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ORIGINAL ARTICLE

Molecular Detection of *Candidatus* Coxiella mudorwiae in *Haemaphysalis concinna* in China

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Abstract

Objective: *Coxiella burnetii* and *Coxiella*-like endosymbionts (CLEs) have been widely discovered in various ticks, animals, and even human beings. To estimate the possible origin of *C. burnetii* and its relatives CLEs, the prevalence of *C. burnetii* and CLEs has been intensively surveyed all over the world.

Method: In the present study, the possible infection of *C. burnetii* and CLEs in host-seeking *Haemaphysalis concinna* was performed with meta-transcript analysis with tick specimens harvested from Mudanjiang City, Heilongjiang province, China. The meta-transcript results were subsequently confirmed by the specific sequence of partial 16S rRNA.

Results: A total of three arrays of gene transcripts were harvested, including pyrophosphate-fructose 6-phosphate 1-phosphotransferase-*eda*-thiol-disulfide isomerase and thioredoxin-*greA*, *carB-carA-DnaJ-DnaK-grpE-ppnk*, *ropC-ropB*, and *ubiA*-non-canonical purine NTP pyrophosphatase-*hemK-prfA*, which suggest the infection of *Candidatus* Coxiella mudorwiae in *H. concinna*. The high identity of the 16S rRNA gene of *Candidatus* C. mudorwiae achieved in our study strongly supports our meta-transcripts analysis.

Conclusion: The prevalence of *Candidatus* C. mudorwiae in hard ticks has been discovered in China. More detailed surveys are imperative to clarify the emergence of CLEs and their implication in the epidemiologic characteristics of Q fever.

Key words: *Candidatus* Coxiella mudorwiae, Q fever, *Haemaphysalis concinna,* China

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INTRODUCTION

Coxiellae are intracellular bacteria that infect humans, vectorial arthropods, and a variety of vertebrates worldwide [1]. Both symbiotic and pathogenic bacteria in the family *Coxiellaceae* cause morbidity and acute disabling diseases in humans and animals. *Coxiella burnetii*, the only officially recorded species in the genus *Coxiella*, has been well-characterized as the agent of Q fever, which occurs sporadically with occasional outbreaks in humans worldwide annually [2]. The well-known Q fever epidemic in The Netherlands during 2007–2010 has attracted much attention due to the > 4000 human cases and health burden [161-336 million Euro] [3,4]. *C. burnetii* is also a category B potential aerosolized biological weapon [5]. The potential health risk from *Coxiella* is the subject of intensive epidemiologic surveys, which have revealed that diverse species, including various *Coxiella*-like endosymbionts (CLEs), exist within the genus *Coxiella*. CLEs isolated from different tick species [6] and their vertebrate hosts [7] have suggested that the natural occurrence of CLEs

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is by no means fortuitous but bound to commensal parasites in multiple tick species. Moreover, CLEs have been shown to provide host ticks with dietary supplementation, such as vitamin B, when deficient in vertebrate blood [8]. The well-characterized CLEs present close affinity with reference strains of *C. burnetii* with respect to morphologic, biologic, and evolutionary traits; however, the CLEs are genetically distinct. *Coxiellaceae* members have been classified into a minimum of 4 highly-divergent genetic clades (A–D) based on the phylogenetic analysis of 16S rRNA, lifestyle transitions, and maternal transmission traits [9]. Based on the taxonomic hierarchy, clade A includes all *C. burnetii* reference strains and CLEs from *Argasidae* ticks. In clade B, however, CLEs of hard ticks (*Amblyomma* and *Ixodes*) and some soft ticks (*Orinthodoros*) are represented with reduced genome sizes [~1M] [10]. Clade C is comprised of CLEs from ticks in the genus *Rhipicephalus* and their relatives [11]. The representative species, *Candidatus* Coxiella mudrowiae (*Ca.* C. mudrowiae) from *R. turanicus* was shown to have larger genome size, which has a very low protein-coding content and an extremely high number of identifiable pseudogenes [12]. Clade C members have also been shown to be opportunistic agents of human skin infections [8,12–15]. Regarding clade D, CLEs that are mainly derived from various *Haemaphysalis*, *Dermacentor,* and *Amblyomma* ticks were presumably pathogenic *Coxiellae* responsible for horse infections [16,17]. The presence of genetically-divergent CLEs in more tick species converge to support the hypothesis that these CLEs are specific endosymbionts of ticks and clade A CLEs hosted primarily by soft ticks might have served as the ancestor of *C. burnetii*. Thus, more detailed genetic information and rigorous assessment of the risk of infection to vertebrates are required for a comprehensive description.

The discovery that ticks carry *C. burnetii* or CLEs underscores the demand to elucidate the transmission routes of *Coxiellae* in natural cycles. First, it is noteworthy that the highly-virulent reference strain of *C. burnetii*, Nine Mile, was isolated from a guinea pig fed by *Dermacentor andersoni* ticks [18,19]. Second, > 50 tick species have been reported carrying visual *C. burnetii* or CLEs based on microscopic observations [6,20]. Many field studies have focused on ticks for Q fever [21,22]. Together with laboratory evidence, at least 10 tick species, including *D. andersoni*, have been formally confirmed to be competent vectors of *C. burnetii* along with their efficient trans-stadial or trans-ovarial transmission abilities [23–32]; however, it remains controversial whether these ticks have any significant role in the natural spread of *C. burnetii* [9]. Furthermore, *C. burnetii* and various CLEs prevail in different tick species, suggesting that these ticks can serve as a reservoir for *Coxiellaceae* members. Considering possible contamination to host skin and persistence in the environment by excreting high concentrations of *Coxiella* in tick feces [32], the medical significance for ticks in the environmental dissemination of *C. burnetii* and CLEs should not be underestimated [22]. Additionally, the noticeable presence of CLEs in salivary glands, ovaries, and Malpighian tubes of various tick species in almost all life stages [33–35] further suggests the possible vertical transmission via the egg cytoplasm or horizontal transmission across development stages rather than being acquired by blood-sucking on infected vertebrates, which demonstrates the risk of vertebrate host infection, including humans.

In China, the first human case of Q fever was reported in the 1950s when patients with atypical pneumonia were diagnosed serologically using a complement fixation test [CFT] [36,37]. The first human case was definitively confirmed in 1962 when the first Chinese strain of *C. burnetii* (Qi Yi) was isolated [38]. Since that time, nearly 30 seroepidemiologic or molecular studies have described *C. burnetii* infections in 64 cities/municipalities within 19 provinces across China [39]. Moreover, CLEs and *C. burnetii* have also been detected from ticks belonging to 5 genera, including *Ixodes* [40], *Dermacentor* [40,41], *Haemaphysalis* [42], *Hyalomma* [43], *Rhipicephalus* [44,45], wild animals [46,47], and even freshwater shrimp [*Palaemonetes sinensis*] [48]. The presence of CLEs in these ticks confers crucial and diverse benefits to the host ticks, affecting their development, nutrition, chemical defense, or reproduction, and distinctly interfere with the maintenance and/or transmission of some tickborne pathogens by tick-endosymbiont interactions, such as competition or mutual reciprocity [49]. The medical significance of CLEs occurring in ticks from Chinese territory, however, remains to be determined. During our meta-transcriptomic surveys for tickborne pathogens, an emerging CLE was found in *Haemaphysalis concina* collected from forest areas in Mudanjiang city of Heilongjiang province. The CLE was identified as *Ca.* C. mudrowiae in clade C, with molecular evidence from 16S rRNA, 3 arrays of transcripts, including pyrophosphate-fructose 6-phosphate 1-phosphotransferase-*eda*-thiol-disulfide isomerase and thioredoxin-*greA*-*carB*-*carA*-*DnaJ*-*DnaK*-*grpE*-*ppnk*, *ropC*-*ropB*, and *ubiA*-non-canonical purine NTP pyrophosphatase-*hemK*-*prfA*. The discovery of *Ca.* C. mudrowiae infected in *H. concinna* in China might advance our understanding of the epidemiology of Q fever and other tickborne diseases that eventually benefit us with rational prevention and control measures based on the evolutionary history.

MATERIALS AND METHODS

Ethics statement

Protocols for field tick collections and samples processing were reviewed and approved by the Institutional Ethics Review Board of Beijing Institute of Microbiology and Epidemiology (BIME-2020-019).

Tick collection, morphologic identification, and nucleotide acid extraction

A total of 213 adult ticks were randomly harvested on vegetation in Mudanjiang, Heilongjiang province, China (44.60°N 129.59°E) between April 2020 and July 2021. Identification of tick species was based on morphologic

characteristics following taxonomy keys for ticks in China [50]. Following species identification, the ticks were grouped by sex, each with 30 individuals. After sterilization with 70% alcohol, ticks in each pool were crushed with a sterile plastic homogenizer and total DNA and RNA were simultaneously extracted with a MagMAX DNA/RNA Isolation kit (Thermo-Fisher Scientific, Life Technologies, Carlsbad, California, USA). Purified DNA/RNA was quantified using the Qubit High Sensitivity assay (Thermo-Fisher Scientific, Life Technologies, Carlsbad, California, USA) before further processing.

Validation species identification by molecular markers

To verify tick species identification, we extracted genomic DNA from a proportion of the homogenates of each specimen or specimen pool. Two genes were used for tick identification: the partial 18S rRNA gene (~1100 nt); and the partial COI gene (~680 nt). The former target gene was amplified using primer pairs [18S-1 (5′-CTGGTGCCAGCGAGCCGCGGYAA-3′) and 18S-2 (5′-TCCGTCAATTYCTTTAAGTT-3′)], and the primer pairs [LCO1490 (5′-GGTCAACAAA TCATA AAGATATTGG-3′)] and [HCO2198 (5′-TAAACTTCAGGGTGAC CAAAAAATCA-3′)] for the latter. PCR amplifications were performed as described previously [51]. For taxonomic determination, the resulting sequences were compared against the nt database, as well as with all COI barcode records on the Barcode of Life Data Systems (BOLD).

Meta-transcripts sequencing for *Candidatus* Coxiella mudorwiae

The RNA samples from *Haemaphysalis concinna* were reverse-transcribed to cDNA using the PrimeScript™ IV 1st strand cDNA Synthesis Mix (Takara, Shiga, Japan) and ribosomal RNA (rRNA) was removed using the Ribo-Zero Gold Kit (Illumina, San Diego, California, USA) following the manufacturer's instructions. Subsequently, for all rRNA-depleted RNA-samples, the sequencing libraries were constructed using the KAPA Stranded RNA-Seq Kit (KAPA Biosystems, Roche, Boston, Massachusetts, USA) with barcode adapters (Bio Scientific, Phoenix, Arizona, USA) according to the manufacturer's instructions. After cDNA-level quantification by Qubit assays (Thermo-Fisher Scientific, Eugene, Oregon, USA), equimolar amounts of nucleic acids were pooled and submitted for sequencing in each library. Further, all libraries were subjected to next-generation sequencing (NGS) using the Illumina HiSeq 2500 platform on a single lane to generate the 125 bp paired-end reads at the BGI Sequencing Centre (www.genomics.cn). Raw sequencing reads of low quality were excluded, trimmed with trim galore [www.bioinformatics.babraham.ac.uk/projects/trim_galore/] [52], then assembled *de novo* using the Trinity v2.8.5 program [53,54]. The assembled scaffolds were predicted according to the open reading frames by MetaGeneMark, and CD-HIT was used to remove redundancy and obtain the initial unique gene catalog. For determination of gene abundance, the reads were realigned with the gene catalog with Bowtie 2 [55]. Only genes with two or more mapped reads were deemed to be present in a sample [56]. The presence of these genes in the corresponding samples was confirmed by nested RT–PCR and Sanger sequencing (data not shown). The unigenes were also aligned to the NR database (https://www.ncbi.nlm.nih.gov/) of NCBI with DIAMOND [57]. The aligned results of each gene with an e value ≤ the smallest e value × 10 were retained [56], then processed with the Lowest Common Ancestor-based algorithm implemented in MEGAN to ensure the species annotation information of sequences [58].

Confirmation *Candidatus* Coxiella mudorwiae infections by PCR amplification for 16S rDNA

Commercial HotStart PCR Premix Kit (Thermo-Fisher Scientific, Vilnius, Lithuania) was used for PCR amplification. Nested PCR according to the work from Seo et al. [16] was used to amplify the 16S rRNA of *Ca. C. mudrowiae* and other CLEs, and sequencing. Cox16SF1 (5′-CGTAGGA ATCTACCTTRTAGWGG-3′) and Cox16SR2 (5′-GCCTACCCGCTTCTGGTACAA TT-3′) were used to perform the first-round amplifications. Then, nested PCR was performed using the primers, Cox16SF2 (5′-TGAGAACTAGCTGTTGGRRAGT-3′) and Cox16SR2. All PCR amplifications were performed using the following program: pre-denaturation at 93°C for 3 min; 30 cycles of denaturation at 93°C for 30 s, annealing at 56°C for 30 s, and polymerization at 72°C for 1 min; and a final post-polymerization step at 72°C for 5 min. PCR products of the second amplification process were analyzed by electrophoresis, with 10 μl of the reaction mixture and a 100 bp DNA ladder (Bioneer, Daejeon, Korea) using 1.5% agarose gels for 30 min at 100 V and visualized using UV transillumination imaging after ethidium bromide staining. Samples yielding amplicons of the expected size were delivered to Sangon Biotechnology Co., Ltd. (Shanghai, China) for bi-directionally sequencing with the primers, Cox16SF1 and Cox16SR1 [5′-ACTYYCCAACAGCTAGTTCTCA-3′] [16].

Phylogenetic analysis

The obtained sequences were compared with the reference sequences in GenBank with the NCBI-BLAST server (http://blast.ncbi.nlm.nih.gov/blast.cgi) and multiple sequences were aligned with ClustalW with default parameters in MEGA 7.0. The phylogenetic trees of 16S rRNA, pyrophosphate-fructose 6-phosphate 1-phosphotransferase-*eda*-Thiol-disulfide isomerase and thioredoxin-*greA-carB-carA-DnaJ-DnaK-grpE-ppnk*, *ropC-ropB*, and *ubiA*-non-canonical purine NTP pyrophosphatase-*hemK-prfA* of *Ca.* C. mudrowiae were constructed with the maximum likelihood method using the best-fit model of nucleotide or amino acid substitution (LG + I + Γ + F for all alignments) with 1000 bootstrap

replicates in the PhyML v.3 program [59]. All phylogenetic trees were rooted at the midpoint for clarity purposes only.

Data availability

All sequence reads generated in this project are available under the NCBI Short Read Archive (SRA) under accessions SAMN26934337–SAMN26934338 (BioProject ID: PRJNA819490). All sequences were deposited in GenBank under accession numbers: OP863298, OP863299, OP863300 for three RNA transcipts, OPB839488 for 16S rRNA.

RESULTS

Prevalence of *Candidatus* Coxiella mudorwiae in *Haemaphysalis concinna*

The presence of CLEs was prompted by assembly contigs derived from *H. concinna,* then confirmed by PCR amplification targeting the 16S rRNA gene combined with sequence analysis. After annotation, all the transcripts achieved in 30 male or female *H. concinna* individuals showed no difference, suggesting unbiased CLE parasitizing in both sexes. Similar to the transcripts, there were also no differences observed in the16S rRNA genes of the CLE in both sexual groups of *H. concinna*. As a consequence, the 16S rRNA gene of the CLE was shown to be identical to the *Ca.* C. mudrowiae strain, CRt (CP011126.1), with 99.72% similarity over 100% coverage. *Ca.* C. mudrowiae is known to be closely related to pathogenic *Ca.* C. massiliensis isolated from infected samples of human skin [13], one horse blood strain of *Coxiella* (H-JJ-10) in Jeju Island of South Korea [17], and another human-derived *Coxiella* strain manifested with a scalp eschar and neck lymphadenopathy [14]. The phylogenetic tree constructed with partial sequences of 16S rRNA genes of CLEs from 46 tick species and *C. burnetii* reference strains formed four distinctive branches corresponding to clades A-D of *Coxiellaece,* respectively, as expected. Among these branches, all *C. burnetii* reference strains were clustered in clade A, along with other CLEs from *Ornithodoros* (*O. amblus*, *O. moubata,* and *Carios capensis*) species. The *Ca.* C. mudrowiae derived from *H. concinna* in the present study was shown to have high similarity to the *Ca.* C. mudrowiae strain, CRt (CP011126.1), and the *Ca.* C. mudrowiae strain, CRS-CAT (CP024961.1), which formed a distinctive sub-branch falling into clade C, including other CLEs from *Rhipicephalus* and *Dermacentor* ticks. Clade C was also demonstrated to be well-separated from other CLE clades, as shown in the phylogeny tree (Fig 1 panels A and B).

Transcript genes of *Candidatus* Coxiella mudorwiae in *Haemaphysalis concinna*

RNA samples from *H. concinna* yielded an average of 10.96 Gb of data, including $7.1\text{-}7.6 \times 10^7$ or 150-base pair-end reads from male and female ticks. Clean reads were subsequently assembled into contigs and compared to the NCBI Genomes database after host subtraction and quality filtering, resulting in 43 or 63 contigs adults from males and females of *H. concinna*, respectively. A prediction of open reading frames (ORFs) was also implemented for comparison with

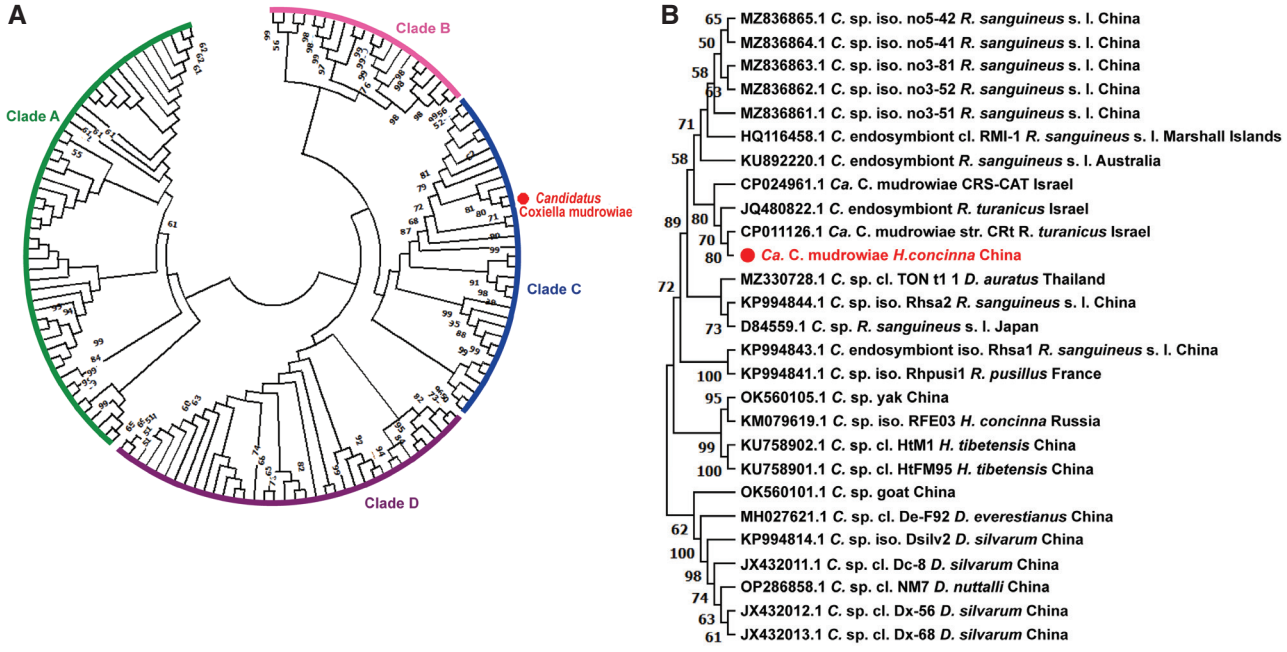

FIGURE 1 | Phylogenetic analysis of Coxiella-like endosymbionts and *C. burnetii* reference strains based on sequences of 16S rRNA in length of 1545 base pairs of nucleotides. Panel A. General view of *Coxiella* bacteria. Panel B. Enhanced region for Clade C of Coxiella-like-endosymbionts. *Candidatus* Coxiella mudrowiae harvested in the present study were marked with red ball. Maximum likelihood tree inferred using the best-fit model of nucleotide substitution (LG + I + Γ + F for all alignments) with 1000 bootstrap replicates.

TABLE 1 | Transcripts of *Candidatus* Coxiella mudorwiae discovered in *Haemaphysalis concinna*.

Genes or Transcripts	Coding length (nt)	Amino acids length	Coverage (%)	Identity (aa%)	Top hit (protein_id)
Pyrophosphate-fructose 6-phosphate 1-phosphotransferase	1765	567	99.0%	89.23%	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase (AKQ33802.1)
eda	644	214	99.0%	92.22%	4-Hydroxy-2-oxoglutarate aldolase (AKQ33803.1)
Thiol-disulfide isomerase and thioredoxin	560	186	100%	93.17%	Thiol-disulfide isomerase and thioredoxin (AKQ33804.1)
greA	480	160	100%	95.28%	Transcription elongation factor GreA (AKQ33805.1)
carA	3294	1096	99.0%	90.79%	Carbamyl-phosphte synthase large chain (AKQ33806.1)
carB	992	329	99.0%	78.37%	Carbamyl-phosphte synthase small chain (AKQ33807.1)
DnaJ	1144	380	97.0%	80.59%	DnaJ (AKQ33808.1)
DnaK	903	301	98.0%	92.33%	DnaK (AKQ33809.1)
grpE	910	203	100%	92.78%	grpE (AKQ33810.1)
ppnK	888	296	100%	93.27%	putative inorganic polyphosphate/ATP-NAD kinase (AKQ33811.1)
rpoC	4130	1376	100%	91.42%	DNA-directed RNA polymerase subunit beta' (AKQ33923.1)
rpoB	2380	794	100%	94.17%	DNA-directed RNA polymerase subunit beta (AKQ33924.1)
ubiA	865	287	100%	78.75%	4-hydroxybenzoate octaprenyltransferase (AKQ33990.1)
non-canonical purine NTP pyrophosphatase	602	198	100%	77.39%	non-canonical purine NTP pyrophosphatase (AKQ33992.1)
hemK	842	276	100%	77.26%	Release factor glutamine methyltransferase (AKQ33993.1)
prfA	2602	865	100%	86.27%	Peptide chain release factor 1 (AKQ33994.1)

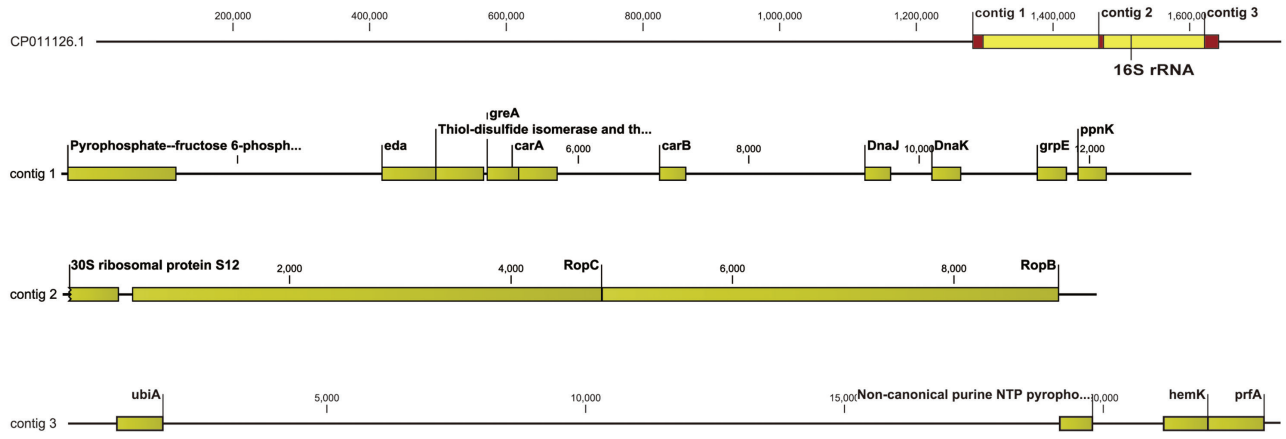

FIGURE 2 | Scheme for genomic information of *Candidatus* Coxiella mudrowiae detected from *Haemaphysalis concinna*. The 3 contigs of transcripts harvested from *H. concinna* in the present study were listed under the reference strain CRt (CP011126.1). Three target contigs were marked with brown color on the similar region of *Candidatus* C. mudrowiae genome (CP011126.1), the intervals failed to achieve between the 3 contigs were marked with bright yellow. Contig 1 to 3 were enhanced to show the annotated genes with yellow blocks.

the protein database of the *Ca.* C. mudrowiae and other CLEs available through BLASTP. Sixteen transcripts of *Ca.* C. mudrowiae were identified from both sexes of *H. concinna*, which included pyrophosphate-fructose 6-phosphate 1-phosphotransferase, *eda*, thiol-disulfide isomerase, *greA*, *carA*, *carB*, *DnaJ*, *DnaK*, *grpE*, *ppnK, rpoC*, *rpoB*, and *ubiA* non-canonical purine NTP pyrophosphatases, *hemK* and *prfA*. Aligned sequences of the 16 genes or transcripts are listed in Table 1 with their identities and coverages of putative amino acids of CLE top hit proteins. Among the CLE top hit proteins, the 10 genes or transcripts, including pyrophosphate-fructose 6-phosphate 1-phosphotransferase, thiol-disulfide isomerase and thioredoxin, *eda*, *greA*, *carA*, *carB*, *DnaJ*, *DnaK*, *grpE*, and *ppnK,* were shown with > 78% identities on > 97% coverages with those of the *Ca.* C. mudrowiae stain, CRt (CP011126.1). The remaining six transcripts of *rpoC-rpoB*, *ubiA*, non-canonical purine NTP pyrophosphatase*, hemK* and *prfA* were demonstrated with higher identities, ranging from 77.26%-94.17%, on 100% coverages with those of the *Ca.* C. mudrowiae stain, CRt (CP011126.1). The results also supported the conclusion that the CLE from *H. concinna* should be identified as *Ca.* C. mudrowiae (Fig 2), which belongs to clade C of family *Coxiellae,* which has a genome reduction and prominent gene decay because of the loss of selection on gene functions [12].

DISCUSSION

Q fever is a widespread zoonotic disease caused by *C. burnetii*, a ubiquitous intracellular bacterium. Due to its highly polymorphic clinical manifestations, Q fever is difficult to prevent, diagnose, and treat in humans and animals [9]. Human exposure to *C. burnetii* may result in asymptomatic-to-mild infections, but also in acute or chronic disease. Although rarely fatal, Q fever can be highly debilitating, even under treatment [60]. This post-infection fatigue syndrome is associated with increased or impaired cellular responses, but with apparently no viable *Coxiella*, low or negligible antibody levels, and clinical expression of a long-lasting fatigue complex involving many body systems [61]. Therefore, Q fever results in a high socioeconomic burden and significant challenges for public health. To prevent and control the possible infections in humans and animals, pioneering studies have been carried out to elucidate the pathogenicity, epidemiology, diagnosis, and treatment aspects of *C. burneti* and > 40 tick species have been shown to naturally infect *C. burnetii* or CLEs [9]. Our NGS approaches have also revealed that *Ca.* C. mudrowiae, but not *C. burnetii*, prevail in *H. concinna* populations, which supports the hypothesis that diverse CLEs rather than presumptive *C. burnetii* predominate in most tick species investigated thus far [62-64]. The reality that ticks carry both *C. burnetii* and CLEs further emphasizes the demand to clearly distinguish *Coxiellae* members, which is necessary to improve our understanding of the epidemiology and evolutionary history of Q fever. The infection of *C. burnetii* in ticks might be rigorously confirmed through an array of impressive assays, including hemolymph tests, isolation in cell-lines [Vero] [65], and multiple-locus variable number tandem repeat (MLVA) analysis [66], multispacer sequence typing [MST] [67], and SNP genotyping [21,68]. Nevertheless, many studies have not been conducted as rigorously, which has led to frequent misidentifications of CLEs. Some strains, primarily assumed to be *C. burnetii* visually, were actually misidentification results of CLEs. CLEs in *A. americanum*, for example, has been repeatedly misidentified as *C. burnetii* in the older literature [69,70], but recent sequence-based detections have confirmed that it is actually a CLE (CP007541) of clade D characterized with a smaller genome size [71]. In our studies, 16 function genes or transcripts beyond 16S rRNA of *Ca.* C. mudrowiae

were achieved, although the percent identities of some genes were quite low compared to the reference strain (Table 1), which may be explanted by the possible sense mutations or different strains from various localities. The limitation of our study was apparent because we failed to achieve a full genome of *Ca.* C. mudrowiae and unambiguously identify the CLE. Future attempts to isolate *Ca.* C. mudrowiae from *H. concinna* would be beneficial in reaching our goal.

Overall, heritable endosymbiotic bacteria are of ecologic and evolutionary importance to the particular arthropod species because the bacteria potentially confer crucial and diverse benefits to the host by affecting development, provision of nutrients, chemical defense, or reproduction [71]. The high frequency of CLE transfer occurring at the tick-vertebrate interface may enhance or decrease the probability of infections not only with *C. burnetiid,* but also with other tick-borne pathogens [72]. For example, *Wolbachia* spp. have recently been shown to be defensive endosymbionts to interfere with the replication and transmission of various pathogens in their arthropods hosts, including mosquitoes and flies [73,74], which eventually limit the vector competence of these blood-sucking arthropods [75,76]. Although most CLEs described to date have been confined to infect ticks, whether commensal CLEs parasitize ticks limit their vector competence to transmit pathogenic organisms, including *C. burnetiid,* remains to be determined. Under natural circumstances, most CLEs in ticks pose a much lower infection risk to vertebrates than *C. burnetii*, which may be explained by the facts that genomic reduction or conjugation machinery might take place in the evolutionary history of CLEs to exchange genetic information, such as Dot/Icm and type IVB secretion system (T4BSS) via horizontal gene transfer [HGT] [49]. Thus, most *Coxiella* progenitors have evolved into avirulent CLEs with reduced genomes and devoid of known virulence genes [CLE of *A. Americanum*] [6] or intermingled pathogens retaining virulent genes (*C. burnetii* or pathogenic CLEs). The infectious *Ca.* C. mudrowiae was also demonstrated to have biologic activity in *H. concinna* along with transcription evidence from 16 genes, which were involved in many essential physiological processes, including energy metabolism (e.g., pyrophosphate-fructose 6-phosphate 1-phosphotransferase), intercellular vesicles formation or transportation (e.g., *DnaJ*, *DnaK*, and *grpE*), and potential intracellular invasion. Whether the transcriptions of these genes participate in the complicated interactions with pathogens or other endosymbiont parasites in host ticks remains to be clarified. In contrast, the occurrence of CLEs, such as *Ca.* C. mudrowiae, also suggest a feasible CLE-based approach to modify pathogen transmission in arthropods, which confirmed our interesting research effort in a direction for intervention of pathogen transmission [77]. Therefore, it is necessary to describe the diversity of CLEs, characterize more fully their genetic relatedness, and assess their potential to cause infections in vertebrates.

In conclusion, *Ca.* C. mudrowiae infected in *H. concinna* has revealed that studies on CLEs can advance our understanding of CLE diversity and epidemiology of Q fever. *Ca.* C. mudrowiae and *C. burnetii* are closely related, but differ in their transmission ecology and infectivity. This phenotypic diversity makes the evolution of genus *Coxiella* a topic of peculiar interest, because there are clearly transitions between avirulent and virulent symbionts residing in confined intracellular environments.

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COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

1. Lang GH. Coxiellosis (Q fever) in animals. In *Q FEVER (Vol. 1) The Disease*. Edited by Marrie TJ. CRC Press; 1990:23-48.
2. Angelakis E, Raoult D. Q fever. Vet Microbiol. 2010;140:297-309.
3. van der Hoek W, Morroy G, Renders NHM, Wever PC, Hermans MHA, Leenders ACAP, et al. Epidemic Q fever in humans in the Netherlands. Adv Exp Med Biol. 2012;984:329-364.
4. van Asseldonk M, Prins J, Bergevoet RH. Economic assessment of Q fever in the Netherlands. Prev Vet Med. 2013;112(1-2):27-34.
5. Madariaga MG, Rezai K, Trenholme GM, Weinstein RA. Q fever: a biological weapon in your backyard. Lancet Infect Dis. 2003;311:709-721.
6. Duron O, Noël V, McCoy KD, Bonazzi M, Sidi-Boumedine K, Morel OJ, et al. The recent evolution of a maternally inherited endosymbiont of ticks led to the emergence of the Q fever pathogen, *Coxiella burnetii*. PLoS Pathog. 2015;11:e1004892.
7. Rousset E. Q fever. In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. Office International des Epizooties (OIE) – World Organization for Animal Health; 2010:13.
8. Ben-Yosef M, Rot A, Mahagna M, Kapri E, Behar A, Gottlieb Y. *Coxiella*-like endosymbiont of *Rhipicephalus sanguineus* is required for physiological processes during ontogeny. Front Microbiol. 2020;11:493.
9. Duron O, Sidi-Boumedine K, Rousset É, Moutailler S, Jourdain E. The importance of ticks in Q fever transmission: what has (and has not) been demonstrated? Trends Parasitol. 2015;31(11):536-552.
10. Nardi T, Olivieri E, Kariuki E, Sassera D, Castelli M. Sequence of a Coxiella endosymbiont of the tick *Amblyomma nuttalli* suggests a pattern of convergent genome reduction in the *Coxiella* genus. Genome Biol Evol. 2020;13(1):1-7.
11. Lalzar I, Friedmann Y, Gottlieb, Y. Tissue tropism and vertical transmission of *Coxiella* in *Rhipicephalus sanguineus* and *Rhipicephalus turanicus* ticks. Environ Microbiol. 2014;16:3657-3668.
12. Gottlieb Y, Lalzar I, Klasson L. Distinctive genome reduction rates revealed by genomic analyses of two *Coxiella*-like endosymbionts in ticks. Genome Biol Evol. 2015;7:1779-1796.

13. Angelakis E, Mediannikov O, Jos S, Bérenger J, Parola P, Raoult D. *Candidatus* Coxiella massiliensis infection. Emerg Infect Dis. 2016;22:285-288.
14. Guimard T, Amrane S, Prudent E, El Karkouri K, Raoult D, Angelakis E. Case report: scalp eschar and neck lymphadenopathy associated with bacteremia due to *Coxiella*-like bacteria. Am J Trop Med Hyg. 2017;97(5):1319-1322.
15. Tsementzi D, Castro Gordillo J, Mahagna M, Gottlieb Y, Konstantinidis KT. Comparison of closely related, uncultivated Coxiella tick endosymbiont population genomes reveals clues about the mechanisms of symbiosis. Environ Microbiol. 2018;20(5):1751-1764.
16. Mediannikov O, Ivanov L, Nishikawa M, Saito R, Sidelnikov YN, Zdanovskaya NI, et al. Molecular evidence of Coxiella-like microorganism harbored by Haemaphysalis concinnae ticks in the Russian Far East. Ann N Y Acad Sci. 2003;990:226-228.
17. Seo M, Lee S, VanBik D, Ouh I, Yun S, Choi E, et al. Detection and genotyping of *Coxiella burnetii* and *Coxiella*-like bacteria in horses in South Korea. PLoS One. 2016;11(5):e0156710.
18. McDade JE. Historical aspects of Q fever. In *Q FEVER (Vol. 1) The Disease*. Edited by Marrie TJ. CRC Press; 1990:5-22.
19. Khavkin T. Q fever studies in the U.S.S.R. In *Q fever: The Biology of Coxiella burnetti*. Edited by Williams JC, Thompson HA. CRC Press; 1991:311-326.
20. Ullah Q, Jamil T, Saqib M, Iqbal M, Neubauer HK. Q fever-a neglected zoonosis. Microorganisms. 2022;10:1530.
21. Pacheco RD, Echaide IE, Alves RN, Beletti ME, Nava S, Labruna MB. *Coxiella burnetii* in ticks, Argentina. Emerg Infect Dis. 2013;19:344-346.
22. Körner S, Makert GR, Ulbert S, Pfeffer M, Mertens-Scholz K. The prevalence of *Coxiella burnetii* in hard ticks in Europe and their role in Q fever transmission revisited—A systematic review. Front Vet Sci. 2021;8:655715.
23. Parker RR, Davis GE. A filter-passing infectious agent isolated from ticks. II. Transmission by *Dermacentor andersoni*. Public Health Rep. 1938;53:2267-2276. Corpus ID: 75146461.
24. Smith DJW. Studies in the epidemiology of Q fever. 3. Transmission of Q fever by the tick *Haemaphysalis humerosa*. Aust J Exp Biol Med Sci. 1940;103-118.
25. Smith DJW. Studies in the epidemiology of Q fever. 8. The transmission of Q fever by the tick *Rhipicephalus sanguineus*. Aust J Exp Biol Med Sci. 1941;19:133-136.
26. Smith DJW. Studies in the epidemiology of Q fever. 10. The transmission of Q fever by the tick *Ixodes holocyclus* (with notes on tick-paralysis in Bandicoots). Aust J Exp Biol Med Sci. 1942;20:213-217. Corpus ID: 209544688.
27. Smith DJW. Studies in the epidemiology of Q fever. 11. Experimental infection of the ticks *Haemaphysalis bispinosa* and *Ornithodoros* sp. with *Rickettsia burneti*. Aust J Exp Biol Med Sci. 1942;20:295-296.
28. Davis GE. American Q fever: experimental transmission by the argasid ticks *Ornithodoros moubata* and *O. hermsi*. Public Health Rep. 1943;58:984-987.
29. Daiter AB. Transovarial and transspermal transmission of *Coxiella burnetii* by the tick *Hyalomma asiaticum* and its role in Q rickettsiosis ecology. Parazitologiya. 1977;11:403-411. Corpus ID: 25934840.
30. Siroky P, Kubelova M, Modry D, Erhart J, Literak I, Spitalska E, et al. Tortoise tick *Hyalomma aegyptium* as long term carrier of Q fever agent *Coxiella burnetii*—evidence from experimental infection. Parasitol Res. 2010;107:1515-1520.
31. González J, González MG, Valcárcel F, Sánchez ME, Martín-Hernández R, Tercero JM, et al. Transstadial transmission from nymph to adult of *Coxiella burnetii* by naturally infected *Hyalomma lusitanicum*. Pathogens. 2020;9:884.
32. Körner S, Makert GR, Mertens-Scholz K, Henning K, Pfeffer M, Starke A, et al. Uptake and fecal excretion of *Coxiella burnetii* by *Ixodes ricinus* and *Dermacentor marginatus* ticks. Parasit Vec. 2020;13:75.
33. Klyachko OS, Stein BD, Grindle N, Clay K, Fuqua C. Localization and visualization of a *Coxiella*-type symbiont within the lone star tick, *Amblyomma americanum*. Appl Environ Microbiol. 2007;73:6584-6594.
34. Machado-Ferreira E, Dietrich G, Hojgaard A, Levin ML, Piesman JF, Zeidner NS, et al. *Coxiella* symbionts in the Cayenne tick *Amblyomma cajennense*. Microb Ecol. 2011;62:134-142.
35. Almeida A, Marcili A, Leite RC, Nieri-Bastos FA, Domingues LN, Martins JR, et al. *Coxiella* symbiont in the tick *Ornithodoros rostratus* (Acari: Argasidae). Ticks Tick Borne Dis. 2012;3(4):203-206.
36. Zhang NC, Zia SH, Liu FT, Zhang NZ. Preliminary study on the problem of Q fever in Beijing [in Chinese]. Chin Med J. 1951;37:235.
37. Zhai SC, Liu SH. Q fever: report of a case [in Chinese]. Chin J Int Med. 1957;5:316.
38. Yu SR, Zhang SL, Li QJ, Zhang BX. Isolation and identification of Q fever rickettsia in Sichuan. In *Monograph on Rickettsiaceae and Chlamydiaceae infections* [in Chinese]. Beijing: Chinese Journal of Epidemiology Press; 1981:18.
39. El-Mahallawy HS, Lu G, Kelly PJ, Xu DH, Li Y, Fan W, et al. Q fever in China: a systematic review, 1989–2013. Epidemiol Infect. 2014;143:673-681.
40. Jiao J, Lu Z, Yu Y, Ou Y, Fu M, Zhao Y, et al. Identification of tick-borne pathogens by metagenomic next-generation sequencing in *Dermacentor nuttalli* and *Ixodes persulcatus* in Inner Mongolia, China. Parasit Vectors. 2021;14:287.
41. Li N, Li S, Wang D, Yan P, Wang W, Li M, et al. Characterization of the Rickettsia-like and *Coxiella*-like symbionts in the tick *Dermacentor everestianus* Hirst, 1926 (Acari: Ixodidae) from the Qinghai-Tibet Plateau. Syst Appl Acarol. 2019;24:106-117.
42. Zhuang L, Du J, Cui X, Li H, Tang F, Zhang P, et al. Identification of tick-borne pathogen diversity by metagenomic analysis in *Haemaphysalis longicornis* from Xinyang, China. Infect Dis Poverty. 2018;7:45.
43. Ni J, Lin H, Xu X, Ren Q, Aizezi M, Luo J, et al. *Coxiella burnetii* is widespread in ticks (Ixodidae) in the Xinjiang areas of China. BMC Vet Res. 2020;16:317.
44. Machado-Ferreira E, Vizzoni VF, Balsemão-Pires E, Moerbeck L, Gazêta GS, Piesman JF, et al. *Coxiella* symbionts are widespread into hard ticks. Parasitol Res. 2016;115:4691-4699.
45. Jiao J, Zhang J, He P, Ouyang X, Yu Y, Wen B, et al. Identification of tick-borne pathogens and genotyping of *Coxiella burnetii* in *Rhipicephalus microplus* in Yunnan Province, China. Front Microbiol. 2021;12:736484.
46. Qi Y, Ai L, Zhu C, Ye F, Lv R, Wang J, et al. Wild Hedgehogs and their parasitic ticks coinfected with multiple tick-borne pathogens in Jiangsu Province, Eastern China. Microbiol Spectr. 2022;10:e0213822.
47. Fu M, He P, Ouyang X, Yu Y, Wen B, Zhou D, et al. Novel genotypes of *Coxiella burnetii* circulating in rats in Yunnan Province, China. BMC Vet Res. 2022;18:204.
48. Lu X, Ma H, Liu J, Sun N, Yu J, Yang Z, et al. First case of *Coxiella burnetii* infection in *Palaemonetes sinensis* cultured in Liaoning, China. Dis Aquat Org. 2022;148:145-151.
49. Brenner AE, Muñoz-Leal S, Sachan M, Labruna MB, Raghavan R. *Coxiella burnetii* and related tick endosymbionts evolved from pathogenic ancestors. Genome Biol Evol. 2021;13(7):1-15.
50. Teng KF, Jiang ZJ. Economic insect fauna of China. Fasc 39, Acarina: Ixodidae. Beijing: Science Press, Academia Sinica; 1991:1-359. [in Chinese]
51. Machida RJ, Knowlton N. PCR primers for metazoan nuclear 18S and 28S ribosomal DNA sequences. PLoS One. 2012;7(9):e46180.

52. Krueger F, James FO, Ewels PA, Afyounian E, Schuster-Boeckler B. 2021. FelixKrueger /TrimGalore: v0.6.7. DOI: 10.5281/ZENODO.5127899.
53. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011;29:644-652.
54. Haas BJ, Papanicolaou A, Yassour M, Grabherr MG, Blood PD, Bowden JC, et al. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat Protoc. 2013;8:1494-1512.
55. Langmead B, Salzberg S. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9:357-359.
56. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.
57. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat Methods. 2015;12(1):59-60.
58. Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. Genome Res. 2007;17(3):377-386.
59. Guindon S. From trajectories to averages: an improved description of the heterogeneity of substitution rates along lineages. Syst Biol. 2013;62(1):22-34.
60. Raoult D, Marrie TJ, Mege JL. Natural history and pathophysiology of Q fever. Lancet Infect Dis. 2005;5(4):219-226.
61. Marmion BP, Sukocheva OA, Storm PA, Lockhart M, Turra M, Kok T, et al. Q fever: persistence of antigenic non-viable cell residues of *Coxiella burnetii* in the host--implications for post Q fever infection fatigue syndrome and other chronic sequelae. QJM: Monthly Journal of the Association of Physicians. 2009;102(10):673-684.
62. Andreotti R, Pérez de León AA, Dowd SE, Guerrero FD, Bendele KG, Scoles GA. Assessment of bacterial diversity in the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* through tag encoded pyrosequencing. BMC Microbiol. 2010;11:6.
63. Williams-Newkirk AJ, Rowe L, Mixson-Hayden T, Dasch GA. Characterization of the bacterial communities of life stages of free living lone star ticks (*Amblyomma americanum*). PLoS One. 2014;9:e102130.
64. Qiu Y, Nakao R, Ohnuma A, Kawamori F, Sugimoto C. Microbial population analysis of the salivary glands of ticks; a possible strategy for the surveillance of bacterial pathogens. PLoS One. 2014;9:e103961.
65. Glazunova OI, Roux V, Freylikman O, Sekeyová Z, Fournous G, Tyczka J, et al. *Coxiella burnetii* genotyping. Emerg Infect Dis. 2005;11:1211-1217.
66. Arricau-Bouvery N, Hauck Y, Béjaoui A, Frangoulidis D, Bodier CC, Souriau A, et al. Molecular characterization of Coxiella burnetii isolates by infrequent restriction site-PCR and MLVA typing. BMC Microbiol. 2005;6:38.
67. Svraka S, Toman R, Škultéty L, Slabá K, Homan WL. Establishment of a genotyping scheme for *Coxiella burnetii*. FEMS Microbiol Lett. 2006;254:268-274.
68. Huijsmans CJ, Schellekens JJ, Wever PC, Toman R, Savelkoul PH, Janse I, et al. Single-nucleotide polymorphism genotyping of *Coxiella burnetii* during a Q fever outbreak in The Netherlands. Appl Environ Microbiol. 2011;77:2051-2057.
69. Parker RR, Kohls GM. American Q fever: the occurrence of *Rickettsia diaporica* in *Amblyomma americanum* in eastern Texas. Public Health Rep. 1943;58:1510-1511.
70. Philip CB, White JS. Disease agents recovered incidental to a tick survey of the Mississippi Gulf coast. J Econ Entomol. 1955;48:396-400.
71. Smith TA, Driscoll TP, Gillespie JJ, Raghavan R. A *Coxiella*-like endosymbiont is a potential vitamin source for the lone star tick. Genome Biol Evol. 2015;7:831-838.
72. Clay K, Klyachko OS, Grindle N, Civitello DJ, Oleske DA, Fuqua C. Microbial communities and interactions in the lone star tick, *Amblyomma americanum*. Mol Ecol. 2008;17:4371-4381.
73. Hamilton PT, Perlman SJ. Host defense via symbiosis in *Drosophila*. PLoS Pathog. 2013;9:e1003808.
74. Brownlie JC, Johnson KN. Symbiont-mediated protection in insect hosts. Trends Microbiol. 2009;17:348-354.
75. LePage D, Bordenstein SR. Wolbachia: can we save lives with a great pandemic? Trends Parasitol. 2013;29:385-393.
76. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, et al. A Wolbachia symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and Plasmodium. Cell. 2009;139:1268-1278.
77. Ahantarig A, Trinachartvanit W, Baimai V, Grubhoffer L. Hard ticks and their bacterial endosymbionts (or would be pathogens). Folia Microbiol (Praha). 2013;58:419-428.