

<https://doi.org/10.1038/s42003-025-07538-6>

# Gut microbiota profiles of sympatric snub-nosed monkeys and macaques in Qinghai-Tibetan Plateau show influence of phylogeny over diet

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The unique environment of the Qinghai-Tibetan Plateau provides a great opportunity to study how primate intestinal microorganisms adapt to ecosystems. The 16S rRNA gene amplicon and metagenome analysis were conducted to investigate the correlation between gut microbiota in primates and other sympatric animal species living between 3600 and 4500 m asl. Results showed that within the same geographical environment, *Macaca mulatta* and *Rhinopithecus bieti* exhibited a gut microbiome composition similar to that of Tibetan people, influenced by genetic evolution of host, while significantly differing from other distantly related animals. The gut microbiota of plateau species has developed similar strategies to facilitate their hosts' adaptation to specific environments, including broadening its dietary niche and enhancing energy absorption. These findings will enhance our comprehension of the significance of primate gut microbiota in adapting to specific habitats.

The Qinghai-Tibetan Plateau, the highest plateau in the world, is also known as “the roof of the world” or the “water tower of Asia”<sup>1</sup>. High-altitude environments pose a unique challenge to the physiology of indigenous Tibetan people and non-human primates (NHPs), who are exposed to extreme cold and low oxygen<sup>2</sup>. In cold environments, homothermal animal require additional energy to sustain their core body temperature. For instance, when rodents live at 4 °C, their daily food intake can double, suggesting that 60% of their total energy expenditure is utilized for thermogenesis<sup>3</sup>. The low-oxygen environment of the plateau prompts an increase in the body's basic metabolic rate, leading to elevated energy expenditure as a means of adapting to the thin oxygen atmosphere<sup>4</sup>. The relatively homogeneous vegetation types in the temperate coniferous forests of the Tibetan Plateau exacerbates the scarcity of primate food resources and poses challenges for their energy acquisition<sup>5,6</sup>. However, primates can be well-adapted to this harsh environment by genetic adaptation<sup>7</sup>.

The gut microbiota is an essential component of acclimatization to extreme environments<sup>2,8</sup>. Evidence has shown that the gut microbiota is critical to the well-being of hosts<sup>9</sup>; as an internalized “microbial organ”<sup>10</sup>, trillions of microbes colonize a host's gut tract, forming a complex microecosystem<sup>11</sup>. Gut microbial composition has myriad effects on

nutrient intake, energy metabolism, immune function, physiological regulation, pathogen resistance, toxin transformation, behavior, and even adaptation of their hosts<sup>12,13</sup>. For instance, the gut microbiota of higher-altitude mammals displays a higher ratio of Firmicutes to Bacteroidetes (F/B ratio) than those of lower-altitude mammals<sup>2,6</sup>. The main functions of Firmicutes and Bacteroidetes are to help the host digest and absorb nutrients and to degrade carbohydrates and proteins, respectively<sup>6,14</sup>. In addition, *Prevotella* may play a crucial role in facilitating energy supply and adaptation to hypoxic conditions. The relative abundance of *Prevotella* in Tibetan populations has been consistently observed to be higher compared to lowland populations<sup>8</sup>.

The gut microbiota also influences on niche differentiation within host feeding strategies<sup>15</sup>. Niche differentiation is the basis for maintaining species diversity because resource partitioning promotes sympatric coexistence<sup>16</sup>, particularly in environments characterized by limited food resources such as the Tibetan Plateau. The Gut microbiota of sympatric animals can be transmitted through direct or indirect contact; however, variation in species-specific activity rhythms and dietary preferences contribute to the diversification of gut microbial communities in these animals<sup>16,17</sup>. The gut microbiota serves as a valuable tool for gaining novel insights into the

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interrelationships among diverse species, particularly regarding the mechanisms underlying the coexistence of similar niches<sup>15,18</sup>.

There have been numerous studies conducted on the gut microbiota of primates inhabiting the Qinghai-Tibetan Plateau, however, most of these studies have focused on a single species of primates, and these study sites did not reach the maximum altitude range for the species<sup>2,6</sup>. At present, there are scarce comparative studies regarding the gut microbiota of diverse primates within the Tibetan Plateau and between primates and other co-occurring animals in the same area.

As one of the world's highest-living primates, the black-and-white snub-nosed monkey (*Rhinopithecus bieti*) inhabits an altitudinal range of 3600–4500 m<sup>19</sup>. Within their home range, near the natural northernmost population (Zhina Group) there is also a traditional Tibetan village (altitude 4000 m) and a population of rhesus macaque (*Macaca mulatta*) as well. This area represents the highest distribution point for both *R. bieti* and *M. mulatta* and approaches the upper limit for human survival<sup>19–21</sup>. This unique setting offers favorable conditions for studying the composition and inter-relationships of gut microbes in different species under extreme altitude conditions. In this study, we have chosen Tibetan people, *M. mulatta*, and *R. bieti* as representatives of primates; yaks (*Bos mutus*) and goat (*Capra hircus*) as representatives of domestic animals; tufted deer (*Elaphodus cephalophus*) and Chinese forest musk deer (*Moschus berezovskii*) as representatives of wild herbivores; and the ubiquitous white eared-pheasant (*Crossoptilon crossoptilon*) as a representative of the Galliformes. The fact that *R. bieti* is monogastric, like humans and *M. mulatta*, is worth noting. However, the *R. bieti* possesses a specialized S-shaped and partitioned stomach<sup>22,23</sup>. This adaptation enables them to eat food containing high levels of structural polysaccharides, i.e., cellulose and related compounds<sup>24</sup>. On the other hand, *B. mutus*, *C. hircus*, *E. cephalophus* and *M. berezovskii* are all ruminants characterized by possessing a complex stomach structure<sup>17</sup>. The digestive system of *C. crossoptilon*, by contrast, is typical of herbivorous birds and comprises the esophagus, crop, proventriculus, gizzard, intestines, and cloaca<sup>25</sup> (Fig. 1).

We aimed to investigate (1) the factors influencing the gut microbiome composition of coexisting primates and other animals at extreme altitude in the Hengduan Mountains on the eastern margin of the Qinghai-Tibetan Plateau, (2) the contribution of the gut microbiome to primate adaptation to

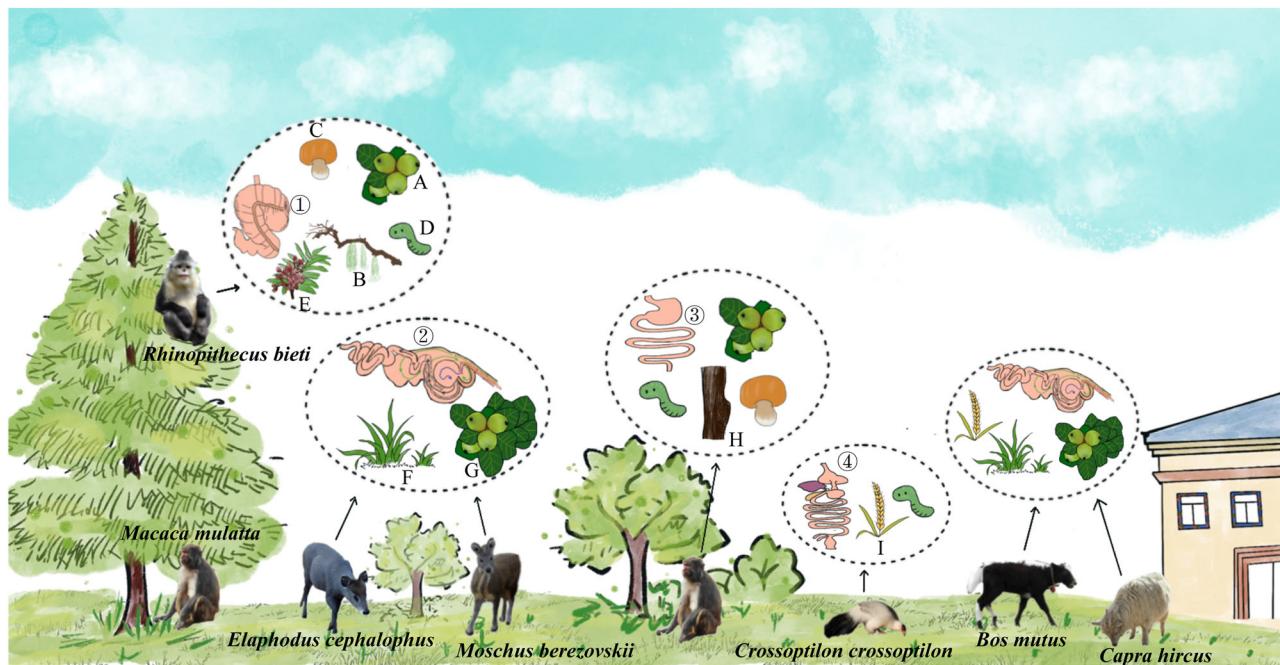
cold, alpine environments and to their ecological niche differentiation. We hypothesized that: (1) The composition of gut microbiota is primarily determined by host phylogeny and dietary niche; (2) The gut microbiota of plateau species have developed similar strategies to facilitate their hosts' adaptation to specific environments, including broadening its dietary niche and enhancing energy absorption.

## Results

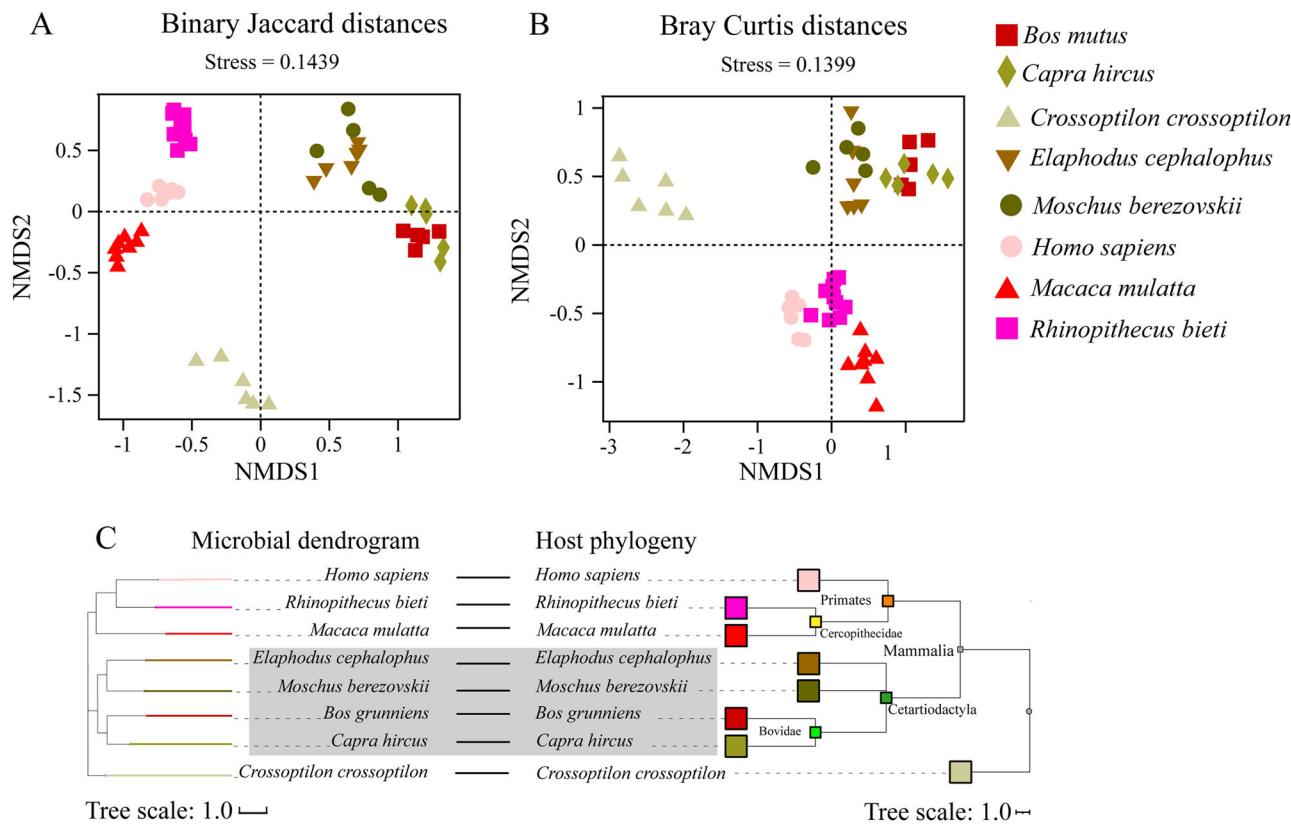
### Gut microbiome structure

In total, we collected 51 fecal samples for 8 sympatric species (Supplementary Table 1). Each species harbored a structurally unique gut microbiome (Fig. 2). Non-Metric Multi-Dimensional Scaling (NMDS; Fig. 2A) binary jaccard and Fig. 2B bray curtis distances of 16S rRNA gene amplicon data illustrates stronger clustering of 8 species fecal samples by host phylogenetic clade (binary jaccard: PERMANOVA  $R^2 = 0.271$ ,  $P < 0.001$ ; bray curtis: PERMANOVA:  $R^2 = 0.486$ ,  $P < 0.001$ ) than diet (vegetarian vs omnivory, binary jaccard: PERMANOVA  $R^2 = 0.053$ ,  $P < 0.001$ ; bray curtis: PERMANOVA:  $R^2 = 0.101$ ,  $P < 0.001$ ). Notably, there was higher similarity of microbiome communities among more closely related host species. The Gut microbiota of Primates, Cetartiodactyla, and Galliformes each form a distinct region, characterized by intra-group convergence and inter-group variations (Fig. 2A, B). The cluster analysis of the gut microbiota in host organisms aligns with their respective phylogenies (Fig. 2C). Our analysis revealed that the phylogenetic relationships of hosts were a vital factor influencing the composition of animal gut microbiota at the OTU level (Fig. 2).

The Pielou evenness index of microbiota in the Cetartiodactyla were significantly higher than those in the Primates ( $t$ -test,  $t = -7.849$ ,  $df = 43$ ,  $P < 0.001$ ) and Galliformes ( $t$ -test,  $t = 9.119$ ,  $df = 28$ ,  $P < 0.001$ ) (Supplementary Fig. 1). The predominant bacterial phylum isolated from the 8 species were Firmicutes and Bacteroidota (Supplementary Fig. 2). Firmicutes were most abundant in *E. cephalophus* (68.79%), intermediately abundant in *M. berezovskii* (57.64%), *B. mutus* (54.04%) and *R. bieti* (52.59%), and least abundant in *C. crossoptilon* (20.14%). In contrast, Bacteroidota were most abundant in *C. hircus* (42.54%) and Tibetan people (39.14%), intermediately abundant in *B. mutus* (36.44%), and least abundant *C. crossoptilon* (3.93%). *C. crossoptilon* had a significantly higher F/B



**Fig. 1 | Niche differentiation of 7 sympatric species.** ①–④: the gastrointestinal morphology of animals. A–I is illustrations of their dietary composition. A Bush fruits; B lichens; C mushrooms; D invertebrates; E Bush flowers and young leaves; F grass; G Bush fruits and mature leaves; H bark; I highland barley.



**Fig. 2 | Phylogeny and Gut microbiome structure in 8 sympatric species based on 16S rRNA gene amplicon.** Depicted are measures of beta diversity by Non-Metric Multi-Dimensional Scaling (NMDS), including Binary Jaccard distances graphed (A) and Bray Curtis distances graphed (B). C Comparison between gut

microbiota tree and host phylogeny relationships, based on Unweighted Pair-group Method with Arithmetic Mean and National Center for Biotechnology Information Taxonomy database. Vegetarian are shaded in the phylogenetic tree.

ratio than other species (*t*-test,  $t = 5.176$ ,  $df = 6$ ,  $P < 0.01$ ), and wildlife exhibited a higher F/B ratio compared to Tibetan people and domesticated animals (Kruskal-Wallis,  $z = -2.236$ ,  $P < 0.05$ ) (Supplementary Table 2). *M. mulatta* (11.94%) and *R. bieti* (8.18%) had the greatest proportion of Spirochaetota, and the relative abundance of Proteobacteria in *M. mulatta* (16.73%) was second only to that of *C. crossoptilon* (Supplementary Fig. 2).

At the family level (Fig. 3), Prevotellaceae were most abundant in Tibetan people (33.46%) and *C. hircus* (21.58%), and were similar in *R. bieti* (14.86%), *B. mutus* (13.98%) and *M. mulatta* (11.64%). Oscillospiraceae were present in large proportions in all Cetartiodactyla species ( $16.32\% \pm SD 3.1\%$ ), and least abundant in *C. crossoptilon* (0.19%). The relative abundance of Lachnospiraceae in the gut tract of mammals ( $9.42\% \pm SD 1.95\%$ ) was also higher than that of *C. crossoptilon* (0.78%).

In total, only 11 (0.04%,  $n = 27615$ ) OTUs were shared by our 8 sympatric species (Fig. 4A). However, there were 35 (0.13%) OTUs shared between Cetartiodactyla and three primates, and 56 (0.20%) OTUs were shared among the three primates (Fig. 4B, C). Notably, the prevalence of OTUs shared between sympatric heterospecifics often indicates the horizontal transmission of gut microbes<sup>26</sup>. These horizontally transmitted bacterial taxa spanned 6 phyla and 8 genera, including notable genera such as *Bacteroides*, *Prevotella*-9, *Faecalibacterium*, *Subdoligranulum*, *Treponema* (Supplementary Table 3).

#### Interspecific differences in microbiomes

The LEfSe results showed that the 8 sympatric species harbored 69 significantly different taxa (Fig. 5, LDA  $> 4$ ,  $P < 0.05$ ). Lachnospiraceae Muribaculaceae, *Prevotella*-7, and *Christensenellaceae* were markedly more abundant in the *R. bieti* gut microbiota. *Succinivibrio* and *Treponema* were the most abundant taxa in the *M. mulatta* gut microbiota. Tibetan people had high relative abundance of the genera *Prevotella*-9, *Faecalibacterium*,

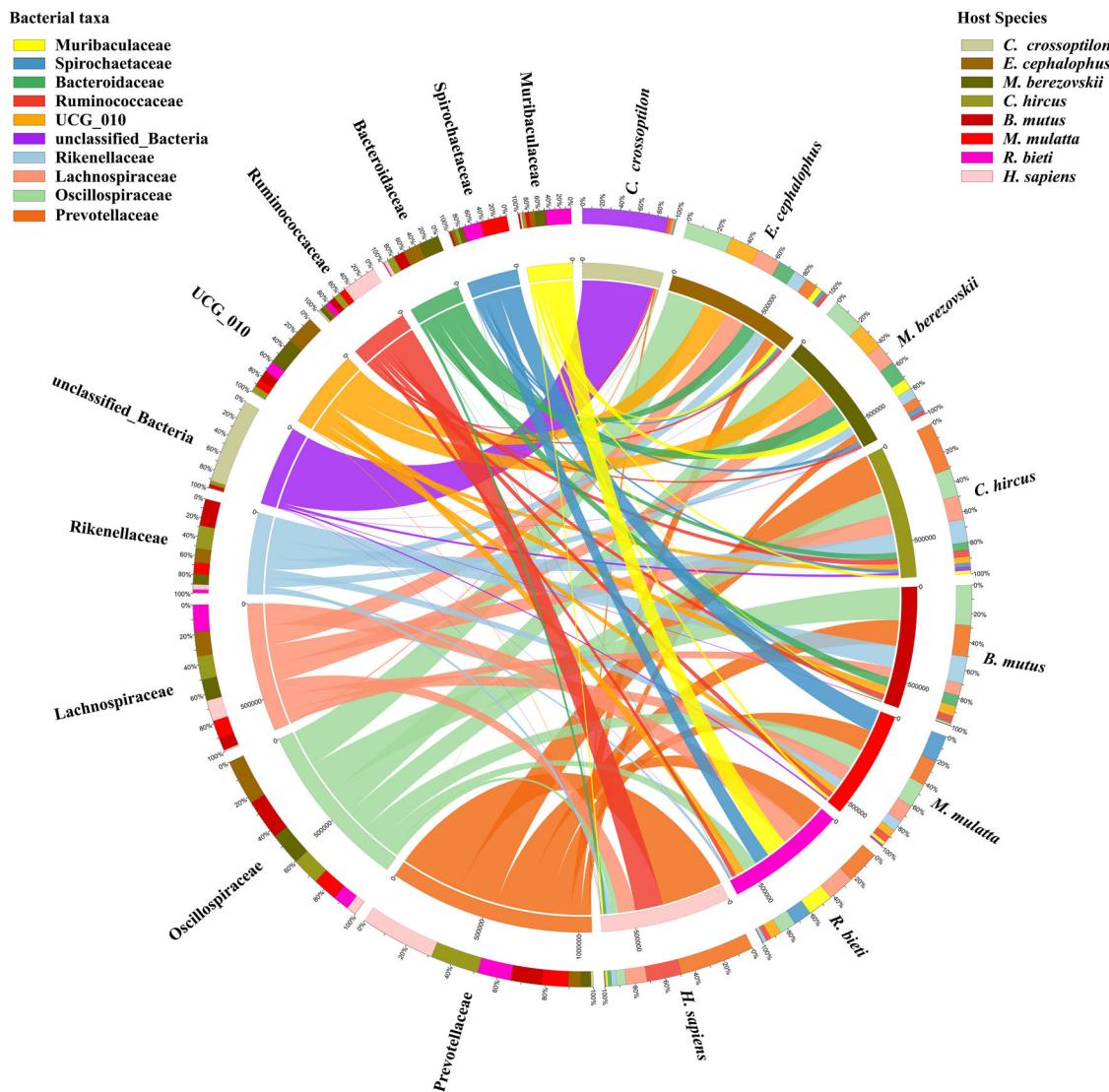
and *Phascolarctobacterium*. On the other hand, ruminants had a higher relative abundance of Oscillospiraceae and Rikenellaceae, and the genera *Akkermansia*, *Prevotella*, and *Prevotellaceae*. The Gut microbiota of *C. crossoptilon* were the most specialized, and with a different flora mainly composed of *Escherichia*, *Shigella*, *Streptococcus*, and *Rickettsiella*.

In addition to bacteria, some Archaea were also detectable using 16S rRNA gene sequencing. The majority of detected Archaeal OTUs in this study could be taxonomically assigned as methanogenic Archaea (methanogens), which are a phylogenetically diverse group of microorganisms. The relative abundance of Methanobacteria within archaea was higher in domestic animals compared to wild animals (*t*-test,  $t = -8.805$ ,  $df = 37$ ,  $P < 0.001$ ) and humans (*t*-test,  $t = -3.671$ ,  $df = 13$ ,  $P < 0.01$ ) (Fig. 6).

#### Gut microbiome function in primates

The Zhina group of *R. bieti* in the area occupied home range of 22.15 km<sup>2</sup> from March 2023 to December 2023 (ranging from 3600 to 4500 m above sea level), with *M. mulatta* occurring. These NHPs in the study area shared space and similar activity patterns with local Tibetan people (Fig. 7). Both species are diurnal, with activity concentrated between 8:00 and 9:00 and between 13:00 and 16:00 (Supplementary Fig. 3).

We obtained a total of 1,241,233,976 clean reads remained from 16 primates after metagenomic sequencing and quality control. After optimizing the read assembly, 5,958,394 contigs were obtained, and 5,452,782 microbiota non-redundant gene data were obtained after gene prediction and de-redundancy. Analysis of Bray Curtis distances indicated an effect of species on pathway composition (PERMANOVA:  $R^2 = 0.704$ ,  $P < 0.001$ ; Fig. 8A). A Kruskal-Wallis test confirmed that the three primates exhibited notable disparities in the relative abundance of KEGG level 3 categories (Fig. 8B). Among these pathways, Tibetan people showed significantly higher enrichment in pathways such as “Butanoate metabolism”, “beta-



**Fig. 3** | Relative abundance of gut bacterial taxa in different animals on the family level based on 16S rRNA gene amplicon analysis.

Alanine metabolism”, “Starch and sucrose metabolism”, “Thiamine metabolism”, “Folate biosynthesis”, “Carbapenem biosynthesis”. However, NHPs showed significantly higher enrichment in pathways such as “Carbon fixation pathways in prokaryotes”, “DNA replication”, “Nucleotide excision repair”, “Mismatch repair”, “Homologous recombination”. Then, we note that some modules are enriched in three primates, such as: Gluconeogenesis (M00003), Acetyl-CoA pathway (M00422), Methionine biosynthesis (M00017, M00338) and Phosphate acetyltransferase-acetate kinase pathway (M00579) (Fig. 9).

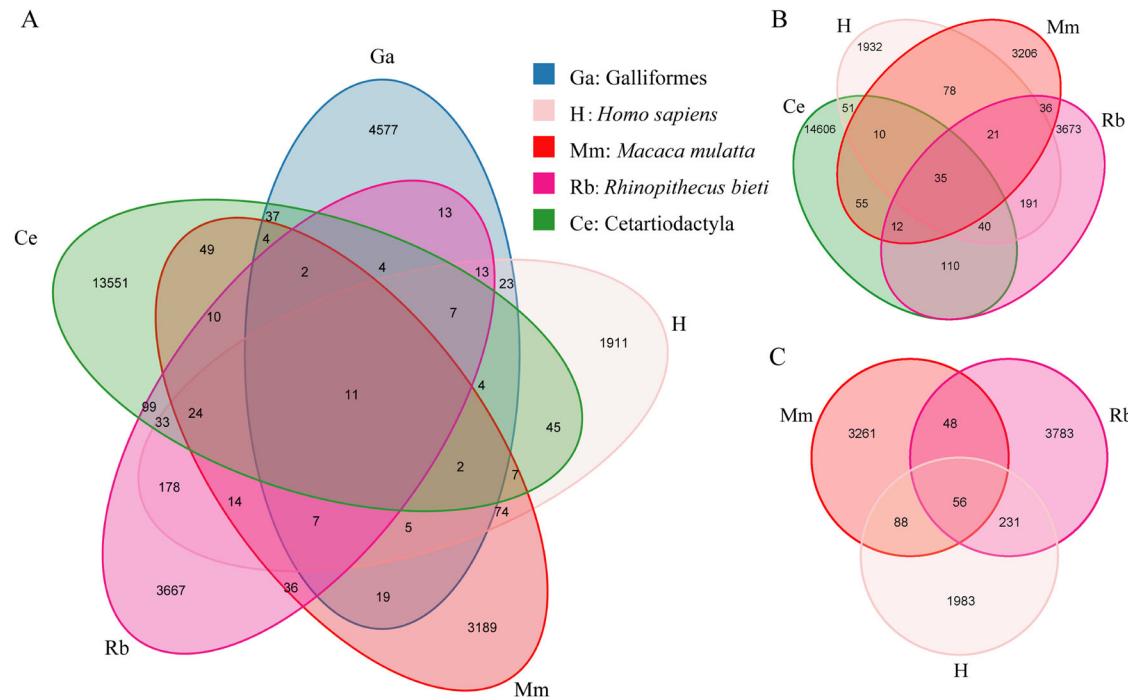
According to the functional annotation of the CAZy database, at family level, two NHPs are more alike (Fig. 8D). They exhibited enrichment in acetyl xylan esterase (EC 3.1.1.72), pectin methylesterase (EC 3.1.1.11), endoglucanase (EC 3.2.1.4), beta-glucosidase (EC 3.2.1.21), lysozyme (EC 3.2.1.17), cellobiose phosphorylase (EC 2.4.1.20), diacetylchitobiose deacetylase (EC 3.5.1.-). On the other hand, the relative abundance of glucosidase (EC 3.2.1.3) and beta-galactosidase (EC 3.2.1.23) in humans is higher than that of the two NHPs.

## Discussion

By comparing gut microbiota across 8 sympatric species, we show that hosts with similar habitat harbor strikingly different gut microbiomes. The impact of host phylogeny and physiology on the gut microbiota of distantly related species was significantly more pronounced than that of the host’s dietary

niche. The gut microbiomes of closely related species, such as various animals within the ruminantia, demonstrated notable resemblances. Traditional Tibetan people and NHPs showed parallel gut microbiome adaptations to analogous ecological conditions. This might be the result of long-term co-evolution between the host and environment and suggests that the composition of gut microbiota in sympatric species is influenced by both host phylogeny and physiology as well as dietary niches<sup>15,17,18</sup>. Interestingly, *R. bieti* are foregut fermenters with a multi-compartmental, sac-like stomach that is divided into fundus I and fundus II from the beginning of lactating larva<sup>22,23</sup>. This unique structure resembles that of ruminant stomachs, enabling them to efficiently digest high-fiber food<sup>22,23</sup>. However, our findings demonstrate that the gut microflora of *R. bieti* exhibits a higher degree of similarity to that of humans and *M. mulatta*, while displaying distinct dissimilarities from ruminants. This represents an additional indication that evolutionary trends in host phylogeny exert a greater influence than dietary niche and physiology on the structuring of gut microbiomes<sup>27</sup>.

Shifts in dietary substrates provided to gut microbes as a result of host diet changes could result in the same processes, explaining the dual impact of host phylogeny and dietary niche that we observed<sup>15,28</sup>. The differentiation of ecological niches is crucial for the maintenance of species diversity, as it facilitates resource partitioning and enables sympatric coexistence, particularly in environments characterized by limited food availability, such as the Qinghai-Tibetan Plateau<sup>16</sup>. Dietary partitioning represents a mechanism



**Fig. 4 | The Venn diagram of the OTUs in all samples among groups via 16S rRNA gene amplicon analysis. A** 11 OTUs were shared by 8 sympatric species. **B** 35 OTUs were shared among the Cetartiodactyla and three primates. **C** 56 OTUs were shared among the three primates.

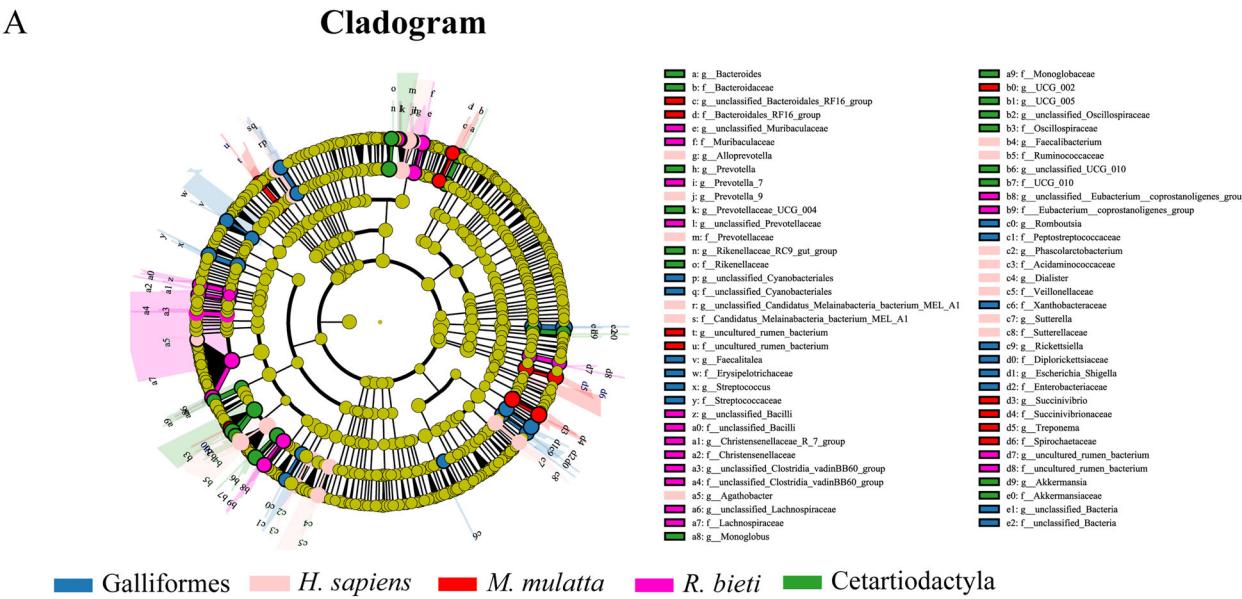
for sympatric species to avoid competition, and is typically demonstrated by documenting diets with minimal overlap and describing the morphological traits that facilitate dietary specialization<sup>29</sup>. In our study, higher relative abundance of Rikenellaceae and *Akkermansia* were observed in ruminants than in Primates and Galliformes. Rikenellaceae exhibited a positive correlation with food intake and body weight, while also facilitating lipid metabolism<sup>30</sup>. Increased *Akkermansia* relative abundance indicates a response of this gut microbe to high fiber and low protein diets<sup>31</sup>. The arginine and fatty acid anabolic pathways encoded by *Akkermansia* can significantly enhance the energy and nitrogen utilization efficiency of ruminants, thereby aiding their survival during periods of severe nutritional stress in cold environments<sup>31</sup>. The abundance of evidence indicates that ruminants possess a highly efficient gut microbial phenotype.

The plant species present in *R. bieti* habitat of our study are chiefly *Abies squamata*, *Picea likiangensis*, and *Larix gmelinii*, accompanied by hard-leaved broadleaf forests and some other evergreen/deciduous shrubs<sup>32</sup>. The plant diversity within their habitat is relatively low. Lichen constitutes the main food source in the annual diet (74.8%), while the proportions of other food sources are significantly lower: buds and leaves account for 15.9%, flowers, fruits, and seeds constitute 2.5%, bark, roots, resin, grass, etc. amount to 4.9%, and invertebrates make up 2.0%<sup>33</sup>. The consumption of lichens gradually increases from south to north in the *R. bieti* diet, with the northern Xiaochangdu group (near our study population) relying on lichens as their primary food source during winter, accounting for 98.2%<sup>33</sup>. In contrast, the central Samage group shows a decrease in lichens consumption at 67%<sup>34</sup>, while the southern Jinsichang group only consumes approximately 5% throughout the year<sup>35</sup>. It is worth noting that lichens contain a higher proportion of hemicellulose compared to other plant parts, making them an unfavorable dietary choice for *R. bieti*<sup>36</sup>. The significant intake of lichens may serve as an adaptive strategy for *R. bieti* facing food limitations at high altitudes<sup>33</sup>. On the other hand, the *M. mulatta* in this study were exclusively confined to a single vegetated habitat, while their diet primarily consisted of bark, leaves, and the fruiting bodies of fungi. The gut microbiota plays a crucial role in facilitating NHPs adaptation to specific dietary requirements<sup>37</sup>. The metagenomic results concerning CAZymes demonstrated that NHPs possess a considerable ability to digest complex

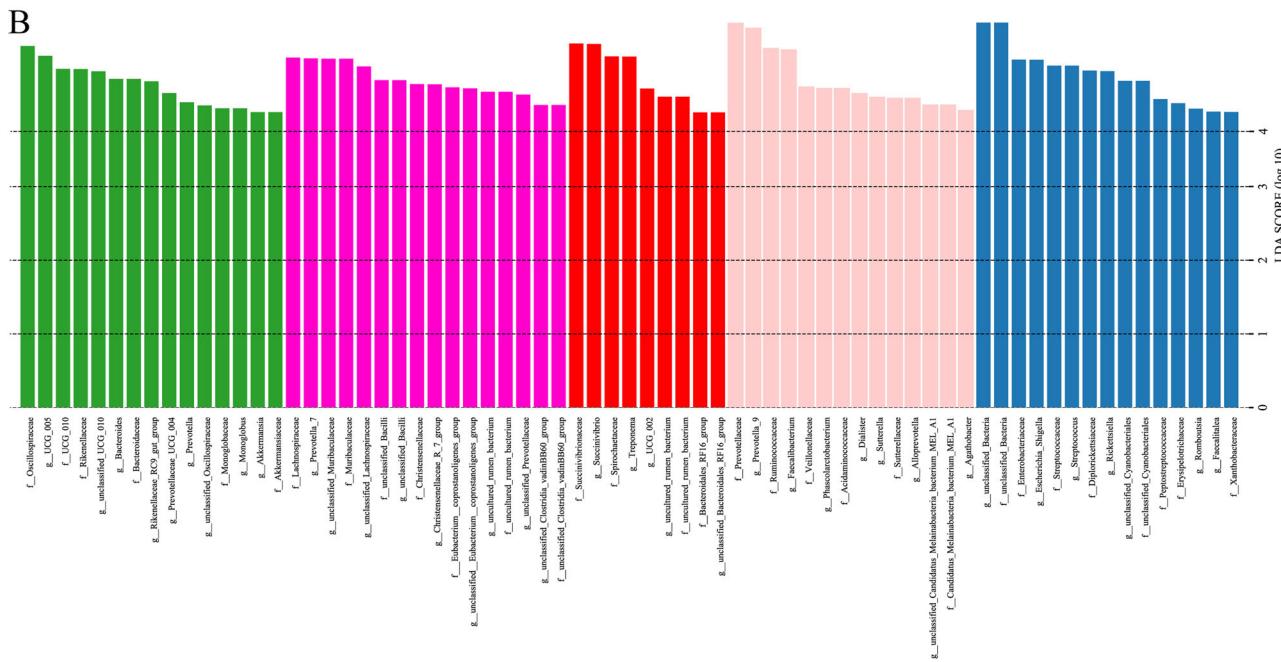
carbohydrates, such as cellulose, hemicellulose, and pectin. Acetylxylosidases (AcXE; EC 3.1.1.72) are components of microbial xylanolytic and cellulolytic systems, role of which is to remove acetyl groups esterifying d-Xyl p residues of xylan main chain at position 2<sup>38</sup>. Endo- $\beta$ -1,4-glucanase predominantly acts upon the non-crystalline regions of cellulose and decomposes the cellulose by randomly severing the beta-1,4-glycosidic bonds, liberating short-chain oligosaccharides of varying lengths, such as fructooligosaccharides and cellobiose<sup>39</sup>. Cellobiose phosphorylase (EC 2.4.1.20), a glycosyltransferase and hexosyltransferase, pertains to the transferase category of enzymes. Its principal function is to catalyze the reversible phosphorylation of cellobiose, which converts it into  $\alpha$ -D-glucose 1-phosphate and D-glucose<sup>40</sup>. Diacetylchitobiose deacetylase (EC 3.5.1.-) exerts a crucial role in the chitin degradation pathway, which is prevalently abundant in the insect exoskeleton, thereby reflecting the insectivorous diet of NHPs<sup>41,42</sup>. Conversely, the results of CAZy database annotation showed that Tibetan People have a strong ability to digest starch (glucoamylase, EC 3.2.1.3)<sup>43</sup> and lactose (beta-galactosidase, EC 3.2.1.23)<sup>44</sup>, which is in correspondence with their diet consisting of highland barley and butter<sup>8</sup>.

Meanwhile, eight species exhibited a shared presence of 11 OTUs, with a notable occurrence of 47 OTUs among mammalian taxa. Notably, increased prevalence of OTUs shared between sympatric species relative to heterospecific species often indicates the horizontal transmission of gut microbes, and the phenomenon has been observed in sympatric chimpanzees and gorillas, as well as in sympatric Malagasy mammals<sup>28</sup>. The presence of symbiotic relationships enhances the microbial diversity within the gastrointestinal tract of animals<sup>45</sup>, and hosts acquire gut microbiota from other species to maintain a significantly greater species richness and biodiversity than in non-gut environments<sup>46,47</sup>. The acquisition of bacterium by animals may have occurred through cohabitation within their shared habitat, thereby enhancing their level of health<sup>6,17</sup>. For instance, *Faecalibacterium*, regarded as a next-generation probiotic or live biotherapeutic product, is a bacterial genus with promising human health applications<sup>48</sup>. And the prevalence of *Subdoligranulum* is significantly associated with the diversity of the gut microbiota, serving as an indicator of the host's metabolic well-being. Additionally, a high level of gut microbial diversity often enhances the host's adaptive capacity by broadening the dietary niche of

A



B



**Fig. 5 | Linear discriminant analysis effect size (LEfSe) analysis of gut microbiota composition among the sympatric species based on 16S rRNA gene amplicon analysis (LDA > 4, P < 0.05).** A taxonomic representation of statistically and biologically consistent differences between different geographical populations. Differences are represented by the color of the most abundant class. Each circle's diameter is proportional to the taxon's abundance and represent phylum, class, order, and

family. **B** Histogram of the LDA scores computed for features differentially abundant among the sympatric species. LEfSe scores can be interpreted as the degree of consistent difference in relative abundance between features in the two classes of analyzed microbial communities. The histogram thus identifies which clades among all those detected as statistically and biologically differential explain the greatest differences between communities.

wild animals, as a diverse gut microbiota confers an extensive capability to handle the intricate compounds found in plant-based food sources<sup>49</sup>. Such as *Bacteroides*, *Treponema* and *Prevotella-9*, they aid the host in breaking down complex carbohydrates, produce SCFAs, and enhance digestion, nutrient absorption, metabolic regulation, and immune response<sup>50,51</sup>. In conclusion, the presence of symbiotic relationships enhances the microbial diversity within the gastrointestinal tract of animals<sup>45</sup>, and hosts acquire gut microbiota from other species to maintain a significantly greater species richness and biodiversity than in non-gut environments<sup>46,47</sup>.

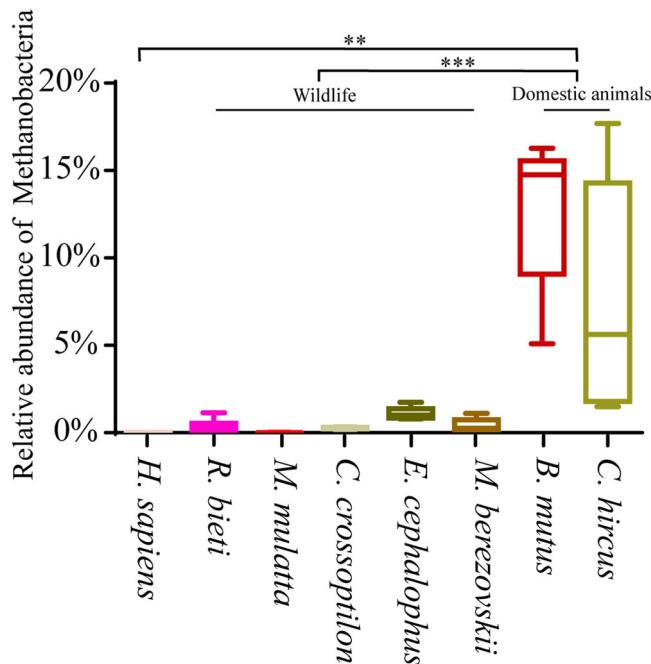
Concurrently, low temperatures intensify the energy requirements of the animals. The austere environment of the Qinghai-Tibet Plateau poses a substantial challenge to the survival of animals, demanding that they possess

effective means of energy acquisition<sup>31,52,53</sup>. In this study, we found that the gut microbiota of these 8 species predominantly consists of Firmicutes and Bacteroidetes at the phylum level, which aligns with findings from previous research<sup>54,55</sup>. Moreover, wild animals displayed higher F/B ratios in comparison to domestic animals and humans. Firmicutes possess a multitude of genes encoding enzymes involved in energy metabolism and exhibit the ability to synthesize diverse digestive enzymes, thereby facilitating nutrient digestion and absorption by their hosts<sup>56</sup>. In contrast, Bacteroidetes primarily specialize in the degradation of carbohydrates and proteins<sup>57</sup>. The higher F/B ratio in a microbial community thus should be positively associated with increased absorption of food energy, facilitating the maintenance of energy homeostasis and core body temperature in challenging

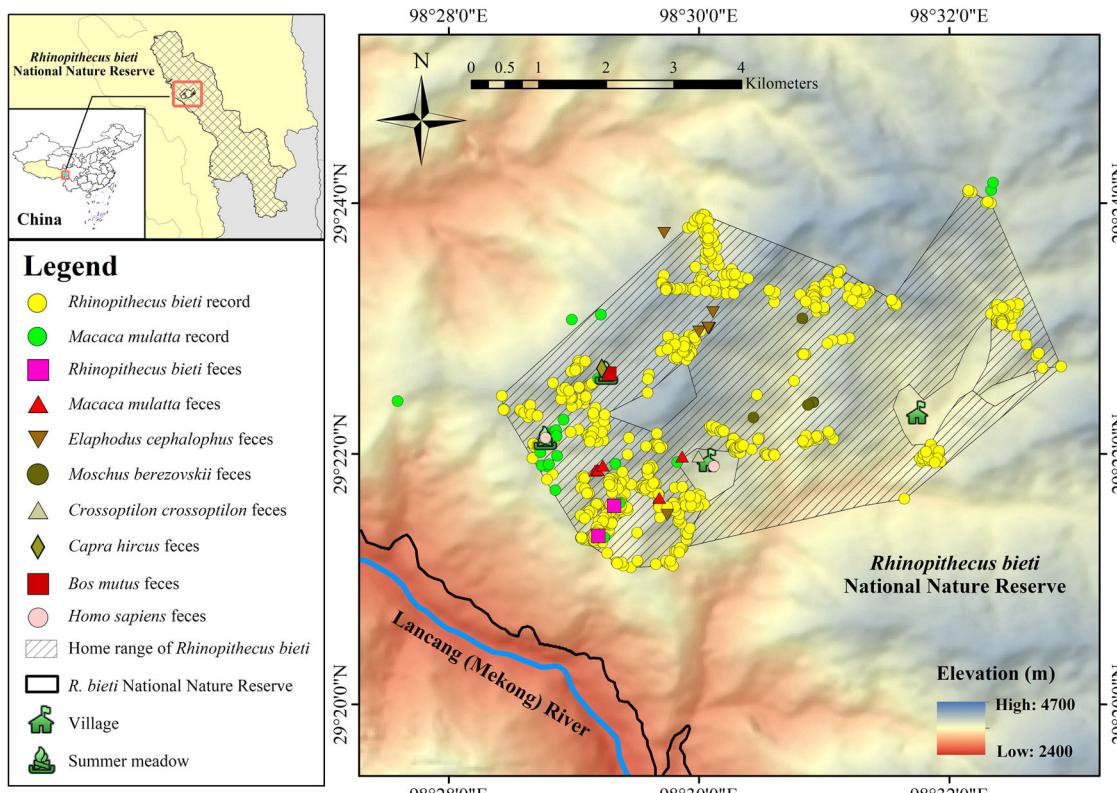
environments<sup>6,14,58</sup>. At the family level, the bacteria with the highest relative abundance among mammals were Prevotellaceae, Oscillospiraceae, and Lachnospiraceae. The relative abundance of Prevotellaceae in Tibetan

human populations is commonly higher than that in lowland populations and thus positively correlated with altitude<sup>8</sup>. Prevotellaceae is a saccharolytic anaerobe associated with the production of short-chain fatty acids (SCFAs), which can serve as a source of energy for the host<sup>59–61</sup>. In addition, SCFAs produced by the gut flora might influence blood pressure regulation by acting on SCFAs receptors, such as olfactory receptor 78 and G-protein couple receptor 41<sup>8</sup>. On the other hand, it was reported that the Oscillospiraceae was more abundant in leaner individuals. A higher relative abundance of Oscillospiraceae might lead to decreased weight, which facilitates animal movement to forage more food resources in high-altitude mountains, thus allowing them to gain more energy to adapt to high-altitude mountains<sup>6</sup>. Additionally, previous research in Tibetan populations has demonstrated that the richness of Lachnospiraceae is higher than in groups living at lower altitudes<sup>8</sup>. In our research, we also observed a high relative abundance of Lachnospiraceae in mammals. These taxa are known to produce SCFAs, which serve as an important energy source and can also regulate the blood pressure of the host. This might help plateau animals maintain a healthier gut environment and adapt to life at higher altitudes<sup>46,62</sup>.

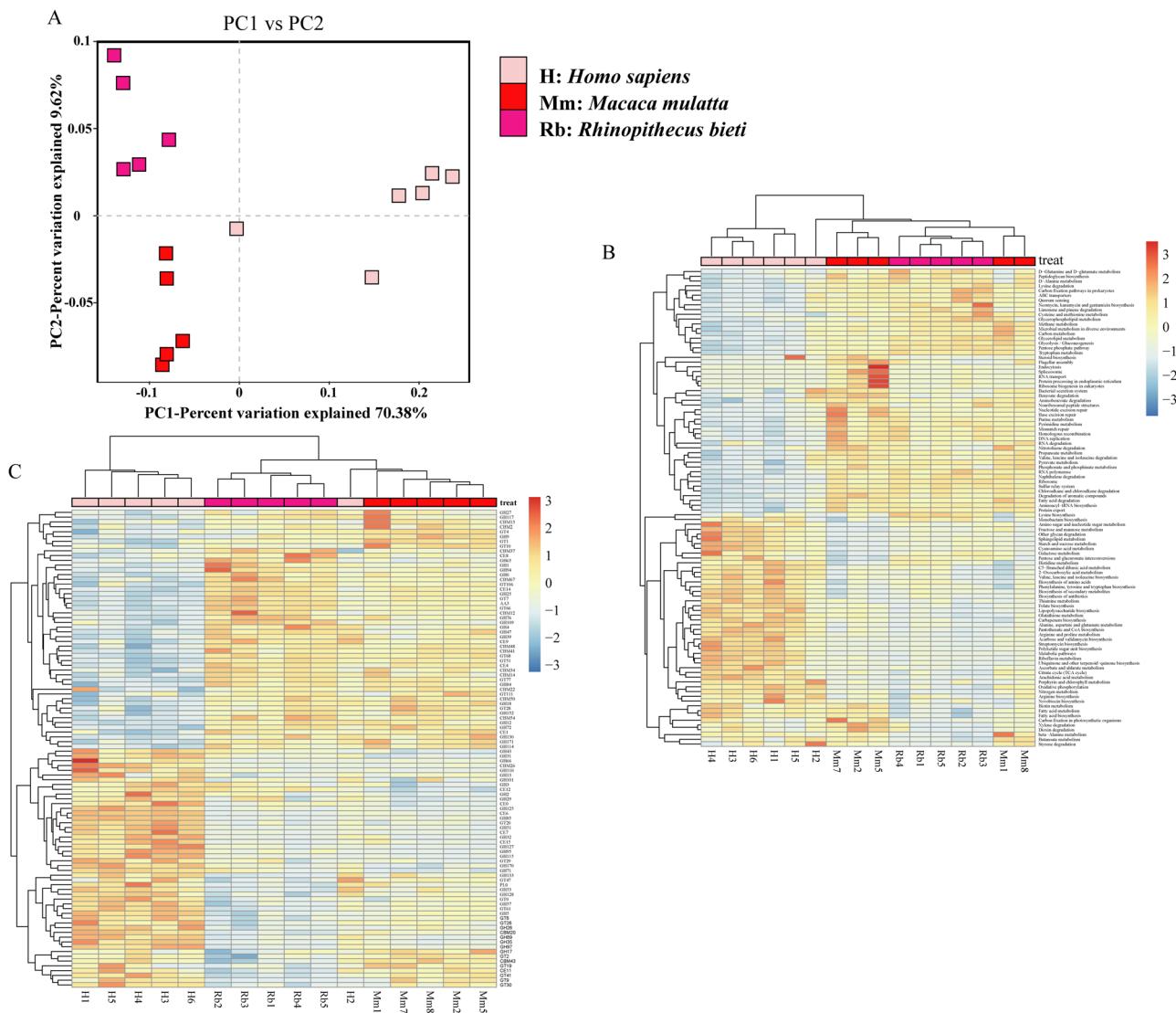
Meanwhile, in our metagenomic analysis, we observed pathways crucial for SCFAs synthesis, encompassing the M00422, M00579, and M00003 modules. The gut microbiota's fermentation process yields SCFAs, primarily acetate, propionate, and butyrate, which are swiftly absorbed by the host's gut epithelium, supplying energy and enhancing the host's metabolic efficiency in food utilization<sup>37</sup>. Among these SCFAs, butyrate emerges as a preferred energy source; however, its production is largely dependent on the availability of intestinal carbohydrates<sup>37,63</sup>. In human gut microbiota, diverse fermentation strategies have evolved to harness additional energy. Pyruvate, a key intermediate, can be broken down and converted into succinate, lactic acid, or acetyl-CoA<sup>37</sup>. These compounds can subsequently be further metabolized by the host to generate acetate, propionate, and butyrate<sup>37,63</sup>. Herein, the modules related to SCFAs can generate the energy substrates acetyl-CoA and pyruvate, mediate the energy metabolism of the host and offer energy compensation to the host. This enhancement in energy metabolism has been instrumental in improving the



**Fig. 6 | Relative abundance differences of Methanobacteria within archaea by species based on 16S rRNA gene amplicon analysis.** The boxes represent 25–75th percentiles, the lines in the box indicate the median and whiskers extend to the maximum and minimum values within 1.5× the interquartile range. Significant differences were marked as “\*\*” ( $P < 0.01$ ), “\*\*\*” ( $P < 0.001$ ) by  $t$ -test.



**Fig. 7 | Map of sampling sites for 8 sympatric species in Markam, Tibet, China.**



**Fig. 8 | Gut microbiome function of primates by metagenome analysis.** A analysis of Bray Curtis distances indicated an effect of species on pathway composition; B a comparison of the microbiome gene functions among primates in the KEGG 3

classification was conducted; C the functional annotations of primates based on the CAZy database, at family level.

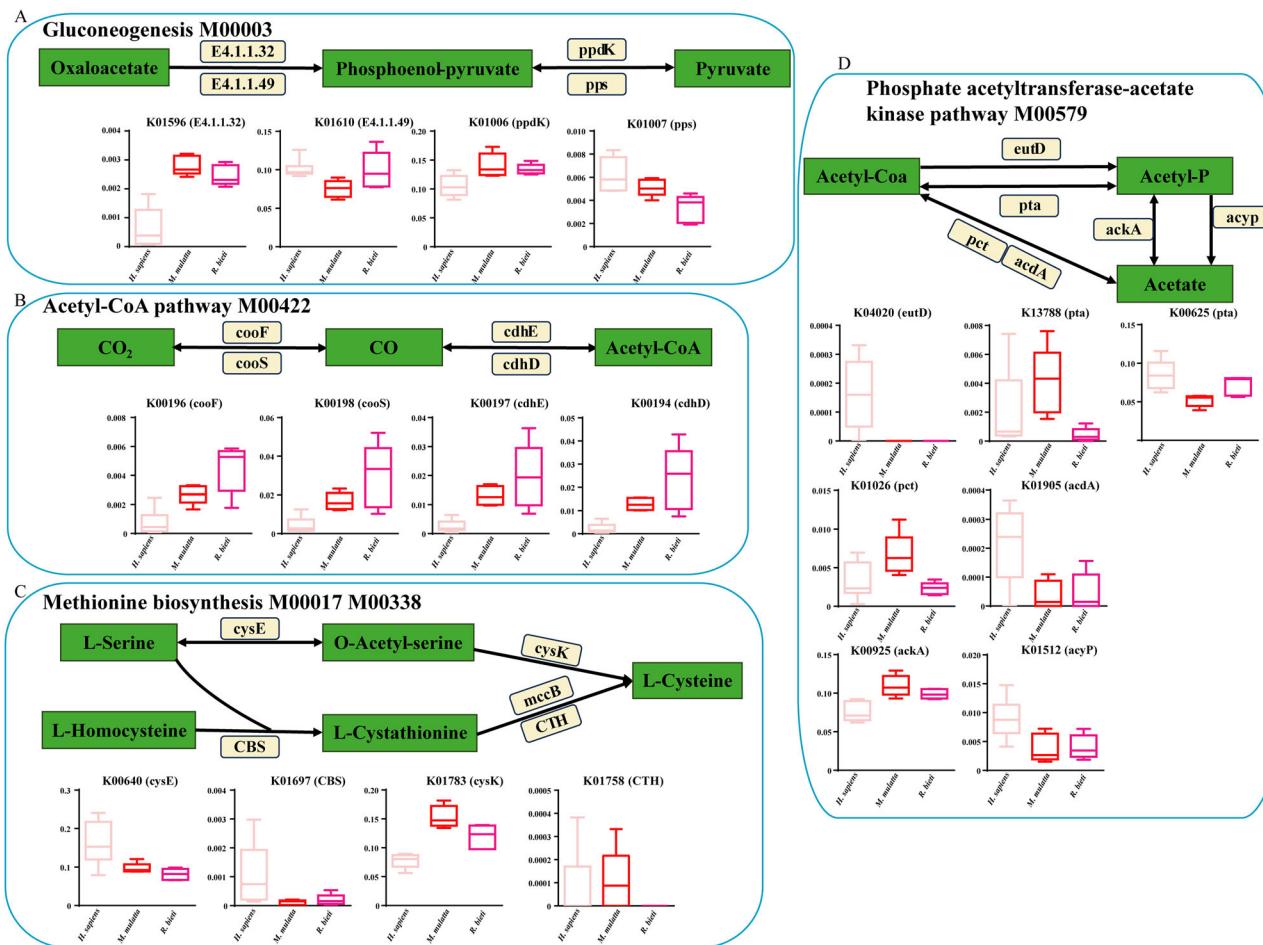
host's adaptation to the challenging environmental conditions of high-altitude plateaus, characterized by low temperatures and food scarcity<sup>37</sup>. In addition, we found that the enrichment rate of genes related to the DNA replication, nucleotide excision repair and mismatch repair pathways was significantly higher in NHPs. These pathways are enriched in animals at high altitudes, which have enhanced DNA repair capacity<sup>37</sup>.

In addition, our results show that Methanobacteria relative abundance in primates, wild ruminants, and *C. cressoptimon* is comparatively lower than that observed in domestic animals. Methanobacteria are predominantly found in the rumen of ruminant animals, accounting for their limited presence in non-ruminants<sup>8,31</sup>. Methane, a by-product of rumen fermentation by methanogenic archaea, leads to energy loss<sup>64</sup>. Low-methane production in the gut microbiota might help maintain highly efficient energy metabolism to survive in the harsh high-altitude environment<sup>65</sup>. The evidence thus suggests that wildlife might possess more efficient gastrointestinal functions compared to domestic animals, enabling them to minimize energy loss and adapt effectively to challenging natural environments.

In addition, the gut microbiota can play a pivotal role in the degradation of environmental chemicals and toxic pollutants, thereby mitigating their potential harm and enhancing host adaptability to the surrounding environment<sup>66</sup>. Our study demonstrates that the gut microbiota of *M.*

*mulatta* exhibits functional enrichment in degrading hazardous substances such as atrazine and styrene. Atrazine is a commonly used herbicide that has been shown to be harmful to the health of animals<sup>67</sup>. Styrene and styrene polymers are released into the environment during the process of synthetic rubber and plastic production and incineration<sup>68</sup>. During our field investigation, we discovered a significant quantity of plastic bags negligently discarded. Styrene and styrene polymers are released into the environment during the process of synthetic rubber and plastic production and incineration<sup>68</sup>. *M. mulatta* frequently traverse and forage in close proximity to human settlements, thereby heightening the potential for exposure to hazardous substances. Functional enrichment of harmful substance degradation in the gut microbiota of *M. mulatta* can protect against the ingestion of such substances and enhance adaptability to worsening habitat pollution.

The aforementioned data collectively emphasize that host phylogeny is the vital influence on the gut microbiota in both human and NHPs residing at high altitudes. In the same geographical environment, *M. mulatta* and *R. bieti* exhibited a gut microbiota composition similar to that of Tibetans, influenced by genetic evolution of host, while significantly differing from other distantly related animals. Meanwhile, various animals possess distinct dietary niches, offering diverse substrates to the gut microbiome, which in



**Fig. 9 | The main metabolic modules of gut microbiota functional genes in primates by metagenome analysis.** A The gluconeogenesis module (M00003); B the acetyl-CoA pathway module (M00422); C the methionine biosynthesis module (M00017, M00338); D the phosphate acetyltransferase-acetate kinase

pathway module (M00579). Different colors signify diverse species. The boxes represent 25–75th percentiles, the lines in the box indicate the median and whiskers extend to the maximum and minimum values within 1.5× the interquartile range.

turn gives rise to variations in the composition of gut microbiota. For instance, *M. mulatta* and *R. bieti* were more proficient in digesting complex carbohydrates, whereas the gut microbiota of Tibetan People was characterized by the capacity to digest starch and dairy products. This confirms our first hypothesis: The composition of gut microbiota was primarily determined by host phylogeny and dietary niche. On the other hand, shared OTUs expand the dietary ecological niche of animals, and the efficient enzymes produced by gut microbiota can assist the host in digesting food. Additionally, the SCFAs produced through gut microbiota fermentation can be rapidly absorbed by the intestinal epithelial cells of the host to supply energy to the host. This substantiates our second hypothesis: The gut microbiota of plateau species have developed similar strategies to facilitate their hosts' adaptation to specific environments, including broadening its dietary niche and enhancing energy absorption. In addition, the gut microbiota of *M. mulatta* can possibly help them degrade specific harmful substances.

## Methods

### Ethics approval and consent to participate

Sample collection and experimental protocols were performed in accordance with the Institutional Review Board (IRB202303) and the Institutional Animal Care and Use Committee of the Central South University of Forestry and Technology, China under permit number LXY-202201004, as well as Administration for Wild Animal Protection in Tibet Autonomous Region, China and adhered to the American Society of Primatologists Principles for the Ethical Treatment of Primates. All of the participants

provided informed consent to participate in this study under the approval of the Ethics Committee at the Central South University of Forestry and Technology (Approval number: 2012-018). All ethical regulations relevant to human research participants were followed. All samples included in this paper were taken in compliance with the laws of China, and no animal experimentation was involved.

### Study site and animals

The study was conducted from March 2023 to June 2024 in *Rhinopithecus bieti* National Nature Reserve in Markang, Tibet, China (Altitude: 3600–4700 m, Average annual temperature: 2–8 °C, Annual precipitation: 400–800 mm, N: 28°48'–29°40', E: 98°20'–98°59') (Fig. 7). There is a Tibetan village, Zhina in the study site, with a total of five families, raising goats, yaks, horses and other domestic animals, and living in a semi-agricultural and semi-nomadic mode. Although detailed information on local fauna is insufficient, the area is home to two species of NHPs, the *R. bieti* (Zhina group) and the *M. mulatta*. Other wild animals are mainly *E. cephalophorus*, *M. berezovskii* and *C. crossoptilon*. We selected local Tibetan people and the two kinds of domestic animals they raise (*B. mutus* and *C. hircus*), and these five common wild animals for our study.

**Field survey and sample collection.** Prior the collection and analysis on fecal samples, we carried out direct tracking and camera trapping to collect data on rudimentary knowledge on distribution and ecological functions of sympatric animals in the area, particularly two NHPs, *M. mulatta* and *R. bieti* (Zhina group)<sup>69–71</sup>. We carried out direct tracking for

2–5 days (07:30–18:30) per week based on terrain accessibility and weather conditions to search for wild animals (particularly *R. bieti*) and their feces. The duration of the work amounted to 110 days, ranging from March 2023 to June 2024. If we found *R. bieti* or other animals, we recorded the position of the animals via GPS receivers every 30 mins whenever possible<sup>69–71</sup>. We also collected feces samples and determined the freshness and the species who may defecate via viewing feces appearances and local experience. We also deployed 15 camera traps at animal trails to monitor wild animals and their potential defecation behaviors<sup>72</sup>. Images of camera traps were retrieved weekly to identify sympatric animals and to assist location of potential feces.

When we managed to follow *R. bieti*, *M. mulatta* and domestic animals, we observed their defecation behaviors whenever possible. If we found fresh feces (less than 12 h after defecation), we collected 3–4 g fecal samples from each individual's fecal patch non-invasively<sup>73</sup>. The stool samples were also obtained from five adult Tibetan human males who had not received any recent antibiotic treatment. After collection, the samples were stored at ambient temperature in sterile plastic tubes that were pre-filled with 5 ml of DNA preservation solution (Singen Inc., Zhejiang, China). All samples were labeled with collection time and individual information and transported to the laboratory via dry ice, where samples were stored at  $-80^{\circ}\text{C}$ .

**Home range and activity pattern of *R. bieti* and sympatric animals.** We combined all recorded GPS coordinates to estimate home ranges of *R. bieti* via the modified Minimum Convex Polygon method (excluding villages, farmlands and alpine meadow/bare rock  $\geq 4600$  m which *R. bieti* never use)<sup>69,70,74,75</sup>. The daily activity rhythms of *R. bieti* and *M. mulatta* were also collected via camera trap<sup>76</sup>. We identified all photographed animals and chose typical species from different taxa (Cetartiodactyla and Galliformes) for feces collection in the field. Species taxonomic identification followed current checklists of mammals and birds in China<sup>77,78</sup>.

**Amplicon sequencing, bioinformatics, and statistics.** Total DNA was extracted from the samples using the TGuide S96 Magnetic Stool DNA Kit (Tiangen Biotech (Beijing) Co., Ltd.) according to manufacturer's instructions. The hypervariable region V3-V4 of the bacterial 16S rRNA gene were amplified with primer pairs 338 F: 5'- ACTCCTACGG-GAGGCAGCA-3' and 806 R: 5'- GGACTACHVGGGTWTCTAAT-3'. polymerase chain reaction (PCR,  $98^{\circ}\text{C}$  for 2 min, followed by 30 cycles of  $98^{\circ}\text{C}$  for 30 s,  $50^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 60 s, and  $72^{\circ}\text{C}$  for 5 min.) products were checked on agarose gel and purified through the Omega DNA purification kit (Omega Inc., Norcross, GA, USA). The purified PCR products were collected and the paired ends ( $2 \times 250$  bp) was performed on the Illumina Novaseq 6000 platform.

The V3–V4 region of the archaea 16S-rRNA gene was also amplified by PCR ( $95^{\circ}\text{C}$  for 5 min, followed by 35 cycles of  $95^{\circ}\text{C}$  for 30 s,  $50^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 40 s, and  $72^{\circ}\text{C}$  for 7 min) using the primers Arch349F (50-GYG-CASCAGKCGMGAAC-30) and Arch806R (50-GGACTACVSGGG-TATCTAAT-30). The PCR products were mixed with an equal volume of  $2 \times$  loading buffer and electrophoresed in a 1.8% agarose gel for detection. Samples with a band at approximately 450 bp were mixed in equidensity ratios followed by purification using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, United States). Sequencing libraries were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States) and quantified with a Qubit 2.0 Fluorometer (Thermo Fisher). Finally, paired-end sequencing was conducted on an Illumina NovaSeq 6000 platform (Illumina, Inc., San Diego, CA, United States) by the Biomarker Bioinformatics Technology Co., Ltd. (Beijing, China).

The qualified sequences with more than 97% similarity thresholds were allocated to one operational taxonomic unit (OTU) using USEARCH (version 10.0). Taxonomic annotation of the OTUs was performed based on the Naive Bayes classifier in QIIME2 (version 2020.6.0)<sup>79</sup> using the SILVA database<sup>80</sup> (release 138.1) with a confidence threshold of 70%. Alpha was performed to identify the complexity of species diversity of each sample utilizing QIIME2 software. Beta diversity calculations were analyzed by

Non-Metric Multi-Dimensional Scaling to assess the diversity in samples for species complexity. To evaluate the distances of gut microbiota among various species, we conducted a hierarchical clustering analysis tree based on the Beta diversity distance algorithm. Specifically, the UPGMA (Unweighted Pair-group Method with Arithmetic Mean) algorithm was employed to construct the inter - sample tree structure, thereby reflecting the differences in the composition of various species. At the same time, we constructed the phylogenetic tree of the hosts by utilizing the Taxonomy feature on the NCBI, and then achieved visualization with iTol (<https://itol.embl.de/>). One-way analysis of variance was used to compare bacterial and archaea abundance and diversity. Linear discriminant analysis (LDA) coupled with effect size (LEfSe) was applied to evaluate the differentially abundant taxa. The online platform BMKCloud (<https://www.biocloud.net>) was used to analyze the sequencing data.

**Metagenomic sequencing, bioinformatics, and statistics.** To explore the differences and similarities in the functions of gut microbiota among three sympatric primates, we selected 16 samples from Tibetan people ( $n = 6$ ), *R. bieti* ( $n = 5$ ) and *M. mulatta* ( $n = 5$ ) for metagenomic sequencing. Total DNA was extracted from the samples using the TGuide S96 Magnetic Stool DNA Kit (Tiangen Biotech (Beijing) Co., Ltd.) according to manufacturer's instructions. DNA quality and quantity were determined using Nanodrop (ND-1000) spectrophotometry (Nanodrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis, respectively. DNA samples were stored at  $-20^{\circ}\text{C}$  until use. Shotgun sequencing was performed using an Illumina NovaSeq 6000, with at least 10 Gb per sample. Raw data were filtered using Trimmomatic v0.36<sup>81</sup> to trim low-quality reads: 3' tailing sequences were removed when the average quality over a 4 bp sliding window was less than 20 and reads less than 70 bp were discarded. The metagenome was assembled using MEGAHIT software (version 1.2.6) with the default parameters<sup>82</sup>. QUAST software (version 5.0.2) was used to evaluate the assembly results<sup>83</sup>. MetaGeneMark software (version 3.26) was used to identify the coding regions in the contigs<sup>84</sup>. Non-redundant gene sets were constructed using MMseqs2 software (version 12-113e3)<sup>85</sup>. The similarity threshold was set to 95%, and the coverage threshold was set to 90%.

The non-redundant gene sets were compared with the kyoto encyclopedia of genes and genomes (KEGG, <https://www.genome.jp/kegg/>) databases using the DIAMOND software (version 0.9.24)<sup>86</sup>. The threshold was set to an  $e < 1e-05$ . In the case of multiple alignment results (hits), the best alignment result was selected as the annotation of the sequence. The non-redundant gene sets were compared with the hidden Markov model of each family in carbohydrate-active enzymes database (CAZy, v10\_date20211003) using the hmmer software (version 3.0, Default comparison parameters, default screening threshold "if alignment  $> 80$ aa, use  $E < 1e-5$ , otherwise use  $E < 1e-3$ ; covered fraction of HMM  $> 0.3$ "). Identified all the families that met the filtering threshold. In that way, we could locate the carbohydrate-active enzymes within the non-redundant gene and analyze the number of conservative carbohydrate-related functional domains they contained. Functional genes composition was subjected to analysis of similarity using the R vegan package, respectively. Wilcoxon rank-sum tests were used for nonparametric tests of the difference between groups of functional genes.  $p < 0.05$  were considered statistically significant.

**Statistics and reproducibility.** In all experiments, biological replicates were employed, with each replicate representing an individual animal. We selected 51 samples from *H. sapiens* ( $n = 6$ ), *R. bieti* ( $n = 10$ ), *M. mulatta* ( $n = 8$ ), *E. cephalophus* ( $n = 6$ ), *M. berezovskii* ( $n = 5$ ), *B. mutus* ( $n = 5$ ), *C. hircus* ( $n = 5$ ) and *C. crossoptilon* ( $n = 6$ ) for the 16S rRNA gene amplicon study. For the metagenomic study, 6 *H. sapiens*, 5 *R. bieti* and 5 *M. mulatta* were included. The t-test and Wilcoxon rank-sum tests were used to analyze the differences between the two groups. The significance threshold was set at  $P < 0.05$ .  $^*P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ . Statistical analysis was performed using IBM SPSS Statistics (version 23).

## Data availability

The data presented in the study are deposited in the National Genomics Data Center (NGDC, <https://ngdc.cncb.ac.cn/gsa/>), accession numbers CRA015247 and CRA015267. Source values underlying the figures can be found in supplementary data.

Received: 27 August 2024; Accepted: 13 January 2025;

Published online: 20 January 2025

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## Acknowledgements

Z.F.X. discloses support for the research of this work from the National Natural Science Foundation of China [32171487] and China Huaneng

Group [HY2022-D8]. Q.Q.S. discloses support for the research of this work from the National Natural Science Foundation of China [32400413], the Natural Science Foundation of Hunan [2023JJ41038] and Excellent Youth Project of Hunan Education Department [22B0257]. We express our gratitude for the support received from all aforementioned projects. We would also like to extend our gratitude to Prof. Ming Li and Dr. Xiaochen Wang from the CAS Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, for their substantial support in the preparation of this article.

### Author contributions

Z.F.X. designed the experiments. C.Z. performed the experiments. C.Z., Y.X.C., and L.Y. compiled the data and conducted the analyses. C.Z. and Y.L. collected samples and data. C.Z., Y.X.C., L.Y., and Q.Q.S. wrote the paper. Y.Y., Y.C., and C.G. provided help in analysis. All authors read and approved the final version of the manuscript.

### Competing interests

The authors declare no competing interests.

### Additional information

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s42003-025-07538-6>.

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**Peer review information** *Communications Biology* thanks Samuel Aroney, and Süleyman Yıldırım for their contribution to the peer review of this work. Primary Handling Editors: Sabina Leanti La Rosa and Tobias Goris. [A peer review file is available].

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