International Journal of Microbiology Research and Reviews ISSN: 2329-9800 Vol. 2 (6), pp. 110-117, July, 2013. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Diversity of cultivable actinomycetes in 6 species of herbivore feces

Yi Jiang[1]*, Xiu Chen[1], Li Han[2], Qinyuan Li[1], Xueshi Huang[2], Shumei Qiu[3], Zhongtao Ding[1] and Chenglin Jiang[1]

1*Yunnan Institute of Microbiology, Yunnan University, 650091 Kunming, Yunnan, P. R. China.
[2]Institute of Microbial pharmaceuticals, College of Life and Health Science, Northeastern University, 110819 Shenyang, P. R. China.
[3]Yunnan Wild Animal Park, 650218 Kunming, Yunnan, P. R. China.

Accepted 21 June, 2013

A large number of microbes existed in animal feces. To provide new sources for discovering new leader of drugs, the diversity of cultivable actinobacteria of animal feces have been studied. Fresh fecal samples of 6 species of herbivore feces were collected. The pure cultures of actinobacteria were isolated from these samples by using 5 media. The 16S rDNA gene sequences of 386 selected strains were analyzed, and the phylogenetic analysis was carried out. The study results shown that actinomycete diversity of each animal feces were different from each other; a total of 29 genera of the Class Actinobacteria were identified from the 6 species of animal feces; 15 genera were identified from fecal samples of Sika Deer（*Cervus nippon*）and Chinese Bamboo Rat（*Rhizomys sinensis*）respectively; and 1 new genus was discovered from Rhino（*Rhinoceros sondaicus*） feces. Members of three genera, *Streptomyces, Rhodococcus* and *Microbacterium* and Order Micrococcales were dominant groups in the 6 species of animal feces. This is a distinct feature of cultivable fecal actinomycete community differing from those in soil, plant, and marine environment. Selective isolation methods for fecal actinomycetes are described.

Key words：Actinomecete diversity, herbivores feces, microbes, diversity, species.

INTRODUCTION

Globally, the demand for new drugs is extremely urgent and extensive due to the fast extension of stubborn disease (cancer, AIDS) and common ailment (hypertension, diabetes, hyperlipidemia and skin disease), germination of new disease (Avian influenza virus) whose cause cannot be seen, and the resistance of pathogens against drug is spreading fast (Appelbaum, 2012; Jiang et al., 2008; Payne et al., 2007; Sommer et al., 2011).

Success rate of synthetic compounds developing into drug was only 0.005%, the rate of all natural product was 0.6%, while the rate of microbial natural product was 1.6% based on the latest statistics in 2012 (Berdy, 2012).

Actinomycetes (Actinobacteria) have been paid a great attention owing to their production of various natural drugs and other bioactive metabolites including antibiotics, enzyme inhibitors and enzymes for a long-term. Over 22,000 bioactive secondary metabolites (including antibiotics) were published in scientific and patent literature, and about a half of them were produced by actinomycetes. About 160 antibiotics have being applied in human therapy and agriculture now; 100-120 of them were produced by actinomycetes (Berdy, 2005). Actinomycete is still an important source for new natural drugs development. So Baltz showed a proposition of “Renaissance in antibacterial discovery from actinomycetes” (Baltz, 2008). However, the development of new drugs from actinomycetes in common habitats is more and more difficult (Jiang et al., 2009). In order to overcome these challenges, some new concepts based on genome was described, that is “new habitats, new methods, new kind, new gene cluster, new products and new use” (Goodfellow and

Corresponding author. E-mail: jiangyi@ynu.edu.cn. Tel. 86-871-65034073. Fax: 86-871-65173878.

Fiedler, 2010; Jensen et al., 2010; Jiang et al., 2009; Xu et al., 2010). In other words, novel microbial type should contain new gene cluster which could synthesize new secondary metabolites, so far as getting new type is an important premise for obtaining new compounds (Jiang et al., 2009). Many laboratories and companies focused on new actinomycete resources from new habitats, such as oceans, extreme environment and plants, for development of new drugs.

It is known early, animal intestinal and fecal microorganisms (Fecal Microbiota) have been studied for decades (Savage, 1977). A large number of microbial typies exist in animal gastrointestinal tract and feces. The intestinal microbial community was composed of 10^{13} to 10^{14} microorganism which outnumbers the somatic and herm cells by at least an order of magnitude (Simpson et al., 2002). However, the most part of these microorganisms are un-cultivable yet (Simpson et al., 2002; Daly et al., 2001; Durso et al., 2010; Gao et al., 2010; Greetham et al., 2002; Ozutsumi et al., 2005; Ritchie et al., 2008; Suchodoski et al., 2004; Suchodoski et al., 2008; Zhang et al., 2010). To explore and utilize the enormous beneficial microbial resource is a very tempting challenge. Intestinal Actinomycete, as a pathogen of human and animal, had been studied widely before (Beman, 1983). But up till now, the research work on fecal actinomycetes as a source for discovering novel drug leads is very few in the world. In our view, only learning the existence of un-cultivable actinomycetes is not enough completely, we have to isolate them into pure cultures. This is one of the new hope for getting new drug leads.

In order to get much more unknown actinomycetes from animal feces for discovering new bioactive metabolites, 6 species of herbivores were selected. The actinomycetes in feces samples were isolated, cultivated and identified. Results are reported here.

MATERIALS AND METHODS

Collection and preparation of fecal samples

6 species of herbivores were selected (Table 1). Fresh fecal samples were collected from the 6 species of herbivores which lives in the Yunnan Wild Animal Park, Kunming, China. But a part of samples of Sika deer (*Cervus Nippon***)**, in grazing state, was collected from Soltau, Germany; and some samples of Indian elephant **(***Elephas maximus*) were collected from “Elephant Valley” in Xiaomengyang National Natural Protect area, Xishuangbanna and Yunnan Wild Animal Park. 2 to 13 health individuals of each species of animal were chosen, for collecting the fresh feces, and mixed into one sample. Each mixed sample was put in sterile dish immediately, and dried for 10 days at 28 ºC. 2 g of each dried sample were pre-treated at 80 ºC for 1 hour, and respectively put in 18 ml sterile water with 0.1 % $Na_4P_2O_5$, and shaken for 60 min at 220 rpm/min. The suspension was treated by ultrasound wave for 40’ at 150W (Jiang et al., 2010), and diluted from 10^{-1} to 10^{-8}.

Isolation media for actinobacteria

Following media were used for isolating actinobacteria in fecal samples:

HV medium (Hayakawa and Nonomura, 1987).

YIM 171: Glycerol 10 g, asparagine 1 g, $K_2HPO_4{\cdot}H_2O$ 1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCO_3$ 0.3 g, Vit mixture of HV medium 3.7 mg (Hayakawa and Nonomura, 1987), agar 15 g, water 1000 ml, pH 7.2.

YIM 212: Mycose 5 g，proline 1 g，$(NH_4)_2SO_4$ 1 g, NaCl 1 g, $CaCl_2$ 2 g, K_2HPO_4 1 g, $MgSO_4{\cdot}7H_2O$ 1 g, Vit mixture of HV medium 3.7 mg, agar 15 g, water 1000 ml, pH 7.2.

YIM 47: Soy bean flour 0.2 g, lignin 1 g, Na_2HPO_4 0.5 g, KCl 1.7 g, $MgSO_4{\cdot}7H_2O$ 0.05 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, $CaCl_2$ 1 g, Vit mixture of HV medium 3.7 mg, soil extract 100 ml, water 900 ml, pH 7.5.

YIM 601: Soluble starch 10 g, casein 0.3 g, KNO_3 2 g, $MgSO_4{\cdot}7H_2O$ 0.05 g, NaCl 2 g, K_2HPO_4 2 g, $CaCO_3$ 0.02 g, $FeSO_4$ 10 mg, Vit mixture of HV medium 3.7 mg, agar 15 g, water 1000 ml, pH 7.2~7.4.

All media were supplemented with 4 groups of sterilized mixture inhibitors to inhibit fungi and Gram negative bacteria: 1, 50 mg cycloheximide, 50 mg nystatin, 20 mg nalidixic acid, 3 mg penicillin; 2, 100 mg cycloheximide, 100 mg nystatin, 40 mg nalidixic acid, 5 mg penicillin; 3, 50 mg $K_2Cr_2O_7$, 5 mg penicillin; 4, 75mg $K_2Cr_2O_7$, 5 mg penicillin for 1000 ml medium.

Plate dilution method was used for isolating actinobacteria. 0.1ml of suspensions of 10^{-5}, 10^{-6}, 10^{-7} dilutions for each sample were coated on the medium plates, and cultivated for 7 to 35 days at 28 ºC, then take count of colonies, and pick up single actinobacteria colony to slant with the same isolation medium.

Identification of pure cultivated actinobacteria

Total 1228 pure strains were isolated from 6 species of animal feces samples, 386 strains were selected for identification after throwing away the duplicates strains based on morphological and cultural characteristics for identification and taxonomy. The DNA of the 386 of pure strains was extracted for 16S rDNA analysis (Orsini and V. Romano-Spica, 2001). PCR amplification of the 16S rDNA, purification and sequence of the PCR products were done as described previously (Cui *et al.*, 2001). The forward primer F8 (8±27), 5’-GAG AGT TTG ATC CTG GCT CAG-3’ and the reverse primer (1510±1492), 5’-GGT TAC CTT GTT ACG ACT T-3’ were used. The resultant sequences were manually aligned with available sequences from public

databases. Phylogenetic trees were inferred by using the neighbour-joining (Saitou and Nei, 1987) and maximum-likelihood methods (Felsenstein, 1981). All pure cultivated strains were identified at a genus level.

RESULTS AND DISCUSSION

Selective isolation for fecal actinomycetes

The mean cfu (Colony-Forming Units) of actinomycetes for 6 species animal feces were about 10^{10}, other bacteria were 10^9, and no fungi were grown, on the isolate plates of YIM 171 medium containing inhibitors (Table 2).

A large number of Gram negative bacteria is a main problem for isolatioing fecal actinobacteria. In order to avoide Gram negative bacteria and fungi, and obtaining much more un-known actinobacteria, the optimum composition were $K_2Cr_2O_7$ 50 mg/L+ penicillin sodium 5 mg/L or mixture solution of nystatin 50 mg/L, nalidixic acid 20 mg/L and penicillin sodium 5 mg/L, most part of Gram negative bacteria were inhibited, and no fungi grown on all five medium plates.

YIM 171, YIM 212 and HV were better for isolating actinomycetes. No fungi were grown, and Gram negative bacteria were less on the 3 media plates.

Communities of Actinomycetes

Table 3 summarized the composition of fecal actinobacteria in the 6 species of herbivores.

1. Alpaca（*Vicugna pacos*）Alpaca is famous for producing finest wool. They are originally living in Andes, Chile, and were imported into Yunnan Wild Animal Park. 2 healthy individuals fecal samples were collected. 268 purified strains were isolated. 72 of them were selected after throwing away the duplicates strains based on morphological and cultural characteristics. 16S rDNA sequences of the 72 strains were determined. The phylogenetic analysis was carried out. Strains were identified at a genus level. Total 11 genera of actinobacteria were identified. They are the members of *Arthrobacter*，*Cellulosimicrobium*，*Dietzia*，*Gordonia, Isoptericola* ，*Kocuria*，*Microbacterium* ，*Nocardiopsis, Saccharomonospora*，*Rhodococcus* and *Streptomyces.* Seven genera of Gram negative bacteria, *Achromobacter*，*Ancylobacter*，*Kurthia*，*Lysobacter*，*Met hylobacterium*，*Shigella* and *Solibacillus*.

2. Common Wildebeest（*Connochaetes taurinus*）
Common Wildebeest is originally living in grasslands, one of the largest number, in Africa, and were imported in Yunnan Wild Animal Park. 4 healthy individual fecal samples were collected. 96 purified strains were isolated. 43 of them were selected for identification. Only five genera of actinobacteria, *Citricoccus*，*Microbacterium, Micrococcus*，*Rhodococcus* and *Streptomyces* were identified. These are common actinomycetes in the natural world. Other bacteria, *Stenotrophomonas* and *Methylobacterium* also identified.

3. Sika deer（*Cervus nippon*）
Sika deer is national rare animal in China, and is listed in directory at I class by China, and IUCN. It is living in the wide area of China, and Southeast Asia, Russia and Europe. Fresh fecal samples of 9 and 4 individuals were collected in Soltau, Germany and Yunnan Wild Animal Park, respectively. Total 238 strains were isolated, 83 pure strains of them were identified. They belonged to 15 genera of actinobacteria, *Actinocorallia*，*Agrococcus, Arthrobacter, Citricoccus*，*Isoptericola*，*Kocuria*，*Leucobacter, Microbacterium*，*Mycobacterium*，*Nocardiopsis*，*Promicromonospora*，*Rhodococcus*，*Salinibacterium*，*Streptomyces* and *Tsukamurella. Actinocorallia, Leucobacter, Mycobacterium, Salinibacterium* and *Tsukamurella* were isolated only from Sika deer feces. Two Gram negative bacteria, *Bosea* and *Stenotrophomonas*, were also identified.

4. Rhino（*Rhinoceros sondaicus*）
Rhino is national rare animal, and is listed in directory at I class by China, and IUCN. 2 individuals were imported from Indonesia. 202 strains were isolated. 66 strains were identified. 6 genera were identified. They are *Dietzia*，*Nocardiopsis*，*Promicromonospora*，*Rhodococcus* and *Streptomyces.* 1 strain (YIM 100770) was characterized with polyphasic taxonomic procedures (Xu et al., 2007), and was a novel genus. Related article will be published on another magazine. Seven genera Gram negative bacteria, *Achromobacter*，*Alcaligenes*，*Ancylobacter*，*Methylobacterium*，*Shigella* (a pathogen), *Solibacillus* and *Stenotrophomonas* were also identified.

5. Indian elephant（*Elephas maximus*）
Indian elephants are living in Southeast Asia, the population of them decreased rapidly, are international rare animal, and listed in directory at I class by China, and IUCN. Fresh fecal samples of 2 individuals were collected from *Elephas maximus* living in Xiaomengyang National Natural Protect area, and Yunnan Wild Animal Park respectively. Total 121 strains were isolated, and 38 of them were identified. Ten genera of actinobacteria, *Arthrobacter*，*Cellulomonas, Cellulosimicrobium*，*Leucobacter*，*Microbacterium*，*Micro monospora*，*Rhodococcus*，*Promicromonospora,Streptomy-ces*，*Verrucosispora* were identified.

Table 1. Names and related data of sampling animals.

Species names of animals	Order	Protection class in China	List of international organization*	Number of strains isolated	Number of strains identified
Vicugna pacos	*Artiodactyla*			268	72
Connochaetes taurinus	*Artiodactyla*			96	43
Cervus nippon	*Artiodactyla*	I	IUCN	238	83
Rhinoceros sondaicus	*Perissodactyla*	LPW	CITES	202	66
Elephas maximus	*Proboscidea*	I	CITES	121	38
Rhizomys sinensis	*Rodentia*			306	104
Total				1228	**386**

* **LPW**= The Law on the Protection of the wildlife of the People's Republic of China**; IUCN**=World Conservation Union; International Union for Conservation of Nature and Natural Resources**; CITES**=Convention on International Trade of Endangered Species**.**

Table 2. cfu /g* of actinobacteria on YIM 171 medium at different dilution.

Dilution times	Actinobacteria		Other bacteria	Fungi
	Mixture fecal samples of 6 species of animal in Table 1	Fecal sample of *Vicugna pacos*		
4th	2023×10^5	1324×10^5	113×10^5	0
5th	186×10^6	188×10^6	66×10^6	0
6th	112×10^7	98×10^7	11×10^7	0
7th	72×10^8	22×10^8	8×10^8	0
8th	21×10^9	7×10^9	3×10^9	0
CK**	26×10^8		511×10^8	8×10^8

cfu /g*= Colony-Forming Units
**CK=without inhibitors at dilution 7^{th}, and cannot pick up the single colony of actinomycetes.

Micromonospora and *Verrucosispora* belonging *Micromonosporaceae* were only found in this animal feces. Three genus of Gram negative bacteria, *Bacillus, Devosia* and *Sphaerobacter* were identified.

6. Chinese Bamboo Rat（*Rhizomys sinensis*）

Chinese Bamboo Rat had been reared owing production of high protein for a long time. Fresh fecal samples of 6 individuals were collected and isolated. Total 306 strains were isolated. 104 strains were identified, and belonged to 13 genera, *Agrococcus*，*Arthrobacter*，*Brachybacterium* , *Corynebacterium* , *Dietzia* , *Gordonia* , *Labedella*，*Microbacterium,* *Oerskovia*，*Rhodococcus*，*Sanguibacter*，*Streptomyces* and *Williamsia.* 6 genera, *Brachybacterium, Corynebacterium, Labedella, Oerskovia, Sanguibacter* and *Williamsia* were identified only in this species. A Phylogenetic tree of part strains isolated from *Rhizomys sinensis* and related valid published type species based on similarity of 16S rDNA sequences is shown in figure 1. 2 genera of Gram negative bacteria, *Comamonas* and *Psychrobacter* were identified.

13 genera of actibobacteria were found from Sika deer（*Cervus nippon*）and Chinese Bamboo Rat（*Rhizomys sinensis*）respectively, and were the most complex in actinomycete community in the 6 species of tested animals. Only 5 genera were identified from Common Wildebeest（*Connochaetes taurinus*）, and was monotonous. Members of 2 genera, *Streptomyces* and *Rhodocoddu* were found from all 6 animals, and were the dominant groups in fecal actinomycetes. *Streptomyces albiaxiali*, *S. cyaneofuscatus, S. griseoflavus, S. rutgersensis, Streptomyces violascens* and *S. violaceoruber,* and *Rhodococcus coprophilus*，*Rh. Corynebacterioides*，*Rh. pyridinivorans* and *Rh. zopfii* were occurred in a high frequency. Distribution of *Microbacterium* was also wide, found from five animal feces, and *Microbacterium aerolatum, M. esteraromaticum, M. foliorum, M. oxydans,* and *M. paraoxydans* were found very easy. But

Table 3. Composition of actinobacteria in 6 species animal feces.

Genus	1*	2	3	4	5	6	Genus	1	2	3	4	5	6
Actinocorallia			√				*Mycobacterium*			√			
Agrococcus			√			√	*Micrococcus*		√				
Arthrobacter	√		√		√	√	*Micromonospora*					√	
Brachybacterium						√	*Nocardiopsis*	√		√	√		
Cellulomonas					√		*Oerskovia*						√
Cellulosimicrobium	√				√		*Promicromonospora*			√	√	√	
Citricoccus		√	√				*Rhodococcus*	√	√	√	√	√	√
Corynebacterium						√	*Salinibacterium*			√			
Dietzia	√			√		√	*Sanguibacter*						√
Gordonia	√					√	*Saccharomonospora*	√			√		
Isoptericola	√		√				*Streptomyces*	√	√	√	√	√	√
Kocuria	√		√				*Tsukamurella*			√			
Labedella						√	*Verrucosispora*					√	
Leucobacter			√		√		*Williamsia*						√
Microbacterium	√	√	√		√	√							
							Total 29	*11*	*5*	*15*	*6*	*10*	*13*

1*=Vicugna pacos; 2=Connochaetes taurinus; 3=Cervus nippon; 4= Rhinoceros sondaicus; 5= Elephas maximus; 6= Rhizomys sinensis.

Table 4. Distribution of different genera in Class *Actinobacteria.*

Corynebacteriales	*Corynebacteriaceae*	*Corynebacterium*
	Dietziaceae	*Dietzia*
	Mycobacteriaceae	*Mycibacterium*
	Nocardiaceae	*Gordonia, Rhodococcus, Williamsia*
	Tsukamurellaceae	*Tsukamurella*
Micrococcales	*Micrococcaceae*	*Arthrobacter, Citricoccus, Kocuria, Micrococcus*,
	Cellulomonadaceae	*Cellulomonas*
	Dermabacteraceae	*Brachybacterium*
	Promicromonosporaceae	*Cellulosimicrobium, Isoptericola, Promicromonospora*
	Microbacteriaceae	*Agrococcus, Labedella, Leucobacter, Microbacterium, Salinibacterium*
	Cellulomonadaceae	*Oerskovia*
	Sanguibacteraceae	*Sanguibacter*
Micromonosporales	*Micromonosporaceae*	*Micromonospora, Verrucosispora*
Pseudonocardiales	*Pseudonocardiaceae*	*Saccharomonospora*
Streptomycetales	*Streptomycetaceae*	*Streptomyces*
Streptosporangiales	*Thermomonosporaceae*	*Actinocorallia*
	Nocardiopsaceae	*Nocardiopsis*
Total 6	**18**	**29**

13 genera, *Actinocorallia, Cellulosimicrobium, Corynebacterium, Labedella, Leucobacter, Micrococcus*，*Micromonospora, Oerskovia, Salinibacterium, Sanguibacter, Tsukamurella, Verrucosispora* and *Williamsia* were rare, only identified from 1 of all 6 species animals.

It is worth to notice that among 1228 strains, 386 strains had been sequenced and the 16S rDNA sequence similarities of 46 strains were below 98.5 % compared with valid published species. In other words, nearly 12%

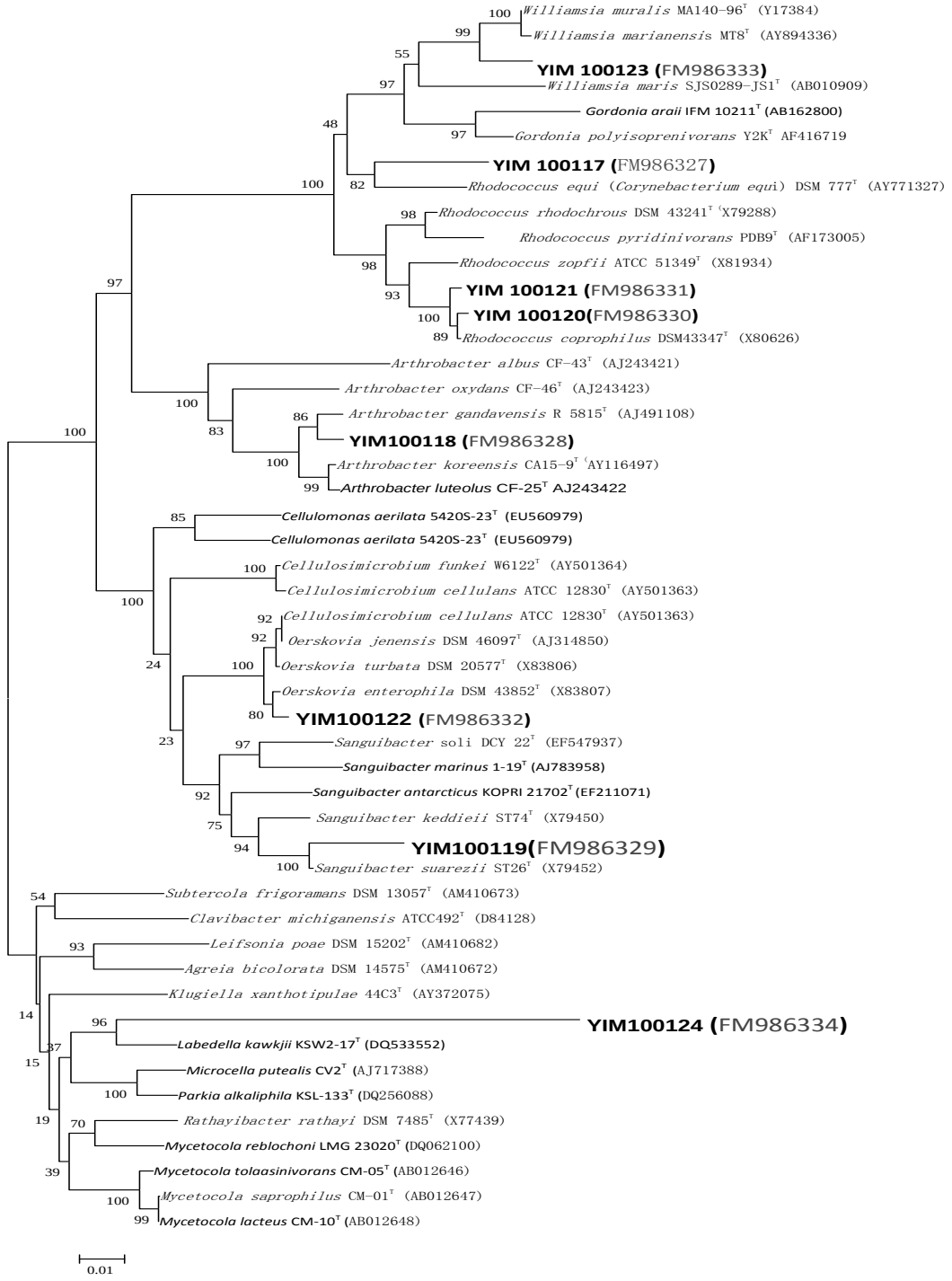

Figure 1. Phylogenetic tree of part strains isolated from *Rhizomys sinensis* and related valid published type species based on similarity of 16S rDNA sequences.

sequencing strains (386 strains) and 3.7% of isolates (1228 strains) were un-known, and they were possible novel species (Stackebrandt et al., 1994; Xu et al., 2007). One strain (YIM 100770) from Rhino（*Rhinoceros sondaicus*）feces was characterized by using polyphasic taxonomic procedures, and was considered a novel genus of actinobacteria. Related article will be published on another magazine. In 2012, one new genus of the family Micrococcaceae, *Enteractinococcus* from *Panthera tigris amoyensis* feces, was published (Cao et al., 2012). These results showed that a large of unknown actinomycetes existed in animal feces. There are several

hundred thousand of higher animal and insects in the whole world. Therefore animal is a tremendous treasure-house of un-known microbial resources.

DISCUSSION

Accoording to Whitmann (2012), Class Actinobacteria was composed by 13 Orders. Table 4 summarized the distribution of different genera from 6 species of herbivores in Class Actinobacteria.

Total 27 genera of the Class Actinobacteria were isolated and identified in 6 species of animal feces. The 29 genera belong to 6 Orders (including *Corynebacteriales, Micrococcales, Micromonosporales, Pseudonocardiales, Streptomycetales* and *Streptosporangiales*) and 18 Families of the Class *Actinobacteria*. Members of Order *Micrcoccales* were composed of 7 Families, *Micrococcuaceae, Cellulomonadaceae, Microbacteraceae, Dermabacteraceae, Promicromonosporaceae, Cellulomonadaceae* and *Sanguibactersceae,* and 16 genera. Members of Order Corynebacteriales included 4 Families, *Mycobacteriaceae, Corynebacteriaceae*, *Dietziaceae* and *Nocardiaceae*, and 6 genera. Order *Streptosporangiales* was consisted of 2 families, *Thermomonosporaceae* and *Nocardiopsaceae,* and 2 genera. Orders *Micromonosporales* had 1 family and 2 genera. Orders *Pseudonocardiales* and *Streptomycetales* had only 1 family and 1 genus respectively.

These results indicated that, first, genera *Streptomyces, Rhodococcus* and *Microbacterium* were the dominant groups in 6 species of animal feces; second, community of Order *Micrococcales* was the richest diversity. This is a distinct feature of cultivable fecal actinomycete community differing from those in soil, plant, and marine environment. Genome sizes of 2 genera, *Streptomyces* and *Rhodococcus,* are up to 9×10^7 base pairs, and are the biggest genome in actinobacteria, and some species of them contains 20 or more natural product biosynthetic gene clusters (Omura et al., 2001; Bentley et al., 2002; McLeod et al., 2006). We hypothesized that first, the function of actinomycetes in intestinal tract of hosts was mainly played by bioactive substances which were produced by members of this 2 genera; second, secondary metabolites with bioactivities produced by fecal actinobacteria, except pathogens, should be no toxic or lower toxic to their hosts. We think these are very important excellence comparing with the metabolites from other microorganisms in other habitats.

Every species of animal forms a special intestinal microbial flora (Fecal Microbiota, Intestinal Microbiota) in the long-term process of co-evolution and natural selection between microbes and its host. However, each individual, before it was born, should be sterile. But once been given birth, it taken its ancestors genetic instruction, meanwhile accepting the combined effects of living environment (climate, food, air, water etc.). The individual and the microorganisms choose each other depending on beneficial, harmful requirements, and adapt to each other, form an extremely complex, relatively stable microbial flora in time and space .The microbial flora will be changes gradually with increase of age and change of living environments. The relationships among the host and microbes, and different microbes are extremely complex and changing (Curtis and Sperandio 2011; Hooper and Gorden 2001). Of course, to learn the relationships and its variation tendency is very important. But in our view, obtaining as much unknown actinomycetes is basic purpose for discovering novel drug leader. In order this purpose, selective isolation methods for fecal actinomycetes should be continually studied and improved. Based on many tests in our laboratory, first, it is best to collect fresh fecal samples from wild animals living in original habitats (this is not easy); second, fresh samples have to dry at 25-28 ºC for 7 to 10 days; third, the dried samples have to be treated for 60 min at 80ºC, and the fecal suspension should be treated with ultrasound wave for 40’ at 150W before isolation; fourth, potassium bichromate 50 mg and 5 mg penicillin, or nystatin 50 mg, nalidixic acid 20 mg and 5 mg penicillin for 1000 ml medium, as inhibitors, have to be added in the isolation medium for inhibiting fungi and Gram negative bacteria; Fifth, in general, the samples should be diluted to 10^{-5}, 10^{-6}, and 10^{-7}, and the optimum dilution concentration for each animal fecal sample should be tested; sixth, YIM 212, YIM 171 and HV medium were better for isolation of fecal actinobacteria, and should be improved and updated; seventh, the whole length of experiment work should be carried out under strict sterile conditions for avoiding spreading pathogens.

ACKNOWLEDGMENT

This work is financed by the National Natural Science Foundation of China (No. 31270001，81072553 and 21062028), National Major scientific and technology special projects (2009ZX09302-003), National Institutes of Health USA (1P41GM086184-01A1).

REFERENCES

Appelbaum PC (2012). 2012 and beyond: potential for the start of a second pre-antibiotic era? J Antimicrob Chemother, 2012, 67(9):2062-8.

approach of research and development on pharmaceuticls from actinomycetes. J. Life Science US, 3(7):52-56.

Baltz RH (2008). Renaissance in antibacterial discovery from actinomycetes. Current Opinion in Pharmacology. 8:1–7.

Beman BL (1983). Actinomycete pathogen. In Goodfellow, M., M. Mordarski and S. T. Williams (ed.). The biology of

the actinomyces. Academic press, London, pp 457-480.
Bentley SD, Chater KF, Cerdeno-Tarraga A-M, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, CroninA, Fraser A, Goble A, Hidalgo J. Hornsby T, Howarth S, Huang CH, Larke KTL, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA (2002). Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). Nature, 417: 141-147.
Berdy J (2005. Bioactive microbial metabolites. J. Antibiot. (Tokyo) 58:1-26.
Berdy J (2012). Bioactive microbial metabolites. J Antibiot. 58, 1-26.
Cao YR, Jiang Y, Jin RX, Han L, He WX, Li YL, Huang XS, Xue QH (2011). *Enteractinococcus coprophilus* gen. nov., sp. nov., of the family *icrococcaceae*
Cui XL, Mao PH, Zeng M, Xu LH, Jiang CL (2001). *Streptomonospora salina* gen. nov., sp. nov., a new member of the family *Nocardiopsaceae.* Int. J. Syst. Evol. Microbiol. 51: 357- 363.
Curtis MM. Sperandio V (2011). A complex relationship: the interaction among symbiotic microbes, invading pathogens, and their mammalian host. Mucosal Immunology. 4: 133–138.
Daly K, Stewart CS, Flint HJ, Shirazi-Beechey SP (2001). Bacterial diversity within the equine large intestine as revealed by molecular analysis of cloned 16S rRNA genes. FEMS Microbial Ecol. 38:141-151.
Durso LM, Harhay GP, Smith TPL, Bono JL, DeSantis TZ, Harhay DM, Anderson GL, Keen JE, Laegreid WW, Clawson ML (2010). Animal-to-animal variation in fecal microbial diversity among beef cattle. Appl. Environ. Microbiol. 76: 4858-4862
Felsenstein J (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17:368- 376.
Gao RC, Huang ZX, Wu CF, Xu B, Wang XY (2010). Culture-independent analysis of microflora in gayals (*Bos frontalis*) feces. African J. Biotechnol. 9:2774-2788.
Goodfellow M, Fiedler HP (2010). A guide to successful bioprospecting: informed by actinobacterial systematics. Antonie van Leeuwenhoek. 98:119–142.
Greetham HJ, Giffard C, Hutson RA, Collins MD, Gibson G.R (2002. Becteriology of the laborador dog gut: a cultural and genotypic approach. J. Appl. Microbiol. 93:640-646.
Hayakawa M, Nonomura H (1987). Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. J. Ferment. Technol. 65:501-509.
Hooper LV, Gorden JI (2001). Commensal host-bacyrtial relationship in the gut. Science. 292: 1115-1118.
isolated from *Panthera tigris amoyensis* feces, transfer of
Yaniella fodinae Dhanjal *et al.* 2011 to the genus *Enteractinococcus* as *Enteractinococcus fodinae* comb. nov. Int J Syst Evol Microbiol ijs. 0.034249-0; published ahead of print January 6, 2012, doi:10.1099/ijs.0.034249-0
Jensen PR (2010). Linking species concepts to natural product discovery in the post-genomic era. J. Ind. Microbiol. Biotechnol. 37:219–224.
Jiang Y, Cao YR, Wiese J, Lou K, Zhao LX, Imhoff JF, Jiang CL (2009). A new
Jiang Y, Cao YR, Zhao LX, Wang Q, Jin RX, He WX, Xue QH (2010). Utrasounic treatment of soil samples for actinomycete isolation. Acta Microbiologica Sinica. 50:1094-1097.
Jiang Y, Xu P, Lou K, Xu LH, Liu ZH (2008). Problem and Countermeasure on Development of Pharmaceuticals from Actinomycete Resources. Microbiology, 35(2): 272-274.
McLeod MP, Warren RL, Hsiao WWL, Araki N, Myhre M, Fernandes C, Miyazawa D, Wong W, Lillquist AL, Wang D. Dosanjh M, Hara H, Petrescu A, Morin RD, Yang G, Stott JM, Schein JE, Shin H, Smailus D, Siddiqui AS, Marra MA, Jones SJM, Holt R, Brinkman FSL, Miyauchi K, Fukuda M, Davies JE, Mohn WW, Eltis LD (2006). The complete genome of Rhodococcus sp. RHA1 provides insights into a catabolic powerhouse. PNAS, 103(42): 15582–15587.
Omura S, Ikeda H. Ishikawa J, Hanamoto A. Takahashi C, Shinose M, Takahashi Y, Horikawa H, Nakazawa H, Osonoe T, Kikuchi H, Shibai T, Sakaki Y，Hattori M (2001). Genome sequence of an industrial microorganism Streptomyces avermitilis: Deducing the ability of producing secondary metabolites. PNAS, 98(21): 12215–12220.
Orsini M, Romano-SpicaV (2001). A micrwave-based method for nucleic acid isolation from environmental samples. Lett. Appl. Microbiol. 33:17- 20.
Ozutsumi Y, Hayashi H, Sakamoto M, IItabashi H, Benno Y (2005). Culture-independent analysis of fecal microbiota in cattle. Biosci. Biotechnol. Biochem. 69:1793-2005.
Payne DJ, Bradley J, Edwards JE, Scheld DG, Bartlett J. G (2007). Drugs from bad bugs: confronting the challenges of antibacterial discovery. Nat. Rev. Drug Discov. 6:29–40.
Ritchie LE, Steiner JM, Suchodoski JS (2008). Assessment of microbial diversity along the feline interatinal trct using 16S rRNA gene analysis. FEMS Microbial Ecol. 66:590-598.
Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406- 425.
Savage, D. C. 1977. Microbial ecology of gastrointestinal tract. Annu. Rev. Microbiol. 31:107-133.
Simpson JM, Martineau B, Jones WE, Ballam JM, Mackie RI (2002). Characterization of fecal bacterial population in canines: effecs of age, breed and dietary fiber.

Microbial Ecology. 44:186-197.
Sommer MO, Dantas G (2011). Antibiotics and the resistant microbiome. Curr Opin Microbiol. 14(5):556-563.
Stackebrandt E, Goebel BM, (1994). Taxonomic Note: A Place for DNA-DNA Reassociation and 16s rRNA Sequence Analysis in the Present Species Definition in Bacteriology. Int J Syst Evol Microbiol. 44(4), 846-849.
Suchodoski JS, Camacho J, Steiner JM (2008). Analysis of bacterial diversity in the canine, duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis. FEMS Microbial Ecol. 66:567-578.
Suchodoski JS, Ruaux CG, Steiner JM, Fetz K, Williams DA (2004). Application of molecular fingerprinting for qualitative asscssment of small-intestinal bacterial diversity in dogs. J. Clin. Mictobiol. 42:4702-4708.
Whitman WB, Goodfellow M, Kämpfer P, Busse HJ, Trujillo ME, Ludwig W, Suzuki KI, Parte A (2012). Bergey's Manual of Systematic Bacteriology: Volume 5: The Actinobacteria (Bergey's Manual/ Systemic Bacteriology (2nd Edition). Springer.
Xu LH, Li WJ, Liu ZH, Jiang CL (2007). Actinomycete taxonomy. Acedemic Press, Beijing, pp. 202-208
Xu LH, Zhang H, Zhang LP, Xue QH, Zhang LX, Xiong Z (2010). Microbial resource Science. 2nd ed. Academic Press, Beijing.
Zhang HH, Chen L (2010). Phylogenetic analysis of 16S rRNA gene sequences reveals distal gut bacterial diversity in wild wolves (*Canis lupus*). Mol. Biol. Res. 37:4013-4023.