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Comparison of the gut microbiotas of laboratory and wild Asian house shrews (*Suncus murinus*) based on cloned 16S rRNA sequences

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Abstract: The Asian house shrew, *Suncus murinus*, is an insectivore (Eulipotyphla, Mammalia) and an important laboratory animal for life-science studies. The gastrointestinal tract of *Suncus* is simple: the length of the entire intestine is very short relative to body size, the large intestine is quite short, and there are no fermentative chambers such as the forestomach or cecum. These features imply that *Suncus* has a different nutritional physiology from those of humans and mice, but little is known about whether *Suncus* utilizes microbial fermentation in the large (LI) or small (SI) intestine. In addition, domestication may affect the gastrointestinal microbial diversity of *Suncus*. Therefore, we compared the gastrointestinal microbial diversity of *Suncus* between laboratory and wild *Suncus* and between the SI and LI (*i.e.*, four groups: Lab-LI, Lab-SI, Wild-LI, and Wild-SI) using bacterial 16S rRNA gene library sequencing analyses with a sub-cloning method. We obtained 759 cloned sequences (176, 174, 195, and 214 from the Lab-LI, Lab-SI, Wild-LI, and Wild-SI samples, respectively), which revealed that the gastrointestinal microbiota of *Suncus* is rich in Firmicutes (mostly lactic acid bacteria), with few Bacteroidetes. We observed different bacterial communities according to intestinal region in laboratory *Suncus*, but not in wild *Suncus*. Furthermore, the gastrointestinal microbial diversity estimates were lower in laboratory *Suncus* than in wild *Suncus*. These results imply that *Suncus* uses lactic acid fermentation in the gut, and that the domestication process altered the gastrointestinal bacterial diversity.

Key words: domestication, Eulipotyphla, insectivore, microflora, symbiosis

Introduction

The Asian house shrew, *Suncus murinus* (Soricidae, Eulipotyphla, Mammalia), has a wide distribution from Southeast to West Asia [17]. In Japan, presumptively

native populations are distributed in the Nansei Islands (the Amami, Okinawa, Miyako, and Yaeyama Islands), whereas introduced populations had distributed in Kyushu [29]. This species has been domesticated as a laboratory bioresource since the 1970s [32, 35, 36], and

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Supplementary Table: refer to J-STAGE: <https://www.jstage.jst.go.jp/browse/exanim>



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several strains captured from different areas have been developed [18, 34]. As a laboratory animal, the domesticated Asian house shrew is called “*Suncus*”. It has been used in various fields of science due to its unique characteristics compared with laboratory rodents, including as models of emesis [7, 54], for *in vivo* motilin studies [42, 51, 52], and for dental studies [20, 57].

These unique characters of *Suncus* owe a great deal to its evolutionary background. The Asian house shrew belongs to the phylum Eulipotyphla (in superorder Laurasiatheria), one of the oldest mammalian groups. Eulipotyphla appeared before the Cretaceous–Paleogene boundary (ca. 66 million years ago) and diverged from the superorder Euarchontoglires (humans, mice, rats, and rabbits) well before the Cretaceous–Paleogene boundary [1, 10]. Animals in this group possess many primitive mammalian morphological characters. The members of Eulipotyphla are insectivorous and lack fermentative chambers such as the cecum and forestomach in their gastrointestinal tracts, meaning they have a very simple gut.

Interestingly, the ratio of the length of the gastrointestinal tract to body size in *Suncus* is very small compared with that in other mammalian taxa [25, 49]. Although the small and large intestines are not perfectly separated because *Suncus* lacks a cecum, the ratio of the length of the large intestine to that of the small intestine is quite small compared with that in other mammals [14, 25]. Histologically, the large intestine extends only 1 cm from the anus [14, 58]. Therefore, *Suncus* has unique gastrointestinal tract characteristics: a very simple structure (with no fermentative chambers), very short total intestine length, and very short large intestine length. Because these features imply that *Suncus* has a different nutritional physiology, we wondered what microbes inhabit their intestines.

The symbiotic microbiota of the mammalian gastrointestinal tract provide the host mammals with valuable substances (e.g., enzymes) for nutritional symbiosis [review in 15, 43]. Recently, it was found that the symbiotic microbiota inhabiting the mammalian gastrointestinal tract play substantial roles in maintaining host homeostasis [3, 19, 37, 50]. The diversity of the gut microbiota has been shaped not only by the host evolutionary background but also by its food habits [5, 26, 27, 30]. Ley *et al.* revealed that the diversity of the fecal microbiota in mammals is higher in herbivores than in omnivores and is lowest in carnivores, based on a net-

work-based analysis of cloned bacterial 16S rRNA gene sequences from 59 mammalian species [26]. This implies that food habits are an important factor in gut microbiota. However, little is known about the gut microbiota of insectivores such as *Suncus*.

In addition, domestication, a prolonged process of captivity over generations, may alter the mammalian gastrointestinal tract microbiota. There are reports that captivity altered the gut microbiota of certain mammalian species [5, 22, 24, 28, 53]. In mammals, the intestinal microbiota is generally transferred vertically from dam to offspring via the birth canal [2, 38]. Furthermore, laboratory animals are usually housed under very stable and clean conditions to avoid infectious diseases and experimental errors that arise from environmental factors, which may prevent the horizontal acquisition of certain bacterial species from the environment. Therefore, we hypothesized that the gut microbiota of laboratory *Suncus* would differ from that of wild *Suncus*.

In this study, we collected the intestinal contents of the small and large intestines of wild and laboratory *Suncus* and compared the microbiota using libraries of cloned bacterial 16S rRNA gene sequences. The main purposes of this study were to characterize the gut microbiota of *Suncus* and compare the microbiota between the small and large intestines and between laboratory and wild *Suncus* to determine how domestication has affected gut microbial diversity. This new knowledge may increase the utility of laboratory *Suncus* and shed light on the evolution of mammalian gut microbial diversity.

Materials and Methods

Animals

We used six (three female and three male) laboratory *Suncus* (the standard KAT strain; originally, 1 male and 2 females were collected from Kathmandu, Nepal in 1991 and domesticated at Nagoya University [34]) animals maintained at the Faculty of Agriculture, University of Miyazaki. The animals were housed in accordance with the standard protocol for laboratory *Suncus* [32, 33] and given fish feed for trout (Masu-Deluxe HI, Nosan, Yokohama, Japan) and tap water *ad libitum*. In addition, 5 (2 females and 3 males) wild *Suncus* individuals were captured in Nishihara in Nakagami District in Okinawa Prefecture using Sherman traps in February 2017. The contents of their small and large intestines were removed

using autoclaved scissors and tweezers into gamma-ray-sterilized 2.0-ml tubes and stored at -80°C until DNA extraction. The small or large intestines of some of the laboratory *Suncus* were empty; we ultimately analyzed samples from three small intestines and four large intestines.

All of the protocols used in this study were approved by the Animal Experiment Committee of the University of Miyazaki (Permission No. 2010-512, 2016-520). The capture of wild animals in Okinawa Prefecture was approved by the Okinawa Prefectural Government (Permit No. Dai 28 Honcho 114, Dai 28 Honcho 115).

Laboratory experiments

Total DNA was extracted from samples using ISOFE-CAL for Beads Beating (Nippon Gene, Tokyo, Japan), according to the manufacturer's protocol. To construct the 16S rRNA gene libraries, we followed the protocols described in Ley *et al.* [26]. In brief, bacterial 16S rRNA genes (ca. 1300 bp) were amplified separately for individuals and intestinal sections using the following universal primer pair: 8F (5'-AGAGTTTGATCCTGGCTC-AG-3') and 1391R (5'-GACGGGCGGTGWGTRCA-3'). The PCR conditions were 94°C for 2 min, followed by 20 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 2 min, with a final 20-min extension at 72°C . The amplified PCR products were pooled for each experimental group: i.e., the large intestines of laboratory *Suncus* (Lab-LI), the small intestines of laboratory *Suncus* (Lab-SI), the large intestines of wild *Suncus* (Wild-LI), and the small intestines of wild *Suncus* (Wild-SI). The PCR products were then gel-purified using a GENECLEAN II kit (Funakoshi, Tokyo, Japan) and cloned using the pGEM-T Easy vector system (Promega, Tokyo, Japan). From each library, more than 196 colonies containing cloned amplicons were processed for sequencing. The plasmid inserts were sequenced using vector-specific primers, a Big Dye Terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA), and a genetic analyzer (Model 3730, 3130, Applied Biosystems).

Data analyses

The data analyses were performed using the 16S rRNA gene analysis software package Mothur v. 1.40.3 [44]. First, the obtained cloned sequences (ca. 1300 bp; 179 from Lab-LI, 184 from Lab-SI, 215 from Wild-LI, and 226 from Wild-SI samples) were aligned as SILVA alignments using align.seqs [40] with SILVA reference file

release 132 (released in June, 2018), and then putative chimeras were checked using chimera.slayer [13] with the SILVA-based alignment of the template file. The SILVA reference and template files were both obtained from the Mothur web site (https://mothur.org/wiki/Silva_reference_files). As a result, 3, 10, 20, and 12 sequences were identified as putative chimeras from the Lab-LI, Lab-SI, Wild-LI, and Wild-SI samples, respectively, and removed from the dataset. The final dataset included 759 sequences (176 Lab-LI, 174 Lab-SI, 195 Wild-LI, and 214 Wild-SI sequences), which were deposited in the DNA Database of Japan under accession numbers LC463303 to LC464061. The classifications of the 759 cloned sequences were estimated using classify.seqs with SILVA reference file release 132.

Next, pairwise genetic distances were calculated using dist.seqs (set countends=F and output=lt options) following filter.seqs (set vertical=T option). Then, the data were clustered into operational taxonomic units (OTUs) with 98.7% similarity thresholds (according to Stackebrandt and Ebers [48]) using the cluster command for OTU-based analyses. From these clustered data, we estimated Good's coverage [12], the Shannon (H') [45] and Simpson (1-D) [47] biodiversity indexes, and the Chao1 [4] species richness estimator using summary.single to compare the alpha diversity estimates among the 4 samples. We also ran rarefaction.single to execute rarefaction analyses of the number of OTUs observed and the coverage index. Finally, the similarity indexes for community membership (Jaccard similarity coefficient) and community structure (θ -YC [59]) among the four samples were estimated using dist.shared following the make.shared command. We also estimated quantitative (weighted UniFrac) and qualitative (unweighted UniFrac) beta diversity measurements based on the phylogenetic tree constructed using clearcut [8].

The correlation between the numbers of sequences and OTUs was analyzed using the Rcmdr package v2.5-1 [11] with R v3.5.1 [41].

Results

We obtained 176 (Lab-LI), 174 (Lab-SI), 195 (Wild-LI), and 214 (Wild-SI) chimera-free 16S rRNA sequences from the small and large intestine contents of laboratory and wild *Suncus*. Using a 98.7% similarity threshold, the cloned sequences clustered into 12 (Lab-LI), 11 (Lab-SI), 46 (Wild-LI), and 39 (Wild-SI) OTUs (Table

1). We found no significant correlation between the numbers of sequences and OTUs (adjusted $R^2=0.541$, $P=0.167$). The Good's coverage for all samples was very high (89.2–99.4%; Table 1), and the rarefaction curve reached a plateau (Fig. 1A), indicating that our sequencing effort was sufficient to obtain perspectives on each microbial community. However, the rarefaction curve analyses of the number of OTUs implied that unsequenced bacteria still existed (Fig. 1B). The results indicate that we successfully revealed the major members of the gastrointestinal microbial communities of laboratory and wild *Suncus*, but we still cannot reject the existence of minor species. All of the alpha diversity estimates were much lower for laboratory *Suncus* than for wild *Suncus* (Table 1).

In all samples, Firmicutes was the predominant bacterial phylum detected (59.5–87.4%; Fig. 2). Within Firmicutes, Streptococcaceae was the major family detected in laboratory *Suncus* (53.1% in Lab-LI and 89.5% in Lab-SI), whereas it was less prevalent in wild *Suncus* (19% in Wild-LI and 38.1% in Wild-SI) (Fig. 3). Of note, all of the Streptococcaceae sequences from laboratory *Suncus* were identified as *Streptococcus* spp., whereas

most from wild *Suncus* were identified as *Lactococcus* spp. (Supplementary Table 1). Peptostreptococcaceae was a second major family group detected within Firmicutes: 34.6% in Lab-LI, 44.8% in Wild-LI, and 39.6% in Wild-SI. Most of these sequences were identified as *Paeniclostridium* spp. (which were formerly classified

Table 1. The microbial diversity and species richness of the large (LI) and small (SI) intestinal contents from laboratory *Suncus murinus* and wild *Suncus murinus* based on 16S rRNA gene libraries

	Laboratory		Wild	
	LI	SI	LI	SI
Number of clones	176	174	195	214
Number of OTUs (98.7%)	12	11	46	39
Good's Coverage (%)	97.7	99.4	89.2	93.9
Shannon index (H')	1.75	1.22	3.22	3.05
Simpson index (1-D)	0.77	0.49	0.94	0.93
Chao1	15	11	69.3	54.6

Number of clones=number of cloned sequences after removing putative chimeras; Number of OTUs (98.7%)=number of operational taxonomic units (OTUs) detected with 98.7% similarity thresholds [48]; Good's coverage=read depth of each library [12]; Shannon and Simpson indexes=diversity indexes [45 and 47, respectively]; Chao1=species richness estimator [4].

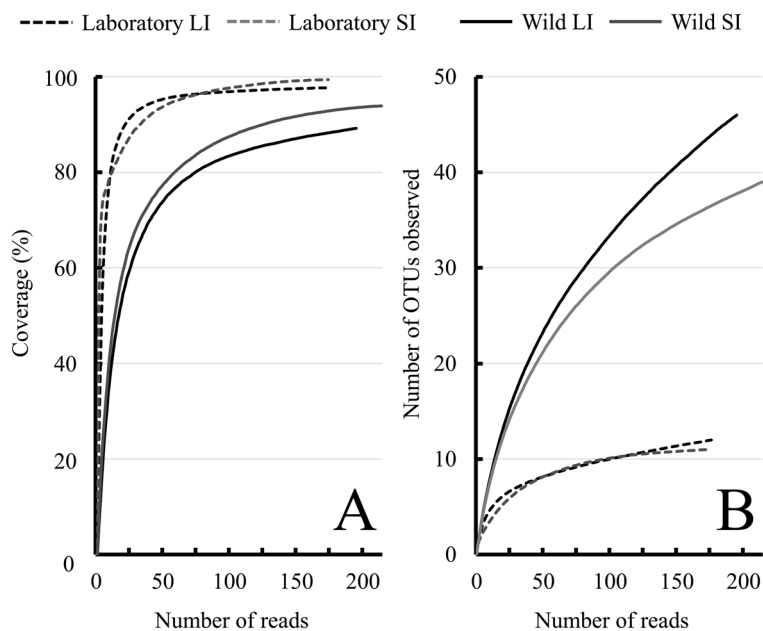


Fig. 1. Rarefaction curves for Good's coverage (A) and the number of OTUs observed (B) for bacterial 16S rRNA gene sequences in the large (LI) and small (SI) intestinal contents from laboratory *Suncus murinus* and wild *Suncus murinus* with a cut-off threshold of 98.7% similarity. Good's coverage indicates read depth of each library [12], and the number of OTUs observed were detected with 98.7% similarity thresholds [48].

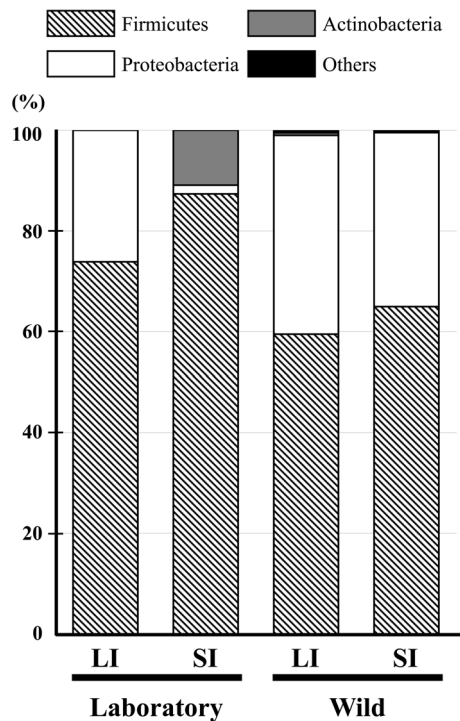


Fig. 2. Relative abundance of bacterial 16S rRNA gene sequences in the large (LI) and small (SI) intestinal contents from the laboratory *Suncus murinus* and wild *Suncus murinus* classified at the phylum level.

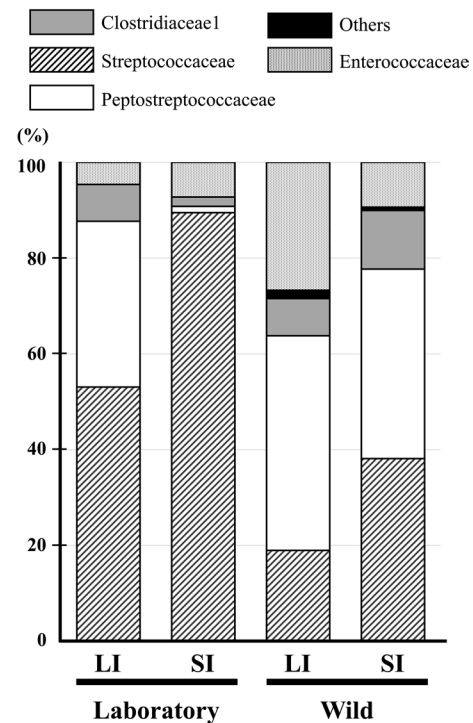


Fig. 3. Relative abundances of bacterial 16S rRNA gene sequences in the large (LI) and small (SI) intestinal contents from the laboratory *Suncus murinus* and wild *Suncus murinus* classified at the family level within Firmicutes.

in the genus *Clostridium*) and *Romboutsia* spp. (Supplementary Table 1). Other groups detected were Clostridiaceae (all sequences were identified as *Clostridium [sensu stricto]*) and Enterococcaceae (including *Enterococcus* spp. and *Vagococcus* spp.) (Supplementary Table 1). These bacterial groups are common in animal gastrointestinal tracts and feces.

Proteobacteria was another major group detected in Lab-LI, Wild-LI, and Wild-SI (26.1–39.5%; Fig. 2), most of which were facultative anaerobes in the family Enterobacteriaceae, another major group in animal gastrointestinal tracts. Of these sequences, the *Escherichia/Shigella* group was found in all samples, whereas *Plesiomonas* spp., which is a pathogenic group, was found only in wild *Suncus* (Supplementary Table 1).

The Lab-SI samples, but not the other samples, contained a relatively high percentage of Actinobacteria (10.9%; Fig. 2), including Actinomycetaceae, Corynebacteriaceae, and Propionibacteriaceae (Supplementary Table 1), which are also found in the gastrointestinal tracts of animals. A single sequence belonging to the phylum Tenericutes was found in Wild-LI and was iden-

tified as *Mycoplasma* spp. (Supplementary Table 1). We did not detect any sequences identified as Bacteroidetes, which is a major bacterial group in the gastrointestinal tracts of humans and mice.

The UniFrac test (Table 2) indicated differences in both community membership (unweighted) and community structure (weighted) among most of the samples ($P < 0.001$), except between Wild-LI and Wild-SI (Table 2), which had smaller Jaccard index (for community membership) and θ -YC (for community structure) values. The difference between Lab-LI and Lab-SI was apparently observed in Actinobacteria (Fig. 2), and numbers of sequences detected were different in some Firmicutes (Fig. 3, see also Supplementary Table 1).

Discussion

Suncus is the only experimental animal belonging to Eulipotyphla. In this study, we first revealed the major members of the gastrointestinal microbiota of laboratory *Suncus* and wild *Suncus*. The microbiotas were rich in Firmicutes, with no Bacteroidetes present (Fig. 2).

Table 2. Similarity indices of microbial membership (Jaccard index and UniFrac unweighted) and community structure (θ -YC index and UniFrac weighted) of the large (LI) and small (SI) intestinal contents from laboratory *Suncus murinus* and wild *Suncus murinus*

	Jaccard index	θ -YC index	Unifrac test	
			unweighted	weighted
Between intestinal part				
Laboratory LI vs. Laboratory SI	0.647	0.407	0.634 ($P<0.001$)	0.672 ($P<0.001$)
Wild LI vs. Wild SI	0.583	0.220	0.625 ($P=0.022$)	0.102 ($P=0.034$)
Between laboratory and wild				
Laboratory LI vs. Wild LI	0.885	0.974	0.767 ($P<0.001$)	0.279 ($P<0.001$)
Laboratory SI vs. Wild SI	0.913	0.965	0.797 ($P<0.001$)	0.741 ($P<0.001$)

Jaccard index=traditional similarity coefficient based on the number of shared operational taxonomic units (OTUs); θ -YC index=similarity index employing relative abundance of OTUs [59]; Unifrac test= β -diversity measure which describes whether communities have same generic structure based on the phylogenetic tree. Weighted Unifrac test incorporates proportional abundance of the taxa (qualitative), and unweighted Unifrac does not take (quantative).

These two phyla are the most common phyla in the vertebrate gut microbiota [27]; we did not detect any Bacteroidetes sequences, even though we analyzed 759 clones in total. This was not due to experimental error in our laboratory because we previously detected many Bacteroidetes from rodents using the same strategy [46]. Although our sequencing effort was not perfect for detecting rare species, Bacteroidetes may be scarce in the *Suncus* gut. Characterization of few Bacteroidetes has also been reported in some mammalian species, including bears, cheetahs, giant pandas, red pandas, hedgehogs, and echidnas (see Supplementary Data in Ley *et al.* [26]). These species are carnivorous, herbivorous, and insectivorous. However, the giant pandas and red pandas are “apparent herbivores” because both species evolved from carnivorous to herbivorous (eating bamboo) with loss of the umami taste receptor [16]; as a result they are poorly adapted for digesting bamboo [6, 23, 55, 56]. Therefore, we suggest that the presence of few Bacteroidetes is not common but may be observed in some insectivorous and carnivorous mammals.

Ley *et al.* [26] reported that fecal microbiota diversity in mammals was high in herbivores, low in carnivores, and intermediate in omnivores. In this study, we detected only 11–46 OTUs from 174–214 clones, implying that the gut microbiota diversity of *Suncus* is very low. Although we do not have definitive evidence, we speculate that the low diversity is due to the morphological features of the gastrointestinal tract in *Suncus*, i.e., its simplicity, short total intestine relative to body size, very short large intestine, and lack of fermentative chambers such as the forestomach and caecum [14, 25,

58]. These characteristics may limit the physiological space and physical area where microbes occur. The carnivorous species containing few Bacteroidetes listed above also had very low alpha diversity estimates compared with other mammals (see the Supplementary Data file in Ley *et al.* [26]), and each has at least one of the four abovementioned anatomical features [49]. Unfortunately, we could not identify all members of the gut microbiota. A future study with deep-sequencing technology using a next-generation sequencer will provide more detailed analyses.

We did identify 4–10 genera within Firmicutes. The lactic acid bacteria *Enterococcus*, *Lactococcus*, *Streptococcus*, and *Vagococcus* were abundant (27.2–84.5%) in the *Suncus* gut, implying that lactic acid fermentation is important in *Suncus*, as in many mammals. Lactic acid bacteria contribute to the complexity of the gut microbiota and have many beneficial effects on metabolism and immunomodulation, as revealed in human studies [39]. Although *Suncus* has a short and simple gastrointestinal tract, lactic acid fermentation is probably essential, and the lactic acid bacteria found in this study are likely autochthonous, as reported in carnivores [9]. However, these lactic acid bacteria and other members of Firmicutes are not the core members of the human or mouse gut microbiotas [31], indicating that the gut microbiota in *Suncus* is quite different from that in humans and mice.

Comparing the gut microbial diversity between wild *Suncus* and laboratory *Suncus*, the diversity was lower in the laboratory animals. Although our experimental design was not a strict comparison between captive and

wild animals because the original collection locality of the KAT strain (Kathmandu, Nepal [34]) is far from Okinawa, where we captured the wild samples, studies have found that captivity (including domestication) lowers gut microbial diversity in mammals [21–23, 28]. In addition, the members of Firmicutes differed between laboratory *Suncus* and wild *Suncus*, with *Streptococcus* being the major genus in the former but not the latter. A recent study that compared fecal microbiotas between wild and captive animals from 41 mammalian species also found a higher abundance of *Streptococcus* spp. in captive animals [28]. Considering this evidence, our results imply that the gut microbiota of *Suncus* has changed with domestication. Furthermore, there was differentiation between the large and small intestines in laboratory *Suncus*, unlike in wild *Suncus* (Table 2). Moreover, the reduction in gut microbial diversity in the phylum Proteobacteria was remarkable (see Supplementary Table 1); more than 10 genera were found in wild *Suncus* and only 2 genera in laboratory *Suncus*. Although we do not have a good explanation for this, it is notable that putative pathogenic bacteria (e.g., *Salmonella*, *Plesiomonas*, *Kluyvera*, and *Morganella*) were found only in wild *Suncus*. It is possible that these putative pathogenic bacteria are exogenous, and not autochthonous. An alternative hypothesis is elimination. Because these putative pathogens might be an obstacle to domestication as experimental animals, it is possible that they were eliminated without being noticed by keeping them in stable and clean environment (see for trial history of breeding environment [32, 33]). Further study of captive *Suncus* from Okinawa will provide more insight into these hypotheses.

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