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Diversity and novelty of the gut microbial community of an herbivorous rodent (*Neotoma bryanti*)

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Abstract Mammalian herbivores host diverse microbial communities to aid in fermentation and potentially detoxification of dietary compounds. However, the microbial ecology of herbivorous rodents, especially within the largest superfamily of mammals (Muroidea) has received little attention. We conducted a preliminary inventory of the intestinal microbial community of Bryant's woodrat (*Neotoma bryanti*), an herbivorous Muroidea rodent. We collected woodrat feces, generated 16S rDNA clone libraries, and obtained sequences from 171 clones. Our results demonstrate that the woodrat gut hosts a large number of novel microorganisms, with 96% of the total microbial sequences representing novel species. These include several microbial genera that have previously been implicated in the metabolism of plant toxins. Interestingly, a comparison of the community structure of the woodrat gut with that of other mammals revealed that woodrats have a microbial community more similar to foregut rather than hindgut fermenters. Moreover, their microbial community was different to that of previously studied herbivorous rodents. Therefore, the woodrat gut may represent a useful resource for the identification of novel microbial genes involved in cellulolytic or detoxification processes.

Keywords 16S rDNA · Detoxification · Intestinal microbes · Mammalian herbivore · *Neotoma bryanti*

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1 Introduction

Mammalian herbivores face several challenges when consuming plant material as a primary food source. First, up to 60% of plant biomass may be comprised of indigestible cell wall material such as cellulose, hemicellulose, and lignin, which are refractory to digestion by endogenous mammalian enzymes (Karasov and Martinez del Rio 2007). Additionally, plants produce a wide array of defense chemicals known as plant secondary metabolites (PSMs) to discourage consumption by herbivorous animals (Rosenthal and Berenbaum 1991). These chemicals affect mammalian herbivores through various negative physiological effects such as reducing the efficiency of digestion or altering homeostasis (Dearing et al. 2005).

In order to persist on a poor quality diet, many herbivores maintain a consortium of symbiotic microbes (Van Soest 1994). The primary role of these microbes involves digestion and fermentation of food; the process by which organic polymers such as cellulose are hydrolyzed and converted into short-chain fatty acids that are easily absorbed by the host (Karasov and Martinez del Rio 2007). Additionally, gut microbes are hypothesized to play a role in the detoxification of plant secondary metabolites (Freeland and Janzen 1974). Ideally, detoxification would take place in a pregastric chamber, such that biotransformation of plant secondary metabolites could occur prior to

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absorption in the small intestine (Freeland and Janzen 1974). Indeed there are examples of microbial detoxification of PSMs in domesticated ruminants (Jones and Megarrity 1986; Smith 1992; McSweeney and Mackie 1997). However, microbial detoxification of PSMs is only beginning to be investigated in wild mammalian herbivores (Hiura et al. 2010; Sundset et al. 2010)

To date, the most extensive effort to characterize the microbial diversity of the mammalian gastrointestinal tract involved analysis of 16S rDNA sequences from fresh feces of 59 species of non-human mammals (Ley et al. 2008). In this study, dietary strategy, gut morphology, and taxonomic order all strongly influenced the microbial community structure. Within the 33 species of herbivores included in this study, a distinct difference existed in the microbial communities of foregut and hindgut herbivores. Notably, the order Rodentia, which is the most diverse and abundant mammalian order (Musser and Carleton 2005), was underrepresented in this study. Only two herbivorous rodents were sampled, the capybara (*Hydrochoerus hydrochaeris*) and naked-mole rat *(Heterocephalus glaber),* both of which are outside the largest superfamily of mammals, Muroidea (Musser and Carleton 2005).

In this study, we aimed to generate the first inventory of the microbial diversity of an herbivorous rodent species within Muroidea, and to place the data within the context of the other mammalian herbivores inventoried by Ley et al. (2008). To conduct our initial inventory, we chose Bryant's woodrat *(Neotoma bryanti)*. Populations of Bryant's woodrat in the Sonoran desert readily consumes creosote bush (*Larrea tridentata*), which contains high levels of phenolics, a class of PSMs (Hyder et al. 2002), and indigestible material (Meyer and Karasov 1989). To deal with the high fiber content, woodrats, like many rodents, maintain large hindgut fermentation chambers, known as ceca (Fig. 1). Interestingly though, woodrats also have highly segmented stomach morphology (Fig. 1; Carleton 1973). This structure is unique from the capybara and naked-mole rat surveyed by Ley et al. (2008), both of which have simple, uncompartmentalized stomach morphology (Stevens and Hume 2004; Kotze et al. 2010). It has been hypothesized that the segmentation in *N. bryanti* may facilitate the growth microorganisms in the pregastric stomach (see Carleton 1973 for discussion), but this has never been sufficiently tested.

Our goal was to conduct a preliminary inventory of the intestinal microbes of an herbivorous rodent within the superfamily Muroidea, and to compare this diversity with the known microbial community structures of other herbivorous mammals as described in Ley et al. (2008). To accomplish this, we inventoried the microbial community of two randomly chosen individuals of *N. bryanti*. This sample size is on par with the interspecific study of microbial diversity conducted by Ley et al. (2008) where most species were represented by a single fecal sample (average=1.6; mode=1 individuals per species of herbivorous mammal). We recognize that a sample size of two individuals does not capture the variability in microbial communities of this species. However, this study was not intended to be a comprehensive intraspecific comparison, but rather to cast the results in an interspecific comparison with a sample size similar to Ley et al. (2008). We hypothesized that *N. bryanti* has a microbial community similar to previously studied hindgut-fermenting rodents. Additionally, based on the represented microbial taxa, we can speculate on the functional significance of the woodrat intestinal microbial community. In addition, we wished to determine the potential for future use of herbivorous rodents in studies investigating microbial detoxification of plant diets. This may be of interest to researchers, as rodents within Muroidea are often small and amenable to laboratory conditions, making them easier to study than captive or wild ruminants.

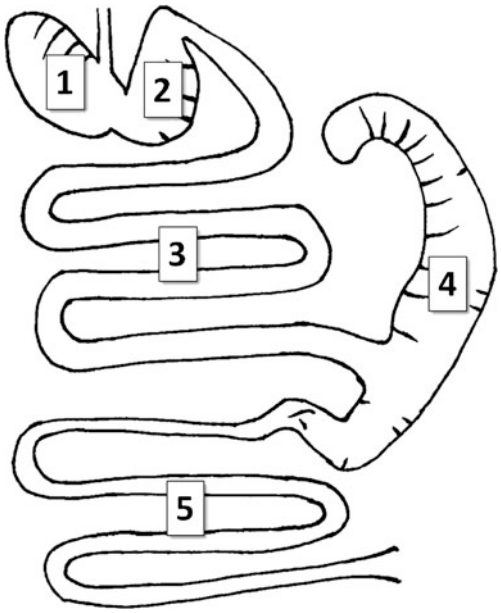

Fig. 1 Diagram of the woodrat gastrointestinal tract. Numbered segments represent 1. Pregastric stomach 2. Gastric stomach 3. Small intestine 4. Cecum 5. Large intestine

2 Methods

Animal collection and housing Neotoma bryanti were collected in April 2009, outside Palm Desert, CA (33°68′ N, 116°36′ W) in the Sonoran desert. Woodrats were transported to the University of Utah Department of Biology Animal Facility and housed in individual cages (48×27×20 cm) under a 12:12-hr light:dark cycle, with 28°C ambient

temperature and 20% humidity. Woodrats were maintained on a diet of high-fiber rabbit chow (Harland Teklad formula 2031) for 7 months prior to experimentation. This chow is nutritionally similar to a natural diet consumed by woodrats, but lacks PSMs (Karasov 1989; Meyer and Karasov 1989). The captive conditions experienced by the woodrats are comparable to those of zoo animals studied in Ley et al. (2008). Additionally, Ley et al. (2008) found that host species, rather than environmental effects, largely influences microbial diversity, as evidenced by two baboon individuals (one from Namibia, one from St. Louis Zoo), which had very similar microbial communities. Likewise, two red pandas housed at different zoos had similar microbial communities (Ley et al. 2008). All procedures were approved under University of Utah's Institutional Animal Care and Use Committee protocol number 10–01013.

Dietary treatment Prior to fecal collection for microbial inventories, PSMs from creosote were added to the maintenance diet to better mimic the woodrat's natural diet. We used creosote resin because creosote bush is a common shrub in this habitat; captured woodrats readily consumed creosote clippings added to their cage. Although the actual amount of creosote consumed in the wild was unknown for this population, the diet of *Neotoma lepida*, the sister taxa to *N. bryanti,* consists of greater than 75% creosote bush in the wild (Karasov 1989). We gradually increased the level of creosote resin in the diet over a 10-day period to permit adaptation to the new compounds. Animals were fed a diet consisting of 1% creosote resin for 2 days, 2% for 3 days, 4% for 3 days, and 6% for 2 days. Individuals did not reduce food intake or lose body mass throughout this feeding schedule.

To extract resin, creosote leaves were collected from trapping sites and frozen at −20°C prior to resin extraction. Resin was extracted by soaking leaves in acetone (1:6, wet leaf mass:volume solvent) for 45 min. Solvent and resin were filtered (Whatman filter paper grade 1) to remove large particles and evaporated using a rotovap until the resin was highly viscous, at which point it was transferred to a vacuum pump for 48 h to remove any remaining acetone. Extracted resin was stored at −20°C prior to use.

Creosote diet was prepared by dissolving resin in a volume of acetone equal to 25% of the dry weight of ground rabbit chow to which it was added. Acetone was evaporated in a fume hood, and complete evaporation was confirmed gravimetrically.

Fecal collection We collected feces from two randomly chosen individuals in order to conduct a preliminary microbial inventory with which to compare to other herbivorous mammals. During the final day of feeding, the bedding was completely changed every hour for 5 h. At each changing, feces were collected and immediately placed on ice and later frozen at −80°C.

DNA isolation and sequencing Feces were thawed on ice and several pellets from each individual were ground with a sterilized mortar and pestle. Fecal material (~25 mg) was incubated with 180 μL enzymatic lysis buffer at 37°C for 30 min to degrade the cell walls of gram-positive bacteria. The lysis buffer consisted of 20 mM Tris·Cl, pH 8.0, 2 mM sodium EDTA and 1.2% TritonX-100 dissolved in deionized water, with 20 mg/ml lysozyme added just before use. DNA was then extracted from fecal material using a QIAGEN DNeasy Blood and Tissue Kit. Bacterial 16S rDNA sequences were PCR amplified using universal primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR products were purified using a GeneJET Gel Extraction kit (Fermentas) and cloned using a TOPO TA cloning kit, following the manufacturer's instructions (Invitrogen Corp.). The success of the cloning procedure was validated by restriction enzyme analysis of recombinant plasmid DNA from several clones.

Plasmid DNA was isolated by an automated procedure in the high throughput sequencing facility at the University of Utah Department of Human Genetics. We isolated plasmid DNA from 144 clones per individual with the goal of obtaining a number of high quality sequences that was within the 33–370 sequences per sample obtained in the analysis of herbivorous mammals by Ley et al. (2008). Moreover, each woodrat sampled needed to exceed the 10–40 microbial sequences deemed sufficient for community comparisons (Lozupone et al. 2010). After overnight growth, plasmid DNA was isolated by alkaline lysis, RNase treatment and ethanol precipitation. The cloned 16S rDNA genes were then sequenced using the primer 27F and ABI BigDye Terminator v3.1 cycle sequencing reagents, followed by capillary electrophoresis and detection with an ABI 3730xl DNA analyzer.

Sequence analysis Potential base-calling errors were removed by trimming sequences to remove bases with a PHRED quality score <20. Sequences were aligned to known 16S rDNA sequences using the Near Alignment Space Termination (NAST) algorithm on the GreenGenes website (http://greengenes.lbl.gov) (DeSantis et al. 2006b; DeSantis et al. 2006a). Bellerophon3, with default parameters, was used to identify and remove any chimeric sequences (Huber et al. 2004). Chimera-free sequences were deposited in GenBank and are available under accession numbers HQ700956-HQ701126. Aligned and chimera-free sequences from Ley et al. 2008 were also obtained from the GreenGenes database for comparative analysis. Only sequences from herbivorous mammals were

selected for our interspecific data analysis (n=53 samples from 33 species; Ley et al. 2008), as well as microbial sequences from cattle feces (n=3 samples; Ozutsumi et al. 2005). Although there were other rodents in the complete Ley et al. (2008) data set that are more closely related to woodrats than the naked mole rat or capybara, these species (Prevost's squirrel, rat) were classified by Ley et al. (2008) and others as omnivores (Marshall et al. 2009; Landry 1970). Therefore these two species were not included in the analysis of herbivorous species.

We used NCBI BLAST (Altschul et al. 1997) to determine the percentage of sequence identity between the woodrat gut 16S rDNA sequences and other microbial 16S rDNA sequences in GenBank. It is widely accepted that the cut-offs for sequence identity at the genus and species level are 95% and 97.5%, respectively (Stackebrandt and Goebel 1994; Ludwig et al. 1998). Woodrat microbial sequences were also classified using the Ribosomal Database Project (RDP), with the standard minimum support threshold of 80% (Wang et al. 2007).

To compare the microbial communities of the woodrat with those of herbivorous mammals reported by Ley et al. (2008), we constructed a phylogenetic tree containing all sequences. To decrease the number of sequences used in creating the tree, microbial communities of each mammalian individual were dereplicated using FastGroupII to group sequences with 97% sequence similarity (Yu et al. 2006). A phylogenetic tree was created using FastTree with Gamma20 likelihoods (Price et al. 2010).

Diversity and community structure of mammalian intestinal microbes were determined and compared using Fast UniFrac (Hamady et al. 2010). This program measures phylogenetic beta diversity between environmental samples (in this case, different hosts) with the UniFrac distance metric. UniFrac distances are based on fractions of shared branch lengths between environmental samples using the phylogenetic tree created from all 16S rDNA sequences (Lozupone and Knight 2005). We calculated UniFrac distance metrics between the microbial communities of all herbivorous rodent species (woodrat, capybara, naked-mole rat) and all other herbivorous mammals using unweighted trees (to investigate differences in community membership) and weighted trees (community structure) (Lozupone and Knight 2005). Average distance metrics for each non-rodent species were calculated first so that each mammal species, and not each individual, represented an independent unit. We then compared average distance metrics from rodent species to the communities of foregut vs. hindgut fermenting mammals. These averages were compared using a Student's t-test with JMP 8. To visualize similar communities, UniFrac was used to conduct Principal Coordinates Analysis (PCoA) using an abundance-weighted tree.

3 Results

Microbial diversity of the woodrat gut We obtained a total of 171 high quality, chimera free sequences (77 and 94 per individual) from the feces of *N. bryanti*, with an average sequence length of 1,045 bp. When comparing woodrat individuals to each other, roughly two-thirds of the sequences from each individual were unique at a 97% sequence identity cut-off (Table 1). The microbial phylum Firmicutes was dominant, comprising an average of 94.0±4.6% of sequences for each individual. The remaining sequences belonged to the phylum Bacteroidetes (4.8±4.8%) and the uncultivated phylum TM7 (1.2±0.1%). Approximately half of the sequences were identified at the genus level using RDP (~56%), resulting in nine genera being identified (Table 1). The majority of sequences that could not be identified at the genera level were identified as members of the families Lachnospiraceae and Ruminococcaceae.

BLAST analysis revealed that most sequences (~96%) shared <97.5% sequence identity with their closest relative in the GenBank database, indicating that the majority represented novel species (Fig. 2). Additionally, 38% of sequences shared <95% sequence identity with their closest relative in the GenBank database, indicating that they represented novel genera to the woodrat gut.

Table 1 Identification of 16S rDNA sequences from woodrat feces. Bold taxa represent phyla and italicized represent genera

Animal Taxon	303 % of sequences	310 % of sequences
Firmicutes		
Lactobacillus	24.6	25.6
Ruminococcus	5.2	12.8
Coprococcus	6.5	3.2
Anaerotruncus	1.3	–
Allobaculum	–	20.2
Acetivibrio	–	2.1
Unclassified	61.0	25.5
Bacteroidetes		
Barnesiella	–	3.2
Tannerella	–	1.1
Unclassified	–	5.3
TM7		
TM7 genera incertae sedis	1.3	1.1
Total sequences	77	94
% of sequences unique to sample[a]	63.7	69.7

[a] Sequences were deemed unique if they had <97% sequence identity with any sequences from the other woodrat sample. Inventories from Ley et al. 2008 were not used in this analysis

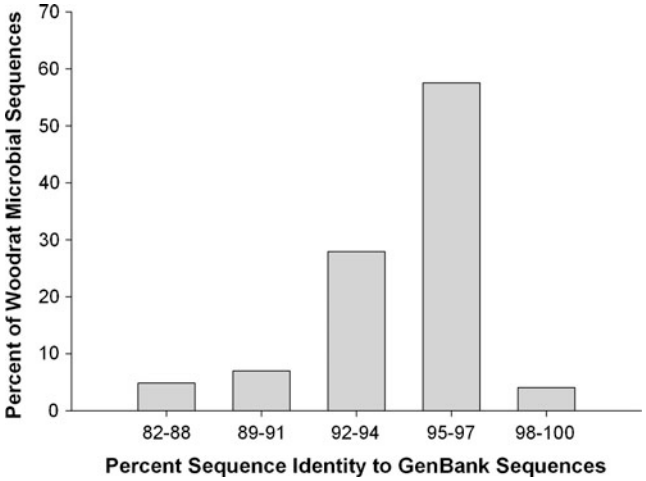

Fig. 2 Distribution of 16S rDNA sequences with varying percent identity to previously reported GenBank sequences. Most sequences represent novel genera or species

Comparison of woodrat microbial diversity with other mammals UniFrac distance metrics were calculated comparing rodent species with all other mammals (Table 2). UniFrac distances comparing Bryant's woodrat and foregut fermenting mammal communities were significantly less than hindgut fermenting mammalian communities, when investigating both community membership and community structure (Students *t*-test: $p=0.034$ and 0.049 respectively; Table 2). UniFrac distances for other rodent species (capybara and naked-mole rat) showed no differences between foregut and hindgut fermenting mammals in terms of community membership or structure. UniFrac distances were larger for Bryant's woodrat compared to other rodent species in all comparisons (Table 2). This is supported by the principle coordinates analysis that shows woodrats cluster far from most mammals, including the other two species of herbivorous rodents (Fig. 3).

Table 2 Mean UniFrac distances from rodent microbial communities to communities of foregut or hindgut fermenting mammals. Values in parentheses represent SE. Sequences for capybara and naked molerat were obtained from Ley et al. 2008. *P*-values in last column represent *t*-test results from horizontal comparisons of UniFrac distances to foregut versus hindgut communities within a given rodent species. Letters following mean values represent Tukey's HSD results from vertical comparisons between rodent species of a given analysis (gut type and non-weighted/weighted distance). Means not sharing the same letter are significantly different at an $\alpha=0.05$

	Foregut unifrac distance	Hindgut unifrac distance	*P*-value
Community membership (unweighted Unifrac distances)			
Bryant's woodrat	0.899 (0.003)^a	0.910 (0.005)^a	0.034*
Capybara	0.812 (0.006)^b	0.805 (0.013)^b	0.664
Naked molerat	0.839 (0.005)^c	0.844 (0.008)^c	0.326
Community structure (weighted Unifrac distances)			
Bryant's woodrat	0.686 (0.014)^a	0.720 (0.014)^a	0.049*
Capybara	0.606 (0.029)^b	0.627 (0.113)^b	0.315
Naked molerat	0.535 (0.021)^b	0.540 (0.025)^c	0.440

4 Discussion

A pervasive interest in the function and ecology of gut microbes has resulted in the cultivation of 10–15% of domestic rumen microbes (Hespell et al. 1997), in comparison to less than 1% of all microbes (Rappe and Giovannoni 2003). There has been a recent call to conduct further research on the microbial ecology of wild ruminants (Kobayashi 2006), and presumably information on other wild herbivores would be beneficial also. In this study we added to the database of Ley et al. (2008) through a preliminary investigation into the intestinal microbial diversity of an herbivorous rodent within the superfamily Muroidea. We found three microbial phyla, and several genera with well studied metabolic processes. Interestingly, Bryant's woodrats were found to harbor a novel microbial community in comparison with other mammals, and this novel community shares more similarity with that of foregut fermenting rather than hindgut fermenting mammals.

We recognize that our microbial community inventories ($n=171$ sequences) originate from two host individuals and from fecal samples. However, the intent of this study was to conduct a preliminary inventory of a new rodent species, and interpret these results in the context of other mammals. The novelty of the microbial species discovered within this species will not be diminished with additional intraspecific sequencing effort. The major aim of this work was to provide data for comparison to a recent interspecific study

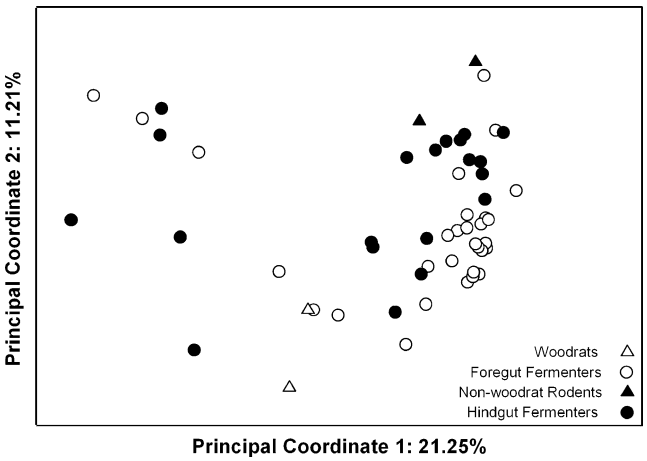

Fig. 3 UniFrac-based Principal Coordinates Analysis of mammalian gut communities using an abundance-weighted phylogenetic tree. All non-woodrat mammal data was obtained from Ley et al. 2008

(Ley et al. 2008). The number of hosts sampled herein was on par with the study conducted by Ley et al. (2008), which sampled the feces, largely from 1 individual per host species of herbivorous mammal. This study was not intended to describe the variability within a species under different conditions or in different regions of the gut.

In our current study, we found that the microbial community of the woodrat gut is comprised mostly of members of the phyla Firmicutes and Bacteroidetes. These are common community representatives in amniote hosts as evidenced by their dominance in the guts of other mammals (Ley et al. 2008), birds (Lu et al. 2003), and reptiles (Costello et al. 2010). The woodrat gut also contains members of the phylum TM7, which has not yet been culitvated in a laboratory setting. TM7 is also present in the guts of some other mammals, comprising between 0% and 2% of all 16 rDNA sequences (Ley et al. 2008).

The known metabolic properties of genera identified in our study provide insights into the putative function of the woodrat gut microbial community. For example, the predominant genus was *Lactobacillus,* a genus known to ferment a wide range of simple sugars. However, *Lactobacillus* does not ferment plant fiber, as it is unable to utilize complex polysaccharides such as cellulose (Barrangou et al. 2006). We also identified the presence of *Ruminococcus*, a genus known to degrade plant polysaccharides into short-chain fatty acids (Forsberg et al. 1997).

Identified genera in the woodrat gut may also play a role in the metabolism of plant secondary compounds. For example, isolates of the genus *Coprococcus* are able to catabolize and utilize phloroglucinol, a phenolic, as a sole carbon substrate (Patel et al. 1981). Also, although the majority of the metabolic properties of TM7 are unknown, members of this phylum are known to be able to degrade toluene, a common aromatic hydrocarbon (Luo et al. 2009). Additionally, members of *Lactobacillus* are able to degrade plant polyphenolics, and seem to be essential for the ability of the Japanese wood mouse (*Apodemus speciosus*) to feed on polyphenolic-rich acorns (Shimada et al. 2006). It is believed that the microbial enzymes involved in aromatic hydrocarbon degradation may have broad substrate specificity (Alvarez and Vogel 1991; Bauer and Capone 1988), and thus members of *Coprococcus,* TM7, and *Lactobacillus* present in the woodrat gut might play a role in degrading the polyphenolics present in creosote leaves. However, further investigations such as metagenomic sequencing, culture of phenolic-degrading microbes, and comparisons of microbial communities across diet treatments are required to rigorously establish whether these microbes biotransform dietary toxins.

The metabolic functions of many of the microbes present in the woodrat gut, though, cannot even be inferred due to the high amount of novelty. According to percent sequence identity taxonomic classification, roughly 38% of the sequences represent novel genera that have not previously been isolated from any environmental sample. This result is noteworthy given the large amount of sequences of mouse (*Mus musculus*) and rat (*Rattus norvegicus*) intestinal microbes deposited in GenBank from various studies (Rawls et al. 2006; Brooks et al. 2003). It is interesting that *N. bryanti* is in the same superfamily as these laboratory rodents, yet does not seem to share microbial species with them. The novel microbes in the woodrat gut may represent a unique community for the mining of novel genes associated with detoxification and cellulolytic processes. For example, a recent metagenomic inventory of the Tammar wallaby (*Macropus eugenii*) foregut uncovered deeply divergent bacterial lineages, as well as microbial polysaccharide utilization genes unique to those found in termites or bovine rumina (Pope et al. 2010).

Comparisons between the gut microbial communities of woodrats and those of other mammals revealed that the woodrat microbial community shares more similarity with that of foregut fermenting mammals in terms of both community membership and structure. Indeed, Fig. 2 shows the microbial community of one woodrat individual superimposed over that of the red river hog (*Potamochoerus porcus)*. Moreover, woodrats cluster closely with the babirusa (*Babyrousa babyrussa)* and springbok *(Antidorcas marsupialis).* Finally, woodrats have a microbial community unlike that of the other herbivorous rodents sampled. These results are surprising given that woodrats have a fermentative cecum in their hindgut (Skopec et al. 2008). It has been suggested that differences between the microbial communities of hindgut and foregut fermenting mammals are a result of the fact that the fermentative microbes of foregut fermenting mammals are emptied into the gastric stomach and digested (Ley et al. 2008). Therefore, future investigations into the microbial community of the woodrat pregastric stomach are warranted. Additionally, further mammalian microbial inventories may illustrate whether this microbial community structure is typical of other herbivorous rodents with segmented stomachs (e.g. voles [*Microtus spp.*]; Stevens and Hume 2004)

Our goal was to conduct a preliminary inventory of the microbial diversity of the woodrat gastrointestinal tract. Interestingly, this study shows that the gut microbial community of herbivorous woodrats is novel, and more similar to other foregut fermenting mammals. It is believed that the number of genes encoded by the gut microbial community outnumbers that of the host by 100-fold (Ley et al. 2006), and thus the woodrat gut may represent a unique community for the identification of novel genes associated with detoxification and cellulolytic processes. Additionally, the fact that woodrats have a community unlike that of other rodents highlights the importance for investigating the

microbial communities of other mammals with unique dietary preferences or digestive physiologies. Woodrats hold promise for a tractable system in which to investigate the function of gut microbial communities under different environmental conditions such as across the regions of the gut or under different dietary treatments.

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