Metagenomic Analysis of the Fecal Microbiomes of Wild Asian Elephants Reveals Microflora and Enzymes that Mainly Digest Hemicellulose^S

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To investigate the diversity of gastrointestinal microflora and lignocellulose-degrading enzymes in wild Asian elephants, three of these animals living in the same group were selected for study from the Wild Elephant Valley in the Xishuangbanna Nature Reserve of Yunnan Province, China. Fresh fecal samples from the three wild Asian elephants were analyzed by metagenomic sequencing to study the diversity of their gastrointestinal microbes and cellulolytic enzymes. There were a high abundance of *Firmicutes* and a higher abundance of hemicellulose-degrading hydrolases than cellulose-degrading hydrolases in the wild Asian elephants. Furthermore, there were a high abundance and a rich diversity of carbohydrate active enzymes (CAZymes) obtained from the gene set annotation of the three samples, with the majority of them showing low identity with the CAZy database entry. About half of the CAZymes had no species source at the phylum or genus level. These indicated that the wild Asian elephants might possess greater ability to digest hemicellulose than cellulose to provide energy, and moreover, the gastrointestinal tracts of these pachyderms might be a potential source of novel efficient lignocellulose-degrading enzymes. Therefore, the exploitation and utilization of these enzyme resources could help us to alleviate the current energy crisis and ensure food security.

Keywords: Wild asian elephant, metagenome, gastrointestinal tract, hemicellulose, enzyme resource

Introduction

The efficient utilization of lignocellulose, which makes up the largest biomass on earth, has been a focal point of research all over the world. Settlement of this aporia would help to relieve the global energy crisis and food security problem [1]. Although lignocellulose degradation enzymes have been studied for many years, there are still many unsolved problems such as single-enzyme systems, unclear degradation mechanisms, and low degradation efficiency [2–5]. Thus, the discovery of novel and efficient lignocellulose

degradation enzymes and other efficient biomass degradation factors, as well as understanding the biodegradation mechanism of biomass, is important for improving the efficiency of lignocellulose degradation.

Asian elephants (*Elephas maximus Linnaeus*) are the largest terrestrial herbivorous mammals in Asia [6]. They have a wide-ranging diet that include leaves, branches, stalks, bark, grass and roots [7]. Similar to other herbivorous mammals, such as giant pandas [8], Asian elephants lack homologues of enzymes needed for cellulose degradation [9]. Therefore, the degradation of cellulose in their food

should depend on their intestinal microflora. Herbivores can get about 70% of their energy from the decomposition of microbial polysaccharides [9–11]. However, Asian elephants are reported to have a low lignocellulose digestibility [6, 12], therefore they must eat a lot of food per day (consuming about 150 kilograms of fresh vegetation a day [13]) to meet their body needs. A high diversity of cellulose-degrading bacteria and Glycoside Hydrolase (GH) family enzymes have been found in Asian elephant feces using metagenomic sequencing analysis, suggesting that Asian elephants have a strong potential for biomass conversion [14]. However, the functional role of gut microbiota in Asian elephants, particularly in cellulose degradation, is still largely unknown.

Our survey showed that the wild Asian elephants in the Wild Elephant Valley in Xishuangbanna, China have a very unique diet. Their main foods are bamboo and wild plantains, which most herbivores choose to avoid. This suggested that the gastrointestinal microbes in the wild Asian elephants might be unique, and might contain novel enzymes for efficiently degrading plant biomass. Herein, in order to explore the diversity of the microbes in wild Asian elephants, and to reveal novel enzymes that are highly effective in degrading lignocellulose contained in their gastrointestinal tract, three wild Asian elephants who live in the same group were selected for study from the Wild Elephant Valley in the Xishuangbanna Nature Reserve of Yunnan Province, China. The fresh fecal samples from the three wild Asian elephants were analyzed by metagenomic sequencing to study the diversity of their gastrointestinal microbes and cellulolytic enzymes.

Materials and Methods

Ethics Statement

This study complied with the protocols established by the China Wildlife Conservation Association, as well as the legal requirements of China. An ethics approval from an Animal Care and Use Committee was received as per the local legislation.

Fecal Sample Collection

Fresh fecal samples from three wild Asian elephants were collected from the Wild Elephant Valley in the Xishuangbanna National Nature Reserve, Yunnan Province, China. The tracks of the wild Asian elephants were followed until they defecated, and the samples were immediately collected from the middle part of the feces. The fresh fecal samples were transported to the laboratory in liquid nitrogen and then stored at -80°C until DNA extraction.

DNA Extraction and Illumina Sequencing

Genomic DNA were extracted from the fecal samples with the Soil DNA Kit (Omega Bio-Tek, USA) following the protocol provided by the supplier (0.25 g of each fecal sample was used). The quality and quantity of the DNA were determined with a Nanodrop (ND-1000) spectrophotometer (Nanodrop Technologies, USA) and through agarose gel electrophoresis, respectively. DNA samples were stored frozen (-20°C) until use. The DNA libraries for sequencing were constructed after testing for the qualified samples followed BGI's previous work on human gut microbe metagenomic sequencing [15]. Finally, the qualified DNA libraries were conducted for cluster preparation and sequencing with Illumina HiSeq2500.

Raw Data Processing and *de novo* Assembly

Before further analysis, the raw data from Illumina HiSeq2500 sequencing was processed to: (1) remove paired reads with adapter; (2) remove paired reads with base in single-end sequencing reads sequenced to N (N shows unclear base information) over 10%; (3) remove the paired reads when the number of inferior-quality bases ($\text{sQ} \leq 5$) was over 50% of the total base number of the reads. Then the metagenomic sequence assembling software MEGAHIT [16] (<https://github.com/voutcn/megahit>) was used to assemble high-quality reads of every sample to obtain longer contigs. Statistics of reads number, length and N50 of the metagenomic sequences were conducted with the local perl script. The high-quality reads were blasted against the assembled sequences using SOAP software [17], and the reads utilization of assembly was calculated. According to these statistical data, the effect of assembly could be evaluated.

Open Reading Frames (ORFs) Prediction and Abundance Analysis

Prokaryote gene prediction software MetaGeneMark (MGM, version 2.10, default parameters <http://exon.gatech.edu/GeneMark/metagenome/Prediction/>) that is suitable for analyzing metagenomes was used to predict ORFs for the assembly results [18], and then collation and statistics of the results were conducted through local perl script. The genes predicted from all the samples were integrated and redundancy removed by using Cd-Hit software. Then the high-quality data obtained from each sample sequencing was compared with the gene sets after removing redundancy (SOAP), and the relative abundance of genes was calculated by comparing the reads number of genes, the length of genes, and the total reads number of samples [19].

Taxonomic Assignment and Functional Classification in Metagenomic Database

The known sequences from Bacteria, Eukaryota, Archaea, and Viruses in the NT database were first extracted utilizing an internal process to construct a reference database. Blastn was used to blast the nucleotide sequences from the gene group with the constructed NT database. Blastx was used to blast the amino acid

sequences from the gene group with the constructed NR database. Then according to LCA arithmetic, species annotation was conducted [15]. Based on the gene relative abundance spectrum, a relative abundance spectrum of species at all levels was constructed and statistical analysis was carried out. R software was used to perform two dimensions of sample and species abundance cluster analysis (heatmap) [19]. KRONA software (<http://sourceforge.net/projects/krona/>) was used to visualize data for species annotation of a single sample [20]. Functional classification of predicted genes was performed by BLAST (version 2.2.21) against the KEGG database [21–23] and the eggNOG (COG) database [24]. CAZymes were searched from the CAZy database [25, 26].

PUL Analysis

Prediction of PUL was carried out following a protocol similar to PULDB [27]. Using the data already generated (comparison of all protein predictions to CAZy), *SusC/SusD* pairs in all assembled contigs were first identified. Then a simple moving window consisting of five protein predictions upstream and downstream was used to search for proteins with a predicted homologue in the CAZy database. If one was found, the sliding window was moved in that direction, and the search was repeated. The search ended when no more homologues from the CAZy database were found. The PUL were drawn using BioPython [28] and GenomeDiagram [29].

Sequence Data Submission

Sequence data have been submitted to the NCBI Short Read Archive (SRA) database under accession number PRJNA492972. BioSample accessions: SAMN10141518, SAMN10141519, SAMN10141520.

Results

Sample Source

The feces were sampled from three wild Asian elephants from the same living environment with nearly the same diet in the Wild Elephant Valley in Xishuangbanna, Yunnan, China (747–1055 meters above sea level, 22°10′22.68″N, 100°51′29.39″E). The Asian elephants were characterized by Asian elephant observers in the protected areas. They were a 4-year-old calf (weaned half a year), a 10-year-old subadult (sexually immature), and a 25-year-old adult (normal). Semi-wild means that the workers in the protected area will provide water for wild Asian elephants at fixed locations, and wild Asian elephants will return to these places to drink and rest after eating in the wild.

Metagenome Sequencing, Assembly, and Annotation

DNA samples recovered from the 3 Asian elephants' feces were subjected to high throughput sequencing using Illumina Hiseq, and 16.65 Gb of data were obtained from

the 3 samples. With an insert size of 350 bp, 14.5 Gb of clean data were obtained after filtering low quality data, and the percentage of clean data was around 87% (Table S1). The clean data of all 3 samples were assembled, and the average number of contigs was 714,640, the average genome size was 514,553,566 bp, and the average of N50 was 788 bp (Table S2).

Then, the assembly was predicted for an ORF. The ORF sequence of the 3 samples was merged and redundancy was removed after the prediction. The number of genes of non-redundant gene sets was 3,054,637, of which the total length was 1,442.49 Mbp and the average length was 472.23 bp. All non-redundant genes were annotated in 4 databases (NR, COG, KEGG, CAZy), and a total of 4,962,359 predicted genes was annotated in the 4 databases, accounting for 89.41% of the total genes. About 85.25% of the gene annotations were in the NR database, 47.13% in the COG database, 27.43% in the KEGG database, and 4.07% in the CAZy database (Table S3).

Microbial Diversity in the Fecal Metagenomes of the Three Asian Elephants

The results of the NR annotation were classified. In the sequences of the annotations of all 3 samples, the proportion of bacteria was the highest, with an average accounting for 98%. The average proportion of archaea, fungi, and viruses was 1.06%, 0.61%, 0.27%, respectively, and there was no significant difference between the 3 samples at the kingdom level. The taxonomic information was further classified by phylum, class, order, family, genus, and species. The highest proportion by phylum was *Firmicutes* (average 42.9%), followed by *Bacteroidetes* (average 26.8%), *Proteobacteria* (average 8.46%), *Fibrobacteres* (average 7.68%), *Spirochaetes* (average 5.55%), and *Actinobacteria* (average 2.12%). The highest proportion by genus was *Fibrobacter* (average 12.13%), followed by *Treponema* (average 7.3%), *Prevotella* (average 7.26%), *Bacteroides* (average 6.52%), *Bacillus* (average 4.95%), *Butyrivibrio* (mean 3.76%), and *Ruminococcus* (average 2.52%). These microflorae were directly or indirectly related to lignocellulose degradation. Compared with ruminant animals such as cows [2] and reindeer [30], the gastrointestinal microflorae of wild Asian elephants were significantly diverse, suggesting that the mechanism of lignocellulose degradation by the gastrointestinal microflora in wild Asian elephants might be different.

At both of the phylum and genus levels, it was found that with the increase of wild Asian elephant age, the proportion of *Fibrobacteres* increased gradually. At the

Fig. 1. (A) Phylogenetic analysis of the three wild Asian elephant fecal samples at phylum level. (B) Relative abundance of different genera in the feces of different age elephants. (C) Based on the species annotation and abundance information of all samples at the genera level, the genus of interest (the top 35 in abundance) were selected and their abundance information in each sample was shown in the heat map.

genus level, the relative abundance of *Bacillus* also increased with the increasing elephant age (Fig. 1C). In contrast, the relative abundance of *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* at phylum level (Fig. 1A), as well as *Ruminococcus*, *Ruminiclostridium* and *Bacteroides* et al. at genus level (Figs. 1B and 1C), gradually decreased with the increasing age of the wild Asian elephants. The above microflorae were closely related to the digestion of

lignocellulose [3, 31–36]. The gradually change in abundance of these microflorae might give hints for the subsequent study of the ability of wild Asian elephants to digest lignocellulose as they grow. In addition to the top 10 genera, the relative abundance of other genera was high (Fig. 1B). However, each genus within the group had a very low relative abundance (more than 90% of them had a relative abundance of less than 0.001, Table S4). Furthermore,

Table 1. Shannon index.

Sample name	Phylum	Class	Order	Family	Genus	Species
4-year old	1.677157	2.329577	2.667309	3.909472	4.710428	5.837153
10-year old	1.776088	2.475587	2.812012	3.862292	4.631579	5.70086
25-year old	1.58855	2.108946	2.365569	3.27689	3.901922	4.791691

with the growth of the wild Asian elephants, the relative abundance of other genera gradually declined (Fig. 1B), as did the number of genera in the group (Table S4).

The alpha diversity Shannon index (Table 1) was calculated based on the relative abundance of different taxonomic classes of species to evaluate the species diversity of the samples. In the Shannon index results, the 10-year-old wild Asian elephant was higher than others in phylum, class and order, and the 25-year-old wild Asian elephant was the lowest, which indicated that the diversity of higher taxonomic grades may fluctuate in the establishment of microbial flora in the gastrointestinal tract of the wild Asian elephants. From the family level, the Shannon index of the wild Asian elephants gradually decreased with the increasing age of the wild Asian elephants, indicating that the diversity of the microflora decreased with the growth of the wild Asian elephants.

The Repertoire of Carbohydrate Active Enzymes in the Fecal Metagenomes of the Three Wild Asian Elephants

The ability of the gastrointestinal microbiomes of Asian elephants to convert lignocellulosic materials to short-chain fatty acids (VFAs) depends on their genomic composition of carbohydrate active enzymes (CAZymes). CAZy is a carbohydrate enzyme database, mainly covering Glycoside Hydrolases (GHs), Glycosyl Transferases (GTs), Polysaccharide Lyases (PLs), Carbohydrate Esterases (CEs), and auxiliary enzymes such as Auxiliary Activities (AAs) and Carbohydrate-Binding Modules (CBMs). In the present work, 267,080 genes were annotated to 6 first-class classification and 286 two-level classification of CAZys. Among them, 46.72% were predicted as novel (with an identity <40% to any CAZy database entry), while genes with identities >80% to any CAZy database entry were only 1.22% (Table S5). GHs accounted for 50.93% of the first-class classification, followed by GTs (22.87%), CBMs (18.71%), CEs (6.08%), PLs (1.29%), and AAs (0.13%). In the two-level classification, we obtained 117 GH families, 67 GT families, 60 CBM families, 15 CE families, 17 PL families, and 10 AA families, with GT2, GH2, CBM50, GH3 and GT4 being the top 5 CAZymes (Table S6).

Glycoside hydrolases (GHs). The GH enzymes encoded by the three metagenomes in this study fell into 117 families, and 65% of the identified GHs showed 50% or lower identity with known proteins, of which GH2 and GH3 were the most prevalent. Eighteen families (GH13, GH43, GH39, GH109, GH31, GH92, GH95, GH78, GH23, GH5, GH94, GH36, GH33, GH20, GH28, GH1, GH73, and GH53) accounted for 50% of the total sequences (Table S6).

The putative GHs were classified into cellulases, endo-hemicellulases, xyloglucanases, debranching enzymes, and oligosaccharide degrading enzymes, according to their main substrates and mode of action (Table S7). When compared with CAZymes from the gut metagenomes of other herbivores [2, 14, 30, 37, 38], the wild Asian elephants in this project had a richer diversity of GH family enzymes. The GH families in this study were rich in GH2, GH3, GH5, GH13, GH23, GH31, GH39, GH43, GH78, GH92, GH95 and GH109, which was more diverse than that reported [14]. The GH2, GH3, GH5, and GH43 family genes are also widely found in other intestinal microbial genomes of herbivores such as giant pandas [37], cows [2], reindeer [30], and camels [38]. However, the GH58, GH68, GH75, GH90, GH96, GH100, GH101, GH104, and GH132 found in this work were rarely reported. These families of enzymes are not present in the feces of bovine [2], moose [39] and camel [38], elephant [14], or even biogas fermenters [40, 41]. Such abundance and diversity of GH family hydrolases in the wild Asian elephant suggested that their gastrointestinal tracts might be sources of novel lignocellulose degrading enzymes.

Carbohydrate-binding modules (CBMs). The CBM domains promote the binding of CAZymes to their carbohydrate substrates, thereby affecting the catalytic activity of the enzymes. The most abundant CBMs that interact with cellulose in the metagenomes of the gut microorganisms in the wild Asian elephants were CBM37, CBM13, CBM2 and CBM6 (Table S6), which was similar to that in camel [38] and bovine rumen [42], and Asian elephants raised in zoos [14]. As for the xylan-bound domain, CBM35, CBM9, CBM54 and CBM4 were abundant, which was also similar to that in the above-mentioned samples, as well as anaerobic biogas fermentation. The proportion of CBM54 was higher than others in this study and was more similar to that in anaerobic biogas fermentation [40]. There were also some less studied CBM families such as CBM51, CBM57, CBM61, CBM62 and CBM66, among which CBM57 accounted for a much larger proportion in the wild Asian elephants than other samples. The rich diversity of CBM families in the wild Asian elephants also suggested that the gastrointestinal tracts of the wild Asian elephants might be potential sources of novel enzymes.

Carbohydrate esterases (CEs), polysaccharide lyases (PLs) and auxiliary activities (AAs). The CEs represent a class of esterases responsible for removing the -O or -N ester-bonded modifications to mono-, oligo-, and polysaccharides, facilitating the action of glycosyl hydrolases

on complex polysaccharides [43]. The CEs predicted in this work belonged to 15 families, of which CE11 and CE1 were the most frequently present in the gene sequences. CE1, CE2, CE3, CE4, CE6, CE7, CE8, and CE12 were relatively rich (Table S6), which were known to play a key role in hemicellulose degradation by enhancing the dissolution of xylan [43]. In addition to CE5, CE13 and CE16, other CE family members were more abundant than that in the camel rumen [38]. The richness and diversity of the CE family enzymes in the wild Asian elephants might be beneficial to the degradation of hemicellulose.

PLs are capable of recognizing uronic acid residues in polysaccharides, catalyzing beta-terminal elimination reactions by releasing sugar residues with unsaturated double bonds at non-reducing ends, ultimately forming unsaturated oligosaccharides. These oligosaccharides often have novel physiological activities while overcoming many of the drawbacks of macromolecular polysaccharides themselves. Therefore, PLs have broad application prospects in scientific research, food industry, medicine and agricultural production. There were 17 families of PLs predicted in this work. Among them, PL1, PL9, and PL11 were the most frequently occurring. Meanwhile, PL6, PL7, PL8, PL10, PL12, PL17, and PL21 accounted for about 20% (Table S6). PLs were not found in camels and other projects, probably because there is little of them. Thus, the gastrointestinal tracts of the wild Asian elephants have the potential for mining PLs.

The AAs associated with lignin degradation were also abundant in the samples of the wild Asian elephants. AA4 was the most abundant AA family (accounting for 50%), while that in camel rumen, Asian elephants raised in zoos, and bioreactors AA6 were the most abundant [38]. However, AA6 accounts for only 20% in this work (Table S6). Since AA4 contains vanillyl-alcohol oxidases (VAO) that catalyze the conversion of a wide range of phenolic compounds bearing side chains at the para-position of the aromatic ring, the ability of the wild Asian elephants to degrade lignin might be higher, and their degrading enzymes might turn out to be special.

Analysis and Comparison of Lignocellulose Degrading Enzymes

Endoglucanase (E.C. 3.2.1.4), Cellobiohydrolase (E.C. 3.2.1.91), and beta-glucosidase (E.C. 3.2.1.21) were on the annotation of cellulose degradation, corresponding CAZy members were GH1, GH3, GH5, GH6, GH8, GH9, GH12, GH44, GH45, GH48, GH51, GH74, GH116, GH124, and among them the most abundant top 5 were GH3, GH5,

GH1, GH51, and GH9. The enzymes that degrade pectin were annotated with pectinesterase (E.C. 3.1.1.11), endopolygalacturonase (E.C. 3.2.1.15), exopolygalacturonase (E.C. 3.2.1.67), pectate lyase (E.C. 4.2.2.2), exopectate lyase (E.C. 4.2.2.9), and pectin lyase (E.C. 4.2.2.10), corresponding CAZy members were CE8, GH4, GH28, PL1, PL3, PL9, PL10, with CE8 and GH28 being the top 2 families. Endo-1,4-beta-D-xylanases (E.C. 3.2.1.8), beta-D-xylosidases (3.2.1.37), acetylxylan esterases (E.C. 3.1.1.72), alpha-L-arabinofuranosidases (E.C. 3.2.1.55), alpha-D-glucuronidases (E.C. 3.2.1.139), ferulic acid esterases (E.C. 3.1.1.73), p-coumaric acid esterases (E.C. 3.1.1.-) were on the annotation of hemicellulose degradation, the families of the corresponding CAZys were CE1, CE2, CE3, CE4, CE5, CE6, CE7, CE12, CE13, CE15, GH1, GH2, GH3, GH5, GH8, GH10, GH11, GH30, GH39, GH43, GH51, GH52, GH54, GH62, GH67, GH116, GH120, and the top 5 families were GH2, GH3, GH43, GH39 and GH5. The relative abundance of lignin hydrolases was too low to be shown (Table S8).

Taxonomic Origin of the Predicted CAZymes

Sequence-based CAZy and NR database annotations suggested that there were 42 phyla of microbes in the taxonomic origin of CAZymes in the fecal samples of the wild Asian elephants. The taxonomic distribution of the predicted CAZymes was shown in Fig. 2. An inspection of the cellulase sequences (families GH1, GH3, GH5, GH9, and GH51, etc.) indicated that the contribution of *Firmicutes*, *Bacteroidetes*, and *Fibrobacteres* in the cellulase repertoire of the wild Asian elephants' metagenomes was 25.8%, 16.0%, and 7.3%, respectively (Fig. 2C). At the genus level, the *Fibrobacter*-encoding cellulase genes were the most abundant (Fig. 2H). This indicated that *Fibrobacter* was the most important cellulose-degrading bacterium in the wild Asian elephants. An analysis of endohemicellulases (families GH43, GH39, GH5, GH28, and GH53, etc.) revealed that > 45% of these enzymes were produced by *Firmicutes* and *Bacteroidetes*, accounting for more than 75% of the hemicellulase-matching gene sequences (shown in Fig. 2D), which suggested that the wild Asian elephants might have strong ability to digest hemicellulose. The origins of the genes encoding carbohydrate-debranching enzymes (families GH2, GH3, GH43, GH51, and GH78, etc.) were shown in Fig. 2E. A similar distribution was observed with respect to the genes encoding oligosaccharide-degrading enzymes (families GH1, GH3, GH5, GH13, GH31, and GH43, etc.) (Fig. 2F). As for the sequences encoding pectinase (families CE8 and GH28, etc.), they were largely from *Firmicutes* (23.9%, Fig. 2G).

Fig. 2. (A) Phylum level taxonomic distribution of the predicted and unclassified CAZymes; (B) Genus level taxonomic distribution of the 39% predicted CAZymes. Phylum level taxonomic origin of the predicted and unclassified cellulases (C), endohemicellulases (D), carbohydrate-debranching enzymes (E), oligosaccharide-degrading enzymes (F), and pectinase (G). (H) Genus level taxonomic origin of the predicted cellulases.

The data also showed that >40% of the gene sequences that encode CAZymes had no species source at the phylum

level, and >60% have no species source at the genus level (Figs. 2A and 2B). This implied that there were a large

number of potentially unknown lignocellulose-degrading enzymes in the gastrointestinal tracts of the wild Asian elephants, which might be the new enzymes we need.

The large contribution of Firmicutes and Bacteroidetes to the coding of CAZymes suggested that in the gastrointestinal tracts of the wild Asian elephants, *Firmicutes* and *Bacteroidetes* might work together to degrade lignocellulose, since members of Bacteroides and Firmicutes are known as effective lignocellulose degradants [42]. This was consistent with the fact that the diet of the wild Asian elephants in the wild mainly includes materials with high levels of lignocellulose, such as bamboo and wild plantain.

Discussion

Asian elephants (*Elephas maximus* Linnaeus) are the largest terrestrial herbivorous mammals in Asia. They are divided into calves (under 5 years old), subadults (between 5 and 15 years old) and adults (over 15 years old) [44, 45]. The three samples in this study were distributed at different ages. At both of the phylum and genus levels, the

relative abundance of their microflorae gradually changed with the age of the wild Asian elephants. Among them, *Fibrobacteres* and *Bacillus* were closely related to the digestion of lignocellulose. This suggested that wild Asian elephants might become more capable of digesting lignocellulose as they age. Though analysis of more samples in each age group was needed to statistically test the relationship, it nevertheless provided a hint for the change of ability of wild Asian elephants to digest lignocellulose as they grow.

It is reported that Asian elephants in a German zoo have common dominant bacteria: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fibrobacteres*, and *Actinobacteria* [14]. However, in this study the microbial diversity was more abundant. In addition, there were big differences in gut microbial abundance among wild Asian elephants and other herbivores, such as pandas [37], cows [2], and reindeer [30]. The structure of the gut microbial community in panda is fairly unitary, mainly containing 79% of *Firmicutes* and 21% of *Proteobacteria*. Cow and reindeer have similar structures of gut microbial community, though *Bacteroidetes*

Fig. 3. Physical map of selected putative polysaccharide utilization loci (PULs) from the fecal samples of the three wild Asian elephants.

The NA represents other genes of non *SusC*/*SusD*/*cazy*.

take a larger portion than *Firmicutes*. From the perspective of rumen and non-ruminal herbivores, the structures of microbial community in Asian elephants and pandas were similar, with *Firmicutes* occupying a larger proportion than *Bacteroidetes*. The larger proportion of *Firmicutes* in panda might be due to the fact that the main food of pandas is bamboo. It is reported that bamboo is more difficult to digest than normal lignocellulose, and giant pandas must have a strong ability to digest hemicellulose to supply energy for their bodies [46]. Thus, we speculated that Asian elephants, like giant pandas, might digest hemicelluloses more efficiently than digest cellulose.

Accordingly, the diversity and abundance of hemicellulose-degrading related hydrolases in the wild Asian elephants of all ages were higher than that of cellulose-degrading related hydrolases (Table S8). The CAZy data combined with Sus-like genes (SusC or SusD) clustering revealed 17 identity groups (polysaccharide utilization loci, PULs) in this study, which encodes all the components required for binding, transportation, and depolymerization of a specific sugar chain structure. Similar to the analysis of novel polysaccharide utilization loci in *Castor canadensis* fecal microbiomes [47], in this study a majority of the enzymes (GH3, GH36, GH39, GH43, GH63, GH92, GH95, GH110,

Fig. 4. The abundance of phylum- and genus-level microbial communities corresponding to hydrolytic enzymes that degrade cellulose, pectin and hemicellulose in the 3 fecal samples.

(A) The abundance of phylum-level microbial communities corresponding to hydrolytic enzymes that degrade cellulose. (B) The abundance of phylum-level microbial communities corresponding to hydrolytic enzymes that degrade pectin. (C) The abundance of phylum-level microbial communities corresponding to hydrolytic enzymes that degrade hemicellulose. (D) The abundance of genus-level microbial communities corresponding to hydrolytic enzymes that degrade cellulose. (E) The abundance of genus-level microbial communities corresponding to hydrolytic enzymes that degrade pectin. (F) The abundance of genus-level microbial communities corresponding to hydrolytic enzymes that degrade hemicellulose.

CE15 etc.) in PULs targeted hemicellulose degradation, only GH9 was responsible for cellulose degradation, GH28 and GH32 mainly degraded pectin (Fig. 3). These implied that wild Asian elephants might mainly digest hemicellulose to supply energy.

Furthermore, at both the phylum and genus levels, by comparing the abundance and diversity of the corresponding bacteria of hemicellulose-degrading enzymes with those of cellulose-degrading enzymes, it was not difficult to find that the abundance and diversity of hemicellulose-degrading bacteria were very high (Fig. 4), which also implied that wild Asian elephants might provide more energy for their bodies by degrading hemicellulose than by degrading cellulose.

Finally, the repertoire of carbohydrate active enzymes and taxonomic origin of the predicted CAZymes in the fecal metagenomes of the three wild Asian elephants showed low identity with the CAZy database entry, and about half of the CAZymes had no species source at the phylum and genus level. This suggested that the wild Asian elephant gastrointestinal tracts might contain a large amount of novel lignocellulose-degrading enzymes, which could be a potential source of novel efficient lignocellulose-degrading enzymes. The exploitation and utilization of these enzyme resources could help us to alleviate the current energy crisis and ensure food security.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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