JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2013. Vol. 7(2), p. 1325-1330

Microbial Community Analysis in the Fresh Panda (*Ailuropoda melanoleuca*) Excrement of Six Months Old

Zhen Zhao[1], Li Wei[1]*, Fang Ma[1],Chun-ying Li[2] and Xin-yan Liu[1]

[1]State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin - 150 090, China.
[2]School of Energy and Civil Engineering, Harbin University of Commerce, Harbin - 150 028,China.

(Received: 07 April 2013; accepted: 28 May 2013)

Microbial community analysis of Panda (*Ailuropoda melanoleuca*) intestinal system is instructive to control Panda intestinal disease, evolve Herbivory intestinal system and research degradation flora rich in high efficiency fiber. The massively parallel sequencing technology, 454 pyrosequencing technique, was adopted to probe microbial community on Panda fresh excrement for six months. With dominant phyla belonging to *Proteobacteria* (14.29% of total bacteria), *Firmicutes* (51.80%), *Bacteroidetes* (14.19%), *Actinobacteria* (5.71%), *Synergistetes* (0.09%), *Spirochaetes* (0.37%), TM7 (0.18%), *unclassified Bacteria* (13.36%). At genera level, *unclassified "Porphyromonadaceae"*, *unclassified Lachnospiraceae*, *unclassified Clostridiaceae* , *Clostridium* XI, *unclassified Clostridiales*, *Advenella* and *unclassified Bacteria* (relative abundances > 2.0%). *Clostridium* having cellulose degradation function accounted for about 20% microbial population in panda excrement. The panda is generally from the carnivorous intestinal tract to grazing.

Keywords: *Ailuropoda melanoleuca*; 454 pyrosequencing technique; Microbial community; Cellulose decomposition bacteria.

The giant panda (*Ailuropoda melanoleuca*) is one of the most critically endangered species in the world, and began to analysis in multidisciplinary research from 1869[1].Intestinal flora are beneficial to the body, they make up the microecological balance and have a relatively stable function with the body[2-5]. The major reason of endangered panda is disease. The most serious disease is intestines problem[6,7]. There is a close relationship between intestines problem and intestinal flora disturbance. Panda is the herbivores, their main food is bamboo. However, their digestive system are still a part of carnivores characteristics, such as the shor digestive tract, lipotyphla, the rapidly passed food, the scarce enzyme and bacteria. They are hardly to digest crude fibre in bamboo. Compared with feed intake, their feces output are high, in the same time, they have the low absorptivity with the protein and carbohydrate[6].There are obvious differences between panda intestinal flora and other animals in the structure[8~10]. Zhang *et al*., found most advantage bacterium group of anaerobic of panda was different from the people and ape, but the same as pig, horse and dog.

Because the panda have a short digestive tract, as the long time eating, the content of oxygen is increasing, which cause anaerobe hard to survive. The unique microbial community structure could have a hold on the characteristics of panda digestion physiology and behavioral ecology. It is significance to protect the panda by the intestinal microecology research, and the panda excrement have the better cellulose degradation bacteria group

* To whom all correspondence should be addressed.
E-mail: weilihit@126.com

to bamboo[11,12]. The research is important to the abandoned bamboo degradation and recycling. Thus, the fresh excrement of six months old panda is used to analyse the microbial community compositon and cellulose degradation function flora.

MATERIALS AND METHODS

Sample Collection

The fresh excrement of six months old panda were used for the research. Fecal samples from wild and captive giant pandas were collected immediately after defecation, snap-frozen in liquid N_2, and shipped to the laboratory on dry ice. All samples were obtained from inside the feces, where there was no contact with soil.

Microbial community analysis

Whose genomic DNA was extracted using the Power Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions, quantified the DNA with a nanodrop spectrophotometer, and documented its yield and purity (characterized by 260/280 nm absorbance ratio). We normalized the DNA to the same concentration for amplifying use. Fragments of 16S rRNA genes containing variable V3 regions were amplified from the extracted DNA with primer sets, BSF341 broad-range forward primer 5'-NNNNNNNACTCAATCCT ACGGGAG GCAGCAG-3' and the USR534 universal reverse primer 5'- NNNNNNNACT CAATATTACCGC GGCTGCTGG-3' with 7 unique barcodesto sort each sample from the mixed pyrosequencing outcomes. Sample PCR mixtures were prepared in 50 mL volumes andincluded 1×High Fidelity PCR buffer (Invitrogen, Carlsbad, CA,USA), 0.2 mM deoxyribonucleoside triphosphates, 0.6 mM each of forward and reverse primers, 1.5 mM $MgCl_2$, 0.4 mg/mL bovine serum albumin, 5 U Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) and 100~200 ng

DNA template. Reactions were run on a GenAmp PCR System 9700 (PerkineElmer Applied Biosystems, Foster City, CA, USA) under the following cycling conditions: 5 min initial denaturationat 95! followed by 20 cycles of denaturing at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72 °C for 60 s, anda final extension at 72°C for 7 min. Negative controls (ultrapure water only) were included for the amplification reactions. After PCR amplification, the amplicons were purified by onetime gel electrophoresis/isolation and two-times purifications using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA). Amplicon pyrosequencing was performed using a 454 Life Sciences GS-FLX sequencer (Roche, NJ, USA). After a sequencing run and basecalling, we sorted the sequences by unique tags using the 454 script to separate and group all data and then trimmed the sequences using the 454 script for downstream analysis. Tag sequences were screened for quality as recommend by Huse *et al*.,[13,14].

After removing sequences of poor quality, distance matrices, cluster, rarefaction analysis and two indices of diversity (ACE and Chao) were computed using the program MOTHUR[15]. Representative sequences from each operational taxonomic units (OUT) were phylogenetically assigned with taxonomic classifications obtained from the RDP-II Classifier[16], the National Centre for Biotechnology Information (NCBI) BLAST, and the Greengenes databases.

RESULTS AND DISCUSSION

Community diversity ananlysis

The total sequences were 1085, The sequences were clustered into 590, 526, 481 and 403 operational taxonomic units (OTUs) respectively at 1%, 2%, 3%, 4% and 5% distance thresholds (Table 1).

These clusters served as OTUs for generating rarefaction curves (Fig.1) and for making calculations with the abundance-based coverage estimator ACE, the Chao richness estimations and Shannon diversities. Despite examining nearly 1085 tags identified as bacterial, the ACE, Chao and Shannon indices indicated that the bacterial community on Panda excrement was high-diversity and our sampling of bacterial richness was incomplete (Table.1). The rarefaction curves at different cutoffs described unprecedented levels of bacterial complexity for Panda excrement samples, yet none had reached the curvilinear or plateau phases (Fig.1). They maybe represent underestimates of the number of different kinds of bacteria in Panda excrement sample. It is supported by observation of significant variation among tags,

Table 1. Similarity-based OTUs and species richness estimates

Cluster distance	OTUs	Ace	Chao	Shannon	Simpson	Coverage
unique	623	4569.633	2390.9	5.715591	0.017692	0.541014
0.01	558	2547.219	1487.659	5.590481	0.018187	0.626728
0.02	482	1751.777	1090.012	5.378531	0.020149	0.703226
0.03	416	1249.535	847.8	5.188192	0.022055	0.764977
0.04	385	1008.509	719.5	5.07641	0.024618	0.79447
0.05	354	855.7295	665.3871	4.96839	0.02614	0.818433

Note: The species richness estimates were determined using the program MOTHUR as described in Methods

with closest matches to the same sequence in V3 reference database. The deeper sequencing may be required to avoid underestimation of microbial diversity in our samples.

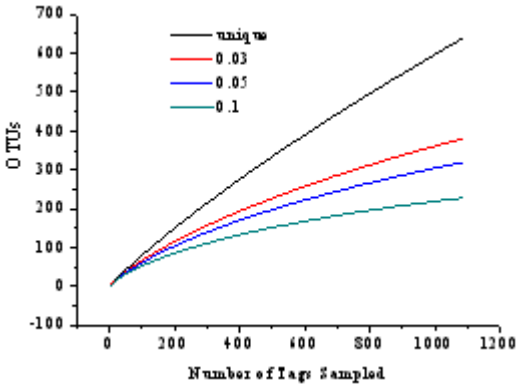
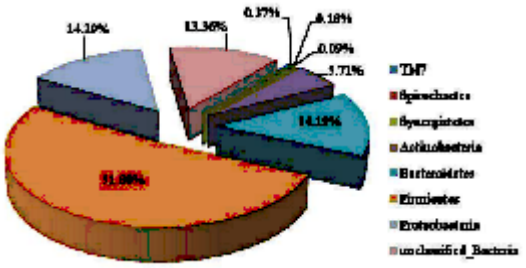


Fig. 1. Rarefaction analysis of Panda excrement sample based on pairwise distance

Rarefaction is shown for OTUs that contain unique sequences, OTUs with differences don't exceed 1%, 3%, or 5%. Pairwise sequence identity of OTUs (≥97% and ≥95%) are arbitrarily assumed to form the same species and genus, respectively.

Community composition analysis

Ribotypes were identified phylogenetically and grouped by phylumor in the case of Proteobacteria, class, using the Global Alignment for Sequence Taxonomy approach as described previously. The total frequency for a given phylogenetic group was calculated (Fig. 2), When grouped at the 97% similarity level. Of the classifiable sequences, 8 phyla were identified across the sample set (Fig. 2).



Pie charts show the Phylum distribution for taxonomically assigned tags that occurred more than 8 times; the remaining tag sequences are grouped into "Other bacteria."

Fig. 2. Phylum distribution for taxonomically assigned tags

The dominant phyla were *Proteobacteria* (including 0.37% *Deltaproteobacteria*, 2.86% *Gammaproteobacteria*, 10.23% *Betaproteobacteria*, 0.83% *Alphaproteobacteria* (Fig.3)), *Firmicutes*(including 0.74% *Negativicutes*, 1.75% *Bacilli*, 2.12% *Erysipelotrichia*, 44.33%

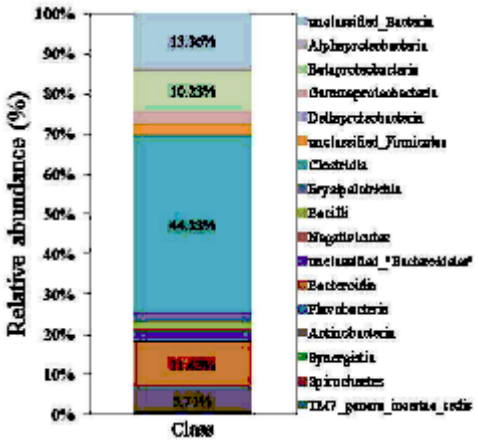


Fig. 3. Class distribution for taxonomically assigned tags

Table 2. Phylogenetic classification of the clusters (relative abundancee≥0.2%) in the Panda excrement sample

Order	Family	Genus	Relative Abundance(%)	Number of phylotypes
TM7	TM7	TM7 genera incertae sedis	0.38	4
Spirochaetales	*Spirochaetaceae*	*Unclassified Spirochaetaceae*	0.19	2
Actinomycetales	*Actinomycetaceae*	*Trueperella*	0.19	2
Actinomycetales	*Microbacteriaceae*	*Unclassified Microbacteriaceae*	0.85	9
Actinomycetales	*Micrococcaceae*	*Yaniella*	0.19	2
Actinomycetales	*Unclassified Micrococcineae*	*Unclassified Micrococcineae*	0.47	5
Actinomycetales	*Dietziaceae*	*Dietzia*	0.76	8
Actinomycetales	*Corynebacteriaceae*	*Corynebacterium*	0.47	5
Actinomycetales	*Unclassified Actinomycetales*	*Unclassified Actinomycetales*	1.61	17
Coriobacteriales	*Coriobacteriaceae*	*Olsenella*	0.38	4
Coriobacteriales	*Coriobacteriaceae*	*Unclassified Coriobacteriaceae*	0.57	6
Flavobacteriales	*Flavobacteriaceae*	*unclassified_Flavobacteriaceae*	0.47	5
Bacteroidales	*Prevotellaceae*	*Prevotella*	0.28	3
Bacteroidales	*Porphyromonadaceae*	*Parabacteroides*	0.47	5
Bacteroidales	*Porphyromonadaceae*	*Proteiniphilum*	0.66	7
Bacteroidales	*Porphyromonadaceae*	*Petrimonas*	0.28	3
Bacteroidales	*Porphyromonadaceae*	*Unclassified Porphyromonadaceae*	7.01	74
Bacteroidales	*Unclassified Bacteroidales*	*Unclassified Bacteroidales*	2.84	30
Unclassified Bacteroidetes	*Unclassified Bacteroidetes*	*Unclassified Bacteroidetes*	2.37	25
Selenomonadales	*Acidaminococcaceae*	*Succiniclasticum*	0.38	4
Selenomonadales	*Unclassified Selenomonadales*	*Unclassified Selenomonadales*	0.28	3
Lactobacillales	*Unclassified Lactobacillales*	*Unclassified Lactobacillales*	0.19	2
Bacillales	*Unclassified Bacillales*	*Unclassified Bacillales*	1.23	13
Erysipelotrichales	*Erysipelotrichaceae*	*Turicibacter*	2.09	22
Clostridiales	*Peptococcaceae*	*Peptococcus*	0.38	4
Clostridiales	*Eubacteriaceae*	*Alkalibaculum*	0.19	2
Clostridiales	*Peptococcaceae*	*Unclassified Peptococcaceae*	0.19	2
Clostridiales	*Lachnospiraceae*	*Unclassified Lachnospiraceae*	7.2	76
Clostridiales	*Ruminococcaceae*	*Ruminococcus*	0.28	3
Clostridiales	*Ruminococcaceae*	*Clostridium III*	0.28	3
Clostridiales	*Ruminococcaceae*	*Unclassified Ruminococcaceae*	3.41	36
Clostridiales	*Clostridiaceae*	*Anaerobacter*	2.56	27
Clostridiales	*Clostridiaceae*	*Unclassified Clostridiaceae 1*	5.02	53
Clostridiales	*Unclassified Clostridiaceae*	*Clostridium XI*	18.39	194

Clostridiales	*Unclassified Clostridiaceae*	*Unclassified Peptostreptococcaceae*	1.52	16
Clostridiales	*Unclassified Clostridiales*	*Unclassified Clostridiales*	5.69	60
Unclassified Clostridia	*Unclassified Clostridia*	*Unclassified Clostridia*	0.19	2
Unclassified Firmicutes	*Unclassified Firmicutes*	*Unclassified Firmicutes*	2.94	31
Deltaproteobacteria	*Deltaproteobacteria*	*Deltaproteobacteria*	0.19	2
Xanthomonadales	*Xanthomonadaceae*	*Thermomonas*	0.28	3
Xanthomonadales	*Xanthomonadaceae*	*Unclassified Xanthomonadaceae*	0.38	4
Pseudomonadales	*Pseudomonadaceae*	*Unclassified Pseudomonadaceae*	0.38	4
Pseudomonadales	*Moraxellaceae*	*Acinetobacter*	0.76	8
Oceanospirillales	*Halomonadaceae*	*Halomonas*	0.66	7
Burkholderiales	*Burkholderiaceae*	*Burkholderia*	0.19	2
Burkholderiales	*Alcaligenaceae*	*Oligella*	0.19	2
Burkholderiales	*Alcaligenaceae*	*Advenella*	6.64	70
Burkholderiales	*Alcaligenaceae*	*Unclassified Alcaligenaceae*	2.46	26
Burkholderiales	*Comamonadaceae*	*Hydrogenophaga*	0.19	2
Burkholderiales	*Comamonadaceae*	*Unclassified Comamonadaceae*	0.57	6
Caulobacterales	*Caulobacteraceae*	*Phenylobacterium*	0.19	2
Rhizobiales	*Unclassified Rhizobiales*	*Unclassified Rhizobiales*	0.28	3
Unclassified Bacteria	*Unclassified Bacteria*	*Unclassified Bacteria*	13.74	145

Clostridia, 2.86% *unclassified Firmicutes*), *Bacteroidetes*(including 0.46% *Flavobacteria*, 11.43% *Bacteroidia*, 2.30% *unclassified Bacteroidetes*), *Actinobacteria*, *Synergistetes*, *Spirochaetes*, TM7, unclassified Bacteria, representing approximately 14.29%, 51.80%, 14.19%, 5.71%, 0.09%, 0.18%, 0.37% and 13.36% of the sequences. They could be classified below the domain level, respectively.

The first 55 most abundant taxa (relative abundances ≥0.2%) in the Panda excrement data set are listed in Table 2. A distinctive feature of the Panda excrement community was the predominant (relative abundances >5.0%) groups of the genus *Unclassified Porphyromonadaceae* (relative abundance,7.01%), *unclassified Lachnospiraceae* (7.2%), *unclassified Clostridiaceae* (5.02%), *Clostridium* XI(18.39%), *unclassified Clostridiales*(5.69%), *Advenella*(6.64%) and *unclassified Bacteria*(13.74%) (Table 2). Subsequently, the subdominant taxa (relative abundances at 1.0-5.0%) included the genus of *unclassified Actinomycetales*, *unclassified Bacteroidales*, *unclassified Bacteroidetes*, *unclassified Bacillales*, *Turicibacter*, *unclassified Ruminococcaceae*, *Anaerobacter*, *unclassified Peptostreptococcaceae*, *unclassified Firmicutes* and *unclassified Alcaligenaceae.*

Because the panda intestinal tract is anerobic, the efficient cellulose degradation bacteria group have the less separation. At present, The major bacteria groups are *Clostridium lentocellu*, *Clostridium cellobioparum*, *Clostridium papyrosolvens*, *Clostridium* sp. *Cellulomonas*.sp., *Lysinibacillus* sp. *Paenibacillus* sp and *Bacillus subtilis*. The separated bacteria groups are major belonged to *Clostridium*, *Bacillus* and *Paenibacillus*. *Clostridium* include 0.28% *Clostridium* III, 18.39% *Clostridium* XI, 0.19% *unclassified Clostridiales* and some unkonwn *Clostridium*, which can constitute of 20% in panda excrement. *Bacillus* and *Paenibacillus* only are about 0.1%, which have two sequences. The other flora might exist a certain cellulose degradation bacteria group, which will be researched in the following works.

CONCLUSIONS

The research work about panda intestinal

micro ecological are focus on captive population, the research content and range are limited. It is late to start the research work about the panda intestinal microecological, especially about the wild giant panda. The microbial community are rich in panda excrement by High-throughput sequencing, which have a lot of cellulose degradation bacteria group, belonged to facultative anaerobe. The panda intestinal have the microorganism to degrade the bamboo. It is increasing clearly that giant pandas possess a suite of evolutionary adaptations for the highly specialized herbivory, which will make the panda from the carnivorous intestinal tract to grazing.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the support of the National Natural Science Funds of China (50908063), the Project supported by the Funds for Creative Research Groups of China (Grant No. 51121062).

REFERENCES

1. Jin-Chu Hu,Jie Hu. The giant panda research and progress. *Journal of xihua normal university* 2003; **24**: 253-257.
2. Zhihe Zhang,Guangxin He,Anju Zhang. The giant panda intestinal normal flora research, *Acta Theriologica sinica* 1995; **15**: 170-175
3. Chunying Li , Weiguang Li,Li Wei, Removal of Ammonia from Aaqueous Solution using Copper-Incorporated Chitosan. *Energy Education Science and Technology* Part A 2012; **30**: 223-230
4. Li Wei, Chun-ying Li, Wu Qin, Guo-chen Zheng,Zhen Zhao. Analysis of the Composition of Electrical Fixtures and Microbial Communities in Electrical Dehydrators. *Energy Education Science and Technology Part A* 2012; **30**: 231-238
5. Jie Zhang,Li Wei, Chun-ying Li, Bin-song Wang.Real-time PCR-based, quantitative detection of sulfate-reducing bacteria in oilfield sewage using a TagMan-LNA probe. *Energy Education Science and Technology Part A* 2012; **30**: 281-286
6. Xinghuai Zhou,Lujun Zeng,Zhongwu Sun. The giant panda disease death factor analysis and its protection countermeasures. Journal of Northeast Forestry University 1998; **26**: 53-56
7. Wei He, Ling Lin, Fujun Shen,Wenping Zhang ,Zhihe Zhang, Emily King, Bisong Yue.Genetic diversities of the giant panda (*Ailuropoda melanoleuca*) in Wanglang and Baoxing Nature *Reserves.Conserv Genet* 2008; **9**: 1541–1546
8. Muthu S S, Li Y, Hu J Y, Mok P Y.A hot-button societal issue: Biodegradation studies (soil burial test) of grocery shopping bags. *Energy Educ Sci Technol Part* A 2012; **29**:31-40.
9. Qian G, Qin Z, He J, Tang R. Research on runoff discharge and treatment system of expressway across water sensitive areas. *Energy Educ Sci Technol Part* A 2012; **29**: 235-246.
10. Ozdemir C, Sen N, Kalipci E.Reaction kinetics and removal of COD with treatment of tce with the synthetic wastewater in UASB reactors. *Energy Educ Sci Technol Part* A 2012; **28**: 689-698.
11. Guifang Wei, Haifeng Lu, Zhihua Zhou, Huabiao Xie, Aishan Wang, Karen Nelson3 and Liping Zhao.The Microbial Community in the Feces of the Giant Panda (*Ailuropoda melanoleuca*) as Determined by PCR-TGGE Profiling and Clone Library Analysis. *Microbial Ecology* 2007; **54**: 194-202.
12. Lifeng Zhu, Qi Wu, Jiayin Dai, Shanning Zhang, and Fuwen Wei,Evidence of cellulose metabolism by the giant panda gut microbiome. *PNAS* 2011; 1-6.
13. Bo Ding, Ya-ping Zhang, Oliver A. Ryder. Extraction, PCR Amplification, and Sequencing of Mitochondrial DNA From Scent Mark and Feces in the Giant Panda. *Zoo Biology* 1998; **17**: 499–504.
14. Huse, S.M., Huber, J.A., Morrison, H.G., Sogin, M.L., Welch, D.M.. Accuracy and quality of massively parallel DNA.pyrosequencing. *Genome Biology* 2007; **8**: R143.
15. Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M.,Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F.. Introducing MOTHUR: opensource, platform-independent, community supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* 2009; **75**: 7537-7541.
16. Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J.,Kulam-Syed-Mohideen, D.M., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M.. The ribosomal database project:improved alignments and new tools for rRNA analysis. *Nucleic Acids Research* 2008; **37**: D141-D145.