composition and abundance across different segments. A total of 52 bands were identified across all samples. Fecal samples exhibited the richest bacterial diversity with 32 bands, followed by stomach and small intestine samples (duodenum and jejunum) with 27, 27, and 28 bands respectively. Large intestine samples (ileum, cecum, colon, and rectum) showed relatively lower bacterial richness with 13, 13, 16, and 15 bands respectively. These differences in microbiota composition may be associated with host health status or variations in digestive function and bacterial colonization across gastrointestinal segments.

Figure 1. PCR-DGGE profiles and cluster analysis of bacteria in gastrointestinal tract. Sto, Duo, Jej, Ile, Cec, Col, Rec and Fae represent bacteria from stomach, duodenum, jejunum, ileum, cecum, colon, rectum, and feces of wild *Rhinopithecus roxellana*, respectively. Numbers (1 to 21) with arrows indicate band numbers. The same abbreviations apply below.

2.2 Diversity Analysis of Gastrointestinal Microbiota PCR-DGGE Fingerprints

Microbiota diversity analysis revealed significant variations across gastrointestinal segments ([Figure 2: see original paper]). Fecal microbiota exhibited the highest diversity index (3.47), evenness (0.88), and richness (32.00). Stomach, duodenum, and jejunum showed relatively high diversity indices (3.30, 3.30, and 3.33), evenness values (0.83, 0.83, and 0.84), and richness values (27.00, 27.00, and 28.00). Large intestine microbiota showed lower diversity, with ileum displaying the lowest values (diversity index: 2.56; evenness: 0.65; richness: 13.00). Microbiota diversity demonstrated a high-low-high trend along the anterior-posterior axis of the gastrointestinal tract, consistent with the cluster analysis results.

Figure 2. Diversity index, evenness, and richness of bacteria in gastrointestinal tract.

2.3 Principal Component Analysis of Gastrointestinal Microbiota PCR-DGGE Fingerprints

Principal component analysis of DGGE profiles showed that samples with different microbiota structures and compositions were distributed consistently with cluster analysis results ([Figure 3: see original paper]). Principal components 1 and 2 explained 42.90% and 29.79% of the variance, respectively, clearly separating gastrointestinal microbiota into three clusters: stomach/duodenum/jejunum, ileum/cecum/colon/rectum, and feces. This indicates high similarity in microbiota structure and composition among adjacent intestinal segments, while fecal samples from the terminal gut region differed substantially from other samples.

Figure 3. PCA of PCR-DGGE profiles of bacteria in gastrointestinal tract.

2.4 Sequence Detection of Dominant DGGE Bands

Eighteen bands were excised from the PCR-DGGE profiles (bands 1-18 indicated by arrows in [Figure 1: see original paper]) and sequenced. BLAST analysis against NCBI revealed that wild golden snub-nosed monkey gastrointestinal bacteria primarily belonged to five phyla: *Proteobacteria* (38.89%), *Firmicutes* (22.22%), *Bacteroidetes* (5.56%), *Actinobacteria* (5.56%), *Verrucomicrobia* (5.56%), and uncultured bacteria (22.22%). *Proteobacteria* and *Firmicutes* were distributed throughout the entire gastrointestinal tract, while *Bacteroidetes* and *Verrucomicrobia* were primarily found in the stomach, small intestine, and feces, and *Actinobacteria* mainly in the stomach and small intestine.

Proteobacteria were dominated by four *Pseudomonas* species (*Pseudomonas proteolytica*, *Pseudomonas cerasi*, *Pseudomonas ficuserectae*, and *Pseudomonas veronii*), two *Escherichia* species (*Escherichia fergusonii* and *Escherichia coli*), and *Shigella sonnei*. *Firmicutes* were represented by *Clostridium sartagoforme*, *Ruminococcus torques*, *Flavonifractor plautii*, and *Enterococcus faecalis*. Notably, *Akkermansia muciniphila*, associated with intestinal barrier function and obesity prevention, was detected in the stomach, small intestine, ileum, and feces. Importantly, unculturable bacteria were identified throughout the gastrointestinal tract, indicating numerous undetected and unidentified bacterial species.

Phylogenetic tree analysis ([Figure 4: see original paper]) revealed that among four uncultured bacteria, only band 4 showed evolutionary similarity to identified *Enterococcus faecalis*, suggesting it may represent an *Enterococcus* strain from *Firmicutes*. Other uncultured bacteria showed substantial divergence from known taxa, requiring further identification and confirming that extensive microbial information remains uncharacterized in wild golden snub-nosed monkeys.

Table 1. Sequencing and BLAST analysis results of DGGE profile bands.

Band No.	Closest Relatives in GenBank Database	Accession No.	Similarity/%	Location	Phylum
1	Uncultured *Bacteroidetes* bacterium	GU958406.1	-	Stomach, duodenum, jejunum, feces	*Bacteroidetes*
2	Uncultured bacterium	GU619482.1	-	Stomach, duodenum, jejunum	Uncultured
3	Uncultured bacterium	GU198356.1	-	Stomach, duodenum, jejunum	Uncultured
4	Uncultured bacterium	KC338352.1	-	Stomach, small intestine	Uncultured
5	*Pseudomonas proteolytica*	NR_{025588}.1		Stomach, duodenum, jejunum, feces	*Proteobacteria*
6	*Olsenella scatoligenes*	NR_{134781}.1		Stomach, duodenum, jejunum, feces	*Actinobacteria*
7	*Pseudomonas cerasi*	NR_{146827}.1		Stomach, duodenum, jejunum	*Proteobacteria*
8	*Clostridium sartagoforme*	NR_{026490}.1		Large intestine	*Firmicutes*
9	*Ruminococcus torques*	NR_{036777}.1		Feces	*Firmicutes*

Band No.	Closest Relatives in GenBank Database	Accession No.	Similarity/%	Location	Phylum
10	*Pseudomonas ficuserectae*	NR_{040798}.1		Stomach, duodenum, jejunum, feces	*Proteobacteria*
11	*Akkermansia muciniphila*	NR_{074436}.1		Stomach, small intestine, feces	*Verrucomicrobia*
12	*Flavonifractor plautii*	NR_{043142}.1		Stomach, small intestine, feces	*Firmicutes*
13	*Pseudomonas veronii*	NR_{028706}.1		Feces	*Proteobacteria*
14	Uncultured bacterium	FJ036720.1	-	Stomach and small intestine	Uncultured
15	*Escherichia fergusonii*	NR_{104826}.1		Gastrointestinal tract	*Proteobacteria*
16	*Shigella sonnei*	NR_{112558}.1		Stomach and small intestine	*Proteobacteria*
17	*Escherichia coli*	NR_{040789}.1		Stomach, duodenum, jejunum, feces	*Proteobacteria*

Band No.	Closest Relatives in GenBank Database	Accession No.	Similarity/%	Location	Phylum
18	*Enterococcus faecalis*	-	-	Stomach, duodenum, jejunum, large intestine, feces	*Firmicutes*

Figure 4. Phylogenetic tree of bacteria in gastrointestinal tract of wild *Rhinopithecus roxellana*.

Discussion

Wildlife represents invaluable biological resources within Earth' s biosphere. Over the past 40 years, global wildlife populations have declined by half [11], accelerating species extinction and increasing zoonotic disease emergence [12]. Microbial homeostasis and diversity promote host health [13], while health status, environment, host genotype, diet, and gender influence both physiological functions and gastrointestinal microbiota structure [6-7]. Although wildlife protection makes gastrointestinal samples difficult to obtain, increasing conservation awareness has enabled characterization of microbiota from deceased wild animals [3,8], providing essential baseline data for survival and conservation. This study represents the first application of PCR-DGGE fingerprinting to analyze microbiota diversity throughout the stomach, small intestine, large intestine, and feces of a deceased wild golden snub-nosed monkey, with cloning, sequencing, and phylogenetic analysis revealing abundant microbiota with segment-specific diversity patterns and *Proteobacteria* dominance, thereby enriching our understanding of wild golden snub-nosed monkey gastrointestinal microbiota.

PCR-DGGE fingerprinting provided rapid, intuitive visualization of microbiota structure and diversity at the molecular level. The identified microbiota primarily comprised *Proteobacteria* (38.89%), *Firmicutes* (22.22%), *Bacteroidetes* (5.56%), *Actinobacteria* (5.56%), and *Verrucomicrobia* (5.56%), with *Proteobacteria* and *Firmicutes* distributed throughout the gastrointestinal tract and *Proteobacteria* as the dominant phylum. These findings differ from Liu et al. [8], who used Illumina MiSeq sequencing of 16S rRNA to analyze partial intestinal segments from a semi-wild male golden snub-nosed monkey that died from pneumonia in Hubei Shennongjia, reporting similar taxa but with *Firmicutes* as the dominant phylum. Similarly, metagenomic sequencing of gastric contents from a healthy captive female golden snub-nosed monkey at Beijing Zoo identi-

fied *Firmicutes* as dominant [3]. These discrepancies likely reflect differences in health status, environment, individual characteristics, gender, diet, and methodology. Our PCR-DGGE analysis of a female wild golden snub-nosed monkey from Sichuan Pingwu that died from heart disease revealed *Proteobacteria* dominance. Notably, *Proteobacteria* abundance correlates with health status and represents a major source of inter-individual microbiota variation [14]. Heart failure in humans is associated with increased *Escherichia* from *Proteobacteria* [15], and McKnney et al. [16] reported that healthy rhesus macaques harbor *Firmicutes*-dominated fecal microbiota, while colitis-afflicted individuals show *Campylobacter* (*Proteobacteria*) dominance. Wang et al. [17] similarly found that healthy golden snub-nosed monkeys harbor more beneficial *Bifidobacterium* and *Lactobacillus*, while diarrheic individuals show increased pathogenic Enterobacteriaceae and *Clostridium* cluster I. Aivelo et al. [18] demonstrated gender effects on primate gut microbiota structure. Despite both our study and Zhou et al. [3] examining females, microbiota differences persisted, likely due to individual variation, intestinal segment differences, and methodological approaches. Compared to high-throughput sequencing capable of parallel analysis of thousands of DNA molecules [19], PCR-DGGE provides simple, rapid, intuitive visualization of microbiota abundance and diversity [10]. Zhou et al. [3] studied a captive monkey, while ours was wild, and captivity is known to cause significant gut microbiota loss [20]. Nevertheless, our study, together with Liu et al. [8] and Zhou et al. [3], collectively enriches golden snub-nosed monkey gastrointestinal microbiota knowledge using different methodologies. In contrast, Yildirim et al. [21] identified *Firmicutes* as the dominant fecal microbiota in colobus monkeys, while *Firmicutes* also dominates in macaques [22] and langurs [23], likely reflecting species-specific and health status differences.

Cluster and PCA analyses ([Figure 1: see original paper] and [Figure 3: see original paper]) revealed distinct microbiota clustering: stomach/small intestine, large intestine, and feces. Each gastrointestinal segment harbors both characteristic shared microbiota and segment-specific communities adapted to distinct microenvironments and physiological functions [24], indicating that fecal microbiota alone cannot fully represent entire gastrointestinal microbiota structure. The stomach initially processes and digests food, serving as an important organ for nutrient absorption. Although the golden snub-nosed monkey' s stomach has a dilated cavity and thin wall that limits mechanical grinding, its low-positioned acid-secreting pyloric region prevents acid from affecting commensal microbes in the higher-positioned body and fundus, ultimately facilitating cellulose decomposition [25]. As herbivorous primates, golden snub-nosed monkeys have evolved 69.7% of olfactory genes rapidly, potentially associated with fruit and plant odors, and gastric *Firmicutes* correlate with carbohydrate metabolism [3]. Our identification of *Firmicutes* as a major gastric microbiota suggests carbohydrate metabolism involvement. The small intestine primarily metabolizes monosaccharides and amino acids, favoring *Proteobacteria* and *Lactobacillales* [26], while the large intestine metabolizes complex polysaccharides with dominant *Bacteroidetes* and *Clostridiales* [27]. The golden snub-nosed

monkey' s small intestine measures 255 cm and large intestine approximately 182 cm. Our DGGE band sequencing revealed that small intestine microbiota were dominated by *Pseudomonas proteolytica*, *Pseudomonas cerasi*, and *Pseudomonas ficuserectae*, along with *Escherichia fergusonii*, *Escherichia coli*, and *Shigella sonnei*—all reported as common enteric pathogens [28-29]. Large intestine microbiota were dominated by *Clostridium sartagoforme* and *Enterococcus faecalis*, common digestive tract bacteria. While most *E. faecalis* are beneficial, strains harboring gene cassettes (part of pathogenicity islands) are pathogenic and detrimental to animal health [30]. Detection of pathogenic bacteria may relate to host health status; our samples came from an aged wild monkey that died from heart disease, and both Wang et al. [17] and Jian et al. [31] demonstrated age and health effects on gut microbiota structure.

Cloning and sequencing of PCR-DGGE bands revealed numerous unculturable bacteria, primarily from the stomach and small intestine, indicating substantial uncharacterized microbial diversity, likely because most studies have focused on easily collected fecal samples due to the species' endangered status. Updated phylogenetic trees show many uncultured bacteria cluster with *Firmicutes* [32], and our phylogenetic analysis ([Figure 4: see original paper]) similarly suggests uncultured bacteria primarily belong to *Firmicutes*, including *Ruminococcus torques*, *Clostridium sartagoforme*, and *Enterococcus faecalis*. Zhao et al. [33] identified *Firmicutes* as the dominant gut microbiota across six wildlife species, with *Ruminococcus* being differentially abundant between monogastric herbivores (kangaroos and elephants) and ruminants (giraffes and alpacas), showing higher abundance in the former. Our finding of *Firmicutes* throughout the wild golden snub-nosed monkey gastrointestinal tract suggests that *Firmicutes*, particularly *Ruminococcus* and *Enterococcus*, should be prioritized in future research. This study provides the first analysis of gastrointestinal microbiota diversity in an aged wild golden snub-nosed monkey that died from heart disease, representing the microbiota characteristics of deceased aged wild individuals and substantially enriching our knowledge. Future studies should employ metagenomic approaches for deeper analysis of microbiota composition and function to guide health and conservation efforts for wild golden snub-nosed monkeys.

Conclusion

This study analyzed gastrointestinal microbiota diversity in an aged deceased wild golden snub-nosed monkey using PCR-DGGE, revealing microbiota characteristics associated with its health status, gender, age, and methodology. Microbiota diversity exhibited a high-low-high trend along the gastrointestinal tract, with *Proteobacteria* (38.89%) and *Firmicutes* (22.22%) as dominant phyla. However, substantial uncultured bacteria (22.22%) remain to be investigated.

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