Postprint: Analysis of Gastrointestinal Microbiota Diversity in Red Pandas by Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis

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Abstract

This study aimed to investigate the diversity of gastrointestinal microbiota in red pandas. Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) technology combined with band clone sequencing, cluster analysis, and principal component analysis (PCA) was employed to examine the structure and diversity of red panda gastrointestinal microbiota. The results showed that: 1) PCR-DGGE profiles revealed abundant microbiota throughout the red panda gastrointestinal tract, with microbial community structure varying among different regions while showing certain similarities in adjacent intestinal segments. Microbiota diversity was higher in colon and fecal samples, followed by stomach and rectal samples, whereas it was lower in jejunum and ileum samples. 2) Most sequenced bands from the PCR-DGGE profiles of red panda gastrointestinal microbiota were classified into Firmicutes, Bacteroidetes (Bacteroides), Proteobacteria, and Verrucomicrobia. Common bands primarily corresponded to uncultured Bacteroidetes bacterium, Enterococcus faecalis, uncultured Clostridium sp., Lactococcus lactis, and Weissella cibaria, with Firmicutes being the dominant microbiota; specific bands were mainly Comamonas sp., Clostridium sp., and Akkermansia. These findings demonstrate that the red panda gastrointestinal tract harbors abundant microbiota, and its diversity exhibits a high-low-high trend along the anterior-to-posterior axis of the gastrointestinal tract.

Full Text

Bacterial Diversity in the Gastrointestinal Tract of *Ailurus fulgens* Analyzed by Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis

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Abstract

This study aimed to investigate the diversity of gastrointestinal microbiota in the red panda (*Ailurus fulgens*). Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) technology, combined with band cloning and sequencing, cluster analysis, and principal component analysis (PCA), was employed to examine the structure and diversity of gastrointestinal bacterial communities. The results demonstrated that: (1) PCR-DGGE profiles revealed abundant bacterial populations in the red panda gastrointestinal tract, with distinct differences in community structure across different anatomical sites, though adjacent intestinal segments showed certain similarities. Colonic and fecal samples exhibited the highest bacterial diversity, followed by gastric and rectal samples, while jejunal and ileal samples displayed the lowest diversity. (2) Sequencing of bands from the PCR-DGGE profiles showed that most bacteria belonged to the phyla Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia. Common bands were primarily identified as uncultured Bacteroidetes bacterium, *Enterococcus faecalis*, uncultured *Clostridium* sp., *Lactococcus lactis*, and *Weissella cibaria*, with Firmicutes representing the dominant phylum. Specific bands corresponded mainly to *Comamonas* sp., *Clostridium* sp., and *Akkermansia*. These findings indicate that the red panda gastrointestinal tract harbors a substantial bacterial community, with diversity following a high-low-high pattern from the anterior to posterior regions.

Keywords: *Ailurus fulgens*; gastrointestinal microbiota; PCR-DGGE; diversity; cloning sequencing

Introduction

The red panda (*Ailurus fulgens*) is a small, rare wildlife species endemic to the Himalaya-Hengduan Mountains, distributed exclusively in China, Nepal, Bhutan, Sikkim, India, and northern Myanmar. Classified as a national second-

class protected animal in China, the red panda belongs to the order Carnivora and family Ailuridae. Despite its carnivorous classification, it shares remarkable similarities with the giant panda in geographic distribution, physiological structure, and behavioral ecology, representing a highly specialized herbivorous carnivore with significant scientific value in taxonomy and ecology. However, due to human activities and habitat destruction, China' s red panda population has declined by 40% over the past half-century. Since 1936, Chinese zoos have initiated captive breeding and conservation programs, and with the prohibition of capture permits in 2003 coupled with scientific conservation efforts, red panda reproduction and population numbers have improved.

Recent research increasingly demonstrates that animal physiological metabolism and growth development are closely associated with gastrointestinal microorganisms. Similarly, gut microbes play a crucial role in bamboo digestion for red pandas. While preliminary reports have described the fecal microbiota structure and composition in red pandas, comprehensive studies on microbiota across the entire gastrointestinal tract remain unreported, with most information about resident microorganisms and their functions still unknown. Research and utilization of gastrointestinal microbiota have become important measures for wildlife reproduction and population conservation. Therefore, investigating gastrointestinal microbiota structure and composition provides essential guidance for red panda husbandry, disease prevention, and population protection.

Modern molecular biology techniques offer powerful tools for studying gut microbiota structure. Since its first report by Muyzer et al. in 1993, PCR-DGGE has been widely applied as a rapid and reliable fingerprinting technique for comparing microbial diversity in animal gastrointestinal tracts. This study employed PCR-DGGE combined with band cloning and sequencing to analyze red panda gastrointestinal microbiota structure and composition, providing practical guidance for husbandry management, disease prevention, and conservation efforts.

Materials and Methods

Sample Collection

Gastric, duodenal, jejunal, ileal, colonic, and rectal samples were obtained from a clinically deceased red panda at Chengdu Zoo, Sichuan Province (July 2016; died from unsuccessful treatment after tail amputation due to fighting). Fresh contents from each gastrointestinal segment were aseptically collected. Additionally, fresh feces were collected from three healthy red pandas in the same enclosure and mixed uniformly as a fecal sample. All specimens were aliquoted into sterile centrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C.

Reagents and Instruments

The following materials were used: QIAamp® DNA Stool Mini Kit (Qiagen, Germany); NanoDrop® ND-1000 spectrophotometer (Wilmington, USA); MJ Research PCR-200 and DGGE electrophoresis system (Bio-Rad, Canada); universal primers for *E. coli* 16S rDNA V3 region (Sangon Biotech, Shanghai); 2×Taq Master Mix (Cwbiotech, Beijing); Gel Extraction Kit (Omega, USA); DGGE reagent kit (Bio-Rad, Canada); pMD® 19-T vector and DH5α competent cells (Takara, Dalian); and analytical-grade silver staining reagents.

Extraction of Total Bacterial DNA

Total bacterial DNA was extracted from gastric and intestinal content samples using the QIAamp® DNA Stool Mini Kit according to the manufacturer' s protocol. DNA concentration was measured using the NanoDrop® ND-1000 spectrophotometer (OD_{260}/OD_{280} = 1.7–1.9), and samples were stored at -20°C.

PCR Amplification of 16S rDNA V3 Region

Bacterial 16S rDNA V3 region sequences were amplified using universal primers. The upstream primer was 5 -CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGG
3 (with the underlined "GC" clamp), and the downstream primer was 5 -ATTACCGCGGCTGCTGG-3 . The 25.0 µL reaction mixture contained 12.5 µL 2×Taq Master Mix, 1.0 µL each of upstream and downstream primers (10 µmol/L), 1.0 µL template DNA, and 9.5 µL double-distilled water (ddH_2O). Thermal cycling conditions were: 94°C for 4 min; 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min; and a final extension at 72°C for 10 min. PCR products were verified by 1.0% agarose gel electrophoresis.

PCR-DGGE Gel Electrophoresis and Band Cloning

PCR-DGGE was performed using the Bio-Rad Dcode system with a 35%–65% denaturing gradient consistent with the electrophoresis direction. Electrophoresis was conducted 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. Common and specific bands from the PCR-DGGE profiles were excised, recovered, and subjected to cloning and sequencing. Obtained sequences were compared against the GenBank database to identify the closest bacterial relatives or clones.

Data Analysis

Bacterial diversity analysis, principal component analysis, and other statistical evaluations of PCR-DGGE profiles were performed using Excel 2010, SPSS 19.0, and NTSYS 2.1 software.

Results

PCR-DGGE Profiles and Cluster Analysis

In PCR-DGGE profiles, dark bands represent dominant bacterial populations, while band number and position reflect richness and species composition. Red panda gastrointestinal samples yielded varying numbers of bands with distinct intensity and positional differences [Figure 1: see original paper]. The profiles showed 15, 10, 6, 13, 19, 16, and 17 bands from stomach, duodenum, jejunum, ileum, colon, rectum, and feces, respectively. Gastrointestinal location significantly influenced microbiota structure and composition, with colonic and fecal samples showing the highest diversity, followed by gastric and rectal samples, while jejunal and ileal samples exhibited the lowest diversity.

Cluster analysis [Figure 1: see original paper] revealed that duodenal and ileal samples, despite being non-adjacent segments, clustered together with a high similarity coefficient of 0.76. Colonic and rectal samples formed another cluster with a similarity coefficient of 0.68, while gastric and fecal samples clustered at a lower similarity of 0.53. Different intestinal segments separated from fecal and gastric samples with a similarity coefficient of only 0.45, indicating distinct microbiota structures across gastrointestinal locations. Bands 1–16 (indicated by arrows) represent excised bands. Sample abbreviations Sto, Duo, Jej, Ile, Col, Rec, and Fae denote stomach, duodenum, jejunum, ileum, colon, rectum, and feces, respectively.

Diversity Analysis of Gastrointestinal Microbiota

As shown in [Figure 2: see original paper], bacterial diversity varied across gastrointestinal locations. Jejunal samples exhibited the lowest Shannon diversity index (2.20), evenness (0.60), and richness (9), while colonic samples showed the highest values (3.09, 0.84, and 22, respectively). Gastric, rectal, and fecal samples displayed similar diversity parameters (2.89, 0.78, and 18; 2.94, 0.80, and 19; and 3.00, 0.81, and 20, respectively). Following the anterior-to-posterior gastrointestinal axis, microbiota diversity exhibited a high-low-high pattern, likely reflecting functional and environmental differences along the digestive tract. These findings align with the PCR-DGGE profile analysis, showing lower diversity in jejunal samples, higher diversity in colonic samples, and intermediate similarity among gastric, rectal, and fecal samples.

Principal Component Analysis

PCA of PCR-DGGE profiles [Figure 3: see original paper] corroborated cluster analysis results. Principal component 1 (PC1) explained 33.56% of variance, while PC2 explained 24.70%. PC1 clearly separated fecal samples from others, and PC2 distinguished colonic, rectal, and fecal samples from remaining segments, indicating substantial differences in microbiota structure and composition. Conversely, gastric, duodenal, jejunal, and ileal samples clustered together, suggesting that while differences exist among gastrointestinal locations,

adjacent segments share similarities in microbiota structure and composition.

Analysis of Common and Specific Bands

Sequencing results for 16 excised bands [FIGURE:1, arrows] are presented in . Cloning and sequencing revealed that common bands primarily represented Firmicutes, Bacteroidetes, and Proteobacteria, including numerous uncultured Bacteroidetes bacteria, *Enterococcus faecalis*, *Escherichia coli*, uncultured bacteria, *Acinetobacter johnsonii*, uncultured *Clostridium* sp., *Lactococcus lactis*, and *Weissella cibaria*. Eight isolates (50.00%) belonged to Firmicutes, representing the dominant phylum, with three enterococcal isolates (18.75%) being predominant within Firmicutes. Specific bands corresponded to *Comamonas* sp., *Blautia* sp., uncultured bacteria, *Clostridium* sp., and *Akkermansia* (Verrucomicrobia). All sequences showed $ $99% similarity to GenBank entries, indicating close phylogenetic relationships with identified microorganisms.

Discussion

Research and utilization of gastrointestinal microbiota have become crucial strategies for wildlife reproduction and conservation. Although red pandas and giant pandas are phylogenetically distant, comparative genomic analyses reveal convergent evolution in genes and enzymes related to bamboo consumption, enabling adaptation to low-nutrient bamboo diets. Gastrointestinal symbiotic microorganisms are intimately linked to host digestion and physiological function. This study analyzed band distribution, number, and intensity in PCR-DGGE profiles from gastric, duodenal, jejunal, ileal, colonic, rectal, and fecal samples, providing rapid, intuitive insights into gastrointestinal microbiota structure and diversity at the molecular level. The PCR-DGGE profiles [Figure 1: see original paper] demonstrated abundant bacterial populations, though their relationships with the host require further investigation.

Despite challenges in sampling and studying gastrointestinal microbiota in wildlife due to conservation status and ecological characteristics, increasing reports on wildlife gut microbiota have emerged in recent years, contributing significantly to global wildlife husbandry and conservation efforts. The cluster analysis of red panda gastrointestinal PCR-DGGE profiles [Figure 1: see original paper] showed that duodenal and ileal samples, though non-adjacent, clustered together with high similarity (0.76), possibly reflecting red panda small intestinal structural characteristics and short food retention times. The small intestine serves as the primary site for nutrient digestion and absorption, and red pandas possess a well-developed small intestine reaching 350 cm in length without a cecum, resulting in the shortest food retention time among mammals.

PCA of PCR-DGGE profiles [Figure 3: see original paper] and diversity index analysis [Figure 2: see original paper] revealed high microbiota diversity

in colonic samples, following a high-low-high pattern along the gastrointestinal axis, likely related to segment-specific digestive functions and high-fiber diets. Previous studies have reported that intestinal location significantly influences microbiota structure and diversity. In this study, gastric and fecal samples separated from other intestinal segments. The red panda' s large stomach capacity, strong epithelial secretory function, well-developed gastric mucous cells, and thick muscularis layer facilitate digestion and absorption of high-fiber bamboo diets. Since fecal samples were collected from healthy cohabitating pandas rather than the deceased individual, and feces represent terminal digestive products exposed to environmental conditions, their microbiota structure differed from other gastrointestinal locations. However, studies have shown that intra-individual microbiota variation exceeds inter-individual variation within species, suggesting that fecal samples from cohabitating pandas can reasonably represent fecal microbiota structure.

Cloning and sequencing of PCR-DGGE bands identified Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia as the primary phyla, with Firmicutes dominance driven by *Enterococcus faecalis* and *Weissella cibaria*. *Weissella cibaria* participates in dietary fiber digestion and absorption, while *Enterococcus faecalis* represents a major probiotic group in mammalian intestines, though some strains exhibit pathogenicity depending on virulence island detection. Williams et al. reported that giant and red pandas utilize distinct microbial communities for bamboo degradation, with *Clostridium* (producing butyrate through fiber fermentation) dominating red panda feces (75%) and Erysipelotrichaceae dominating giant panda feces (95%), indicating different fiber degradation strategies. In this study, *Clostridium* was detected only in feces, possibly related to the red panda' s cecum-less anatomy with short colon and rectum.

Kong et al. compared wild and captive red panda fecal microbiota, finding higher diversity in wild individuals dominated by Firmicutes, with most bacteria possessing fiber degradation functions crucial for bamboo digestion. *Blautia*, specific to the colon in this study, plays important roles in fermenting dietary prebiotics into short-chain fatty acids and exerting anti-inflammatory and anti-tumor effects. *Akkermansia*, specific to the stomach, improves obesity and diabetes in model systems, suggesting potential importance in fiber fermentation, though this requires further investigation. The detection of opportunistic pathogens *E. coli* and *A. johnsonii* in gastric and ileal samples, respectively, may contribute to gastrointestinal disease during compromised health status, consistent with findings that canine distemper virus infection increases Enterobacteriaceae abundance in giant pandas.

This study provides the first visual representation of gastrointestinal microbiota structure and composition in a clinically deceased red panda using PCR-DGGE. However, numerous uncultured bacteria remain uncharacterized, and their functions and host relationships require further investigation. Nonetheless, these results provide foundational data and practical guidance for red panda husbandry

and disease prevention.

Conclusion

This study demonstrates that: (1) The red panda gastrointestinal tract harbors abundant bacteria, mostly associated with fiber digestion. The 16 identified isolates primarily belonged to Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia, with eight Firmicutes isolates (50.00%) representing the dominant phylum. Enterococci (three isolates, 18.75%) were predominant within Firmicutes, and fiber-degrading taxa included *Clostridium* and *W. cibaria*. (2) Microbiota diversity varied across gastrointestinal locations, following a high-low-high pattern from anterior to posterior regions, likely reflecting segment-specific digestive functions and environmental conditions.

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