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Full Length Research Paper

Study of fecal bacterial diversity in Yunnan snub-nosed monkey (*Rhinopithecus bieti*) using phylogenetic analysis of cloned 16S rRNA gene sequences

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The bacterial diversity in fecal samples from Yunnan snub-nosed monkey (*Rhinopithecus bieti*) was investigated by constructed 16S rRNA gene clone library and restriction fragment length polymorphism analysis. As a result, a total of 156 representative clones for each profile, comprising nearly full length sequences (with a mean length of 1.5 kb) were sequenced and submitted to an on-line similarity search and neighbor-joining phylogenetic analysis. Using the criterion of 97%, these 16S rRNA gene sequences were binned in 129 OTUs. 11 sequences whose similarity is ≥ 97% were affiliated to the cultured bacteria and accounted for 7.05% of the total clones. For 23 sequences (14.74%), the similarity with the database was in the range of 89 - 97%. The remaining 122 sequences (78.21%) were uncultured and unidentified bacteria. Based on the phylogenetic analysis, the fecal bacteria of *R. bieti* distributed mainly in 6 bacteriophyta of *Firmicutes, Proteobacteria, Bacteroidetes, Fibrobacteres, Spirochaetes* and *Actinobacteria,* and belonged to 17 genera. Besides, there were a large number of uncultured and unidentified bacteria. These results illustrate the fecal bacteria diversity of *R. bieti*.

Key Words: *Rhinopithecus bieti*, fecal bacterial diversity, 16S rRNA gene, phylogenetic analysis.

INTRODUCTION

Alias Yunnan snub-nosed monkey or black snub-nosed monkey (*Rhinopithecus bieti*) belongs to *Rhinopithecus, Colobinae, Cercopithecidae,* and *Primates* (Li and Lian, 2007). *R. bieti* is one of the national first-class protected animals and a world-class rare species (Bai et al., 1988), which had been listed among the highly endangered species by the International Union on Conservation of Nature (IUCN) (Reng et al., 2004). The existing natural population of this species is about 1,500 in 13 species groups, which distribute along the narrow terrain between Jingsha River in the east, the eastern bank of Lancang River in the west, Yunlong County of Yunnan Province in the south, and Markam County of Tibetan Autonomous Region of Tibet in the north, China (Long et al., 1996).

The stomach of *R. bieti* is an S-shaped structure (Chen et al., 1995), and consists of the cardiac area, fundus ventriculi+, corpus ventriculi, canalis ventriculi and pylorus. The fundus ventriculi and corpus ventriculi retain a large number of bacteria, which have a similar fermentation function to those in the ruminant rumen, and the pylorus is the area for digestion (Peng et al., 1983). *R. bieti* is the primate who is living on the highest altitude besides human (Long et al., 1996; Zhao, 1998). Unlike other primates, *R. bieti* eats such epiphytes of spruce and fir as usnea, mosses and lichens, and the leaves, flowers and fruits of *Rosaceae, Aceraceae* and other broadleaved trees

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Abbreviations: OUT, Operational taxonomic unit; **IPTG,** isopropyl-β-D-thiogalactopyranoside; **X-Gal,** 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; **LB,** Luria–Bertani; **NCBI,** National Centre for Biotechnology Information; **PCR,** polymerase chain reaction.

as its diet (Zhang et al., 2005). As an herbivorous animal, the feeding habits of *R. bieti* have obvious seasonal variations (Ding, 2003). So far, no other wild animal had been found eating usnea. Such a particular feeding habit with crude fiber foods might be relevant to the special structure of the stomach and intestinal microorganisms. Currently, researches at home and abroad on *R. bieti* has mostly focused on aspects of taxonomy, ecology, anatomy and conservation genetics, but research on fecal bacterial diversity has not been reported.

The large number of microorganisms in the intestine of *R. bieti*, which constitute a complex and dynamic equilibrium of microflora, play an important role in helping the parasitifer digest food and resisting invasion by alien flora (Hooper et al., 2001; Neish et al., 2002). Microbial disequilibrium may lead to physiological dysfunction and diseases (Feng et al., 2005). Traditional classification methods that are based upon purification and culture of the intestinal microbial community are only effective with culturable microorganisms, which means that those intestinal microorganisms that can not be cultured inevitably are ignored (Kocherginskaya et al., 2001). Using traditional research methods, investigations of microbial morphology are so subjective that there are some deficiencies. Moreover, molecular biology studies in recent decades have shown that microbial purification and culture cannot be accomplished for more than 99% of environmental microorganisms (Pace, 1997).

To achieve a comprehensive understanding of the constitution of the intestinal bacterial community of *R. bieti*, we use phylogenetic analysis of cloned 16S rRNA gene sequences. This approach allowed us to establish the diversity of intestinal bacteria in *R. bieti*. Our results may provide a basis for further study of the relationships between the composition of the intestinal microecosystem, the feeding habits of *R. bieti* and the structure of the bacterial communities. They also may lay a foundation for the development and utilization of microbial resources, and for the conservation of this wild animal.

MATERIALS AND METHODS

Experimental material

100 samples of *R. bieti* feces were collected in November 2008 from Longma Mountain in Yunlong County of Dali Prefecture, Yunnan Province, China. Fecal samples (taken ≤12 h after defecation) were collected from 80 - 120 *R. bieti* in a single group with the help of the experts of *R. bieti*, who can distinguish these monkeys' feces from the feces of other animals. Samples of fresh material were soaked in 100% ethanol (Li et al., 2006), transported to the laboratory in an icebox, and stored at –70°C (All research reported in this manuscript complied with animal care regulation, applicable national laws, and the ASP Principles for the Ethical Treatment of Non Human Primates).

Total DNA extraction

To minimize animal-to-animal variations, the aliquots of feces from 100 samples were mixed before DNA extraction. Total DNA was extracted using Bacterial Genomic DNA Extraction Kit (TaKara, Dalian, China). The DNA concentration and its integrity (size > 15 kb) were estimated by agarose gel electrophoresis with 0.8% (w/v) agarose, 1× Tris–borate–EDTA buffer, and 1 ng/mL Gelview (Suau et al., 1999).

16S rRNA gene amplification

Polymerase chain reaction (PCR) amplification of bacterial 16S rRNA gene was performed using the universal primers F27 (5′-AGATTGATCMTGGCTAGGGA-3′) and R1492 (5′-TACGGYTA CCTTGTTACGACTT-3′) (Lane, 1991). Samples were amplified in five replicate reactions to minimize stochastic PCR bias (Polz and Cavanaugh, 1998). The PCR was set up in a 50 µL volume that contained 1.5 µL fecal DNA, 5 µL 10× PCR buffer (25 mmol/L Mg^{2+}), 4 µL dNTP (2.5 mmol/µL), 1.5 µL each primer (10 pmol/µL), 0.5 µL Taq DNA polymerase (TaKara Inc. China) and 36 µL ddH_2O. The amplification conditions were as follows: 4 min of initial denaturation at 94°C; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min 30 s, extension at 72°C for 2 min 30 s, with the last cycle followed by a 20 min extension step at 72°C. The PCR products were combined and visualized on an agarose gel, the bands were excised, and DNA was purified from the gel slices using TaKaRa Agarose Gel DNA Purification Kit. All purified DNA samples were mixed in an eppendorf tube.

Construction of 16S rRNA gene clone library

The purified DNA products were ligated into pMD18T-vectors using a rapid ligation kit according to the instructions of the manufacturer (TaKara Inc. China), and then transformed into competent *Escherichia coli* DH5α cells by heat shock (45 s at 42°C). Clone libraries were established on Luria–Bertani (LB) agar plates with ampicillin (100 µg/ml) and also with IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Colonies that contained a plasmid with an insert could not produce β-galactosidase and degrade X-Gal; consequently, they were white (Suau et al., 1999; Li et al., 2007).

RFLP analysis of 16S rRNA gene

To screen the clones for grouping into similar types, those that contained target 16S rRNA gene were subjected to RFLP analysis. Two vector primers: M13-47 (5′-CGCCAGGGTTTTCCCAGT CACGAC-3′), and RV-M (5′-AGCGGATAACAATTTCACACAGG-3′) (Li et al., 2007) were used to amplify each insert by 30 cycle colony PCR. The products were digested with 1 U each of restriction nucleases *Afa*I and *Msp*I for 6 – 12 h at 37°C. The digested fragments were visualized on a 2.5% agarose gel, and different clones were distinguished according to their RFLP patterns. Clones that produced the same RFLP pattern (DNA fragments of the same size) were grouped together and considered representative of the same operational taxonomic unit (OTU). One clone of each RFLP type was sequenced. The genetic diversity identified by RFLP analysis was subjected to statistical analysis of the percentage of coverage of the library, using the formula (1–(n/N)) ×100% (Good, 1953). Here, n represents the number of clones that occurred only once (16S rRNA gene sequence similarity of < 97%), and N is the total number of clones examined.

Sequencing and sequence analysis

Selected cells were grown overnight in ampicillin-selective LB broth.

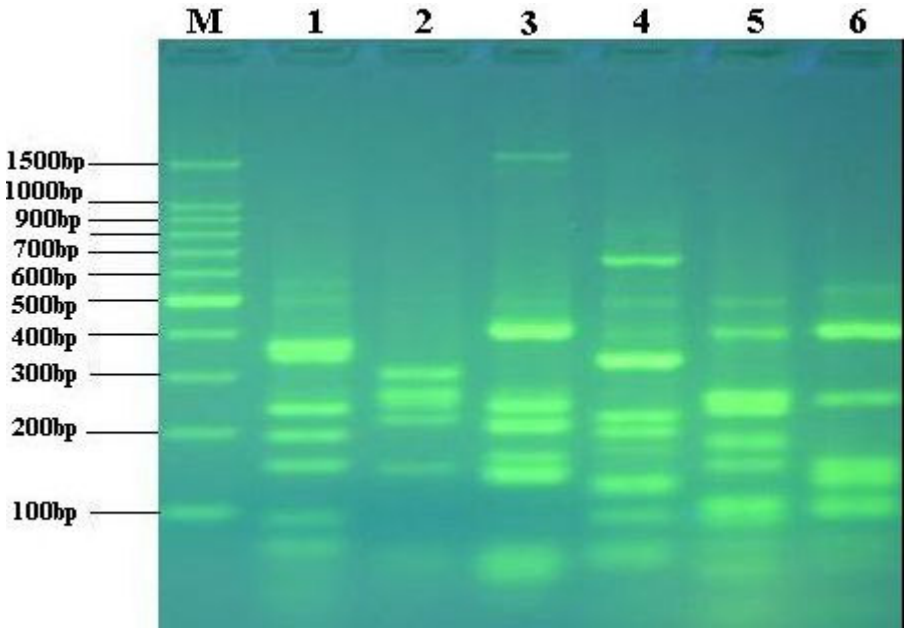

Figure 1. *Afa*I and *Msp*I restriction patterns of 16S rRNA geneM, 100-bp ladder marker; 1– 6, different restriction patterns out of the 156 different patterns.

The clones were sequenced at the Beijing Genomics Institute. The resulting rRNA gene partial sequences were compared with GenBank entries using BLAST to select reference sequences and obtain a preliminary phylogenetic affiliation of the clones. All the sequences obtained were checked for chimeric artifacts using the CHIMERA_CHECK program (Cole et al., 2005). The nearest neighbors were retrieved from NCBI through a BLAST search (http://www.ncbi.nih.gov/BLAST). The most similar sequences were retrieved and aligned with those obtained in this study using Clustal W (Thompson et al., 1994), and the alignments for which homology of residues could not be assumed reasonably were excluded from the phylogenetic analysis. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with the two-parameter model of Kimura (Kimura, 1980). The bootstrap analysis (Felsenstein, 1985) was based on 1000 resamplings.

Nucleotide sequence accession numbers

The sequences from this study were submitted to GenBank under accession numbers GQ451171–GQ451325.

RESULTS

RFLP analysis of the fecal bacterial population

The colony PCR products showed different types of bands after digestion with the restriction nucleases *Afa*I and *Msp*I (Figure 1). At last, we got a total of 156 RFLP groups, which allowed for small variations. Although the RFLP results can not reflect accurately all intestinal microbial groups present in *R. bieti*, they do reflect the richness of the library. Each type was grouped into an operational taxonomic unit (OTU). There were some differences between clones that grouped are within an OTU; however, they are approximated to a phylogenetic taxonomic group (Moyer et al., 1996). Thus, the coverage calculated was 82.69%, which suggested that this study identified almost the whole dominant biodiversity in the given environment. One clone of each RFLP type was sequenced, with which a mean sequence length of 1.5kb. The results of the check for chimeras showed that all of the sequences obtained were normal.

Cloned sequence alignment analysis

We used a 97% level of sequence identity to define the OTUs (Janda and Abbott, 2002). Clones with similarity > 97% were classified into an OTU. 129 OTUs were identified from the 156 cloned sequences (Table 1). The sequence assignment showed that the highest and lowest similarity to those in Genbank was 99 and 84%, respectively.

Of the 156 clones isolated, 40 (comprising 37 OTUs and represent 25.64% of the clones) had ≥ 97% similarity of sequences with known species of bacteria, which included *Pseudomonas* sp., *Pedobacter* sp., *Yersinia enterocolitica, Ruminococcus, Deefgea* sp., *Budvicia aquatica, Sphingobacterium* sp., *Pseudomonas syringae, Pelosinus* sp., *Mycetocola saprophilus, Comamonas* sp. and some uncultured bacteria. Another 105 clones (comprising 81 OTUs and representing 67.31% of the clones) shared a sequence similarity of 90 – 97% with known species of bacteria, and the remaining 11 clones (comprising 11 OTUs and representing 7.05% of isolated

Table 1. Distribution of 16S rRNA gene clones and operational taxonomy units (OTU) retrieved from the feces of *Rhinopithecus bieti*

Items	Clones		OTU[a]	
	Number of clones	%Total clones	Number of OTU	%Total OUT
Similarity				
≥ 97%	40	25.64	37	28.68
90 - 97%	105	67.31	81	62.79
< 90%	11	7.05	11	8.53
Phylum				
Firmicutes	33	21.15	31	24.03
Proteobacteria	14	8.97	12	9.30
Bacteroidetes	7	4.49	7	5.43
Fibrobacteria	8	5.13	5	3.88
Spirochaetes	16	10.26	8	6.20
Actinobacteria	6	3.85	6	4.65
Unclassified	72	46.15	60	46.51
Total	156		129	

a: Clones having > 97% similarity of 16S rRNA gene among each other were defined as 1 operational taxonomy unit (OTU).

clones) have less than 90% similarity with sequences of known bacteria (Table 1).

Phylogenetic analysis

The similarity for most of the sequences with those of known bacteria was too low to identify the sequence as representing a particular taxon, therefore, phylogenetic trees were constructed to investigate the taxonomic placement. Phylogenetic trees reveal that the intestinal bacteria, extracted from the feces of *R. bieti*, are plentiful and they mainly distributed among the *Firmicutes, Proteobacteria*, *Bacteroidetes, Fibrobacteres*, *Spirochaetes*, *Actinobacteria* and unclassified bacteria (Figure 2). And different bacteriophyta have different proportion (Table 1).

Phylogenetic analysis of *Firmicutes*

Of the 156 clones, there were 33 (21.15%) situating at the *Firmicutes* of low G+C gram-positive bacterium (Figure 3). Therein, all bacteria belonged to *Clostridium*, which were the dominant bacteria in the intestine of *R. bieti*. The 33 clones (31 OTUs) had high similarity to the bacteria in the feces and rumen of mammals such as oxen, rats, reindeer, gorillas, and humans. To some extent, this is related to the evolutionary process of mammals. The two sequences J153 and J169 had 95% similarity to the unidentified bacteria in the feces of *Bos frontalis*. J132 had 96% similarity to the uncultured *Ruminococcus* from bovine feces. J169 and J137 shared a 100% bootstrap value with the bacteria from the rumen and feces of cows and the galactophore of dairy cows, which showed high evolutional relativity. J346 had 95% similarity to *Clostridium* separated from the galactophore of dairy cows. J83 and J19 had 96% similarity to the uncultured bacteria separated from the methane tank and dunghill. J260 had 97% similarity to the *Pelosinus* sp. They shared equal distance in the phylogenetic tree. J86 clustered with the uncultured bacteria from feces of gorillas, and shared 97% similarity with a bootstrap value of 100%. There was high evolutional relativity between them. Therefore, the intestinal bacteria of primates are probably similar to each other. J133 had 93% similarity to the bacteria separated from the feces of patients with irritable bowel syndrome and shared a 100% bootstrap value, which indicated the stability of the genetic relationship in evolution.

Phylogenetic analysis of *Proteobacteria*

The sequence alignment and phylogenetic tree structure (Figure 4) showed that, in the 16S rRNA gene clone libraries, there were 34 sequences (28 OTUs) (21.79%) that resembled 16S rRNA gene sequences of the identified bacteria, most of which belonged to the *Proteobacteria*. There were 14 (8.97%) from 156 sequences classified into the *Proteobacteria*, which included the four classes of *Proteobacteria*: α, β, γ and ε, and the eight genera: *Rhodobacter*, *Sphingomonas*, *Rhodopseudomonas*, *Deefgea*, *Comanonas*, *Pseudomonas*, *Yersinia*, and *Campylobacter*. The phylogenetic analysis showed that the bacterium was classified into two clusters.

The three classes β, γ and ε were classified as one cluster, including 8 sequences. J30 and J44 respectively had 99 and 98% similarity with *Yersinia enterocolitica* and

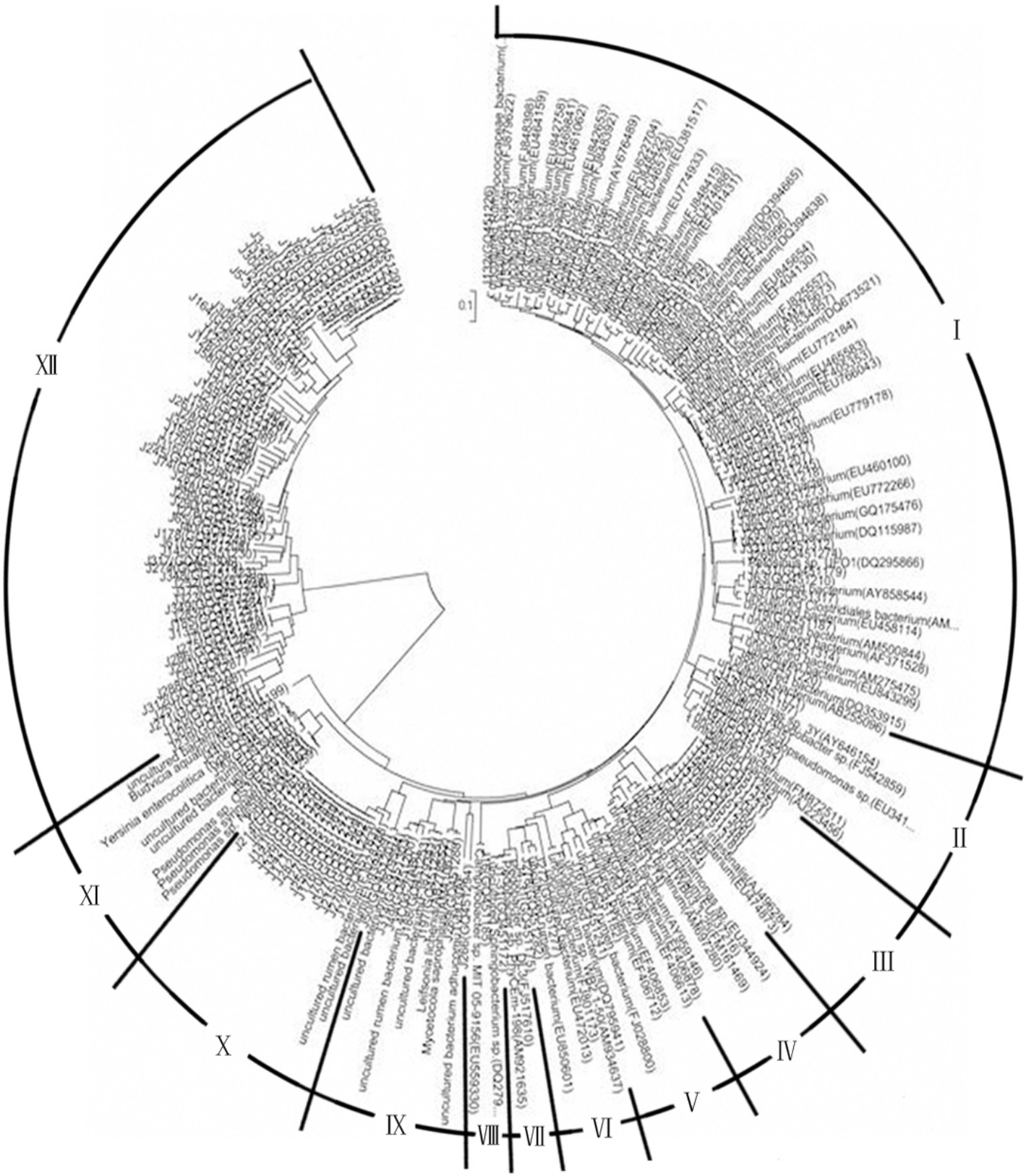

Figure 2. Phylogenetic tree based on 16S rRNA gene sequences from *Rhinopithecus bieti* clones. Numbers in parentheses represent the sequence accession numbers in GeneBank. The bar represents 10% sequence divergence. Roman numerals represent different bacteriophyta. Ⅰ:*Firmicutes*. Ⅱ:α-*Proteobacteria*.Ⅲ:*Fibrobacteres*.Ⅳ:β-*Proteobacteria*.Ⅴ:Bacteroidetes.Ⅵ:Flavobacteria.Ⅶ:Sphingobacteria.Ⅷ:ε-*Proteobacteria*.Ⅸ:*Actinobacteria*.Ⅹ:*Spirochaetes*.Ⅺ:γ-*Proteobacteria*.Ⅻ:Unclassified.

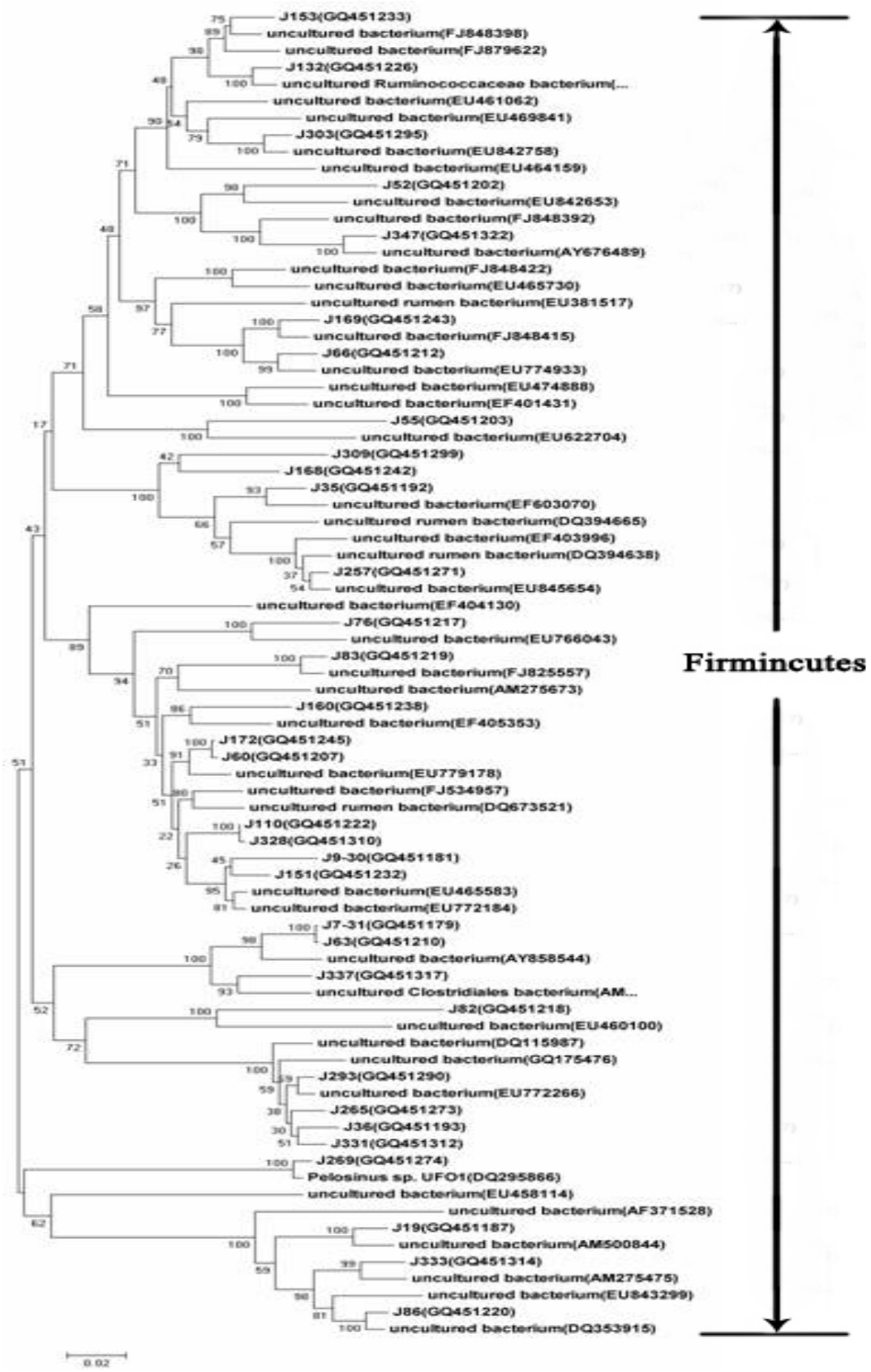

Figure 3. Phylogenetic tree based on 16S rRNA gene sequences from the *Firmicutes* phyla for *Rhinopithecus bieti*(The scale bar represents 2% sequence divergence).

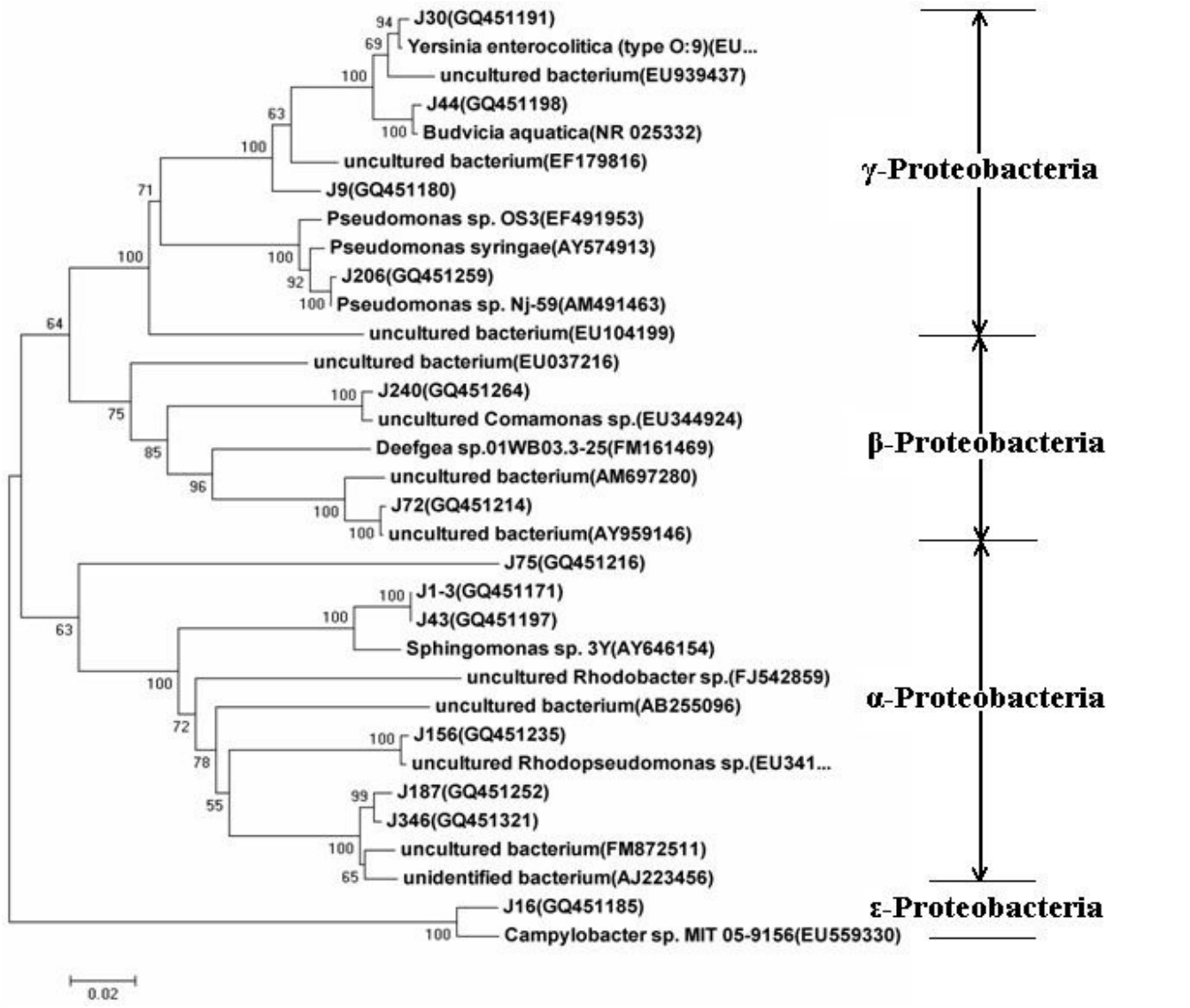

Figure 4. Phylogenetic tree based on 16S rRNA gene sequences from the *Proteobacteria* phyla for *Rhinopithecus bieti* (The scale bar represents 2% sequence divergence).

Budvicia aquatica. Therein, *Yersinia enterocolitica* was the prominent pathogenic bacterium in mammal, and caused enterocolitis and pestilence. J206 was clustered with the three species *Pseudomonas sp. Nj-59*, *Pseudomonas syringae* and *Pseudomonas sp.OS3.* As a result of the close evolutionary distance, it was predicted that J206 belonged to a sub-germline of *Pseudomonas.* J16 had 96% similarity to *Campylobacter* sp. that was isolated from the feces of artificial feeding chimpanzees, and the phylogenetic tree showed that they had a genetic relationship. J240 had 99% similarity with *Comanonas* that was isolated from the intestine of *Hepialus gonggaensis* larvae, and had a bootstrap value of 100% in the phylogenetic tree. J72 had 99% similarity to uncultured bacteria isolated from human vaginal epithelium. In the phylogenetic tree, it clustered with the aerobic bacteria that were isolated from river water in limestone areas, which showed that they were highly related in evolutionary terms.

α-*Proteobacteria* was classified separately as one cluster, which included six clone sequences. J1-3 had 96% similarity with *Sphingomonas* sp. that was isolated from soil polluted with diesel oil. By comparison, J43 shared 99% similarity with unidentified bacteria in human feces, but in the phylogenetic tree, it clustered with *Sphingomonas*. Therefore, in terms of evolution, it had a close genetic relationship with J1-3.

Phylogenetic analysis of *Bacteroidetes*

The *Bacteroidetes* comprised three types of bacteria:

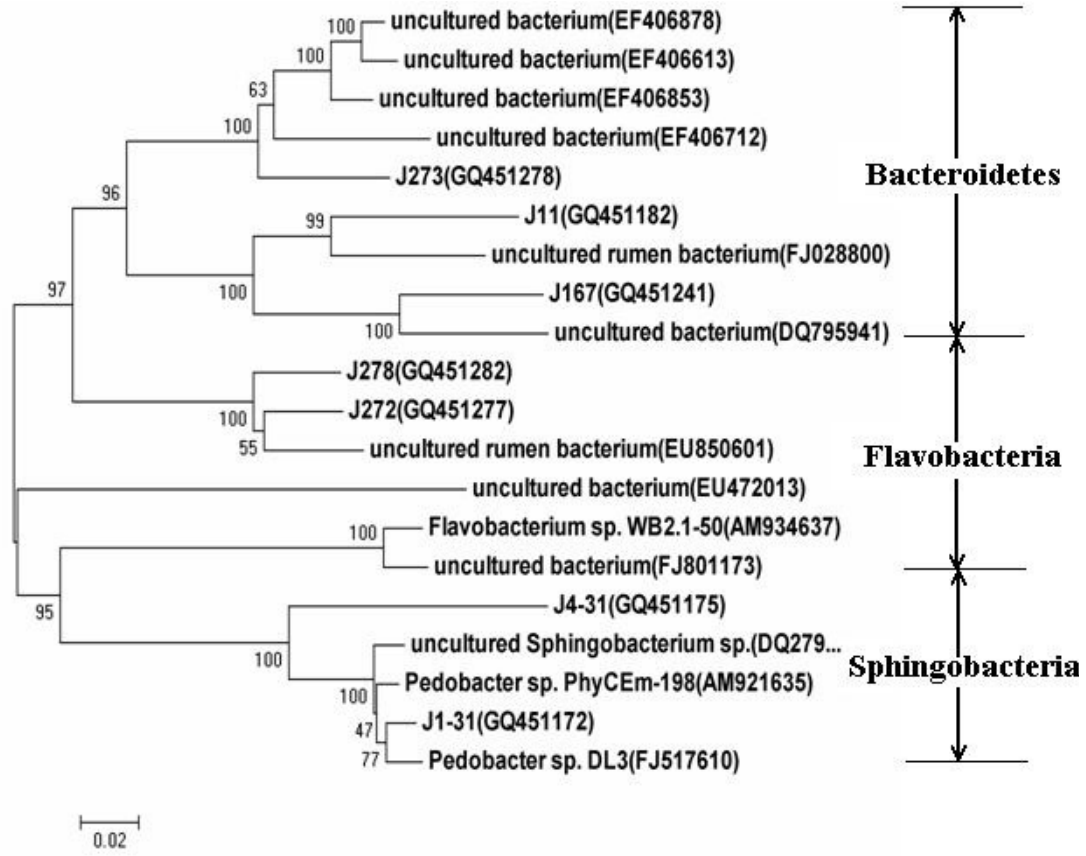

Figure 5. Phylogenetic tree based on 16S rRNA gene sequences from the *Bacteroidetes* phyla for *Rhinopithecus bieti* (The scale bar represents 2% sequence divergence).

Bacteroid, *Flavobacterium* and *Sphingobacterium* classes. By comparison and phylogenetic tree construction, the clone sequences from the intestine of *R. bieti* were classified into three clusters (Figure 5). The three sequences (J273, J11, J167) of the *Bacteroides* class were all uncultured bacteria; two sequences (J272 and J278) belonged to the *Flavobacterium* class; and J4-31 and J1-31 were classified as *Pedobacter* and *Sphingobacterium*, respectively. J273 had 92% similarity with the uncultured bacteria from the mouse colon and they may be relevance to some extent because of their close evolutionary distance. J11 shared a sequence similarity of only 89% with the uncultured rumen bacteria isolated from the rumen of Taiwan water buffalo, but with a 100% bootstrap value, they had a close relationship. As the phenomenon appeared on J11, the J167 though had low similarity to the uncultured bacteria isolated from human feces, they had a close genetic relationship with each other. The research on the bacteria of human feces, which closed genetic relationship to J167, found that *Bacteroidetes* took a relatively lower proportion in the fat men than in the thin men, and *Bacteroidetes* bacteria probably had some link with human obesity (Ley et al., 2006). J272 and J278 had 94 and 93% similarity, respectively, with the uncultured bacteria isolated from rumen fluid of water buffalo, and additionally the phylogenetic tree showed that they had some relativity. Both of them had evolutionary similarity to *Flavobacterium* sp. from river water and uncultured bacteria from marsh water. J4-31 had 89% similarity to *Pedobacter sp. DL3* isolated from south-polar soil. They kept close distance in the phylogenetic tree. J1-31 clustered with *Pedobacter* sp. in the phylogenetic tree, and had 97% similarity to *Pedobacter* sp. isolated from the rhizosphere of *Lolium perenne*, as shown by BLAST analysis.

Phylogenetic analysis of *Fibrobacteres, Spirochaetes and Actinobacteria*

The phylogentic tree was classified into three parts (Figure 6): *Spirochaetes, Fibrobacteres* and *Actinobacteria*, with a total of 30 sequences (20 OTUs). There were seven OTUs (10.9%) classified as *Spirochaetes*. J12 and J215 had 93 and 94% similarity, respectively, to the uncultured bacteria isolated from the rumen fluid of Taiwan water buffalo, and J270 and others had 92% similarity. J50 had 98% similarity to the uncultured bacteria isolated from excrement of *Nycticebus pygmaeus*, with a 100% boorstrap value. The phylogenetic tree

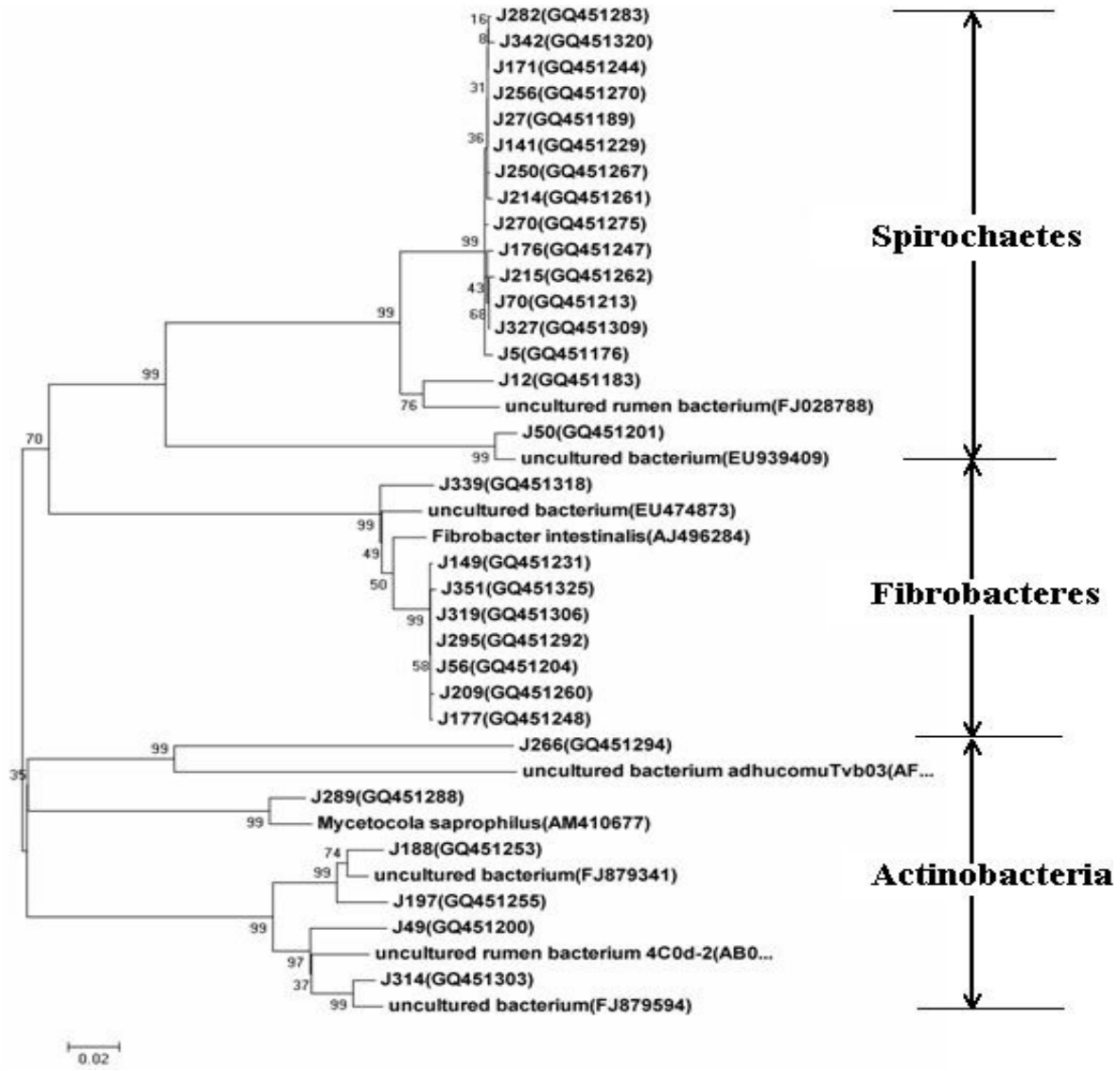

Figure 6. Phylogenetic tree based on 16S rRNA gene sequences from the *Spirochaetes*, Fibrobacteres and *Actinobacteria* phyla for *Rhinopithecus bieti* (The scale bar represents 2% sequence divergence).

showed that these sequences clustered a single branch, which represented their evolutionary relationship.

The second part was classified as *Fibrobacteres* and included eight sequences (7 OTUs), which accounted for 5.13% of the total sequences. The seven sequences J295, J351, J56, J319, J209, 177 and J149 had 96% similarity with *Fibrobacter intestinalis*. J339 had 95% similarity with uncultured bacteria that were isolated from red river hog feces. The phylogenetic tree showed that the eight sequences had a close evolutionary genetic relationship.

The third branch, including five OTUs (0.64%), was classified as *Actinobacteria* (high G+C Gram-positive bacteria). J289 had 97% similarity with *Mycetocola saprophilus*, and the phylogenetic tree demonstrated that they were related in evolution. BLAST showed that J266 shared a sequence similarity of only 89% with *Treponema pectinovorum* (*Treponemas*). However, the phylogenetic tree demonstrated that it had a close genetic relationship with the uncultured bacterium adhucomuTvb03 (AF228819) isolated from human mucous membrane. The four sequences J188, J197, J49 and J314 only matched with uncultured bacteria, and clustered a new branch. This differed from *Mycetocola* which may be a new genus.

Phylogenetic analysis of unidentified *bacteria*

Among the 156 clone sequences, there were 72 sequences (64 OTUs) (46.15%) that did not match with sequences in GenBank, and belonged to uncultured and unclassified bacteria. Independently, they formed a large branch with

an extremely high bootstrap value (Figure 1). They possibly represent a novel sub-class or an even higher class. It is predicted that they are special flora that exist among the intestinal bacteria of *R. bieti*. Therefore, future research and development are directed to the under-developed microbial resources. In the phylogenetic tree, they were classified roughly into four clusters, but their taxonomical position could not be defined specifically.

DISCUSSION

Molecular scatology

The *R. bieti* is a rare and valuable endangered species. Therefore, it is impossible to collect samples by conventional methods that involve destructive or invasive sampling. This problem, however, might be solved to a large extent by means of fecal analysis based on molecular scatology in recent years. With the combination of traditional fecal analysis and molecular technology, biologists could make further studies on free-ranging endangered species without disturbing or even observing them (Wei et al, 2001). Fecal samples can be collected easily, and they contain genetic material that can provide much useful information about the animals, which can be used in studies involving micro-genetics, population ecology and behavioral ecology.

Fecal samples of *R. bieti* used in the experiment were soaked in 100% ethanol (Li et al., 2006), transported to the laboratory in an icebox, and stored at –70°C. Although this ethanol preservation possible had little changes in the microbiota of *R. bieti,* it reflects the fecal bacterial diversity of *R. bieti* to a large extent by the use of fecal analysis based on molecular scatology.

Analysis of bacterial diversity

The total DNA extracted from the feces of *R. bieti* was used to construct a 16S rRNA gene library. After homological comparison with the sequences in GenBank, it was found that the intestine of *R. bieti*s had a large number of bacteria that may be involve in the digestion of cellulose, pectin or lignin, such as *Fibrobacter*, *Treponema*, *Pseudomonas*, *Bacillales*, *Clostridium* and *Ruminococcus*. Such as *Fibrobacter intestinalis* can degrade cellulose and hemicelluloses efficiently and specifically (Béra-Maillet et al., 2004), and *Treponema pectinovorum* is able to degrade the cell-wall pectin. Some researchers have found *Bacillus licheniformis* and *Bacillus subtilis*, which play an especially important role in degrading cellulose (Pang, 2004; Hu et al., 2008; Qu et al., 2008). The endocellulase that is generated by *Ruminococcus* and *Clostridium* genera probably is related closely to fiber degradation (Wang, 2008; Zhang et al., 2008; Si and Jiang, 2003). Therefore, the bacterial community that degrade fiber and lignin efficiently and specifically is linked closely with the process by which *R. bieti* digests crude fiber food, such as Chinese usnea, and then transforms it into glycosides or bacterial protein for absorption and utilization.

In addition, *Pedobacter* sp. which generates phytase was discovered (Shao, 2008). This showed that the intestinal bacteria of *R. bieti* can decompose phytic acid and absorb the phosphorus element to promote growth. We also found bacteria such as *Yersinia, Budvicia and Campylobacter*, which were all pathogenic bacteria for humans and animals. *Yersinia* may lead to diarrhea or acute gastroenteritis (Gao et al., 1985). Campylobacter mainly caused chordapsus and food poisoning, as well as adjuvant arthritis, hepatitis and so on. It was unknown whether *R. bieti* suffers from these diseases. The *Sphingomonas* in feces efficiently degraded macromolecular organic pollutants, such as phenanthrene, which had carcinogenic activity in animals and induced an allergic response in the skin. This may help monkeys degrade polluted or poisoned food. J286 had 96% similarity with uncultured bacteria from soil samples polluted by carbon tetrachloride. Strangely, the intestinal flora of *R. bieti* included the anaerobic photoautotrophs, *Rhodopseudomonas* and *Rhodobacter*. However, their functions in the intestine of monkeys were unknown. These probably resulted from the process in which monkeys eat the Chinese usnea and incompletely digested it, which just were the “passers-by” of intestine attached to the food.

Comparison of microbial diversity in feces of animals with different feeding habits

As with any PCR-based method, clone libraries are subject to biases (Von Wintzingerode et al., 1997). With this in mind, the *R. bieti* clone library was compared with gastrointestinal clone libraries from animals using diverse digestive strategies. Table 1 shows the intestinal bacterial diversity of *R. bieti*, Gorillas, Gayals, Normal humans, Vegetarian women, Yaks, and Manchurian tigers. *Actinomycetes, Bacteroidetes* and *Firmicutes* existed in *R. bieti*, Gorillas, and Humans, but the *Firmicutes* in the intestine of *R. bieti* were much less in number than in Humans and Gorillas, rather than the dominant flora. Although *Proteobacteria* were abundant in the intestine of *R. bieti*, they were not present in Gorillas, and only a few were found in the Human intestine. This demonstrated that there was a resemblance between the intestinal flora of *R. bieti*, Humans and Gorillas, but the different feeding habits leaded to obvious discrepancies in the types and numbers of intestinal bacteria in different primates. In addition, the stomach of *R. bieti* was similar to the rumen of ruminants, therefore, the intestinal flora of *R. bieti* was very similar to that of Gayals and Yaks, but the numbers were significantly different. For instance, *Bacteroidetes,*

Table 2. Comparison of gastrointestinal clone libraries generated from animals with different feeding habits.

Bacterial phylum	% of phylogenetic lineage[a]						
	R. bieti	**Gorilla[b]**	**Gayal[c]**	**Human[d]**	**Vegetarian woman[e]**	**Yak[f]**	**Manchurian tiger[g]**
Actinobacteria	0.64	5.3		0.2	0.5		
Bacteriodetes	4.49	1.1	1.4	47.7	6.0	33.3	
Fibrobacter	5.13					3.3	
Firmicutes	21.15	71.0	57.1	50.8	90.2	58.3	16.3
Fusobacteria				0.08			
Lentisphaerae		3.2					
Planctomycetes		1.1					
Proteobacteria	8.97			0.6	3.3		
Spirochetes	10.9	1.1	0.7			5.1	
Verrucomicrobia		17.2		0.6			
Unclassified	46.15		40.8	0.02			83.7

a: Values are proportions of phylogenetic lineages reported for each clone library.
b: Frey et al. (93 clones from 16 individuals).
c: Deng et al. (147 clones from 6 individuals).
d: Eckburg et al. Data represent the means of the results for three individuals.(11831 clones from 3 individuals).
e: Hayashi et al.（183 clones in total).
f: An et al. (194 clones in total).
g: Tu et al. (15 clones from 2 individuals).

Firmicutes and *Spirochaetes* present in the intestine of Gayals, Yaks and *R. bieti*; however, in the intestine of Gayals and Yaks, *Firmicutes* were the dominant flora. In addition, the *Firmicutes* made up a large proportion of the bacteria in the intestines of *R. bieti*, Gorillas, Gayals, Normal humans, Vegetarian women, Yaks, and Manchurian tigers. This showed that the bacteria of the *Firmicutes* were probably the original flora, which had developed with mammal phylogeny. Whereas, there were a large number of unidentified bacteria in the intestine of *R. bieti* and Gayals. Similarly, there were a large number of unidentified bacteria among the bacterial flora of the Manchurian tiger. These results reveal that the animal intestine bacterial types and numbers differ with the feeding habits of the animals (Table 2).

Conclusion

Based on 16S rRNA gene sequences, we analyzed the composition and distribution of fecal bacteria in *R. bieti*. The bacteria were classified as the following six phyla: *Firmicutes, Proteobacteria, Bacteroidetes, Fibrobacteres, Spirochaetes and Actinobacteria*. The 156 different kinds of 16S rRNA gene sequences were classified into 17 genera and 122 uncultured and unidentified bacteria. This demonstrated the diversity of intestinal bacteria in *R. bieti* and made up for the defects and deficiencies of the traditional culture methods in analyzing microflora. However, 16S rRNA gene sequence analysis is not sufficient to guarantee the clarification of the microbiological environment, because variation always occurs in the extraction of DNA, PCR and cloning selection. Therefore, the culture-independent approach should be combined with direct morphological observation and *in-situ* hybridization to improve the efficiency of research.

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