Microbial Diversity of Ticks And A Novel Tyhpus Group *Rickettsia* Species (*R. bacterium* Ac37b) Detected In Inner Mongolia, China

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Abstract

Background: Ticks are arthropods that can carry multiple pathogens and parasitize on livestock and mammals as well as on humans. Animal husbandry in Inner Mongolia, China, provides a suitable tick habitat. In this study, PacBio full-length 16S rDNA third-generation sequencing was used to analyze the diversity of microbial communities carried by ticks in different regions of Inner Mongolia. The aim of the study is to characterize the microbiome carried by ticks in different geographical locations and to provide theoretical support for regional prevention and control of pathogen populations in the future.

Methods: In this study, a total of 905 *Dermacentor nuttalli* and 36 *Ixodes persuleatus* were collected from the surface of sheep in four main pasture areas in Inner Mongolia. Pooled DNA samples were prepared from three samples from each region and from each tick species. In total the microbial diversity of 12 samples was analyzed by PacBio full-length 16S rRNA third-generation sequencing, and the α and β diversity were determined.

Results: The main bacterial genera we found were *Rickettsia* (35.27%), *Ac37b* (19.33%), *Arsenophonus* (11.21%), *Candidatus* Lariskella (10.84%), and *Acinetobacter* (7.17%). There were significant differences in the microbial composition of ticks from different regions and in different tick species. *Rickettsia bellii* was found in the *I. persuleatus* group. In addition, *Anaplasma* and a novel tyhpus group *Rickettsia* species (*R. bacterium* Ac37b) were found in the sample group of *D. nuttalli* in the city of Ordos.

Conclusions: In this study, *Rickettsia bellii* was first found in *I. persuleatus* in Inner Mongolia, and a novel tyhpus group *Rickettsia* species (*R. bacterium* Ac37b) was found in *D. nuttalli* from the city of Ordos. Our study provides a basis for the prevention and control of tick-borne diseases through the analysis of tick microbial diversity in different regions of Inner Mongolia. Furthermore, we were able to detect a new tick-borne pathogen in D. nutalli.

Background

Ticks are a kind of arachnids (Arachnida: Acari: Ixodida) parasitic on animals or humans. The larvae, nymphs, and male and female adults of ticks are all bloodsucking. The hosts include terrestrial mammals, birds, reptiles, and amphibians. When ticks bite the host, they can not only cause irritation, anemia, local or systemic hypersensitivity but also become the vector for the transmission of various pathogens [1]. The pathogens transmitted by ticks are even more diverse than in mosquitoes [2, 3], including forest encephalitis virus, *Rickettsia*, *Coxiella*, *Borrelia burgdorferi*, *Anaplasma*, and *Babesia* [4, 5, 6, 7, 8]. Depending on the pathogen the severity of tick-borne disease (TBD) can even be life-threatening. As human populations have grown and their interactions with the wild have increased, also human exposure to pathogens carrying ticks has greatly increased [9]. The transmission process of TBD is influenced by many factors, including pathogens, vectors, potential hosts, the environment, and human behavior. In addition, TBD often benefits from human population mobility, animal migration, and global logistics. Especially the global logistics increases the possibility of tick-borne pathogens spreading

across borders [10]. Worldwide, tick-borne diseases (TBD) such as tick-borne encephalitis, Crimean-Congolese hemorrhagic fever, and Rocky Mountain spot fever have posed new threats to public health around the world, and the incidence of TBD is increasing at an alarming rate [11–13]. The United States reported nearly 650,000 cases of vector-borne diseases from 2004 to 2016. More than 75% of these cases were tick-borne diseases. From 2012 to 2017 the United States reported nearly 288,000 cases of seven tick-borne diseases to the Centers for Disease Control and Prevention (CDC). Due to underreporting, the true number of cases may be higher [14]. At the same time, the rate of introduction or late identification of new or unknown tick-borne pathogens is also accelerating [15].

China has a vast territory, complex geography, diverse climate, and a wide variety of ticks. So tick-borne diseases are prevalent in most areas of China, seriously affecting human health [16, 17]. Zhao et al. [18] found that *D. nuttalli* is one of the tick species carrying many different tick-borne pathogens in China. According to the model prediction [18], the habitat suitable for 19 tick species was 14 - 476% larger than the geographical area where these species are currently found, indicating that there are still serious deficiencies in our knowledge of tick distribution. Due to large pasture areas and extensive animal husbandry, Inner Mongolia provides excellent habitat for ticks and the zoonosis incidence is often very high [19]. In 2005 Jia N et al. [20] reported human cases of *Rickettsia raoultii* in northeast China.

Ticks and tick-borne diseases have a substantial impact on the economy and human life in Inner Mongolia. Nevertheless, the community structure and diversity of microbial communities on ticks parasitizing sheep in various regions of Inner Mongolia have not been thoroughly studied so far. The microbial communities of ticks are influenced by many factors, including geographical area, feeding status, blood meal source, and developmental stage [21].

Current techniques for 16S ribosomal RNA (rRNA) gene sequence analysis, based on typical clustering thresholds of operational taxa (OTUs), are insufficient for accurate taxonomic allocation and for addressing the phylogenetic relationships at the species level when only a few hypervariable regions are amplified. Therefore, 16S rRNA gene amplification sequence data using the v3-v4 region can only be explored at the genus level [22]. PacBio full-length 16S rDNA third-generation sequencing technology, however, is more accurate and can be used for precise species identification [23].

Therefore, we collected ticks from the surface of sheep in four main pastoral areas of Inner Mongolia, and analyzed their microbial diversity using PacBio full-length 16S rDNA third-generation sequencing technology. The aim of our investigation was to assess the distribution characteristics of tick microbial communities in different geographical locations in Inner Mongolia, providing information for better environmental management.

Methods

Tick collection and Sample Preparation

In mid-April 2019 a total of 941 ticks were collected from sheep in Hulun Buir City (New Barag Right Banner), Chifeng City (Bayan Wendusumu Area, Tianshan Town, Alukeerqin Banner), Ordos City (Chengchuan Town, Otog Front Banner), and the Forest area of Arshan. According to morphological identification, 905 *D. nuttalli* and 36 *I. persuleatus* (Table 1, Fig. 1, Fig. 2) were collected and stored at -80°C.

Table 1
Tick samples collection information.

Location	Tick species	Longitude	Latitude	Samples
Hulun Buir	*D. nuttalli*	116°82'	48°67'	292
Chifeng	*D. nuttalli*	121°64'	43°46'	543
Ordos	*D. nuttalli*	108°32'	37°70'	70
Arshan	*I. persuleatus*	119°94'	47°17'	36

DNA extraction

The morphologically identified tick samples were disinfected with 75 % ethanol, dried on filter paper, washed three times with PBS, and finally dried on filter paper.A total of 941 tick samples were divided into 206 sample pools. In each sample pool, the same area with the genus 5 ~ 20 not only full blood or 1 ~ 2 only full blood tick were taken and put into a sterile grinding tube, add 5 mm magnetic bead 1 and 3 mm magnetic beads 2, grinding tube into the adapter (ahead - 20 ℃ refrigerator 30 min), install the adapter into the grinding apparatus, set the parameter to 70Hz and 180S.Finally, according to TIANGEN blood / cell / tissue genomic DNA extraction kit instructions to extract DNA, all extracted DNA stored at-20°C standby. Three DNA samples (Table 2) were randomly selected from each area, and 10 μL of each sample was prepared for microbial diversity sequencing.

16S rRNA Amplicon Sequencing and Data Analysis

The V1-V9 region of the bacteria 16S ribosomal RNA gene were amplified by PCR (95°C for 2 min, followed by 27 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s and a final extension at 72°C for 5 min) using primers 27F 5'-AGRGTTYGATYMTGGCTCAG-3' and 1492R 5'-RGYTACCTTGTTACGACTT-3', where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions.SMRTbell libraries were prepared from the amplified DNA by blunt-ligation according to the manufacturer's instructions (Pacific Biosciences). Purified SMRTbell libraries from the Zymo and HMP mock communities were sequenced on dedicated

PacBio Sequel cells using the S/P1-C1.2 sequencing chemistry. Purified SMRTbell libraries from the pooled and barcoded samples were sequenced on a single PacBio Sequel cell. Replicate 1 of the samples was sequenced using the S/P2-C2/5.0 sequencing chemistry, and Replicate 2 of the samples was sequenced with a pre-release version of the S/P3-C3/5.0 sequencing chemistry. All amplicon sequencing was performed by Shanghai Biozeron Biotechnology Co. Ltd (Shanghai, China).

PacBio raw reads were processed using the SMRT Link Analysis software version 6.0 to obtain demultiplexed circular consensus sequence (CCS) reads with the following settings: minimum number of passes = 3, minimum predicted accuracy = 0.99. Raw reads were processed through SMRT Portal to filter sequences for length (<800 or >2500 bp) and quality. Sequences were further filtered by removing barcode, primer sequences, chirmas and sequences if they contained 10 consecutive identical bases. OTUs were clustered with 97% similarity cutoff using UPARSE(version 7.1 http://drive5.com/uparse/) and chimeric sequences were identified and removed using UCHIME. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the silva (SSU132)16S rRNA database using confidence threshold of 70% [24].Subsequently Alpha and Beta-diversity Analyses.The rarefaction analysisbased on Mothur v.1.21.1[25] was conducted to reveal the diversity indices, including the Chao, ACE, and Shannon diversity indices. The beta diversity analysis was performed using UniFrac [26] to compare the results of the principal component analysis (PCA) using the community ecology package, R-forge (Vegan 2.0 package was used to generate a PCA figure).One way analysis of variance (ANOVA) tests were performed to assess the statistically significant difference of diversity indices between samples. Differences were considered significant at $p < 0.05$. Venn diagrams were drawn using online tool “Draw Venn Diagram” (http://bioinformatics.psb.ugent.be/webtools/Venn) to analyze overlapped and unique OTUs during the treatment processes.

For identification of biomarkers for highly dimensional colonic bacteria, LEfSe (linear discriminant analysis effect size) analysis was done [27]. Kruskal-Wallis sum-rank test was performed to examine the changes and dissimilarities among classes followed by LDA analysis to determine the size effect of each distinctively abundant taxa [28].

Results

PacBio sequencing data

A total of 12 samples were sequenced (Table 2). After data screening and deletion, a total of 20,1159 reads were generated and classified. The reads of each sample ranged from 11,550 to 21996. The dilution curve of the Shannon index at OTU level showed a suitable range for sequencing, and the observed Shannon index accumulation curve also reached a plateau (Fig.3).

Table 2 Information of tick samples used for bacterial microbiome analysis.

Sample Name	Tick Species	n
Hulun Buir-1		8
Hulun Buir-2		1
Hulun Buir-3		7
Chifeng-1		8
Chifeng-2	*D. nuttalli*	10
Chifeng-3		1
Ordos-1		1
Ordos-2		1
Ordos-3		1
1. *persuleatus-1*		6
2. *persuleatus-2*	*I. persuleatus*	6
I.persuleatus-3		6

Bacterial microbiome composition

A total of 326 OTUs were detected in 12 samples, and the bacterial microbial components were 11 phyla, 15 classes, 38 orders, 62 families, 104 genera, and 141 species. At the genus level, the abundance of *Rickettsia* was the highest (35.27 %), followed by Ac37b (19.33 %), *Arsenophonus* (11.21 %), *Candidatus* Lariskella (10.84 %), *Acinetobacter* (7.17 %), *Cupriavidus* (2.39 %), and *Romboutsia* (1.00 %). Most Ac37b was found in the *D. nuttalli* of Ordos and Chifeng, while *Candidatus* Lariskella mainly existed in the *I. persulcatus*. At the species level, *Rickettsia raoultii* had the highest abundance (23.23%), followed by Ac37b unclassified (19.33%). *Rickettsia* unclassified (11.94%), *Candidatus* Lariskella unclassified (10.84%), *Arsenophonus* unclassified (10.32%). *Rickettsia raoultii* exists in most samples but is rarely found in Ordos.

Differences of ticks in different areas

There were 180, 182 and 135 OTUs in the samples of *D. nuttalli* from Hulun Buir, Chifeng and Ordos, respectively. According to the α diversity the Hulun Buir region shows a greater microbial diversity than the other two regions (fig. 5). At the genus level, there were *Rickettsia* (35.87%), Ac37b (0.05%) and *Arsenophonus* (41.51%) in the Hulun Buir region. In Chifeng area, *Rickettsia* (57.42%), Ac37b (16.38%) and *Arsenophonus* (3.32%) were found. The Ordos region had *Rickettsia* (0.08%), Ac37b (60.74%) and *Arsenophonus* (0.01%). To further distinguish the composition of the microbial community, weighted UniFrac analyses revealed differences regarding the region, as measured by an analysis of similarity (ANOSIM, R=0.7994, P = 0.011) and visualized by principal component analysis (PCoA) (Fig. 6). PCoA

explained 47.46% (Axis 1) and 28.93% (Axis 2) of the variation, with samples from different regions clustering separately (Fig. 6).

Differences Between *D. nuttalli and I. persuleatus*

North of the Arshan region is adjacent to the New Barag Left Banner and to the Ewenki Autonomous Banner of the Hulun Buir City, close to the Hulun Buir tick collection point. All *I. persuleatus* came from the Arshan region. Whereas in the Hulun Buir area only *D. nuttalli* was found. A total of 123 microbial OTUs were found in ticks, and α diversity indicated that specimens of *D. nuttalli* contained greater microbial diversity than specimens of *I. persuleatus* (Fig. 5). Among the first 10 genera, there were significant differences between the microbial compositions of *I. persuleatus* and *D. nuttalli*. *Rickettsia* (47.71%), *Candidatus* Lariskella (43.34%), and *Acinetobacter* (1.71%) were the major microbial components of *I. persuleatus*. *Rickettsia* (35.87%), *Arsenophonus* (41.51%) and *Cupriavidus* (5.66%) were found in *D. nuttalli*.

Among the first 10 species, the *Rickettsia* unclassified (45.37 %), *Rickettsia raoultii* (1.96 %) and *Candidatus* Lariskella unclassified (43.34 %) were the main microorganisms in *I. persuleatus*, while *Rickettsia raoultii* (34.99 %), *Arsenophonus* unclassified (38.12 %) and *Cupriavidus gilardii* CR3 (5.66 %) were the main microorganisms in *D. nuttalli. The* differences in the microbial composition were significant.

In general, *D. nuttalli* in Hulun Buir area showed the highest microbial diversity. We selected pathogenic bacteria with high abundance and obvious harm to humans at the species level (*Rickettsia raoultii*, *Anaplasma*, *Rickettsia bellii*, *Coxiella*) for further analysis. We found that Ac37b, a new type of *Rickettsia*, was predominant in the Ordos region, and *Anaplasma* was much more abundant in the Ordos region than in other regions. The abundance of *Rickettsia* was very low in Ordos, but it was higher in *D. nuttalli* from Hulun Buir and Chifeng. *Rickettsia bellii* was detected for the first time in Inner Mongolia. It only appeared in the *I. persuleatus* group and there were many unclassified *Rickettsia*. *Arsenophonus* unclassified was also an important component of its microorganisms in Hulun Buir (Fig.7).

Discussion

D. nuttalli is a tick species widely distributed in Inner Mongolia, eastern Siberia, and China. It is parasitic on livestock and can also cause damage to humans [29]. *D.nuttalli* can carry different pathogens including *Babesia*, *Anaplasma vois*, *Rickettsia*, and *Coxellia* [30, 31, 32]. *I. persuleatus* is the dominant tick species in northeast China. Inner Mongolia has a large east-west span, so there are *I. persuleatus* populations at the border with northeast China. This often is associated with the transmission of tick-borne encephalitis virus and can pose a serious threat to human life and safety [33, 34].

D. nuttalli is the dominant tick species in Inner Mongolia, with significant influence on the economy and health of the local human population. Jiao et al. [35] carried out a simple microbial diversity analysis of ticks on cattle in the Hulun Buir area of Inner Mongolia. Beyond this, the microbial community

composition of ticks in other areas of Inner Mongolia was not further investigated. The bacterial diversity on different tick species must be analyzed to further understand the relationships between ticks and microorganisms. Samples collected from multiple regions are more likely to find new pathogens in microbial diversity.

In our investigation, we applied PacBio full-length 16S rRNA third-generation sequencing to the V1-V9 region of the 16S rRNA. In their study on oral microorganisms, Zhang et al. [36] found that OTU sequences generated by PacBio were much larger than those generated on the MiSeq platform. Therefore, we adopted PacBio full-length 16S rRNA third-generation sequencing for the microbial diversity analysis of ticks in Inner Mongolia. According to our analysis, the microbial diversity on *D. nuttalli* samples from different regions of Inner Mongolia was significantly different. The highest microbial diversity was found in *D. nuttalli* samples from Hulun Buir, and *Rickettsia* (35.87%) and *Arsenophonus* (41.51%) were the main microorganisms. *Rickettsia* is an arthropod-associated obligate intracellular gram-negative bacterium that can cause mild to severe disease in humans [37]. *Arsenophonus* is an intracellular symbiotic bacterium of insects with a wide host range and rich biodiversity. An androidal effect on ticks has not been reported so far, and its other biological functions have not yet been identified [38].

Linear discriminant analysis effect size (LEfSe) analysis showed that *Rickettsia raoultii*, Peptostreptococcaceae and Clostridia played an important role in the Chifeng formation. Characteristic of the Ordos formation is *Anaplasma*. Enterobacterale and Xanthomonadaceae belong to Hulun Buir (Fig. 8). *Rickettsia raoultii* is the pathogene of the spotted fever group, which is transmitted vertically in arthropods as a symbiotic bacterium and in vertebrates as a pathogenic bacterium and is a pathogen of human diseases [39]. *Anaplasma* is a gram-negative intracellular obligate parasite, and its pathogenicity poses an important threat to several animals, and even public health security [40]. Currently, there are six species of *Anaplasma* recognized worldwide, including *A. phagocytophilum*, *A. ovis*, *A. capra*, *A. bovis*, *A. marginale* and *A. platys* [41]. In addition to *A. phagocytophilum*, *A. bovis* and *A. capra* have been reported to infect humans [42, 43]. A novel tyhpus group *Rickettsia* species (*R. bacterium* Ac37b) was found in the Ordos region [44]. The *Rickettsia* typhus group is mainly composed of *R.* przewskii and *R. morzewskii*. In Australia, three types of typhus, epidemic typhus, mouse typhus, and tsutsugamushi disease, have been found successively. These are closely related to native wild animals and ticks in Australia [45]. In China, typhus has also been an important cause of human morbidity and mortality in the past decade. The disease was initially identified only in southern China, but now cases of typhus have been reported in northern China, with a wide geographical distribution [46].

R. bellii is the only known species in a third group that differentiated before the spotted fever group and the typhus group separated. *R. bellii* is the most common *Rickettsia* in American ticks and is found in all species of *Ixodes*. Including *Dermacentor* and *Amblyomma*, it is also the only *Rickettsia* that has been found in both *Ixodes*, showing the largest arthropod host range among known *Rickettsia*: It is pathogenic to mammals [47]. But the pathogenic potential for humans is still unknown and should be closely monitored [48].

We can further explore the relationship between *Rickettsia bellii* and other *Rickettsia*. In the Ordos area, there were pathogenic bacteria that are more threatening to humans, and a new classification of *Rickettsia* has emerged. The pathogen prevention and control in this area needs to focus on monitoring and strengthening the popular knowledge of methods for personal protection. The newly discovered pathogens in Inner Mongolia need to be isolated and sequenced in future studies, and the pathogenicity of the organisms should be tested through subsequent animal experiments. At the same time, the sampling sites can be expanded in future investigations, and ticks should be collected from different hosts, such as cattle, camels, and other mammals.

Conclusions

In this study, we analyzed the microbial diversity of two ticks in four regions of Inner Mongolia. A novel rickettsia species (R.bacterium Ac37b) was found in Inner Mongolia for the first time, and Rickettsia Bellii was found in *I.persuleatus*. Ticks carried more potential pathogens in Ordos area,and there were co-infections of Rickettsia and Anaplasma, which may be related to its geographical environment. The prevention and control of tick-borne diseases in this region should be strengthened. In the future research, sampling in the western region of Inner Mongolia can be focused on.

Abbreviations

D. nuttalli:*Dermacentor nuttalli*;*I.persuleatus*:*Ixodes persuleatus*;PCR:

Polymerase chain reaction;*R.bacterium* Ac37b:*Rickettsia bacterium* Ac37b

Declarations

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Authors' contributions

LW,LLX ,ZG and SS performed laboratory analysis, analyzed the data and wrote the frst draft. JFY,DDQ and LM revised the manuscript. XYS participated in sample collection. SYF directed the experiment and helped to revise the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All datasets have been included with this article.

Ethics approval and consent to participate.

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

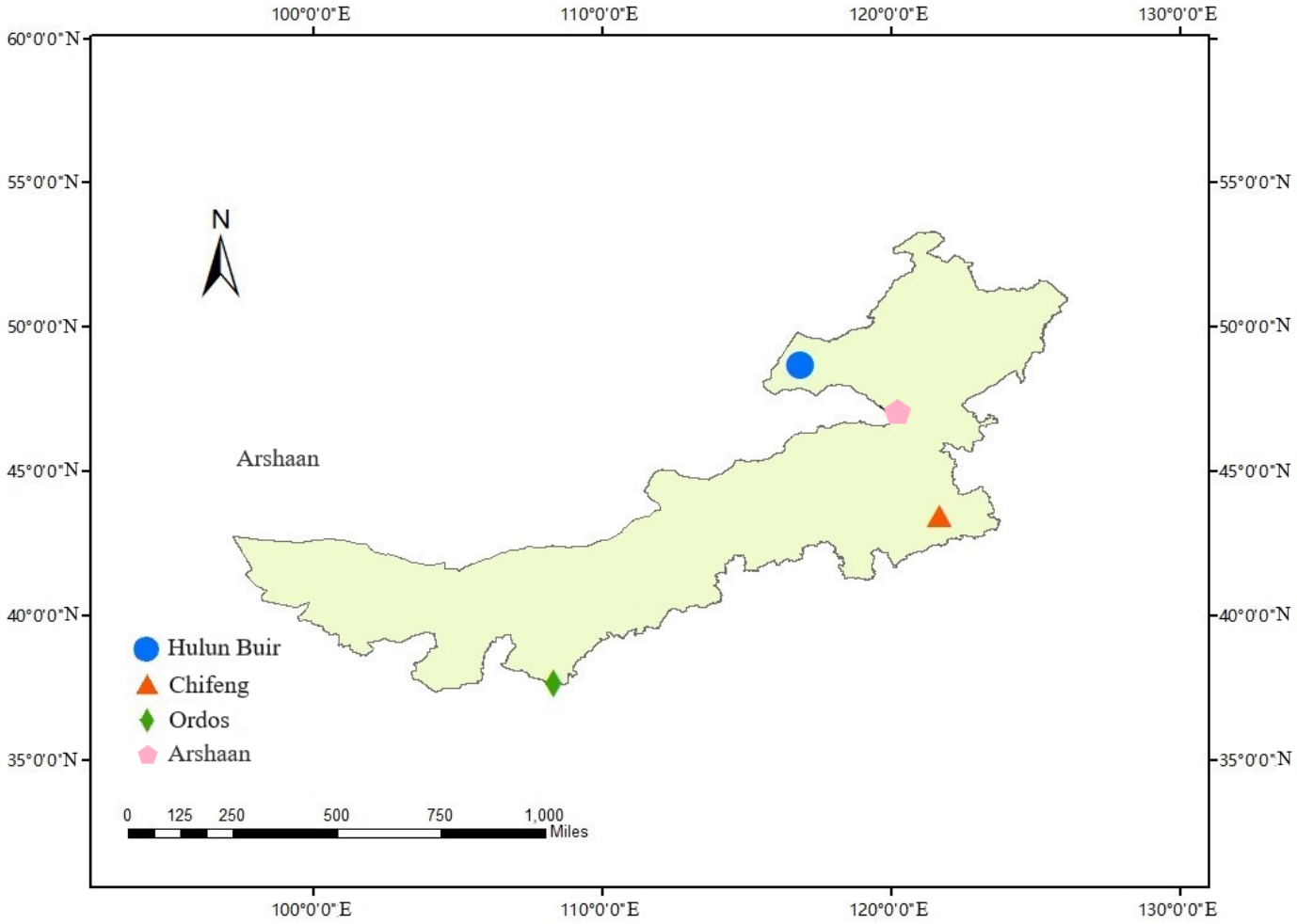
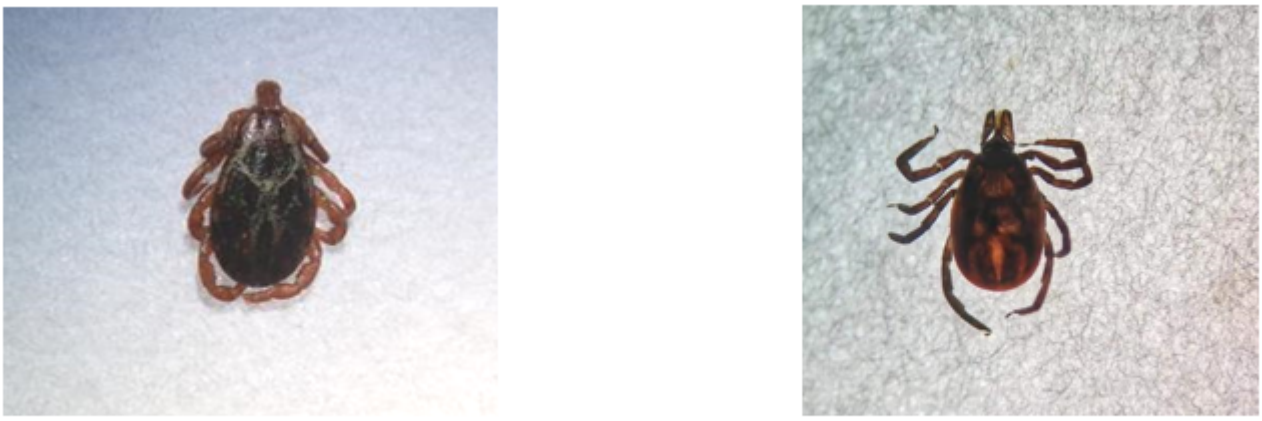


Figure 1

Map of tick collection sites in Inner Mongolia, China.



a b

Figure 2

a Not full of blood D. nuttalli. b Not full of blood I. persuleatus.

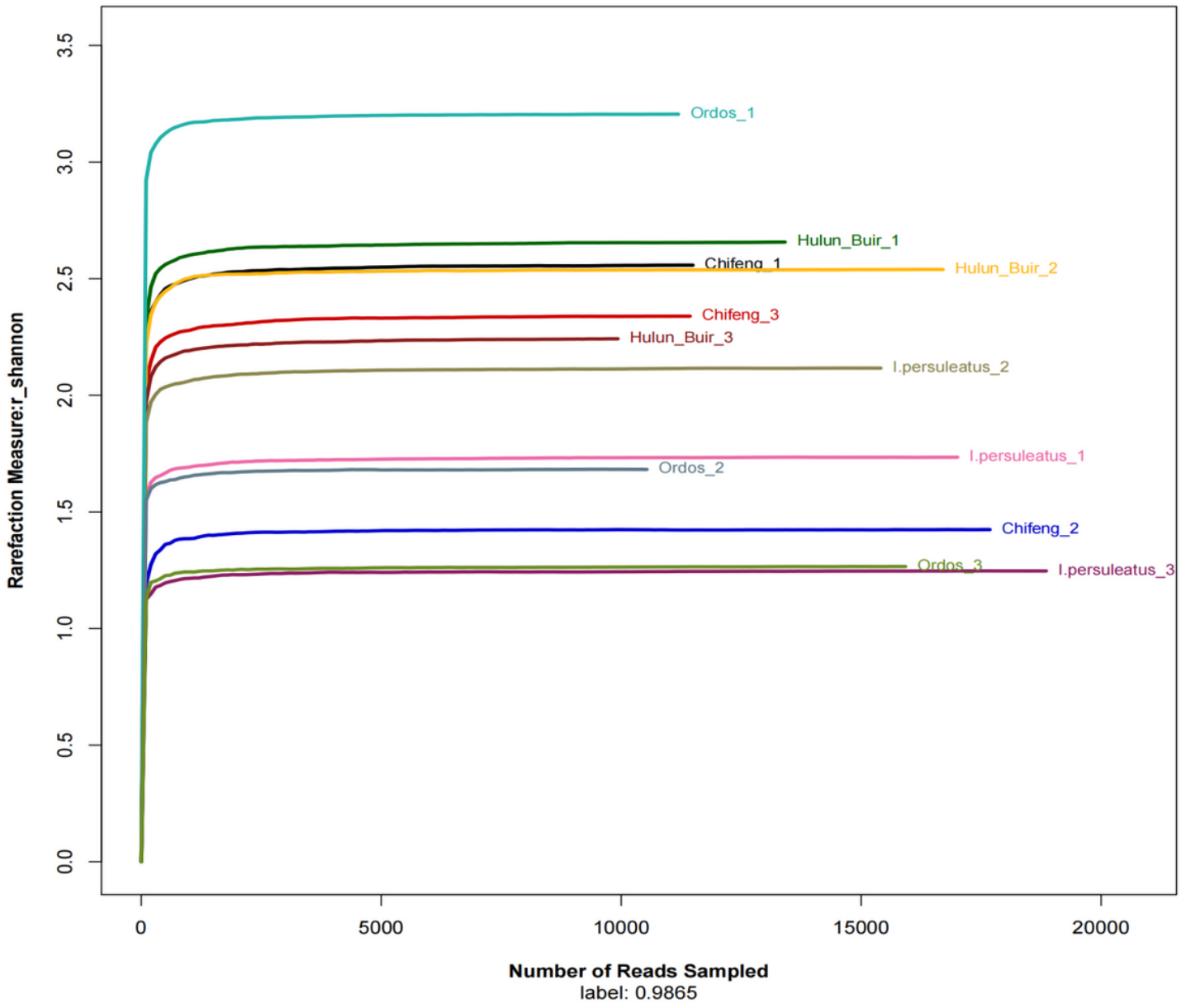


Figure 3

Shannon Wiener curve.

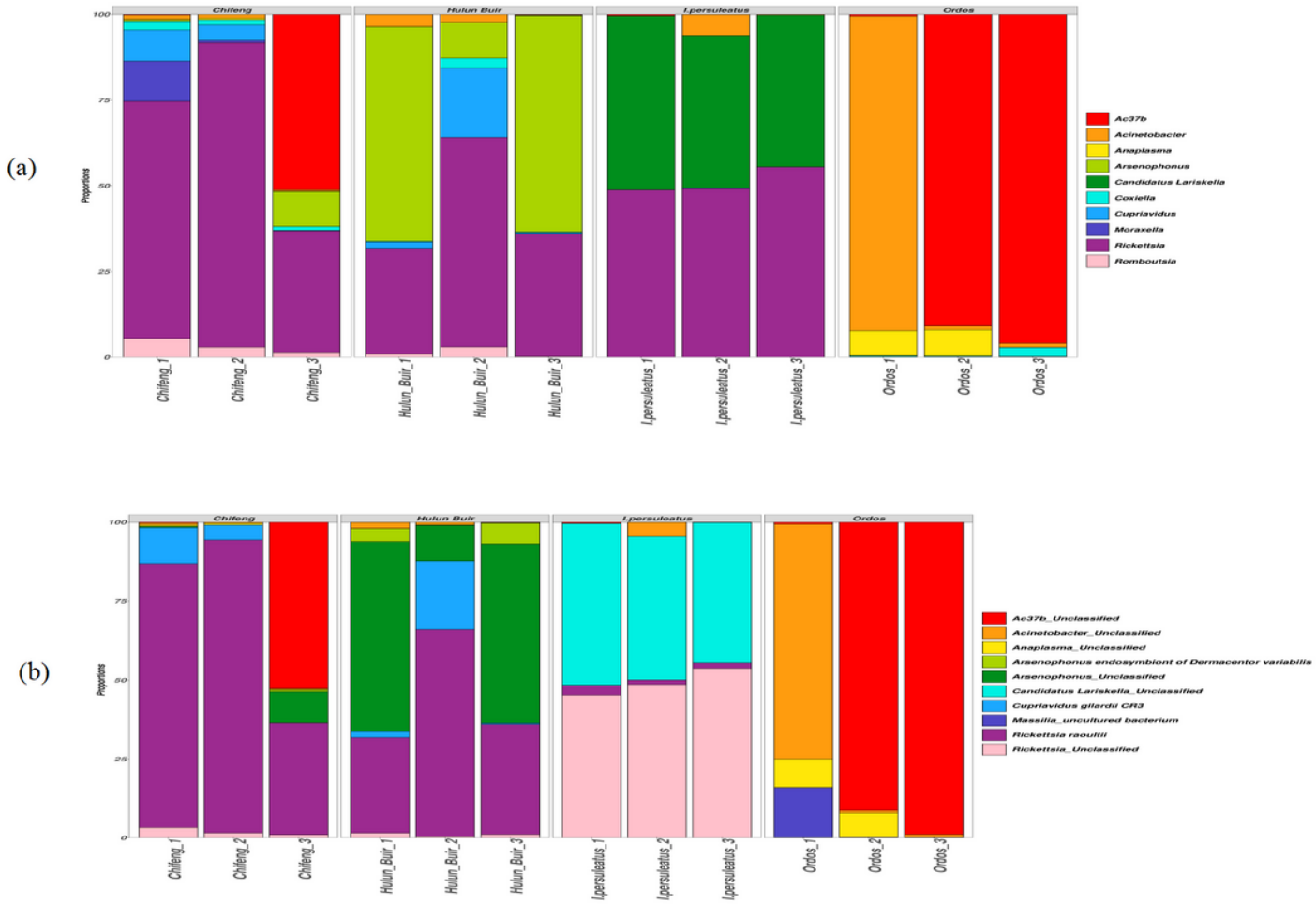


Figure 4

Microbe composition of different regions and species. a Top 10 microbial components at genus level. b Top 10 microbial components at species level.

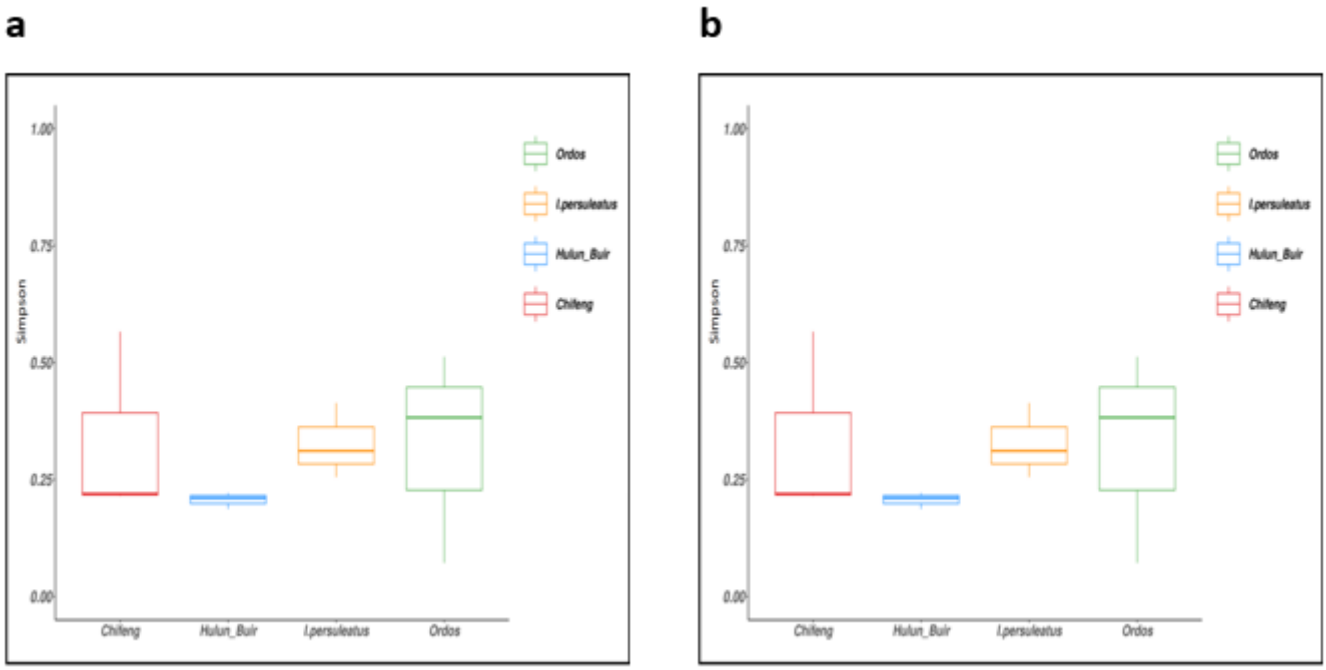


Figure 5

Alpha diversity measures for D. nuttalli and I. persuleatus in three areas. a Shannon's index. b Simpson's index

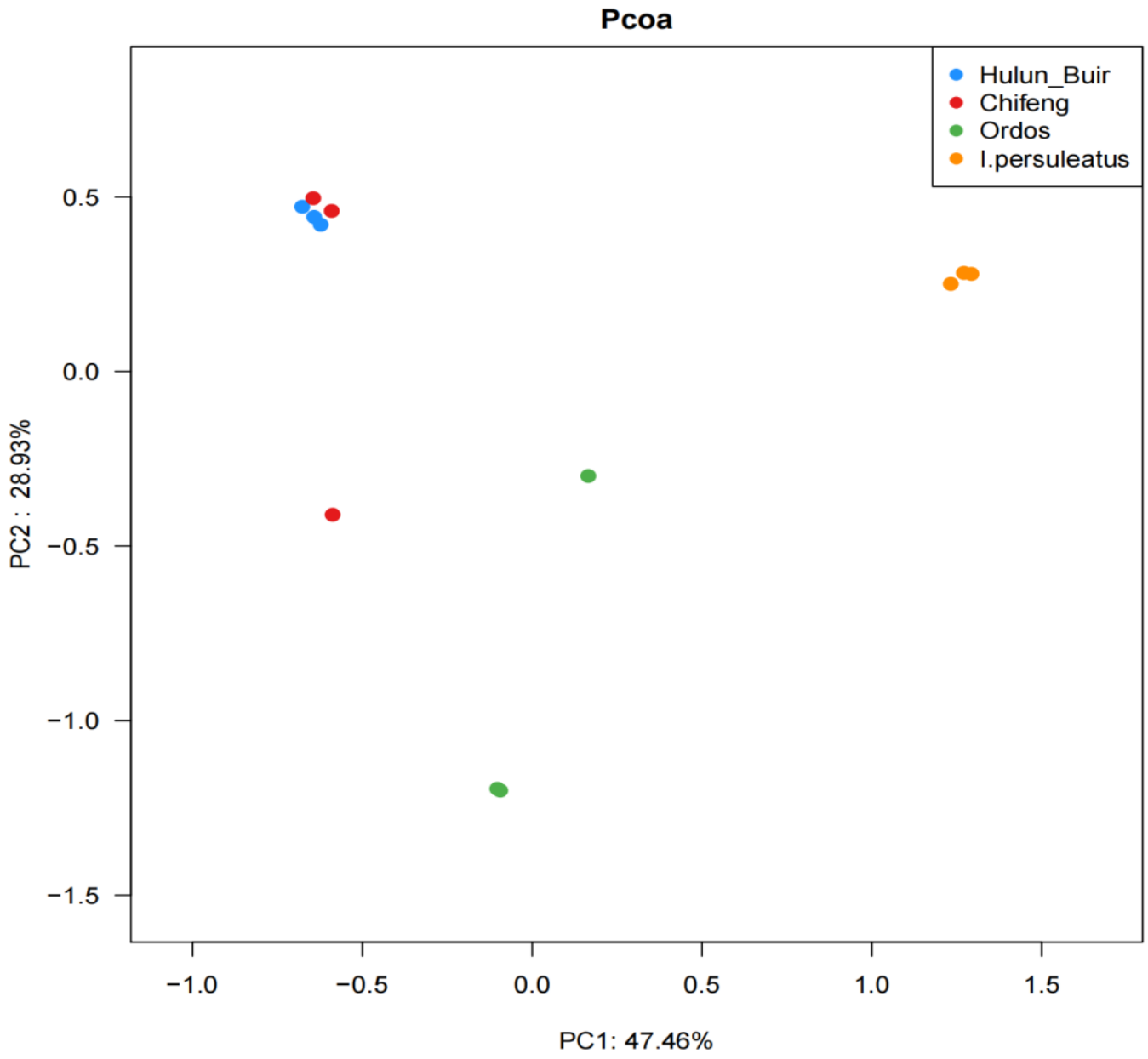


Figure 6

PCoA of β-diversity measures for twelve groups. Weighted UniFrac PCoA graph showing PC1, which accounts for 47.46% variation and PC2 for 28.93% variation. Different color dots represent different regions and species.

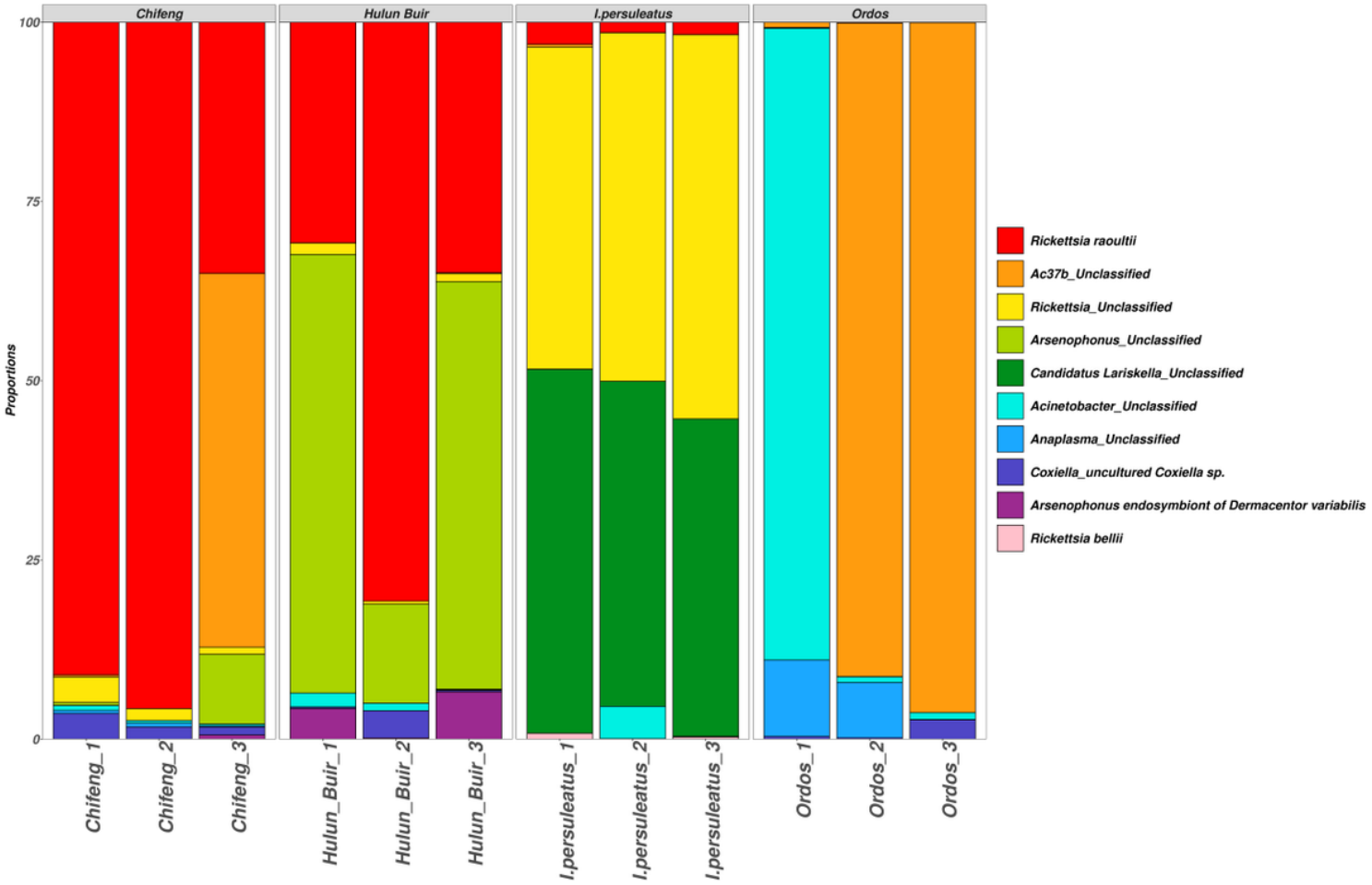


Figure 7

The composition of bacteria and pathogenic bacteria with a high abundance.

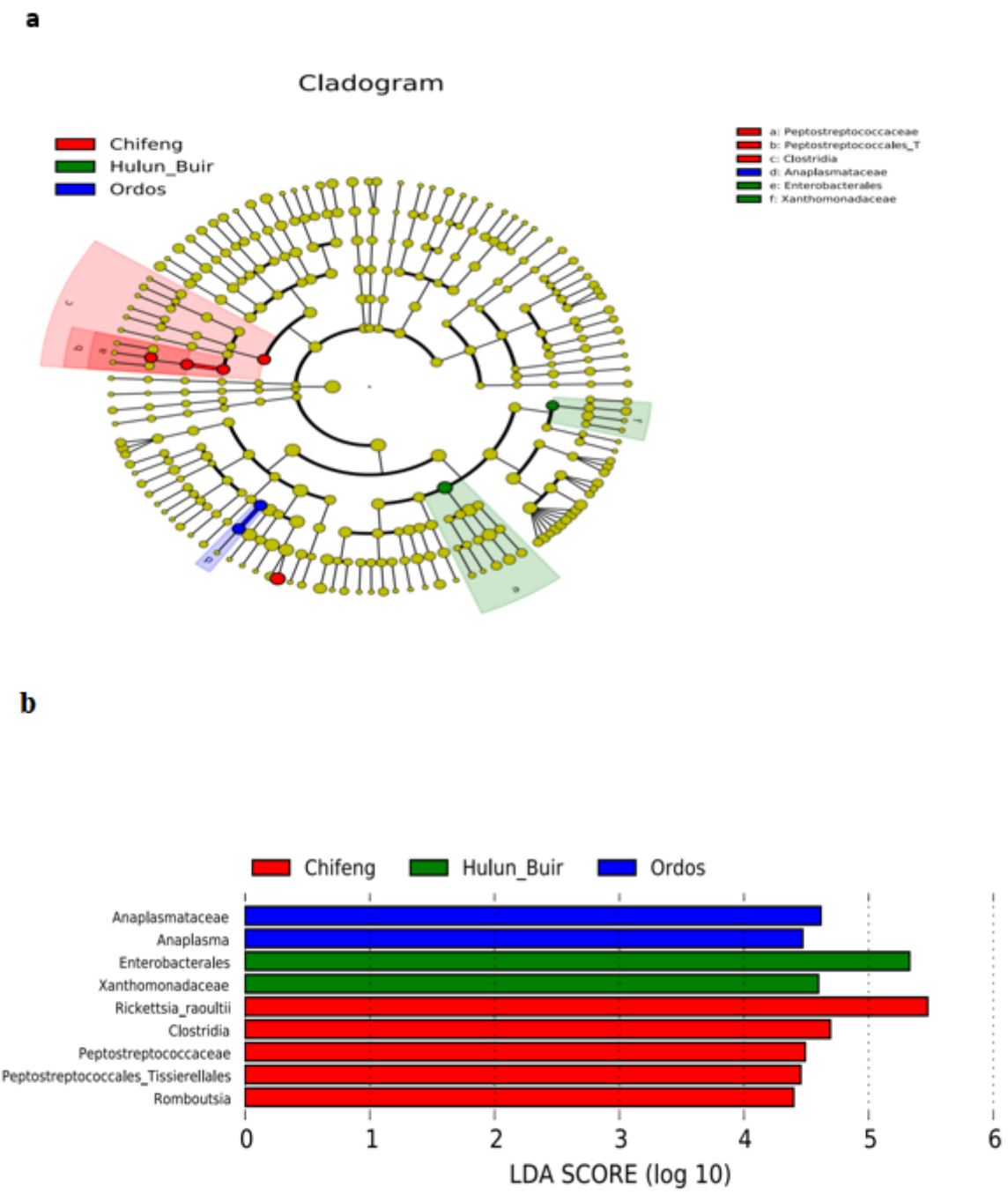


Figure 8

a Clustering tree analysis by Linear discriminant analysis effect size (LEfSe). b Histogram of LDA analysis

Supplementary Files

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