

Molecular Detection of Tick-Borne Pathogens in Ticks Collected From Hainan Island, China

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Research

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Abstract

Background

Kinds of pathogens such as viruses, bacteria and protozoa are transmitted by ticks as vectors, and they have deeply impact on human and animal health worldwide.

Methods

To better understand the genetic diversity of bacteria and protozoans carried by ticks in Chengmai county of Hainan province, China, 285 adult hard ticks belonging to two species (*Rhipicephalus sanguineus*: 183, 64.21% and *R. microplus*: 102, 35.79%) from dogs, cattle, and goats were collected. Rickettsiales bacteria, Coxiellaceae bacteria, Babesiidae, and Hepatozoidae were identified in these ticks by amplifying the 18S rRNA, 16S rRNA (*rrs*), citrate synthase (*gltA*), and heat shock protein (*groEL*) genes.

Results

Our data revealed the presence of four recognized species and two *Candidatus* spp. of Anaplasmataceae and Coxiellaceae in locality.

Conclusions

In sum, these data reveal an extensive diversity of Anaplasmataceae bacteria, Coxiellaceae bacteria, Babesiidae, and Hepatozoidae in ticks from Chengmai county, highlighting the need to understand the tick-borne pathogen infection in local animals and humans.

Introduction

Ticks are vectors for a variety of etiological agents of zoonotic disease including viruses, bacteria and protozoa [1]. Tick-borne diseases such as rickettsiosis, anaplasmosis and babesiosis are of substantial concern for both humans and animals all over the world. In China, tick-borne pathogens have posed great threat to residents, especially those in rural and forest [2]. However, most of these pathogens such as rickettsiosis, anaplasmosis, and babesiosis are still underestimated, because the epidemiological and clinical information about these pathogens is quite limited. In addition, misdiagnosis is very often due to their clinical manifestation's similarity to other syndromes such as hemorrhagic fever with renal syndrome (HFRS) [2]. Furthermore, ticks also act as vectors for a variety of pathogens causing diseases to companion animals and livestock [3, 4]. These include bovine anaplasmosis (*Anaplasma marginale* and *A. bovis*), bovine babesiosis (*Babesia bigemina* and *B. bovis*), canine babesiosis (*B. canis*), canine ehrlichiosis (*Ehrlichia canis*), caprine anaplasmosis (*A. capra*), etc [5, 6–8]. These pathogens have significant economic impacts on livestock production and cause remarkable economic losses each year. On the other hand, infection in these animals also increased the risk of developing tick-borne disease in their owners.

Hainan Island, the second largest island of China, locates in the Southern China Sea. It has a typical tropical climate and plentiful wildlife. Until the year 2018, it has an area 33,900 square kilometers and a population of 9.34 million. In the recent decades, it is becoming a tourist attraction because of its beautiful scenery and geographical position. Previous study indicated that Hainan Island is the natural epidemic focus of North Asia Tick-Borne Spotted Fever (NASF), which is caused by *Rickettsia sibirica* belonged to Spotted Fever Group Rickettsiae (SFGR) [9]. The antibodies investigate of NASF in locality revealed the positive incidence are 38.3% and 53% in local human and mouse, separately [10]. However, the reports on other tick-borne pathogens in Hainan Island have been very few. In this study, to evaluate the potential risk of tick-borne pathogens to local residents, tourists and domestic animals on Hainan Island, we performed a survey of the occurrence and prevalence of bacteria and protozoans in ticks collected from Hainan Island, China.

Materials And Methods

Samples collection and DNA isolation

Tick samples were collected from stray dogs, goats and cattle in Chengmai county locating in the northwest of Hainan Island from September to October, 2019. Only adult ticks were included in this study. Ticks were brought to China CDC alive and stored individually at -80 °C before DNA extraction. The tick species was determined by morphological identification, and then confirmed by PCR and sequencing.

Before DNA extraction, the ticks were washed with 75% ethyl alcohol followed by 0.01M phosphate buffer solution (PBS) and dried. They were divided into pools (3 ticks each) and homogenized in PBS. Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. All DNA extracts were stored at -20 °C.

Detection of tick-borne pathogens

All the tick samples were tested for the presence of Rickettsiales bacteria, Coxiellaceae bacteria, Babesiidae, and Hepatozoidae. PCR detection of Babesiidae and Hepatozoidae species targeting the 18S rRNA gene was performed using primers as described previously [11]. The *rrs* gene of Rickettsiales bacteria and Coxiellaceae bacteria species were amplified by nested PCR using primers as described [12, 13]. For further exploration of phylogenetic positioning of the bacterial strains, the 760-bp fragment of the *gltA* gene encoding citrate synthase and the 614-bp fragment of the *groEL* gene encoding the heat shock protein were amplified for samples that were positive for these bacteria. Both negative and positive controls were added.

PCR amplicons were analyzed by electrophoresis in 1.0% agarose gels. The amplicons shorter than 600 bp were directly subjected to Sanger sequencing by Sangon Biotechnology Company (Shanghai, China). The PCR products longer than 600 bp were cloned into pMD19-T cloning vector (TaKaRa, China), transformed into *E. coli* and plated onto culture dish. Then the obtained clones were picked and sent for Bi-directional sequencing.

Phylogenetic analysis

Nucleotide sequence similarities between the obtained 16S/18S rRNA, *groEL* and *gltA* sequences and those from GenBank were calculated by DNASTar (ver. 7.0). For phylogenetic analysis, the sequences were aligned with reference sequences using ClustalW (default parameters) within MEGA 6.0 [14]. Phylogenetic trees were then estimated using the Maximum Likelihood (ML) method implemented in PhyML, version 3 [15]. Totally, 1000 bootstrap replicates were used under the same procedure to estimate the support for each node. All trees were mid-point rooted.

Results

Tick Collections

The ticks were collected in Fushan town, Chengmai county locating in Northern Hainan Island, China (Fig. 1). In total, twelve dogs, ten goats and fifteen cattle were inspected and 285 ticks were collected during September to October. These ticks were identified by morphological observation and sequence analysis of their 12S rRNA gene, and they were assigned to two tick species, *Rhipicephalus sanguineus* (183, 64.21%) and *R. microplus* (102, 35.79%). All the 132 ticks from dogs and 51 ticks from goats are *R. sanguineus*. They were equally divided into 44 pools and 17 pools, with 3 ticks for each pool. The 102 ticks from cattle are all *R. microplus* and they are divided into 34 pools (Table 1).

Table 1
Detection of tick-borne pathogens from domestic animals in Hainan Island, China, 2019.

Species	Dogs	Goats	Cattle	Total
<i>R. sanguineus</i>	6/44/132 ^a	3/17/51	0/0/0	9/61/183
<i>R. microplus</i>	0/0/0	0/0/0	5/34/102	5/34/102
Total	6/44/132	3/17/51	5/34/102	14/95/285

^a positive pool/total pool/total tick.

Detection of Babesia/Theileria and Hepatozoon

Genetic analysis of the recovered 18S rRNA gene sequences using the blastn with Nucleotide collection nr/nt revealed that they were most closely related to those of *Babesia canis vogeli* (100.0%) and *Hepatozoon canis* (99.54%~99.77%), respectively (Table 2). Phylogenetic analysis of 18S RNA gene sequences revealed the co-circulation of *B. canis* and *H. canis* in the 1 and 3 *R. sanguineus* pools from dogs, respectively (Fig. 2, Table 3). The sequences of *B. canis vogeli* HNRS/dog/B1 exhibited a close relationship to those of the known *B. canis vogeli* found in *R. sanguineus* from domestic and foreign [7, 16–19] in the 18S RNA tree (Fig. 2A). Additionally, the sequences *H. canis* HNRS/dog/A2, *H. canis* HNRS/dog/B6, and *H. canis* HNRS/dog/C12 were closely related to each other, as well as to *H. canis*

strain SK-144 and *H. canis* strain 9992-4 [20, 21] in *Canis lupus familiaris* from Israel and Saint Kitts and Nevis, forming a distinct lineage in the 18S RNA tree (Fig. 2B).

Table 2
Bacterial and protozoal sequences obtained from ticks in Hainan island, China.

Strains	Genes(nt)			Bacteria or protozoon	
	rrs or 18 s rRNA	groEL	gltA		
R. sanguineus					
Dogs					
HNRS/dog/B1	368 (100.0%) ^a	- ^b	-	<i>Babesia canis vogeli</i>	
HNRS/dog/A2/B6/C12	436 (99.77%)	-	-	<i>Hepatozoon canis</i> strain SK-144	
	436 (99.54%)	-	-	<i>Hepatozoon canis</i> isolate TWN1	
HNRS/dog/C6/C23	783 (99.87%)	925 (99.89%)	809 (99.86%)	<i>Anaplasma platys</i> strain S3	
	783 (99.87%)	925 (99.68%)	809 (98.75%)	<i>Anaplasma platys</i>	
Goats					
HNRS/goat/Y5/Y9/Y10	1347 (100.0%)	-	-	Coxiellaceae bacterium PH06	
	1347 (95.70%)	-	-	<i>Coxiella burnetii</i> strain VR145	
R. microplus					
Cattle					
HNRM/cattle/D6	782 (100.0%)	-	-	<i>Anaplasma marginale</i> str. Dawn	
	782 (100.0%)	-	-	<i>A. marginale</i> strain WHBMXZ-130	
HNRM/cattle/D26	782 (100.0%)	-	845 (99.76%)	<i>Anaplasma marginale</i> str. Dawn	
	782 (100.0%)	-	845 (99.41%)	<i>A. marginale</i> strain WHBMXZ-130	

^a The length of sequence amplified from the samples (Nucleotide sequence identity compared to the reference sequences from GenBank).

^b “-”, not available.

Strains	Genes(nt)			Bacteria or protozoon
	<i>rrs</i> or 18 s rRNA	<i>groEL</i>	<i>gltA</i>	
HNRM/cattle/D29	782 (100.0%)	894 (100.0%)	845 (99.76%)	<i>Anaplasma marginale</i> str. Dawn
	782 (100.0%)	894 (100.0%)	845 (99.41%)	<i>A. marginale</i> strain WHBMXZ- 130
HNRM/cattle/E8/E24	781 (100.0%)	971 (99.88%)	848 (99.28%)	<i>Ehrlichia</i> sp. strain WHBMXZ- 40
	781 (100.0%)	971 (91.56%)	848 (81.96%)	<i>Ehrlichia chaffeensis</i> str. Saint Vincent

^a The length of sequence amplified from the samples (Nucleotide sequence identity compared to the reference sequences from GenBank).

^b “-”, not available.

Table 3
Prevalence of tick-borne pathogens in ticks in Hainan island, China.

Species of tick-borne pathogens		<i>R. sanguineus</i>	<i>R. microplus</i>
Anaplastataceae	<i>Anaplasma marginale</i>	0/0/0	3/34/102
	<i>Anaplasma platys</i>	2/61/183 ^a	0/0/0
	<i>Ehrlichia</i> sp.	0/0/0	2/34/102
Coxiellaceae	<i>Coxiella</i> like bacteria	3/61/183	0/0/0
Babesiidae	<i>Babesia canis</i>	1/61/183	0/0/0
Hepatozoidae	<i>Hepatozoon canis</i>	3/61/183	0/0/0
Total		9/61/183	5/34/102

^a positive pool/total pool/total tick.

Detection of bacterial pathogens

Genetic analysis of the *rrs*, *groEL*, and *gltA* gene sequences from ticks revealed that they were most closely related to those of Coxiellaceae, and Anaplastataceae bacteria. Briefly, the *rrs* sequences recovered from the ticks pools sampled from Hainan Island exhibited high sequence similarities to those from species of Coxiellaceae bacterium (100%), *C. burnetii* (95.7%), *A. marginale* (100%), *A. platys* (99.87%), and *Ehrlichia* sp. (100%), respectively (Table 2). Whereas the similarities between the sequences recovered from this study and known reference sequences from GenBank varied from 81.96–99.86% for the *gltA* gene sequences, and from 91.56–100.0% for the *groEL* gene sequences (Table 2). Hence, these

data revealed the co-circulation of *Ehrlichia*, *Anaplasma*, and (the proposed) *Candidatus Coxiellaceae* bacterium in ticks collected from Hainan Island (Fig. 3, Fig. 4, Table 2).

Anaplasmataceae bacteria were identified in 2 *R. sanguineus* pools, and 5 *R. microplus* pools (Table 3). Phylogenetic analysing the sequences of *rrs*, *gltA*, and *groEL* genes revealed the circulation of three species of Anaplasmataceae bacteria in the ticks from Hainan Island. In the *rrs*, *gltA*, and *groEL* gene trees (Fig. 3), the sequences of *A. marginale* HNRM/cattle/D6, *A. marginale* HNRM/cattle/D26, and *A. marginale* HNRM/cattle/D29 were closely related to those of the known *A. marginale* found in ticks and cattle [12, 22, 23]. Notably, *A. platys* HNRS/dog/C6 and *A. platys* HNRS/dog/C23 clustered with those of *A. platys* discovered in ticks, dogs, and camels from China, Portugal, Italy, Japan [12, 24, 25] in all three gene trees (Fig. 3). Finally, the sequence *Ehrlichia* sp. HNRM/cattle/E8 and *Ehrlichia* sp. HNRM/cattle/E24 recovered from 2 *R. microplus* tick pools exhibited a close relationship to those of the candidatus *Ehrlichia* sp. identified in ticks from China (Fujian, Wuhan, Xinjiang, and Shenyang provinces), Niger, and Thailand [12, 26–28] (Fig. 3).

Besides, *Coxiella* like bacterial DNA was mainly found in 3 *R. sanguineus* tick pools (Table 3). In the *rrs* gene tree (Fig. 4), 3 positive samples (*Coxiella* like bacteria HNRS/goat/Y5, *Coxiella* like bacteria HNRS/goat/Y9, and *Coxiella* like bacteria HNRS/goat/Y10) showed 100% similarities to *Coxiellaceae* bacterium PH06 (KM079622), which found from *Pediculus humanus* in Marseille, France in 2014.

Discussion

The previous studies revealed the dominant species are *R. sanguineus* and *R. microplus* in Hainan Island, China [29], as well as in our study. Hainan Island is the natural epidemic focus of North Asia Tick-Borne Spotted Fever (NASF) [9]. The antibodies investigate of NASF in local human and mouse indicated the positive incidence are high [10]. In the current study we evaluated the presence of several pathogens as Rickettsiales bacteria, *Coxiellaceae* bacteria, Babesiidae, and Hepatozoidae in these two ticks. The results revealed the presence of four recognized species and two *Candidatus* sp. of Anaplasmataceae and *Coxiellaceae*. Importantly, *A. marginale*, *A. platys*, *H. canis*, *B. canis*, and *Coxiella burnetii* are known to be animal and/or human pathogens [28, 30]. Hence, the data clearly indicate that multiple bacteria and protozoa co-circulate in hard ticks in Hainan Island. As more species of ticks are present in Hainan Island, it is likely that more additional tick-associated pathogens will be discovered.

Many members in the family Anaplasmataceae are causative agents of tick-borne diseases (i.e. Anaplasmosis and Ehrlichiosis) with a remarkable impact on human and animal health [31]. Several species have been identified in family Anaplasmataceae and some are considered as human or animal pathogens. For example, *A. phagocytophilum*, *A. marginale*, and *A. platys* are the important disease-producing pathogens in the genus *Anaplasma* [5, 32]. *A. marginale* is the common ruminant pathogens infecting buffalo and cattle which is distributed on six continents, especially in tropical and subtropical regions [30, 33, 34]. While *A. platys* is the agent of cyclic thrombocytopenia in dogs and is the only classified Rickettsiales species known to infect platelets [17]. Besides infecting dogs, the infection of this

pathogen has been reported in cats, foxes (*Vulpes vulpes*), cattle, goats, camels, red deer, and humans [8, 25, 35–42]. In our study, two documented bacteria *A. marginale* and *A. platys* were identified in *R. microplus* and *R. sanguineus* ticks separately. The cattle, as the major hosts of *R. microplus*, possessed the high prevalence of *A. marginale*, and these results suggests that more surveillance in cattle in Hainan Island needs to be performed. *R. sanguineus* is considered the primary vector of infection to *A. platys*, which is all founded in dogs in this study. However, the high infection rate of *A. platys* in local dog ticks indicated high risk of cyclic thrombocytopenia to other animals and humans, due to the close exposure between the dogs and others. Therefore, more surveillance and researches in dogs and its pathogens in Hainan Province needs to be performed.

Similar to the *Anaplasma*, several novel *Ehrlichia* bacteria has been discovered in the past decade [17, 31, 33]. Here, a tentative species was identified in *R. microplus* ticks from cattle, which is mainly founded in *R. microplus* ticks from several provinces and countries [12, 26, 27, 43]. Otherwise, the sequence of this *Candidatus* species are close to that of *E. chaffeensis* which is human pathogen bacteria, so more attentions would be paid to this potential novel species.

Coxiella burnetii is the causative agent of Q fever which is a worldwide zoonosis. In common, the infection is mostly persistent in animals, whereas it is often asymptomatic in humans, which can manifest as an acute disease like a flu-like illness, or pneumonia, or as a chronic form like endocarditis, et al [15]. There are not typical symptoms when infection occurred in animals and humans except during pregnancy. The most primary reservoirs of *C. burnetii* are goats, sheep, and cattle [44]. In nature, the ticks, as the main vector and reservoir, play important part in the bacteria maintaining and transmitting, otherwise the cattle and goats play the most important roles in human infections. In this study, *Coxiella* like bacteria were identified in 3 *R. sanguineus* ticks sampled from Hainan. It would be paid more attentions to due to its closer relations with the known *Coxiella burnetii*.

Canine hepatozoonosis is a tick-borne disease distributed worldwide, and *H. canis* is the etiology of hepatozoonosis in dogs [45]. Canine babesiosis is a vector-borne disease caused by several *Babesia* spp. like *B. canis vogeli* et al [46]. In our study, *H. canis* and *B. canis vogeli* were identified in 3 and 1 *R. sanguineus* ticks collected from Hainan Island, separately. These data revealed that the two agents are co-circulating in arthropods, and the infection risk is higher for dogs, as well as other animals in certain regions.

Conclusions

In conclusion, two species (*R. microplus* and *R. sanguineus*) of hard ticks were sampled from dogs, cattle and goats in Hainan province, China. More importantly, four recognized species and two *Candidatus* spp. of Anaplastaceae and Coxiellaceae were discovered from these two hard ticks, indicating that this certain geographic region harbors a considerable diversity of Anaplastaceae bacteria, Coxiellaceae bacteria, Babesiidae, and Hepatozoidae in nature. The data from this study highlight the need for stated

surveillance of local arthropods, mammal, and humans, and this can provide the evidence for several bacteria, and protozoa infection.

Abbreviations

HFRS: hemorrhagic fever with renal syndrome

NASF: North Asia Tick-Borne Spotted Fever

SFGR: Spotted Fever Group Rickettsiae

PBS: phosphate buffer solution

ML: Maximum Likelihood

Declarations

Availability of data and materials

The data sets supporting the conclusions of this article are included within the article or uploaded to GenBank.

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Author Contributions

KL designed the research and supervised the experiments; GT, XB, KL, and XQ collected the samples and performed the experiments; KL, and ML analyzed the data; KL, ML, and WG wrote the manuscript.

Ethics declarations

Ethics approval

This study was reviewed and approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention of the Chinese CDC.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures



Figure 1

Map showing the location of sample collection sites (•) in Chengmai Country, Hainan Province, China.
Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



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Figure 1

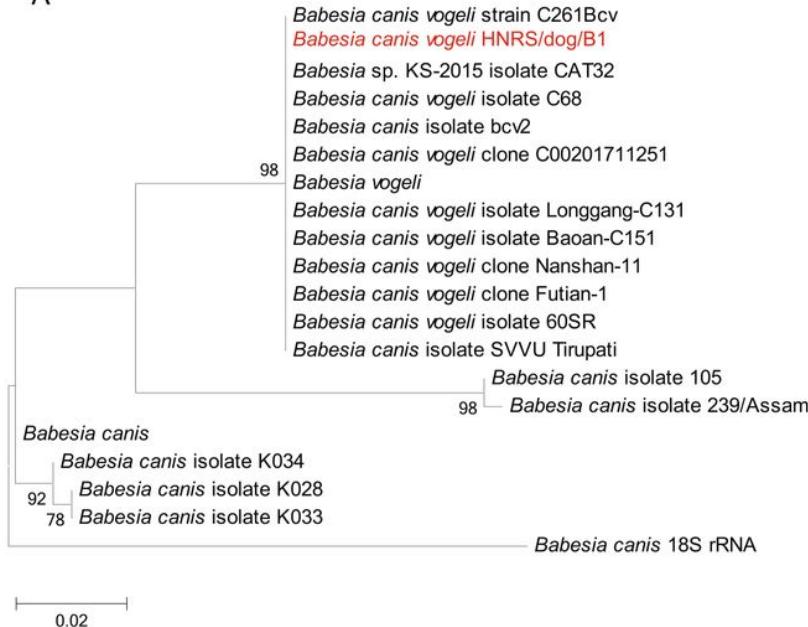
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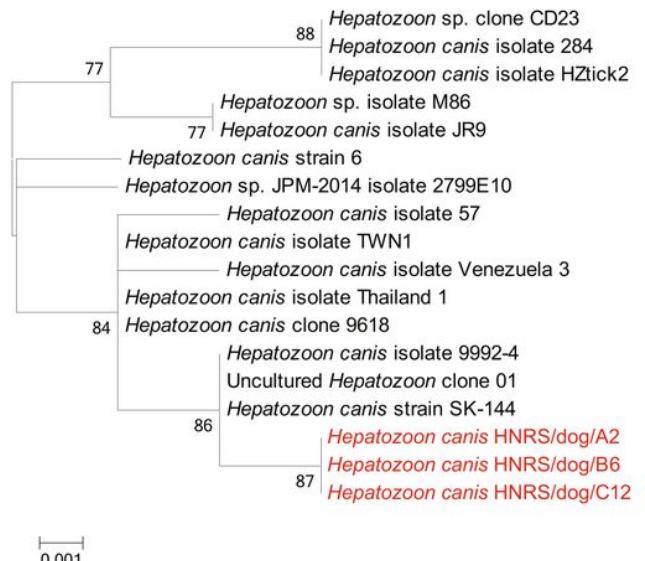
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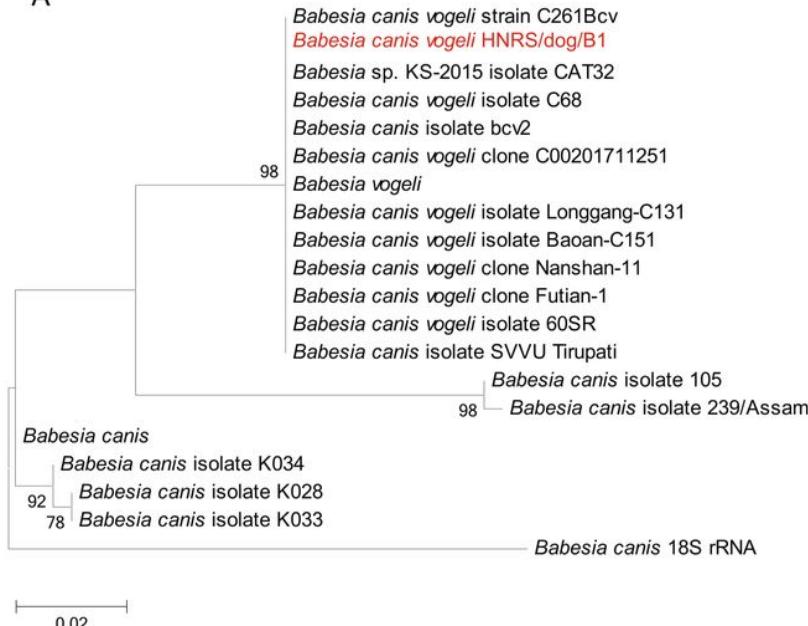


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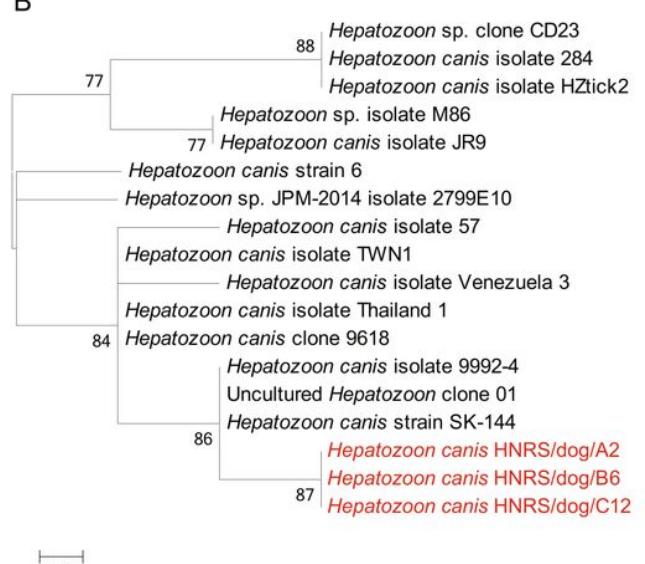
**Figure 2**

Phylogenetic trees based on the partial 18S rRNA gene sequences of *Babesia canis* (A) and *Hepatozoon canis* (B). Both trees were mid-point rooted for clarity only. Bootstrap values (>70%) are shown for appropriate nodes. The scale bar represents number of nucleotide substitutions per site. The positive sequences are marked in red.

A



B

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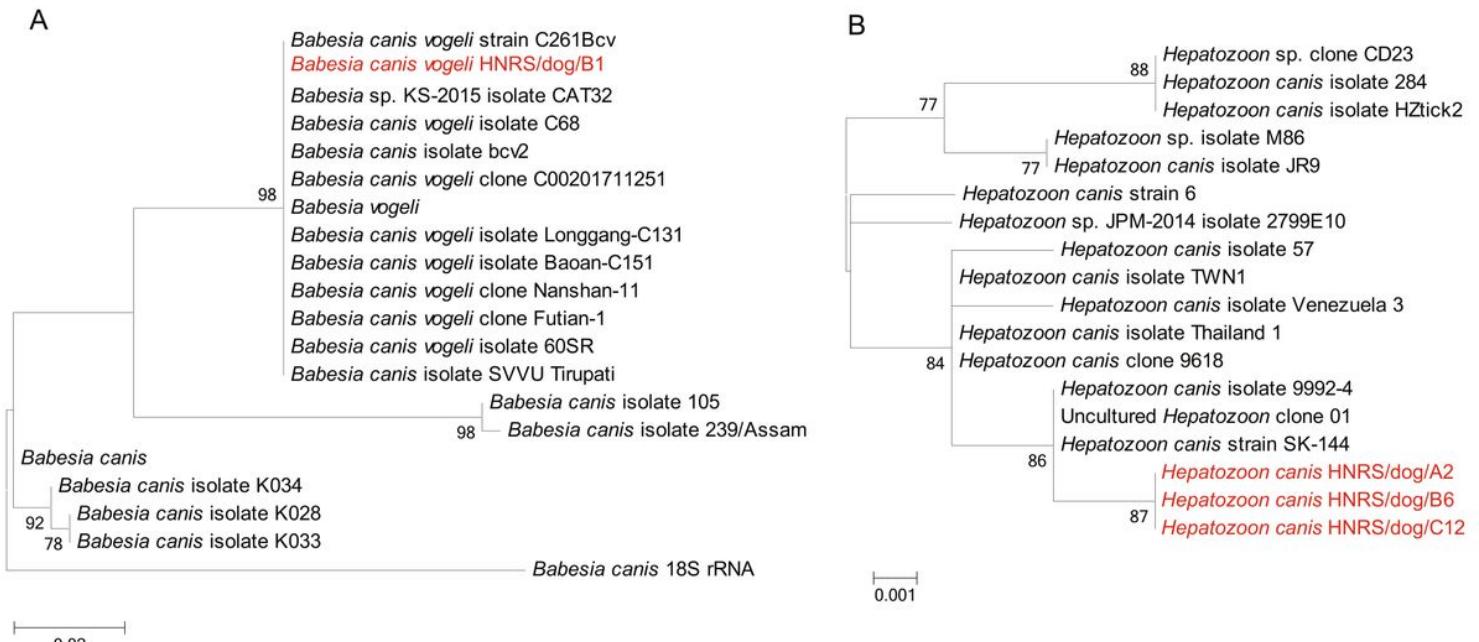


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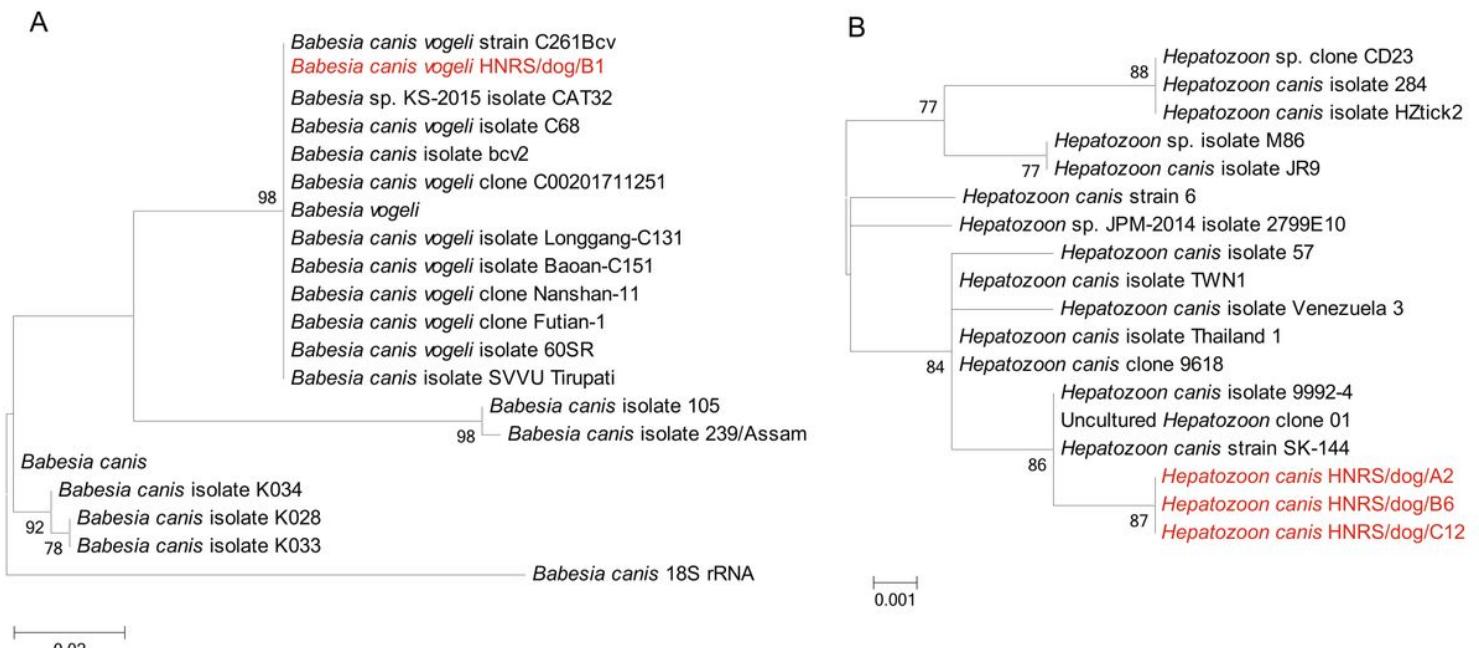


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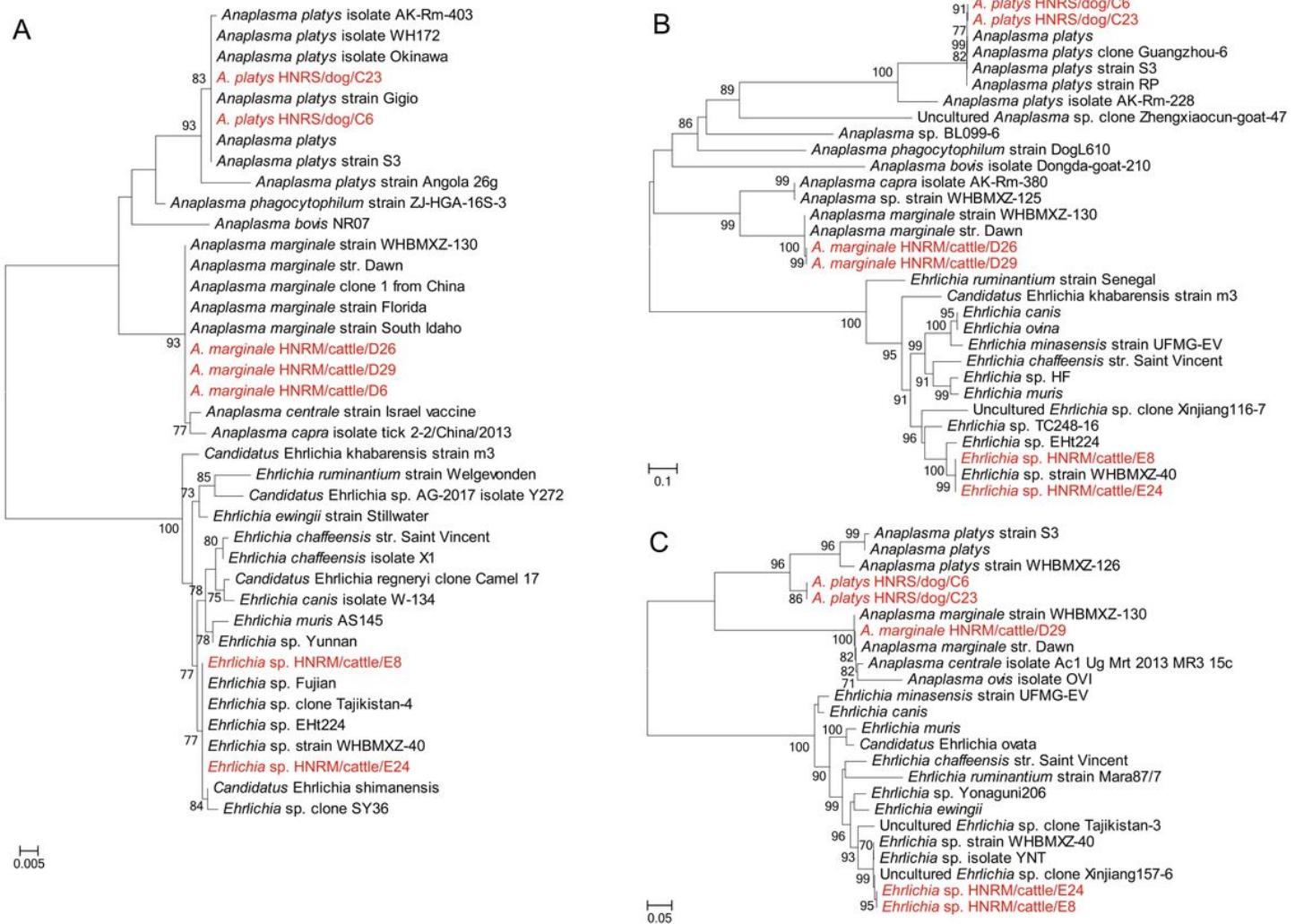


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Phylogenetic trees based on partial Anaplasmataceae rrs (A), gltA (B) and groEL (C) gene sequences. Trees were mid-point rooted for clarity only. Bootstrap values (>70%) are shown for appropriate nodes. The scale bars represent number of nucleotide substitutions per site. The positive sequences are marked in red.

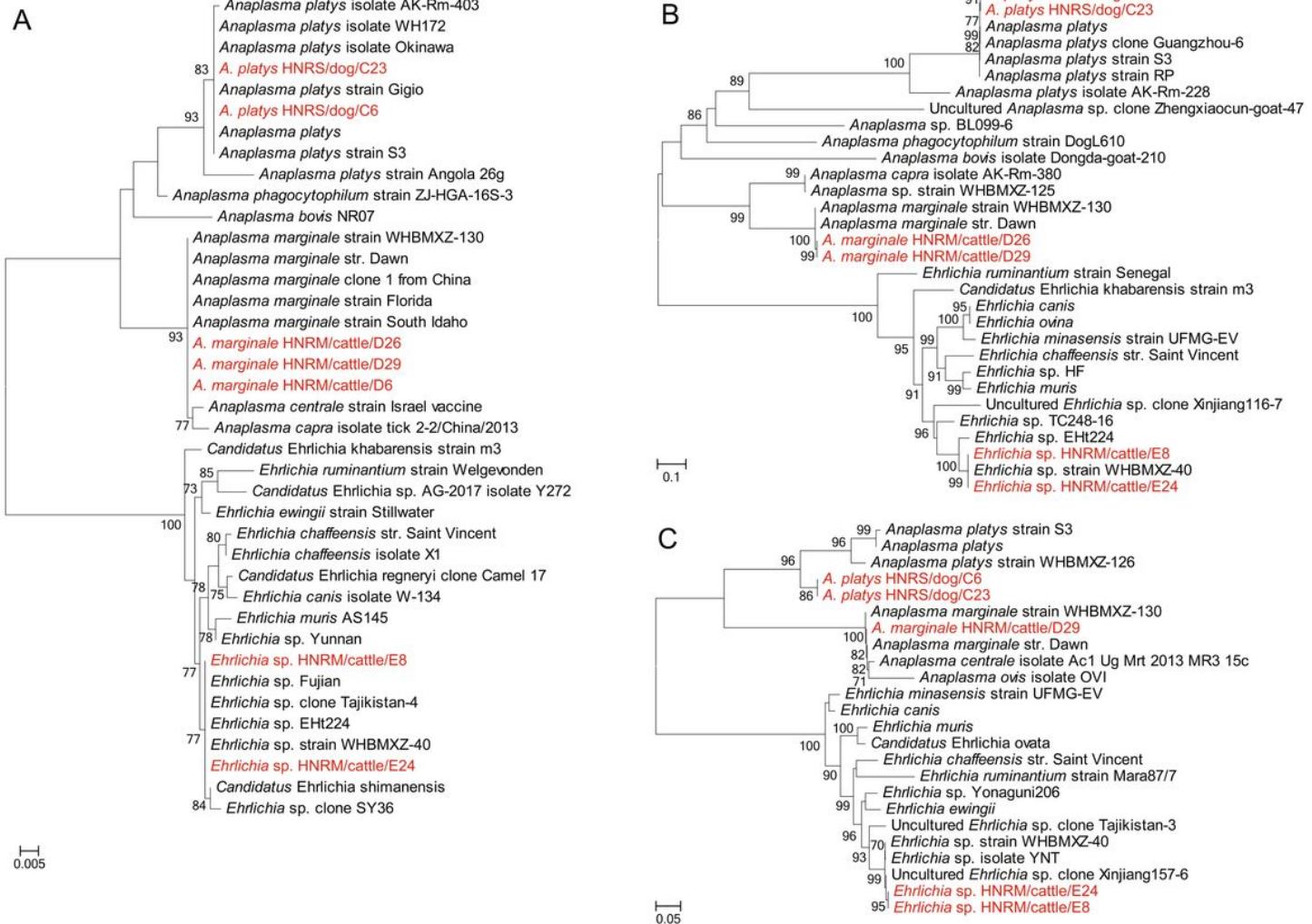


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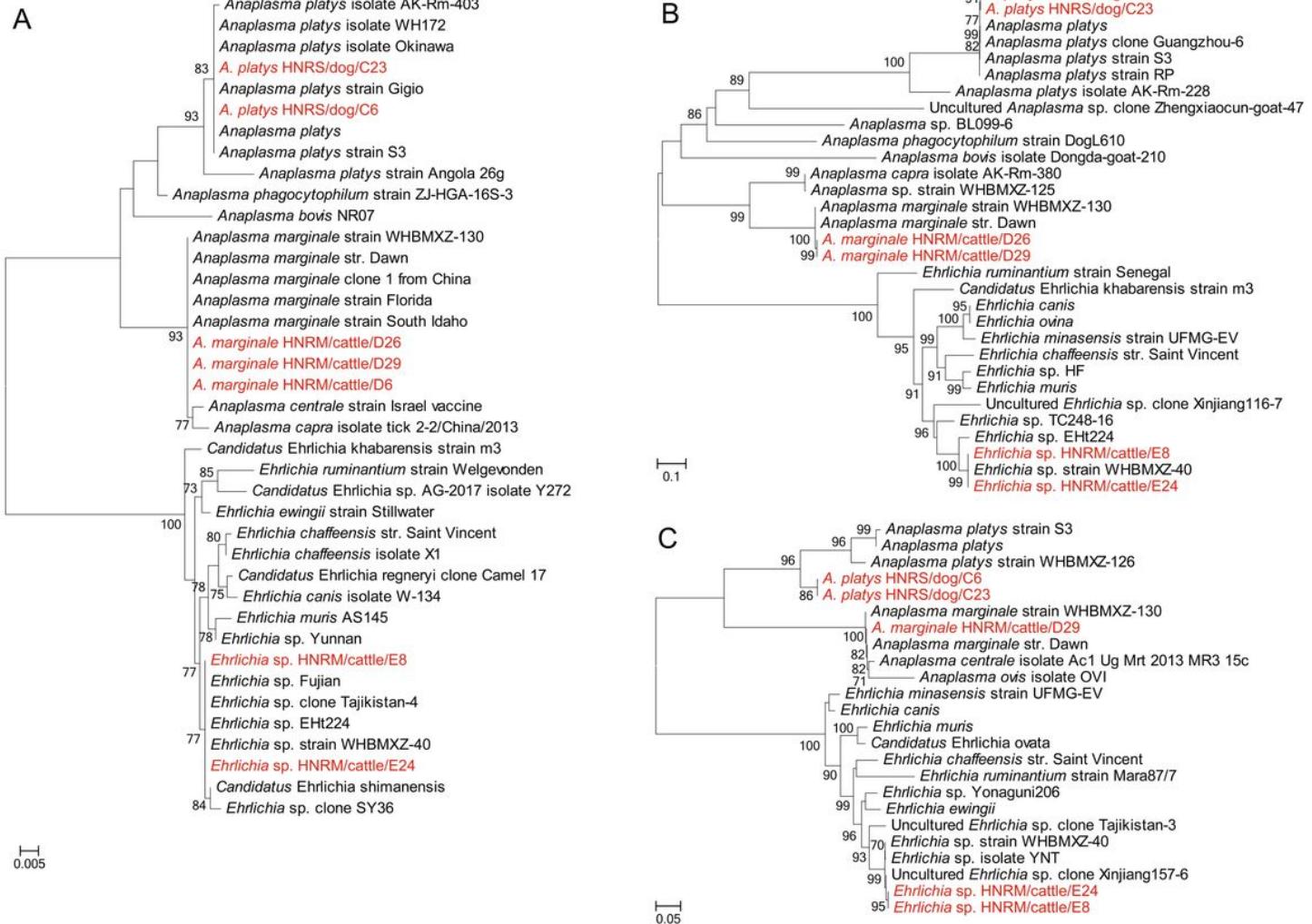


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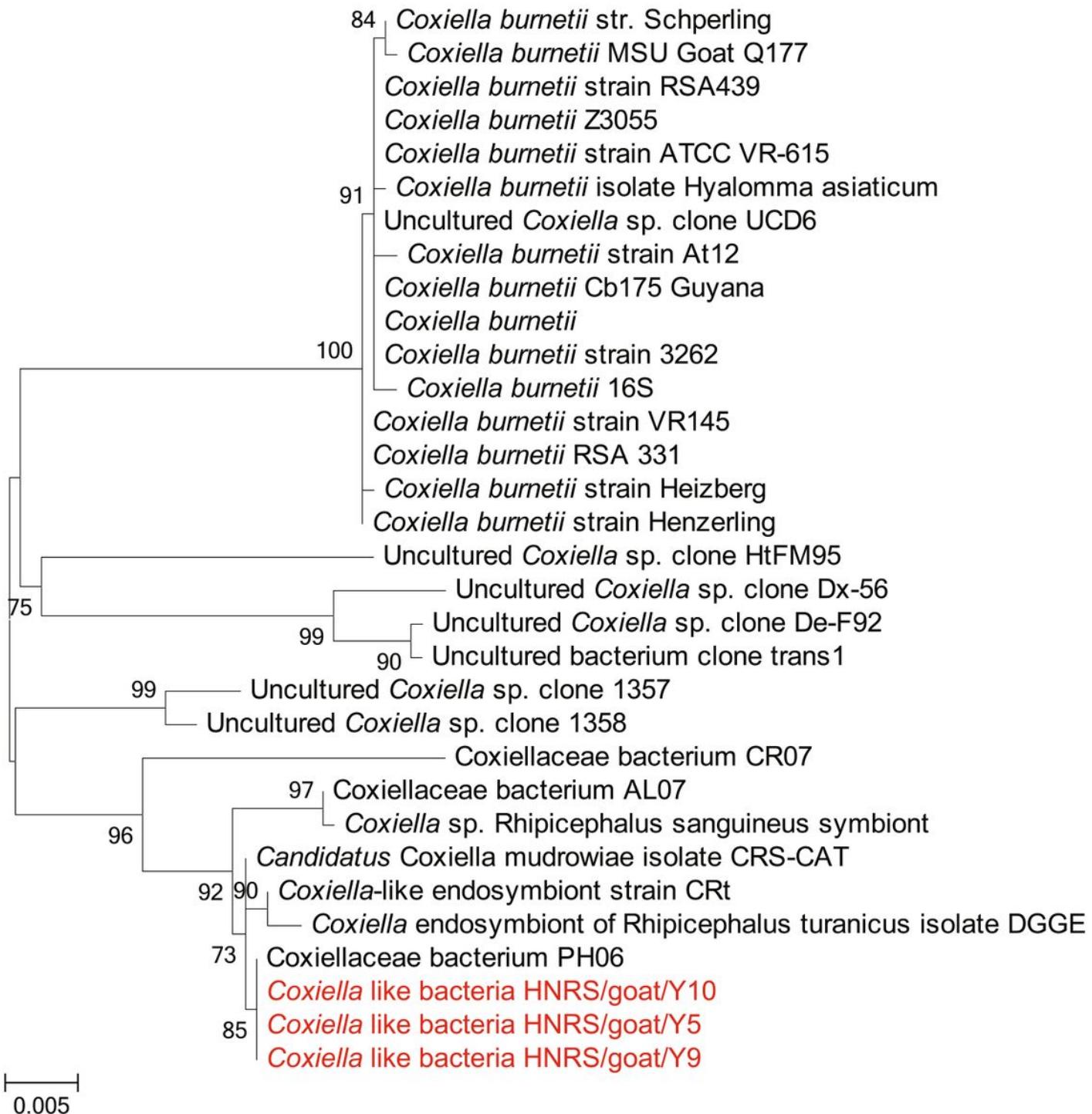


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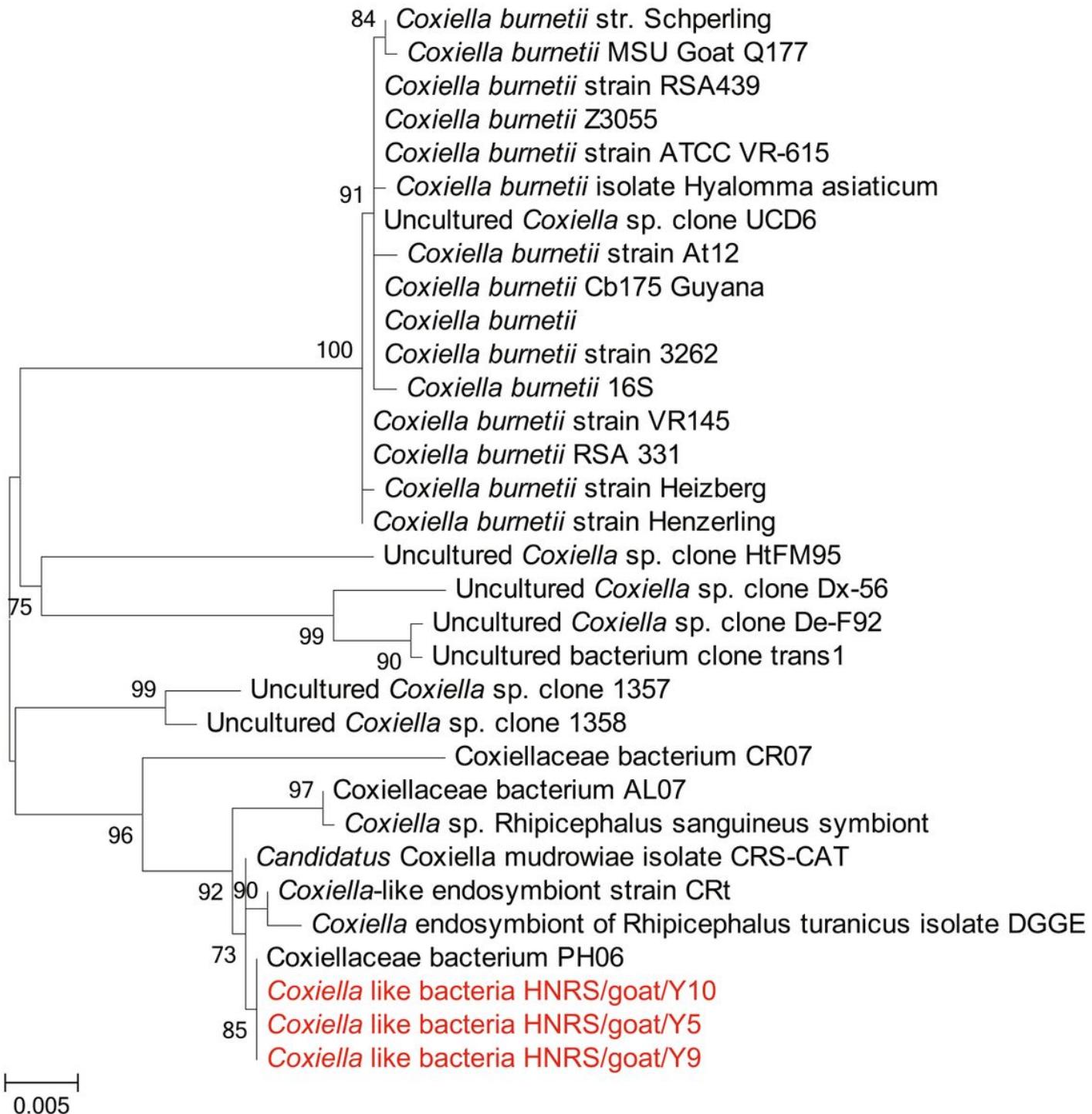


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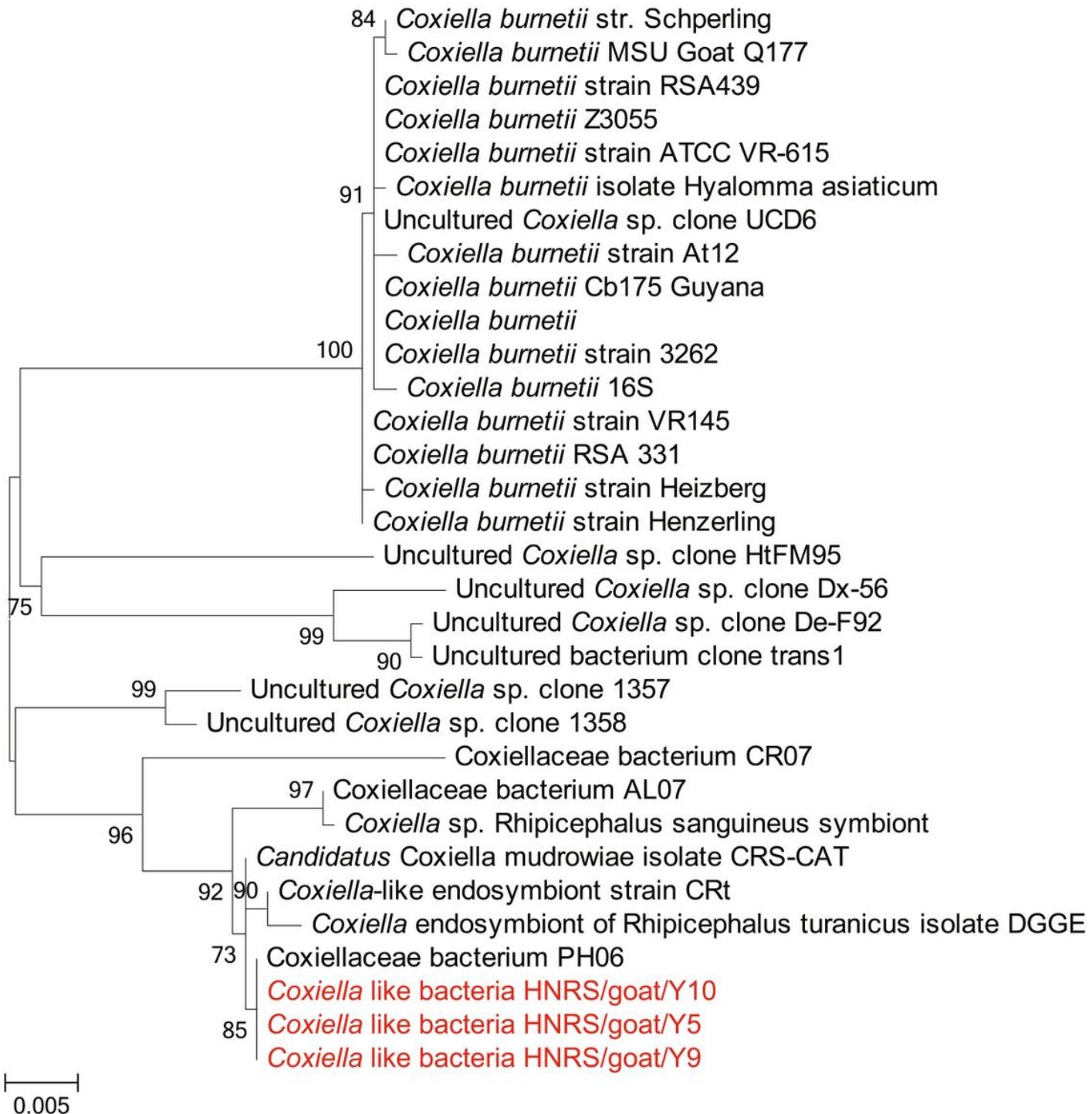


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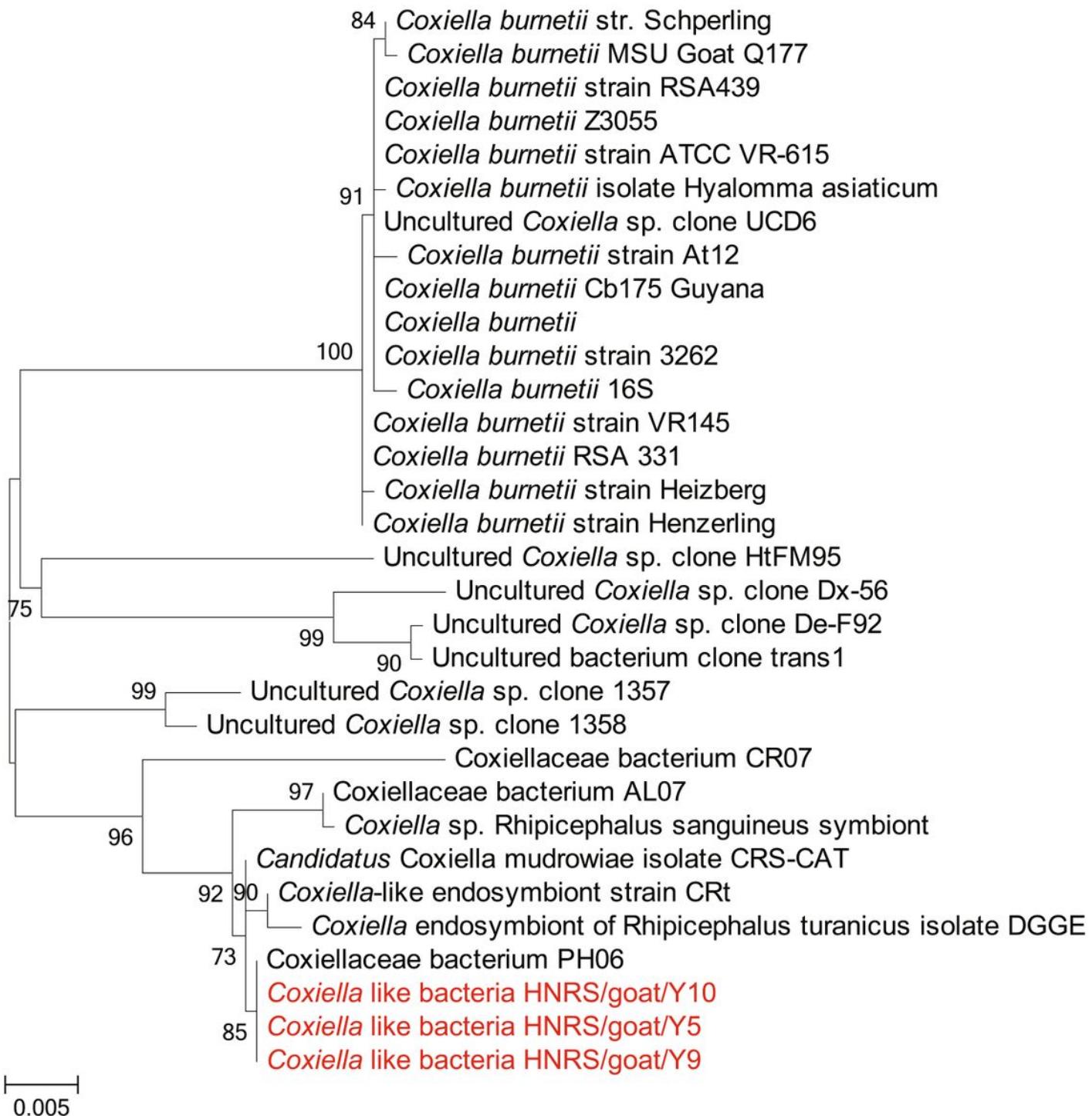


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