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Diversity analysis of the endosymbiotic bacterial community in field-collected *Haemaphysalis* ticks on the tropical Hainan Island, China

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Abstract: Ticks are important vectors of various pathogens that cause infectious diseases in humans. Endosymbiotic bacteria have been explored as targets for tick and tick-borne disease control. However, the tick bacterial community on Hainan Island, which is the largest tropical island in China and has an environment favourable to ticks, has not yet been studied. In this study, we surveyed the bacterial community of ticks collected from grass in one village in Haikou. A total of 20 ticks were morphologically and molecularly identified as *Haemaphysalis* spp. The tick bacterial 16S rRNA hypervariable region amplicon libraries were sequenced on an Illumina MiSeq platform. A total of 10 possible bacterial genera were detected, indicating a low-diversity bacterial community profile. The dominant bacterial genus, *Massilia*, accounted for 97.85% of the population. Some other bacterial genera, including *Arsenophonus* and *Pseudomonas*, have been reported to play a role in tick development and tick-borne pathogen transmission in other tick species. Overall, the study highlights the first descriptive understanding of the tick bacterial community on Hainan Island and provides a basis for deciphering the interactions between the tick microbiome and tick-borne pathogens.

Keywords: endosymbiotic bacteria, bacterial community, 16S rRNA, tropics, Illumina MiSeq

Ticks are obligate blood-sucking arthropods. They are the second most common vectors for human diseases next to mosquitos (Mansfield et al. 2017, Cafarchia et al. 2022). They can transmit numerous bacterial and viral pathogens, including species of *Borrelia*, *Rickettsia*, *Anaplasma*, *Coxiella*, *Francisella*, and cause severe fever with thrombocytopenia syndrome virus (SFTSV) (Luo et al. 2015, Boulanger et al. 2019). With global warming and the growing interest in outdoor activities, the incidence of tick-borne diseases has been on the rise, and it has become a global public health concern (Chavatte and Octavia 2021, Wikel et al. 2021).

In addition to pathogens, ticks also harbour a variety of non-pathogenic microbes, which may play a role in tick development and pathogen transmission (Narasimhan and Fikrig 2015). These microbes could be promising targets for tick and tick-borne disease control. Assessment of tick microbial communities is the first step in developing a symbiont-based method to prevent tick-borne disease transmission. Tick microbial communities are affected by several factors, including tick species, geographical origin, life stage, tick sex, and tick immunity (Narasimhan et al. 2021).

Human disease pathogens, such as *Borrelia burgdorferi*, spotted fever group rickettsiae, and *Anaplasma phagocytophilum*, have been detected in *Haemaphysalis* spp. (Jiang et al. 2011, Stanley et al. 2020). Ticks of the genus *Haemaphysalis* Koch have been reported as vectors of SFTSV, which causes serious disease throughout East Asia (Luo et al. 2015, Zhuang et al. 2018). The microbial communities of *Haemaphysalis* spp. have been investigated in some regions in East Asia (Duan and Cheng 2017, Zhang et al. 2019). However, little is known about the microbial communities of ticks, including *Haemaphysalis* spp. on Hainan Island, which is the largest tropical island in China and has an environment favourable to ticks (Galay et al. 2018, Tufa et al. 2021).

In this study, we sampled *Haemaphysalis* ticks found in a field in Haikou on Hainan Island and characterised their microbial composition using the 16S rRNA amplicon Illumina MiSeq sequencing method. Our results may be useful for the future development of a new strategy for tick and tick-borne disease control.

MATERIALS AND METHODS

Tick collection

Adult ticks were collected in Haikou, Hainan, China (19.9900N, 110.4000E) (Fig. 1A), by flagging over vegetation with a 0.8-m^2 white flannel flag. The ticks on the white flannel

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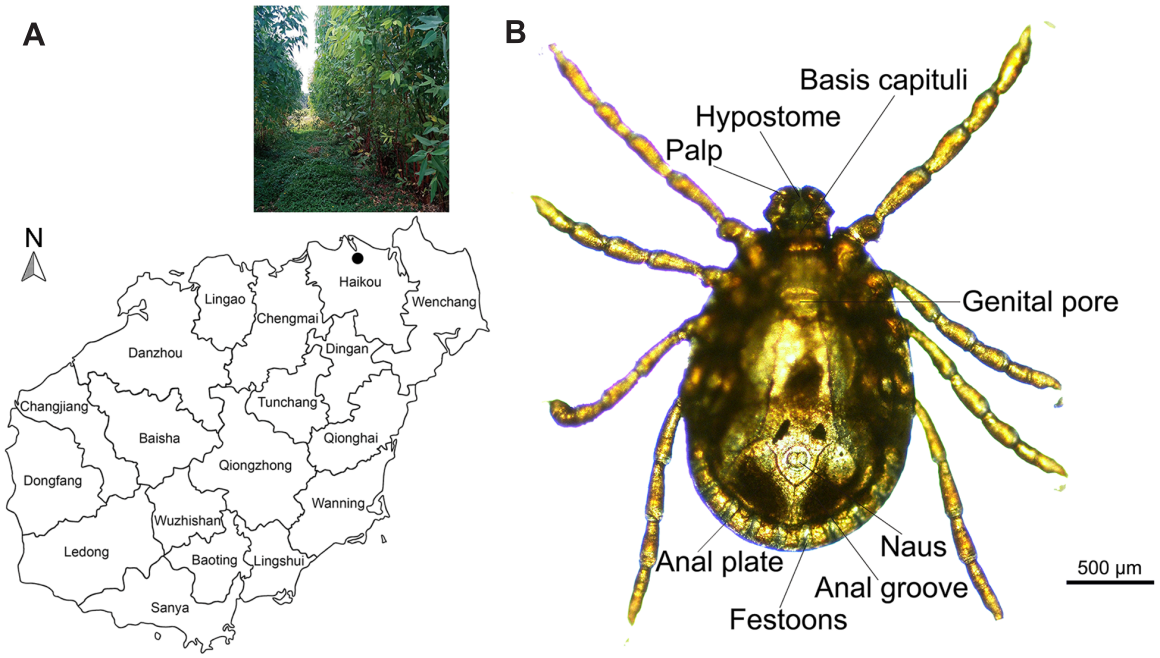

Fig. 1. A – Image of Hainan Island showing the geographic location of the tick collection site (black point). The floating picture represents the environment of the tick collection site. **B** – The morphological characteristics of adult female of a *Haemaphysalis* tick in ventral view.

flag were swept gently into T25 ventilated cell culture flasks using a plush brush.

Morphological classification and molecular identification of ticks

Each tick sample was washed with 75% alcohol three times and then immersed in 75% alcohol for storage. Ticks were observed under a positive research-level Eclipse Ni-E microscope (Nikon Instruments, Tokyo, Japan) and preliminarily identified to the genus level according to specific illustrated taxonomic keys of external morphology (Kohls 1957, Walker et al. 2003).

One tick from the most common species collected was chosen and used for the follow-up study. Genomic DNA was extracted from one leg of a single tick using MightyPrep reagent for DNA (Takara, Dalian, China). The mitochondrial cytochrome c oxidase subunit I (COI) fragment was amplified by polymerase chain reaction (PCR) using the forward primer 5′-GAGTCGGTAAAATGGCGCTAC-3′ and the reverse primer 5′-GCTATCTTTAAGAGGGTAATA-3′ (Gu et al. 2010). PCR was conducted in a 20 µl volume reaction with 10 µl 2×PCR Mix (Bioteke, Beijing, China), 0.8 µl primer F (10 µM), 0.8 µl primer R (10 µM), 4 µl template genomic DNA, 4.4 µl ddH_2O. The PCR reaction profile was 94 °C for 3 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, and then 72 °C for 10 min. PCR products were checked by 1% agarose gel electrophoresis and sequenced by the Beijing Genomic Institute (BGI) Company (Guangzhou, China).

The COI sequence obtained through PCR-Sanger sequencing from the tick sample was identified using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990, Madden et al. 1996). The phylogenetic relationships among the tick sample sequence and the deposited COI sequences available in GenBank with the BLAST algorithm were assessed using the neighbor-joining (NJ) tree by the Kimura 2-parameter (K2P) model with 1,000 replicate bootstrap values in MEGA version 11 software (Tamura et al. 2021).

Illumina deep sequencing of tick bacterial DNA

Bacterial DNA was extracted from a total of 20 ticks as a pooled sample using the phenol-chloroform-isoamyl alcohol (PCI) method. Briefly, after three rounds of washing with sterile saline, 20 adult female ticks were pulverised in liquid nitrogen. We then suspended the tick powder in 1 ml of PBS, added 100 µl of lysozyme solution (50 mg/ml), incubated it at 37 °C for 30 min, added 150 µl proteinase K solution (20 mg/ml) and incubated it at 55 °C overnight. The DNA lysate was treated with a 25 : 24 : 1 (v/v) mixture of phenol, chloroform, and isoamyl alcohol. DNA was precipitated by 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.1 µl glycogen (20 mg/ml) carrier in 0.6 volume of isopropanol.

The dried DNA was suspended in 50 µl Tris-EDTA (TE, pH 8.0) buffer (Campelo Morillo et al. 2022) and used as the template for library construction. A two-step PCR amplification approach was implemented for microbial 16S rRNA amplicon library preparation. The primers were designed as fusion primers composed of an Illumina MiSeq adapter, barcode, and specific primer. The first PCR volume was 50 µl in total, consisting of 10 µl 5 × PCR buffer, 1 µl dNTPs (10 mM), 0.5 µl Phusion ultra fidelity DNA polymerase (NEB, Beijing, China), 1 µl primer F (10 µM), 1 µl primer R (10 µM), 1 µl template DNA, and 35.5 µl ddH_2O.

The reaction was amplified using the universal primer pairs (515F/926R) targeting the V4-V5 region of the 16S rRNA genes in bacteria. The forward primer was 5’-TTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA-3’, and the reverse primer was 5’-GAGTTCCTTGGCACCCGAGAATTCCACCGTCAATTCMTTTGAGTTT-3’ (Li et al. 2019). PCR was performed with the following conditions: 2 min of predenaturation at 94 °C, followed by 20 cycles of 30 s denaturation at 94 °C, 30 s annealing at 56 °C, 30 s elongation at 72 °C, and then a final extension at 72 °C for 5 min. The first PCR

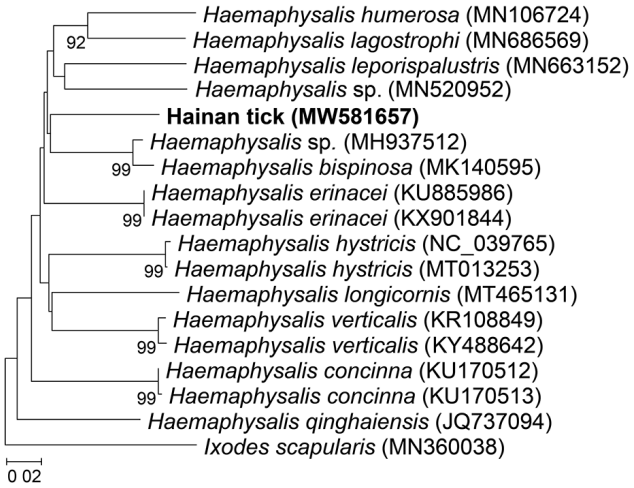

Fig. 2. *Haemaphysalis* spp. The neighbor-joining tree was constructed using 17 sequences of *Haemaphysalis* spp. and 1 sequence of the *Ixodes scapularis* Say (outgroup). The numbers next to the nodes represent bootstrap values from 1000 replicates

products were separated on a 1.2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen, Silicon Valley, USA) obeying the manufacturer's instructions, and then used as a template for the secondary PCR, performed in 40 µl reaction mixture with 8 µl 5×PCR Buffer, 1 µl dNTPs (10 mM), 0.4 µl Phusion ultra fidelity DNA polymerase, 1 µl primer F (10 µM), 1 µl primer R (10 µM), 5 µl template DNA, and 23.6 µl ddH_2O.

The procedure was carried out using two universal primers. The forward primer was 5'-AATGATACGGCGACCACCGAGATCTACAC-barcode-TCTTTCCCTACACGACGCTC-3', and the reverse primer was 5'-CAAGCAGAAGACGGCATACGAGAT-barcode-GTGACTGGAGTTCCTTGGCACCCGAGA-3' (Pichler et al. 2018). Amplification conditions were as follows: predenaturation at 94 °C for 2 min, 8 cycles of 30 s denaturation at 94 °C, 30 s annealing at 56 °C, 30 s extension at 72 °C, and a final extension at 72 °C for 5 min. Then, 16S amplicon sequencing was performed on an Illumina MiSeq platform with a 2 × 300 bp paired-end (PE) reads (TinyGene, Shanghai, China).

Bioinformatic analysis

Illumina MiSeq yielded raw PE reads that were submitted to the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI). Following trimming and filtering for quality control using Trimmomatic and FLASH (Magoč and Salzberg 2011, Bolger et al. 2014), the PE sequences were merged on the basis of the overlap to optimise the sequences using Mothur software (http://www.mothur.org) (Tan et al. 2020) and clustered into operational taxonomic units (OTUs) with a 97% identity threshold using the UPARSE pipeline (https://drive5.com/uparse) (Tyurin et al. 2021). Chimeric read filtration was performed using UCHIME (Edgar et al. 2011). The OTUs with the highest frequency were screened as representative OTUs. Representative OTUs were annotated using Mothur (classify seqs) software with a confidence value of 60% based on the Silva128 database (Verbanic et al. 2020). The OTU clustering and taxonomy were annotated via BLAST (Fosso et al. 2018). The phylogenetic analysis of the OTU sequences and the deposited sequences was assessed by the maximum likelihood phylogenetic tree (ML tree) generated on the IQ-TREE web server (http://iqtree.cibiv.univie.ac.at) using the maximum likelihood method with 1,000 bootstrap replicates (Trifinopoulos et al. 2016). Rarefaction and rank abundance curves were generated using R 3.6.0 software (https://www.R-project.org) (Van Eesbeeck et al. 2021). Alpha diversity, including the Shannon, Simpson, Chao, and ACE indices, was calculated using QIIME (Caporaso et al. 2010).

Table 1. Information for the COI gene of the Hainan tick sample through the BLAST search.

Species	GenBank Accession No.	Query cover (%)	Ident (%)
Hainan tick	MW581657	100	89.52
Haemaphysalis bispinosa	MK140595	97	88.78
Haemaphysalis concinna	KU170512	98	87.02
Haemaphysalis concinna	KU170513	98	86.85
Haemaphysalis erinacei	KU885986	98	88.85
Haemaphysalis erinacei	KX901844	98	88.85
Haemaphysalis humerosa	MN106724	99	87.02
Haemaphysalis hystricis	NC 039765	99	86.98
Haemaphysalis hystricis	MT013253	99	86.98
Haemaphysalis lagostrophi	MN686569	99	86.86
Haemaphysalis leporispalustris	MN663152	96	87.29
Haemaphysalis leporispalustris	MN663153	96	86.96
Haemaphysalis longicornis	MT465131	99	87.12
Haemaphysalis qinghaiensis	JQ737094	99	86.52
Haemaphysalis sp.	MH937512	99	89.61
Haemaphysalis sp.	MN520952	99	86.60
Haemaphysalis verticalis	KR108849	98	88.37
Haemaphysalis verticalis	KY488642	98	87.04

Ident – percent identity of best BLAST alignments.

RESULTS

Morphological and molecular identification of ticks

We selected a total of 20 off-host unfed ticks that were identified using taxonomic keys as adult female *Haemaphysalis* spp. based on their morphological characteristics using taxonomic keys (Fig. 1A). Briefly, the unique characteristic of *Haemaphysalis* ticks is that their palp articles 2 are broad and laterally produced beyond the basis capituli. The genital pore was U-shaped, with the anal groove surrounding the anus and 11 obvious festoons that were nearly square in shape. (Fig. 1B). To identify the tick species, the COI gene was taken as a marker for molecular identification.

Genomic DNA was extracted from the leg of a single representative tick and the COI fragment generated by PCR was approximately 700 bp in size. BLAST analysis of the sequence revealed that the COI sequence had the greatest sequence similarity to that of *Haemaphysalis* sp., with a homology coverage of 99% and sequence identity of 89.61% (Table 1). The K2P distance between the collected ticks and *Haemaphysalis* sp. was 11.59%, indicating that the collected ticks may represent a new species in the genus *Haemaphysalis*. The close relationship between the collected ticks and several other *Haemaphysalis* species was evident in the neighbor-joining phylogenetic trees (Fig. 2).

Endosymbiotic bacterial community

To investigate the bacterial composition of the collected ticks, 20 whole ticks as a single pool were used for sequenc-

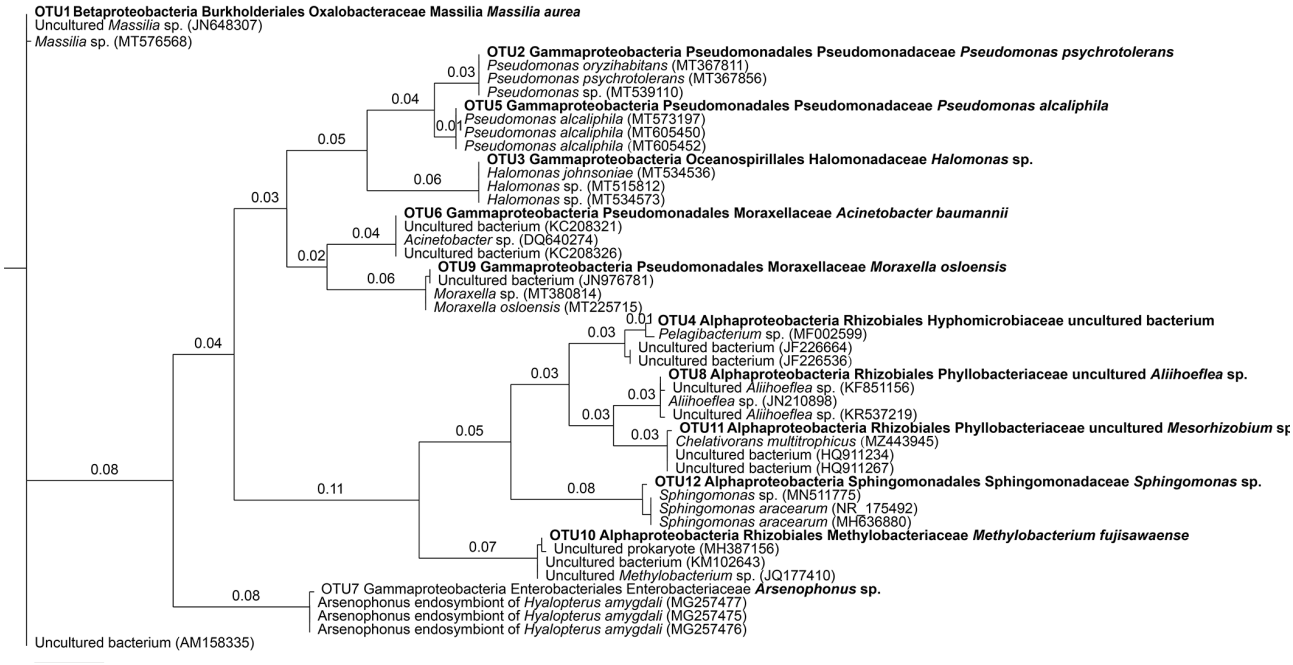

Fig. 3. Phylogenetic analysis of bacterial communities based on the 16S rRNA library at the OTU level of *Haemaphysalis* sp. The phylogenetic tree indicates the distribution of OTUs in 16S rRNA libraries

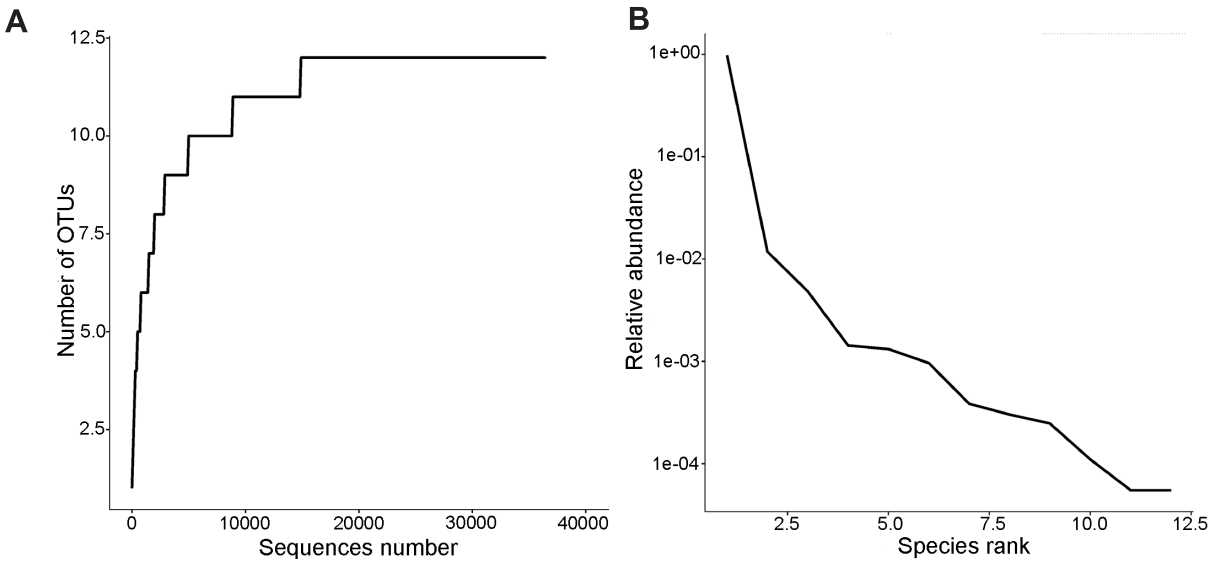

Fig. 4. Rarefaction and rank abundance analysis of samples of *Haemaphysalis* sp. **A** – the rarefaction curve; **B** – the rank abundance curve.

ing. The raw PE fastq-formatted reads were deposited as project number SRR21846545 in the SRA database of the NCBI. Sequencing the amplicons of the V4-V5 hypervariable regions of the bacterial 16S rRNA gene produced 40,582 reads. After quality filtering, 36,410 high-quality sequences (89.72% of raw sequences) were obtained for downstream analysis. OTU cluster analysis presented 12 taxa detected for the adult female *Haemaphysalis* sp. The endosymbiotic bacteria were all Proteobacteria, distributed in three classes (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria), six orders (Burkholderiales, Pseudomonadales, Oceanospirillales, Rhizobiales, Enterobacteriales, Sphingomonadales), ten families (Oxalobacteraceae, Pseudomonadaceae, Halomonadaceae, Hyphomicrobiaceae, Moraxellaceae, Enterobacteriaceae, Phyllobacteriaceae, Moraxellaceae, Methylobacteriaceae, Sphingomonadaceae), ten genera (*Massilia*, *Pseudomonas*, *Halomonas*, *Pelagibacterium*, *Acinetobacter*, *Arsenophonus*, *Aliihoeflea*, *Enhydrobacter*, *Methylobacterium*, *Sphingomonas*) and three unidentified species. A phylogenetic tree of 12 OTUs was built to further display their taxa (Fig. 3).

The rarefaction curve of the *Haemaphysalis* sp. sample reached saturation at 15,000 sequences, indicating adequate sampling depth (Fig. 4A). It was also supported by

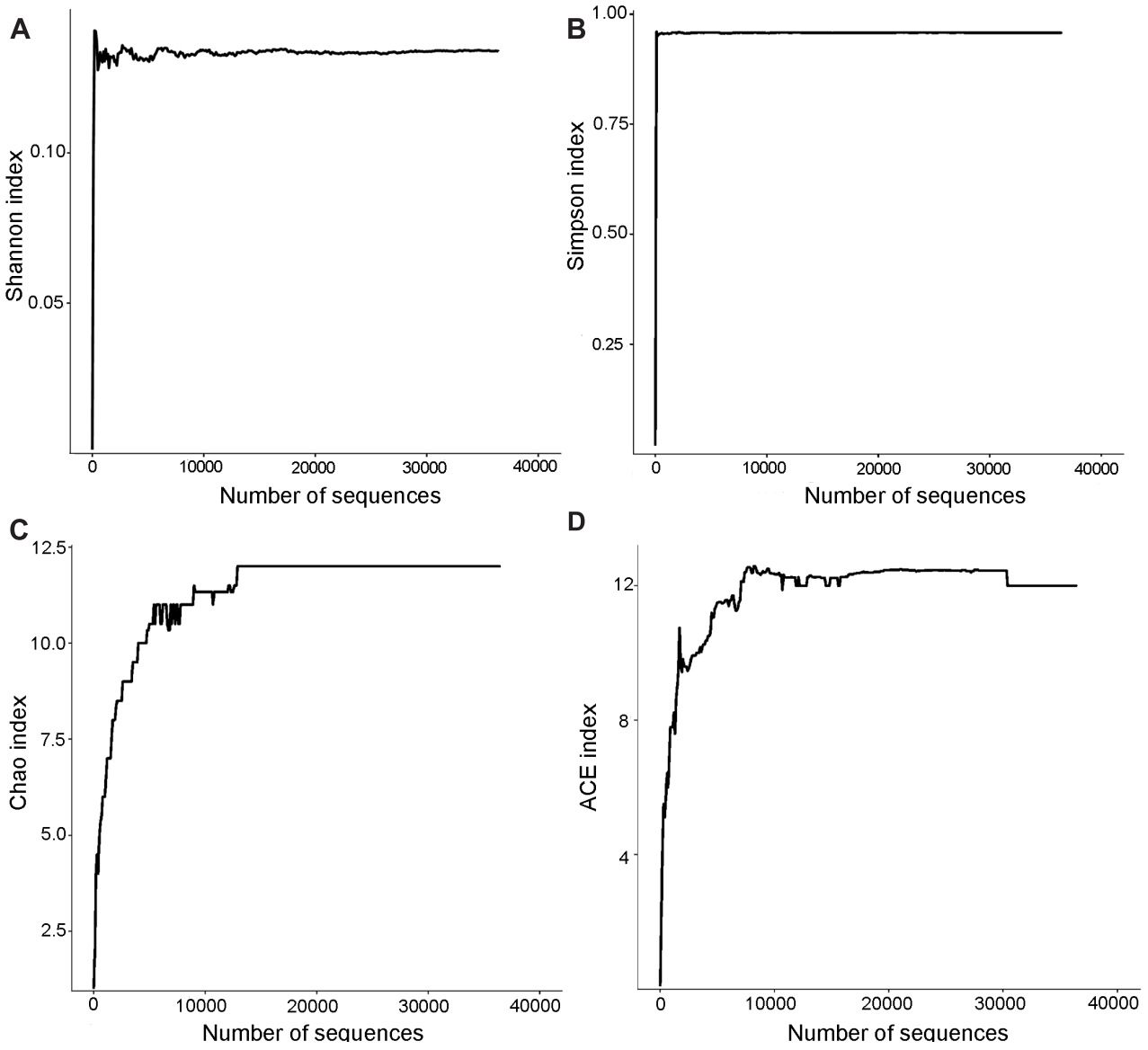

Fig. 5. The alpha diversity analysis revealed differences in the endosymbiotic bacterial species richness and evenness for *Haemaphysalis* Koch tick samples. **A** – Shannon index curve; **B** – Simpson index curve; **C** – Chao index curve; **D** – ACE index curve.

Good's coverage (100%). For the rank abundance curve (Fig. 4B), species richness was estimated by the span of the curve on the horisontal axis, and the uniformity of species is reflected by the smoothness of the curve in the vertical direction. Of the total 10 bacterial genera detected, *Massilia* was found to be the dominant bacterial genus, accounting for 97.85% of the population in the tick samples. The proportion of other genera, including *Arsenophonus*, *Acinetobacter*, *Pelagibacterium*, *Halomonas*, and *Pseudomonas*, ranged from 0.02% to 1.31%.

Alpha diversity analysis

Alpha diversity was assessed by the Shannon, Simpson, Chao, and ACE indices. A larger Shannon index value or a smaller Simpson index value illustrated a higher diversity of the bacterial community, and *vice versa*. In this study, the Shannon index value was 0.13385, close to 0, and the Simpson index value was 0.957619, quite close to 1, consistently indicating a low endosymbiotic diversity of the bacterial community in the samples of *Haemaphysalis* sp. The Chao and ACE indices reflect the richness of the bacterial community. The curves of the Chao and ACE indices rose rapidly to 12 and then tended to flatten with the continuous increase in sequencing depth, illustrating that the sample amount approached the saturation point and that the sequencing depth basically covered all the endosymbiotic bacterial species of the samples of *Haemaphysalis* sp. (Fig. 5).

DISCUSSION

In this study, we report the low-diversity of the bacterial community in field-collected *Haemaphysalis* sp. ticks from Hainan Island. Our finding is in contrast to previous reports that state that there is a high diversity in *Haemaphysalis* ticks as well as other tick species (Wikel et al. 2018, Zhang et al. 2019). However, a limited microbiome has also been reported in some tick species, including *Haemaphysalis leporispalustris* (Packard), *Ixodes pacificus* Cooley et Kohls, and *Dermacentor variabilis* (Say)

(Chicana et al. 2019, Couper et al. 2019). This difference may be caused by different tick species or environmental traits. As previous studies in the literature have indicated that blood meals taken from a mammalian host induced the endosymbiotic microbiome in blood-fed ticks to be more complex than that in unfed ticks and field-collected ticks, ticks that were not blood-fed were used to in the present study to eliminate some confounding factors (Gurfield et al. 2017, Thapa et al. 2019).

The bacterial community in the whole body of *Haemaphysalis* sp. ticks was dominated by the genus *Massilia*. This bacterial genus is commonly reported in soil rather than ticks (Nazipi et al. 2021). Only one study reported that *Massilia* bacteria were found in Neotropical ticks parasitising passerine migratory birds in Louisiana in the United States (Budachetri et al. 2017). Some *Massilia* species are potential pathogens, and they have been isolated from human clinical samples (La Scola et al. 1998, Kämpfer et al. 2012). Further study is warranted to determine whether the *Massilia* reported in our study is a human pathogen.

Numerous studies have revealed that endogenous microbiota can affect insect development and defence against pathogen invasion (Engel and Moran 2013, Cheng et al. 2016, Yuan et al. 2017, 2021). Ticks also have endogenous microbiota and an effective defence system (Yuan et al. 2020, Fogaça et al. 2021). This motivated the characterisation of tick microbiota to develop new strategies against ticks and tick-transmitted pathogens.

In the present study, *Arsenophonus* and *Pseudomonas* bacteria were identified in *Haemaphysalis* sp. *Arsenophonus* bacteria decreased tick motility, while *Rickettsia* bacteria increased motility (Kagemann and Clay 2013). Moreover, infection with *Borrelia burgdorferi*, the Lyme disease pathogen, was found to be associated with an increased abundance of midgut *Pseudomonas* in *Ixodes scapularis* Say (Ross et al. 2018). In the present study, the ticks were sampled from a rural area in Haikou and were identified as *Haemaphysalis* sp. Tick of this genus are which is a potential vectors of the human pathogen Kyasanur Forest disease virus (Atre et al. 2022). The virome of ticks in the same geographic area needs to be investigated in further work.

Microbiomic research has attracted attention across multiple fields in recent years (Weinroth et al. 2022) and has demonstrated unique ecological insights depending on the 16S rRNA high-throughput sequencing method. The dynamic composition of endosymbiotic bacteria is the result of insect vectors being exposed to a constantly changing external environment, demonstrating the association of vector organisms with the external environment. Considering disease-related aspects, endosymbiotic bacteria influence pathogen transmission in mosquitoes (Rodríguez-Ruano et al. 2020) as well as other insect vectors.

A pooled sample, rather than an independent sample, although failing to capture interindividual differences, can track the dynamics of the microbiome at the population level to demonstrate their general characteristics. Taken together, this study contributes to perspectives on the bacterial community of adult female *Haemaphysalis* ticks from Hainan Island. To the best of our knowledge, this is the first study to investigate the tick microbial community on Hainan Island. Our findings establish a baseline microbe composition and diversity in field-collected *Haemaphysalis* sp. ticks on Hainan Island, which can be helpful in future investigations and studies of vector-borne diseases associated with *Haemaphysalis* ticks.

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