# Variance in the Gut microbiota of Wild Rodent along the Spatial Distance and Species Identity Scale

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**Authors’ Contribution**

Wanhong Wei and Yongzhen Wu designed the study; Yongzhen Wu, Taoxiu Zhou, Chen Gu, Baofa Yin collected and processed the data, which were analyzed by Yongzhen Wu and Taoxiu Zhou; Baofa Yin, Shenmei Yang, Yunzeng Zhang and Ruiyong Wu revised the manuscript.

**Data Accessibility**

Molecular sequence data has been deposited in the NCBI Sequence Read Archive (SRA) database (accession number PRJNA1019510 ).

# Abstract

1. The gut microbiota of rodents is essential for survival and adaptation, and has been shown to be susceptible to a variety of factors, ranging from environmental conditions to genetic predispositions. Nevertheless, few comparative studies have considered the contribution of species identity and geographic spatial distance to the variation in gut microbiota.
2. Here, we investigated the gut microbial communities of four wild rodent species (*Rattus norvegicus*, *Apodemus agrarius*, *Cricetulus barabensis*, and *Tscherskia triton*) at five sites in northern China's farming-pastoral transition zone. By performing a cross-factorial comparison, we are able to test whether belonging to the same species, or instead, being in the same capture site dominates in determining gut microbiota composition.
3. Our analysis found that the Amplicon Sequence Variants (ASVs) showed a partial overlap with the species identity and the geographic capture sites, which did not reveal a ‘phylosymbiosis’ pattern.
4. The gut microbiota of these four rodent species adhered to typical mammalian characteristics, predominantly characterised by the Firmicutes and Bacteroidetes phyla. As the geographic distance between populations increased, the shared microbial taxa among conspecific populations decreased. We observed that within a relatively small geographical range, even different species exhibit convergent α-diversity due to their inhabitation within the same environmental microbial pool. In contrast, the composition and structure of the intestinal microbiota in allopatric populations of *A. agrarius* showed marked differences, as well as *C. barabensis*. Additionally, geographical environmental elements, exhibited significant correlations with diversity indices. Conversely, host-related factors had minimal influence on microbial abundance.
5. These findings illuminated that the similarity of the microbial compositions was not determined primarily by the host species, the location of the sampling explained a greater amount of variation in the microbial composition, indicating that the local environment played a crucial role in shaping the microbial composition.

**Keywords:** rodent, 16S rRNA gene sequencing, gut microbiota, distance isolation

# 1 | INTRODUCTION

Microbes are considered an integral component of the animal phenotype (Holmes & Nicholson 2005), exerting an influence on the fitness and, consequently, on ecologically significant traits of their hosts (McFall-Ngai *et al.* 2013; Groussin *et al.* 2017; Greene *et al.* 2020). Despite the increasing number of researchers dedicated to unveiling the mysterious factors determining the structure of host gut microbiota, many gaps persist, especially in various animal hosts within natural systems.

In recent decades, studies based on the combination of bioinformatics analysis and high-throughput sequencing technology have shown that many factors, such as host genetics, diet, season, age, and lifestyle, can strongly affect the compositions of the gut microbiota (Benson *et al.* 2010; Spor, Koren & Ley 2011; Claesson *et al.* 2012; Adriansjach *et al.* 2020; Jiang *et al.* 2021; Kavaliers *et al.* 2021; Reese *et al.* 2021). However, there is still debate about the precise nature of the predominant factors, with some researchers emphasising the role of genetics, while others highlight environmental influences. For instance, research conducted in laboratory settings unveiled a significant correlation between the genetic makeup of the mice and the composition of their gut microbiota, while co-housing had a limited impact on microbiota composition, emphasizing the role of host genetics (Campbell *et al.* 2012). Additionally, studies in natural environments also revealed that the species-specific component of microbial composition stems from the shared diversification of both host and microbes. A study that compared the gut microbiota of small mammals (especially mice, voles and shrews) in various habitats ranged from 2 to 23 km, found that despite factors such as diet and location in the environment influencing the host's microbiota to some extent, species identity was the strongest predictor of microbial composition (Knowles, Eccles & Baltrunaite 2019). This conclusion was consistent with previous studies on wood mice (Weinstein *et al*. 2021)and American pikas (Kohl *et al.* 2018).

Moreover, some studies also emphasise the decisive role of environmental factors*.* Dietary variations can exert a profound influence on the gut microbiota. A study utilising genetically defined mice revealed that dietary intervention, transitioning mice to a high-glucose, high-fat diet, could rapidly reshape the abundance of the gut microbial community into a novel and stable composition, independent of the hereditary distinctions among individual mice (Carmody *et al.* 2015). This finding is consistent with a study involving humans (David *et al.* 2014). Furthermore, insights from nonhuman primates suggest that the gut microbiota of baboons is primarily influenced by soil geological history and exchangeable sodium content, rather than host genetic factors (Grieneisen *et al.* 2019). Similarly, research on humans and four species of hominoids has revealed a weak correspondence between the phylogeny of host systems based on mitochondrial DNA (mtDNA) and the composition of their fecal microbiota. This underscores the significance of the host environment (Ochman *et al.* 2010).

While research involving wild and laboratory animals provides substantial evidence regarding environmental and genetic influences, it is essential to consider several factors that can introduce biases in research conclusions. For example, some studies that emphase genetic dominance in the variation of host-associated microbial communities face challenges due to being restricted to laboratory settings or relatively small geographic scales (Wang *et al.* 2019). These limitations hinder the detection of factors that influence the microbiota of species with strong dissemination capabilities within the intestine (Pascoe *et al.* 2017). On the other hand, studies that emphasise environmental factors often focus on a limited number of species or individual factors, failing to address the interactions among these factors in a comprehensive way. Given the lack of correlation between laboratory model mouse studies and ecological environments, conducting research in natural habitats with greater geographic distances can mitigate the confounding effects of intraspecific gene flow, enabling an examination of the major factors contributing to variation in host gut microbiota (Linnenbrink *et al.* 2013; Lin *et al.* 2020). This approach can significantly improve our understanding of the influences shaping microbial communities and their dynamics.

In this study, we proposed a large-scale geographic population survey that involved four distinct species of natural rodents: *R. norvegicus*, *A. agrarius*, *C. barabensis*, and *T. triton*. We employed 16S rRNA gene sequencing technology in conjunction with bioinformatics analysis to characterise the microbial communities of these organisms at both the individual and population levels. Furthermore, we used principal coordinates analysis (PCoA), permutational multivariate analysis of variance (PERMANOVA), spearman correlation analysis, and other methodologies to quantify the contributions of host-related factors and geographic factors to the variation in gut microbiota communities. Our objective was to demonstrate that as the spatial distance between populations increases, the congruence between the phylogenetic signals of host gut microbiota and the evolutionary overlap among hosts decreases, while community disparities escalate in accordance with the geographic distance between populations. We hope that this study could contribute to a more comprehensive understanding of the interaction between host genetics and environmental factors, as well as tits significance in shaping the composition of gut microbiota.

# 2 | MATERIAL AND METHOD

## 2.1 | Sample and field data collection

In total, 110 rodents of four species (*R. norvegicus, A. agrarius, C. barabensis and T. triton*) were collected during 24 July and 17 August 2020 at five geographic locations in the farming-pastoral transitional zone in northern China (Table 1, Figure. 1). Our survey sites set a distance rule greater than 50km, to minimize the possibility of gene exchange between different populations of the same species. Each day at dusk, a small collapsable aluminium Sherman trap (2 × 2.5 × 6.5 inches) baited with peanut seeds was placed along predetermined transects. These traps were left overnight for two consecutive days. The captured individuals were euthanized using ether and then stored in sterile bags in a vehicle refrigerator at -20°C during transportation to the laboratory. In parallel, we document individual-specific data, including weight, sex, age, and supplemented by geographical coordinates for each sampling point (Supporting Information Table S1), we categorized age groups based on individual body weight (Yang & Zheng 2003; Yang *et al.* 2009). Also, we also obtained meteorological data from the China National Meteorological Information Center (https: //data.cma.cn) for each coordinate point (Supporting Information Table S2).

We used the thorough cleaning of the dissection tools with 75% ethanol and then performed sample dissections. Approximately 10 mg of content from the distal cecum was extracted as a microbial sample. To prevent RNA degradation within the microbial samples, these contents were placed in 4 ml of RNALater. At the end of the fieldwork, the samples were transported while frozen in the laboratory and stored at -80°C before DNA extraction.

## 2.2 | DNA extraction and sequencing

The whole genome DNA of the samples was extracted using the CTAB method and the DNA concentration and purity of 1% DNA in the agarose gels were checked. According to the concentration, DNA was diluted to 1ng/μL using sterile water. Using primers 515F (5’-barcode-GTGCCAGCMGCCGCGGTAA-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’) to amplify the 16S rRNA genes in the V4 region. All PCR reactions were performed using 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs); 2 μM of forward and reverse primers, and about 10 ng template DNA. The thermal cycle aging from 98℃ for 1 minute. Then denature at 98℃ for 30 cycles for 10 seconds, annealing at 50℃ for 30 seconds, and 72℃ tensile strength for 30 seconds. Finally, keep at 72℃ for 5 minutes. After that, we mixed an equal amount of 1X loaded buffer (contained SYB green) with the PCR products and examined by electrophoresis on 2% agarose gel. PCR products was mixed in equidensity ratio, and then mixture PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end target reads were generated.

## 2.3 | 16S rRNA bioinformatics

Quality controls of the already demultiplexed paired-end sequence reads were performed according to the QIIME2 (Caporaso *et al.* 2010). Firstly, the paired-end reads were assigned to samples based on their unique barcodes and were truncated by cutting off the barcodes and primer sequences. The reads were then merged using FLASH (Version 1.2.11, http://ccb.jhu.edu/software/FLASH/) (Magoc & Salzberg 2011), and the splicing sequences were called raw tags. Then, quality filtering on the raw tags was performed using the fastp (Version 0.20.0) software to obtain high-quality clean tags. Clean tags were compared with the reference database (Silva database https://www.arbsilva.de/) using Vsearch (Version 2.15.0) to detect the chimera sequences, and then the chimera sequences were removed to obtain the effective tags (Haas *et al.* 2011). After that, effective tags were denoised with the DADA2 module in the QIIME2 software to obtain initial Amplicon Sequence Variants (ASVs), and then ASVs with abundance less than 5 were filtered out (Marizzoni *et al.* 2020). To study the phylogenetic relationship of each ASV and the differences of the dominant species among different samples, multiple sequence alignment was performed using QIIME2 software. The absolute abundance of ASVs was normalised using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analyses of alpha diversity and beta diversity were performed on the basis of the output normalised data.

# 2.4 | Statistical analyses

Community bar charts were used to plot the relative abundance of bacteria in each caecal sample at the phylum, class, order, family and genus levels with R software (version 4.1.2, ‘stats’ packages). Venn charts was used to analyze the core and unique bacterial in diﬀerent species with the R software. Alpha diversity was calculated from 7 indices in QIIME2, including Observed\_ASVs, Chao1, Shannon, Simpson, Dominance, Good’s coverage and Pielou’s evenness. Wilcoxon’s rank sum test was used to analyse the geological signiﬁcance of Alpha diversity indexes with the R software (‘stats’ packages). The beta diversity on Bray-Curti’s dissimilarity was calculated by QIIME2 software. Principal Coordinate Analysis (PCoA) was performed with the R software (‘vegan’ packages) to get principal coordinates and visualise from complex multidimensional data. Unweighting Pair-group Method with Arithmetic Means (UPGMA) Clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage and was conducted by R software. Beta diversity was compared to host and environmental variables using permutational multivariate analysis of variance based on the distance tables. This was done using the PERMANOVA function in R with sex, age, and trapping location as predictor variables and Bray-Curti’s dissimilarity index as the response. The P values were estimated by comparing the pseudo-F values against 999 random permutations of the data. To investigate whether there are significant differences in the abundance of specific taxa between different groups of conspecific populations as well as heterospecific populations, linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/lefse/) was performed on a normalised relative abundance matrix. Furthermore, within the R environment, we utilised Spearman correlation analysis to evaluate the correlation between the Bray-Curtis similarity matrix of ASV and the geographical distance matrix, and examined the correlations between environmental factors, α-diversity indices, and composition of the microbial community.

# 3 | RESULTS

## 3.1| Identification of Bacterial ASVs

16S rRNA amplicon sequencing of a total of 110 rodent caecal content samples targeting the hypervariable V4 region of the 16S rRNA gene was sequenced and analysed, and 7,111,399 high quality sequences were obtained. These sequences were clustered into 6,308 ASVs, representing 36 phyla, 85 classes, 183 orders, 303 families, and 529 genera (Supporting Information Table S3).

## 3.2 | Phylogenetic Structure and Distribution of ASVs

Analysing the entire dataset of ASV, we observed that the arrangement of branches in the phylogenetic tree of the microbial community based on the unweighted unifrac distance did not completely overlap with the phylogenetic signals of the host species, as well as the geographical locations exhibited a similar pattern (Figure 2a). VEEN diagram illustrates the shared counts of ASVs across different species and populations at various sites. At site1, the *A. agrarius* and *T. triton* shared a total of 1,414 ASVs. Moving on to site2, *A. agrarius* and *R. norvegicus* exhibited an overlap of 1,651 ASVs. At site3, the *A. agrarius* and *C. barabensis* shared 1,426 ASVs. Site4 showed that the *A. agrarius* and C. barabensis shared 1,024 ASVs. At site5, *A. agrarius* shared 1,128 ASVs with *C. barabensis* and 1,021 ASVs with *R. norvegicus*, while *C. barabensis* and *R. norvegicus* have a shared count of 948 ASVs. Additionally, these three species collectively shared 875 ASVs (Figure 2b). Among the five geographic populations of *A. agrarius*, there was an 801 ASVs overlap. For the three geographic populations of *C. barabensis*, the overlap was 492 ASVs. Lastly, the two geographic populations of *R. norvegicus* shared a total of 1,037 ASVs (Figure 2c). Although we could find some conservative ASVs in all comparison groups, the amount of conservative bacteria was consistently higher in the cases of heterospecific populations within the same habitat compared to conspecific populations in different habitats (P < 0.001) (Figure 2d).

## 3.3 Bacterial Composition and Abundance among Populations

Analysis at the phylum level showed that the dominant phyla (mean relative abundance > 10%) in all populations included Firmicutes (33.52-57.05%, with an average relative abundance of 44.12%) and Bacteroidetes (11.53-39.91%, average 24.32%). Rare phyla (mean relative abundance < 5%) included Campilobacterota (average 3.44%), Actinobacteriota (average 3.31%), Desulfobacterota (average 1.87%), Spirochaetota (average 1.6%), Acidobacteriota(average 0.79%), Fusobacteriota (average 0.15%) (Figure 3a). At the genus level, the top 10 genus were *Lactobacillus* (3.8–40.8%, average 12.32%), *Helicobacter* (0.1-8.0%, average 3.4%), *Lachnospiraceae\_NK4A136\_group* (1.1-5.4%, average 3.4%), *Streptococcus* (0.2–6.9%, average 2.4%), *Bacteroides* (0-14.0%, average 1.8%), *Prevotella* (0-14.0%, average 1.7%), *Desulfovibrio* (0.8-2.6%, average 1.5%), *Colidextribacter* (0.7-2.3%, average 1.4%), *Roseburia* (0.2-3.4%, average 1.4%), and *Prevotellaceae\_UCG-003* (0-4.1%, average 1.1%) (Figure 3b).

Comparison of α-diversity indices among all conspecific populations showed that there was no significant difference in Chao1 and Shannon indices between different groups at S1, S2, S3, and S5, but group S4.Ap.a and S4.Cr.bhad a significant difference in the Chao1 indices (*P* = 0.0081) and Shannon indices(*P* = 0.0024) (Figure 3c,3d; Table 2). On the contrary, in cases of conspecific populations at different site, the population of *A. agrarius* at S4 exhibited the highest level of diversity, while S5 showed the lowest (Figure 3c,3d; Table 2). The α diversity level of the *C. barabensis* population at S3 was significantly higher than that of S4 (Shannon, P < 0.001), the observed count of bacterial species was significantly higher at S5 compared to S4 (Chao1, P < 0.001) (Figure 3c,3d; Table 2). However, there were no significant differences in α diversity index between the two geographic populations of *R. norvegicus* (Figure 3c,3d; Table 2).

## 3.4 | Comparison of Gut Microbiota Beta Diversity between Populations.

PCoA plots based on Bray-Curtis dissimilarity showed clear sample clustering not only by host species, but also capture site (Figure 4a-h). At S1, groups S1.Ap.a and S1.Ts.t (PERMANOVA, *F* = 3.08, *P* = 0.007) were differed (Figure 4a, Table 3). At S2, clear differentiation was observed between S2.Ap.a and S2.Ra.n (PERMANOVA, *F* = 2.68, *P* = 0.02) (Figure 4b, Table 3). At S3, the gut microbiota composition of the S3.Ap.a and S3.Cr.b groups did not show clear differentiation, suggesting convergent community structures between these two populations (Figure 4c, Table 3). At S4, the groups S4.Ap.a and S4.Cr.b were clustered separately (PERMANOVA, *F* = 6.1, *P* = 0.001) (Figure 4d, Table 3). At S5, there were no significant differences between the populations of *A. agrarius* and *C. barabensis*, but both the population of *A. agrarius* (PERMANOVA, *F* = 3.68, *P* = 0.005) and *C. barabensis* (PERMANOVA, *F* = 3.52, *P* = 0.023) population were distinct from the population of *R. norvegicus* (Figure 4e, Table 3). When comparing inter-species differences among conspecific populations in the different site, significant distinctions were observed within the five populations of the *A. agrarius* (S1.Ap.a, S2.Ap.a, S3.Ap.a, S4.Ap.a, S5.Ap.a,) (Figure 4f). Similarly, the three heterospecific populations of *C. barabensis* (S3.Cr.b, S4.Cr.b, S5.Cr.b) showed noticeable separation (Figure 4g). However, the two populations of the *R. norvegicus* (S2.Ra.n and S5.Ra.n) appeared relatively dispersed, this might suggest that the within-group differences for both groups were higher than the between-group differences (Figure 4h).

Furthermore, the Linear Discriminant Analysis Effect Size (LEfSe) noted that there were 8 distinct taxa (LDA = 4) between the populations of *A. agrarius and T. triton* at S1, and all of these taxa were enriched within the *T. triton* (Figure 5a); 9 different taxa (LDA = 4) between *A. agrarius* and *R. norvegicus* in S2, mainly from Bacteroidetes and Firmicutes (Figure 5b); In S4 (LDA = 4), the phylum of Proteobacteria and some potential pathogenic genera of bacteria, such as *Morganella* and *Streptococcus* were enriched most within S4.Ap.a, while genera of *Lactobacillus* and *Bifidobacterium* that were considered probiotics were enriched in *C. barabensis* (Figure 5c). The main differences between *C. barabensis* and *R. norvegicus* in S5 (LDA = 4) were found in Firmicutes phylum and Muribaculaceae family (known as S24-7 family) (Figure 5d). It should be note that no taxonomic differences were detected between the *A. agrarius* and *C. barabensis* at site 3. Interestingly, we observed an increase in the number of taxa when comparing conspecific populations within different sites. It was showed that the five isolating geographical populations of the *A. agrarius* were discriminated by a total of 59 taxa (LDA = 4). These taxonomic distinctions were primarily delineated across 8 phyla (including Firmicutes, Actinobacteria, and other core phyla), 9 classes (including Bacilli, Bacteroidia, and others), 10 orders (including Lactobacillales, Bacteroidales, Enterobacterales, Spirochaetales, Clostridiales, and more), 15 families (including Muribaculaceae, Streptococcaceae, Vibrionales, Lactobacillaceae, Enterobacteriaceae, and others), and 12 genera (*Lactobacillus*, *Muribaculaceae*, *Streptococcus*, *Ureaplasma*, and others) (Figure 5e). For the allopatric speciation of *C. barabensis*, we detected 34 significantly distinct taxa (LDA = 4). These groups mainly encompassed 3 phyla (including Campilobacterota, Proteobacteria, and Actinobacteriota), 4 classes (including Clostridia, Campylobacteria, Bacilli, and Actinobacteria), 7 orders (including Lachnospirales, Oscillospirales, Campylobacterales, etc.), 10 families (including Lachnospiraceae, Oscillospiraceae, Prevotellaceae, Helicobacteraceae, etc.), and 8 genera (*Helicobacter,* *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, etc.) (Figure 5f). However, no significant bacterial differences were detected at the taxonomic level between the two geographic populations S2.Ra.n and S5.Ra.n.

## 3.5 | Association of environmental and host factors with microbial diversity and composition

The results of spearman analysis showed that host factors such as age, gender, and BMI have minimal effects on the variation in gut microbiota abundance (Figure 6a). In contrast, external environmental factors displayed distinct associations; altitude exhibited significant positive correlations with the Chao1 index (*R2* = 0.318, *P* = 0.002 ) and species observed index (*R2* = 0.318, *P* = 0.002 ), indicating higher diversity in high-altitude regions. Meanwhile, the annual average temperature showed a certain down-regulating effect on microbial diversity, while the annual average precipitation had an up-regulating effect (Figure 6b).

Furthermore, a correlation analysis between core genera and these influencing factors unveiled significant associations. Regarding host-related factors, BMI displayed a significant negative correlation with *Lactobacillus* (*R2* = 0.318, *P* = 0.002 ) and a significant positive correlation with *Treponema* (*R2* = 0.318, *P* = 0.002 ), whereas gender exhibited a significant positive correlation with *Morganella* (*R2* = 0.318, *P* = 0.002 ) (Figure 6c). In the case of external host factors, annual average temperature (Anu\_Tem) showed a significant positive correlation with *Muribaculaceae* (*R2* = 0.318, *P* = 0.002), *Ureaplasma* (*R2* = 0.369, *P* < 0.001), *Vibrio* (*R2* = 0.359, *P* < 0.001), *Treponema* (*R2* = 0.405, *P* < 0.001), and *Prevotella* (*R2* = 0.577, *P* < 0.001), while displaying a significant negative correlation with *Lactobacillus* (*R2* = -0.408, *P* < 0.001), *Streptococcus* (-0.321, *P* = 0.002), and *Morganella* (*R2* = -0.39, *P* < 0.001). Annual average precipitation (Anu\_Rai) showed a significant positive correlation with *Lactobacillus* (*R2* = 0.408, *P* < 0.001), *Streptococcus* (*R2* = 0.321, *P* = 0.002), and *Morganella* (*R2* = 0.39, *P* < 0.001), while showing a significant negative correlation with *Muribaculaceae* (*R2* = -0.318, *P* = 0.002), *Ureaplasma* (*R2* = -0.369, *P* < 0.001), *Vibrio* (*R2* = -0.359, *P* < 0.001 ), *Treponema* (*R2* = -0.405, *P* < 0.001), and *Prevotella* (*R2* = -0.577, *P* < 0.001). Altitude exhibited a significant positive correlation with *Muribaculaceae* (*R2* = 0.371, *P* < 0.001) and a significant negative correlation with *Streptococcus* (*R2* = -0.415, *P* < 0.001) and *Vibrio* (*R2* = -0.297, *P* = 0.004) (Figure 6d). What’s more, we observed a significant positive correlation between the dissimilarity of gut microbiota and spatial distance ( *R2* = 0.291, *P* < 0.001).

# 4 | DISCUSSION

In this study, we provided a comprehensive analysis of the gut microbiota of rodent populations, shedding light on the diversity, phylogenetic structure, and the influential factors affecting microbial composition. We found the α-diversity among different populations converged if they shared microbial pool (inhabited the same site), while they still possessed certain β-diversity differences. This suggested that the environment determines which specific bacterial species were present in the gut, while the host itself regulated the abundance of these species. Besides, there was significant microbial differentiation observed among different geographic populations of *A. agrarius* and *C. barabensis*. Furthermore, we found that as the geographic distance increased, the microbial dissimilarity among these distinct populations increased. However, the gut microbiota of brown rats appeared minimally influenced by geographic distance, likely due to their strong dispersal ability and omnivorous nature.

Recent work has provided evidence for the formation of species between mammals and their gut microbiota (Amato *et al.* 2016). Compared to clinical studies, microbial research targeting wild animals was limited at that time. Many published studies focused on a single population of a single host species. In our study, we conducted a large-scale geographical survey of natural rodent populations from the farming-pastoral transitional zone in northern China (Chen *et al.* 2008), while the survey area itself was a typical ecologically vulnerable region, highly responsive to environmental changes (Han *et al.* 2018). Therefore, we had reason to believe that the intestinal microbiota of rodents in this region may exhibit considerable plasticity. And to avoid opportunities for gene flow between populations, we increased the distance between sampling points to 50 kilometers.

In our study, the vast dataset generated through 16S rRNA amplicon sequencing provided significant insights into the diversity of bacterial communities within rodent caecal content. And the clustering of sequences into more than 6,000 ASVs, representing a wide taxonomic range, emphasized the richness and complexity of the gut microbiota in these rodents. Our study showed that the phylogenetic arrangement of microbial communities did not entirely mirror the phylogenetic signals of host species, indicated the combined influence of host genetics and environmental factors on gut microbiota composition. And the Venn diagram analysis provided a clear view of ASVs sharing among different species and populations at various sites, supporting that factors beyond host genetics, like spatial distance, play a substantial role in shaping the gut microbiota (Suzuki, Martins & Nachman 2019; Chen *et al.* 2020; Montoya-Ciriaco *et al.* 2020). The similar patterns in geographical locations further underlined the role of environment in microbial composition. A plausible explanation could be that the gut microbiota composition of mammals was influenced by the geographical distance between locations due to bacterial dispersal constraints (Moeller *et al.* 2017). Within smaller geographic ranges, a more likely explanation was the profound impact of shared environment resources (Lavrinienko *et al.* 2021).

Following the QIIME pipeline, we found that the wild rodents gut microbiota was comparable to that of other mammals with two major phyla, the Firmicutes and Bacteroidetes (Benson *et al.* 2010; Amato *et al.* 2016; Ross *et al.* 2018). Our results showed that the relative abundance of Firmicutes was higher than that of Bacteroidetes in *A. agrarius*, *C. barabensis* and *R. norvegicus*, while the relative abundance of Bacteroidetes was higher than that of Firmicutes in *T. triton*. This underscored the potential influence of dietary factors on microbial composition (Zoelzer, Burger & Dierkes 2021). The *A. agrarius*, *C. barabensis* and *R. norvegicus* were not strict herbivores like squirrels and lemurs (Riofrio-Lazo & Paez-Rosas 2015; Shi *et al.* 2017; Greene *et al.* 2020; Liu *et al.* 2020), whereas the *T. triton* were herbivorous, occasionally incorporating insects into their diets (Xiong *et al.* 2009). Firmicutes were common type of bacteria known for their ability to degrade various complex organic compounds, who may be more abundant in omnivorous animals to facilitate digestion and absorption of a diverse range of foods (Guindo *et al.* 2020). Bacteroidetes that typically abundant in the gut of herbivorous animals were known for their ability to break down plant cellulose, and were efficient in breaking down polysaccharides present in plant materials, aiding animals in the digestion of fiber to acquire energy and nutrients (Clauss *et al.* 2020; Fu *et al.* 2021). Why Some bacterial groups are more host specific than others is an interesting open question. One possibility is that Some bacteria are more amenable to host selection via immunity (Benson *et al.* 2010; Kurilshikov *et al.* 2017). Such differences in biology warrant further investigation as potential mediators of host specificity.

By comparing the microbial community variations between heterospecific populations and conspecific populations, we revealed that heterospecific populations within the same habitat had a higher abundance of conservative bacteria, whereas conspecific populations in different habitats had a lower abundance of conservative bacteria. Some other research also indicated that shared habitats significantly promote α-diversity convergence among conspecific symbiotic populations within the same habitat (Grieneisen *et al.* 2019; Grond *et al.* 2020). Additionally, our results suggested that geographical factors play a species-specific role in shaping the α-diversity of host gut microbiota, with *A. agrarius* and *C. barabensis* populations being more susceptible to the influence of geographical factors, whereas this influence was less pronounced in *R. norvegicus* populations. We found that there was no significant difference in β diversity of gut microbiota between the *A. agrarius* and *C. barabensis* at site 3, indicating a higher similarity in gut microbiota between these two species in a homogenized environment, which could be disadvantageous as it may intensify interspecific competition between the two species (Anders *et al.* 2022; Shaner & Ke 2022). Besides, the observed within-group differences being higher than between-group differences in certain cases (such as *R. norvegicus*) suggested unique microbial adaptations within specific populations (He *et al.* 2020).

The correlation analyses between environmental/host factors and microbial diversity revealed intriguing associations. Although we did not observe a statistically significant influence of gender on the detected variations, aligned with findings in research involving small wild mammals like woodrat, vole, and house mice (Goertz *et al.* 2019; Lin *et al.* 2020; Weinstein *et al.* 2021). This consistent pattern suggests that fluctuations in gut microbiota resulting from natural environmental dynamics may overshadow differences attributable to host factors such as sex and age. However, it's noteworthy that in larger mammals, particularly those with a significant relative body size, such as vertebrates, the impact of gender tends to be more conspicuous (Adriansjach *et al.* 2020; Bjork *et al.* 2022). Additionally, controlled experiments in laboratory conditions, where environmental variables are meticulously regulated, also emphasize and demonstrate the presence of sex-based disparities (Kohl, Dearing & Bordenstein 2018; Screven & Dent 2018). Furthermore, we discovered that higher altitude exhibited positive correlations with microbial diversity indices, indicating potential adaptations to high-altitude environments (Quagliariello *et al.* 2019; Suzuki, Martins & Nachman 2019; Li *et al.* 2020). Moreover, the impact of annual average temperature and precipitation on microbial diversity suggests environmental factors significantly influencing gut microbiota. The taxonomic differences identified between populations and their correlation with environmental factors provide valuable insights into potential allopatric speciation events (Baral *et al.* 2018; Khakisahneh *et al.* 2020). The distinct taxa enriched within specific populations indicate potential adaptation and coevolution of the gut microbiota with their host species in different geographic locations (Suzuki *et al.* 2020).

The findings of this study hold broad implications for understanding the dynamics of gut microbiota in rodent populations. The highlighted influence of observed environmental factors underscores the necessity to consider habitat and diet in future investigations. Moreover, a deeper understanding of the ecological and evolutionary factors shaping the gut microbiota could be achieved by incorporating more host-associated information, such as inter-individual social contacts, kinship, social hierarchy, lineage differentiation, and other related aspects.

In conclusion, this study provides a comprehensive understanding of the gut microbiota in rodent populations, elucidating the intricate interplay between host identity, environment, and microbial communities. We revealed that the dominant role of species identity in the environment may not be universal, at least to a certain extent, as the scope of the investigation broadened, the explanatory power of spatial distance concerning the variations in the host's gut microbiota surpassed that of host identity. These findings contribute to our broader understanding of gut microbiota dynamics and their potential implications for ecology, evolution, and health.

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**Figure 1 |** **The geographical location information of the sampling sites and an overview of the habitats.** (a) Specific geographical region of the study area in China, situated in the northern segment of the agro-pastoral transitional zone in northern China. (b) locations of the sampling points (S1: Dongsheng Village, DS; S2: Wanbao Town, WB; S3: Erzhan Town, EZ; S4: Xinzhan Town, XZ; S5: Datong Town, DT). (c) Aerial habitat overviews of the sampling points were captured using a DJI Mavic 2 Pro unmanned aerial vehicle.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Species | Abbreviation | Site | | | | |
| S1 | S2 | S3 | S4 | S5 |
| *Apodemus agrarius* | Ap.a | 10 | 24 | 15 | 10 | 8 |
| *Cricetulus barabensis* | Cr.b | 0 | 0 | 5 | 8 | 4 |
| *Tscherskia triton* | Ts.t | 9 | 2 | 1 | 0 | 0 |
| *Rattus norvegicus* | Ra.n | 0 | 9 | 1 | 1 | 3 |

**Table 1 | Species and Quantity of rodent captured at each site for which the gut microbiota was characterized.** The groups that sample size was less than 3 would not be analyzed.

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**Figure 2 | The systematic phylogenetic structure of ASVs and a comparison of their distribution among different populations.** (a) Hierarchical clustering of samples according to Unweighted unifrac distance dissimilarity by using UPGMA, with bars colored according to host species and capture site that each sample came from. (b) Comparison of the shared and unique ASVs between heterospecific populations from the same site. (c) Comparison of shared and unique ASVs between conspecific populations from different sites. (d) Inter-group Wilcoxon test for the number of shared ASVs within the sympatric populations and between allopatric populations.

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**Figure 3 | Gut microbial composition and abundance of the four rodent species from different sites.** (a) Relative abundance of the top 10 species in phylums level. (b) Relative abundance of the top 10 species in genus level. (c) Variation of α diversity in the gut microbiota of rodents based on Chao1 index and (d) Shannon index.

**Table 2 | Intergroup Wilcoxon rank-sum difference test for α-diversity between conspecifics from the same habitat and heterospecific from different habitats.** Bold indicates significant differences in the compared groups.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Group-Pair** | | **Chao1** | | | **Shannon** | | |
| **Difference** | ***P* value** | | **Difference** | ***P* value** | |
| Same site, heterospecific | S1.Ap.a-S1.Ts.t | 4.233 | | 0.5945 | -2.344 | | 0.8320 |
| S2.Ap.a-S2.Ra.n | -3.222 | | 0.6728 | -13.056 | | 0.2204 |
| S3.Ap.a-S3.Cr.b | -12.733 | | 0.1564 | -19.533 | | 0.1185 |
| S4.Ap.a-S4.Cr.b | 22.175 | | **0.0081** | 35.575 | | **0.0024** |
| S5.Ap.a-S5.Cr.b | -13.875 | | 0.1924 | -24.250 | | 0.1024 |
| S5.Ap.a-S5.Ra.n | -15.458 | | 0.1890 | 10.917 | | 0.5031 |
| S5.Cr.b-S5.Ra.n | -1.583 | | 0.9046 | 35.167 | | 0.0583 |
| Different site, conspecific | S1.Ap.a-S2.Ap.a | 25.900 | | **0.0007** | 25.267 | | **0.0159** |
| S1.Ap.a-S3.Ap.a | -9.767 | | 0.1690 | 2.833 | | 0.7730 |
| S1.Ap.a-S4.Ap.a | -37.400 | | **0.0000** | 5.400 | | 0.6159 |
| S1.Ap.a-S5.Ap.a | 32.025 | | **0.0002** | 11.850 | | 0.3005 |
| S2.Ap.a-S3.Ap.a | -35.667 | | **0.0000** | -22.433 | | **0.0179** |
| S2.Ap.a-S4.Ap.a | -63.300 | | **0.0000** | -19.867 | | 0.0564 |
| S2.Ap.a-S5.Ap.a | 6.125 | | 0.4386 | -13.417 | | 0.2237 |
| S2.Ra.n-S5.Ra.n | -6.111 | | 0.5963 | 10.556 | | 0.5109 |
| S3.Ap.a-S4.Ap.a | -27.633 | | **0.0002** | 2.567 | | 0.7938 |
| S3.Cr.b-S4.Cr.b | 7.275 | | 0.4613 | 57.675 | | **0.0001** |
| S3.Ap.a-S5.Ap.a | 41.792 | | **0.0000** | 9.017 | | 0.3929 |
| S3.Cr.b-S5.Cr.b | 40.650 | | **0.0007** | 4.300 | | 0.7899 |
| S4.Ap.a-S5.Ap.a | 69.425 | | **0.0000** | 6.450 | | 0.5722 |
| S4.Cr.b-S5.Cr.b | 33.375 | | **0.0022** | -53.375 | | **0.0005** |

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**Figure 4 | Clustering of gut microbial communities according species identity and capture site.** (a-c) Principle coordinate (PCoA) plots based on Bray-Curtis dissimilarity for heterospecific populations in different sites and conspecific populations in the same site (d-h).

**Table 3 | Permutation Multivariate Analysis of Variance (PERMANOVA) results for gut microbiota composition between different populations based on bray-Curtis distance.** ∗p < 0.05, ∗∗p < 0.01, and ∗∗∗p < 0.001. ns, not significant.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **group-pair** | **F.Model** | **R2** | **Pr (>F)** |
| **Same site, heterospecific** | S1.Ap.a-S1.Ts.t | 3.0755 | 0.1532(0.8468) | **0.007** |
|  | S2.Ap.a-S2.Ra.n | 2.6805 | 0.12364(0.87636) | **0.02** |
|  | S3.Ap.a-S3.Cr.b | 0.90782 | 0.04801(0.95199) | 0.454 |
|  | S4.Ap.a-S4.Cr.b | 6.1005 | 0.27603(0.72397) | **0.001** |
|  | S5.Ap.a-S5.Cr.b | 1.5191 | 0.13188(0.86812) | 0.136 |
|  | S5.Ap.a-S5.Ra.n | 3.6764 | 0.29002(0.70998) | **0.005** |
|  | S5.Ra.n-S5.Cr.b | 3.5229 | 0.41334(0.58666) | **0.023** |
| **Different site, conspecific** | S1.Ap.a-S2.Ap.a | 2.2012 | 0.09915(0.90085) | **0.035** |
|  | S1.Ap.a-S3.Ap.a | 5.8263 | 0.20212(0.79788) | **0.001** |
|  | S1.Ap.a-S4.Ap.a | 5.3174 | 0.22804(0.77196) | **0.001** |
|  | S1.Ap.a-S5.Ap.a | 2.972 | 0.15665(0.84335) | **0.003** |
|  | S2.Ap.a-S3.Ap.a | 4.2018 | 0.14389(0.85611) | **0.001** |
|  | S2.Ap.a-S4.Ap.a | 5.6334 | 0.21977(0.78023) | **0.001** |
|  | S2.Ap.a-S5.Ap.a | 3.3329 | 0.15623(0.84377) | **0.005** |
|  | S2.Ra.n-S5.Ra.n | 1.5217 | 0.13207(0.86793) | 0.138 |
|  | S3.Ap.a-S4.Ap.a | 8.3219 | 0.26569(0.73431) | **0.001** |
|  | S3.Ap.a-S5.Ap.a | 14.014 | 0.40024(0.59976) | **0.001** |
|  | S3.Cr.b-S4.Cr.b | 11.87 | 0.51903(0.48097) | **0.001** |
|  | S3.Cr.b-S5.Cr.b | 7.8036 | 0.52714(0.47286) | **0.005** |
|  | S4.Ap.a-S5.Ap.a | 15.909 | 0.49857(0.50143) | **0.001** |
|  | S5.Cr.b-S4.Cr.b | 26.571 | 0.72656(0.27344) | **0.003** |





**Figure 5 | LEfSe analysis identified differential bacterial taxa between heterospecific populations in the same site (a-d) and between conspecific populations in the different sites (e-f).** The LDA value distribution histogram shows species with LDA score above the set value (the default setting value is 4). That is, marker species associated with significant difference between the groups. The length of the histogram represents the influence of the different species (LDA Score).

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**Figure 6 | Spearman analysis of the correlation between host-related internal factors and external factors with gut microbiota diversity changes, as well as correlation analysis with genus-level abundance changes.** (a) Host-related internal factors (Age, Sex, BMI). (b) External factors (Anu\_Tem: annual average temperature, Anu\_Rai: annual average precipitation, Altitude: altitude). (c) Correlation between host-related internal factors and the genus-level structural variation of gut microbiota. (d) Correlation between external factors and the genus-level structural variation of gut microbiota.



**Figure 7 | Spearman analysis of the dissimilarity among microbiota and geographic distance.** Linear regression of sample geographical distance against the Bray-Curtis dissimilarity distance matrix, depicted by a straight line in academic terms.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.