Infection, Genetics and Evolution 74 (2019) 103927

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

GroEL gene typing and genetic diversity of *Anaplasma bovis* in ticks in Shaanxi, China

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ARTICLE INFO

Keywords:
Anaplasma bovis
groEL
Detection
Genetic diversity

ABSTRACT

Anaplasma bovis, causative agent of bovine anaplasmosis, is usually identified by nested-PCR amplifying the *rrs* gene. However, it is difficult to determine the genetic relationship among different variants within *A. bovis* using this gene because of high conservation. In this study, two tick species, identified as *Rhipicephalus microplus* and *Haemaphysalis longicornis* based on morphological and molecular methods by analyzing *COI* gene, were collected from cattle, goat or sheep. Subsequently, *A. bovis* was initially detected by PCR amplifying the *rrs* gene in ticks in Shaanxi Province, China. The sequencing and Blast results showed that some false positive samples were found when only based on the amplification of partial *rrs* gene, presenting these sequences resembled those of other Alphaproteobacteria rather than *A. bovis*. Although major surface proteins genes were proposed and used successfully to identify members within Anaplasmataceae, these genes were unavailable for *A. bovis*. Hence, primers targeting the *groEL* gene were designed and a PCR assay was developed. The PCR products were sequenced and similarity and phylogenetic analysis suggested all these sequences are the *groEL* gene of *A. bovis*. In addition, phylogenetic analysis based on the *groEL* gene also revealed the genetic diversity of *A. bovis* worldwide, as well as in Shaanxi Province of China, which wasn't reflected by analyzing the *rrs* gene. In sum, *groEL* gene is important for molecular detection and phylogenetic analysis of *A. bovis.*

1. Introduction

Anaplasma bovis is an obligate intracellular gram-negative bacterium in genus *Anaplasma*, family Anaplasmataceae (order Rickettsiales) (Dumler et al., 2001; Raoult and Parola, 2007). *A. bovis* infects mononuclear cells of infected vertebrate animals, mainly including cattle and buffalo (Dumler et al., 2001). The infection can causes anaplasmosis, and the mainly clinical symptoms were hyperthermia and abnormal weight loss, as well as weakness, pale mucous membranes, prescapular lymph node inflammation and even death (Rar and Golovljova, 2011). Meanwhile, vertebrates play an important role in the life cycle of this organism due to its transstadial transmission in ticks rather than transovarial transmission (Raoult and Parola, 2007). Besides aforementioned two animals, its DNA has been identified in a variety of wild and domestic animals although its pathogenicity to these animals are unknown, and presents worldwide distribution based on the *rrs* gene (Battilani et al., 2017; Goethert and Telford 3rd., 2003; Harrison et al., 2013; Kubo et al., 2015; Lee et al., 2009; Li et al., 2014; Liu et al., 2012; Masuzawa et al., 2011; Rar and Golovljova, 2011; Tay et al., 2015; Yang et al., 2014; Ybañez et al., 2013; 2014).

For Rickettsiales bacteria, species identification by bacterial isolation and serotyping is difficult due to their strictly intracellular bacteria character and cross-reactions among different species within genus (Du Plessis et al., 1987). Hence, they were detected and species identified frequently by different developed DNA-based techniques. Accordingly, amplification of the *rrs* gene has been used to identify *A. bovis* because that it has not been cultivated in vitro to date. However, highly conserved sequences of the *rrs* gene affected the phylogeny to determine the genetic diversity and evolutionary relationship among different strains within this organism. Hence, other genes with enough evolutionary information should be used to study the genetic diversity and evolution of *A. bovis*. Currently, major surface proteins (MSP) genes

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https://doi.org/10.1016/j.meegid.2019.103927
Received 13 March 2019; Received in revised form 12 June 2019
Available online 18 June 2019

were used successfully for molecular detection and genetic diversity analysis within species in Anaplasmataceae. However, these genes were unavailable for *A. bovis* (Rar and Golovljova, 2011). In addition, the *groEL* gene is a highly conserved housekeeping one in both prokaryotes and eukaryotes. Recently, this gene has been used to determine the phylogenetic analysis among Rickettsiales bacteria, including *Ehrlichia*, *Rickettsia* and *Anaplasma* (Chisu et al., 2018; Dumler et al., 2001; Lee et al., 2003; Park et al., 2005).

Hard ticks, as the vector, transmit *A. bovis* to animals through sulking blood. Until now, several ticks speices, such as *Amblyomma variegatu*m, *Am. cajennense*, *Hyalomma* sp., and *Rhipicephalus appendiculatus*, are considered to be its vectors (Battilani et al., 2017; Dumler et al., 2001; Rar and Golovljova, 2011). In addition, *A. bovis* has also been detected in other ticks genera although their role in the lifecycle of this bacteria is unclear, including *Haemaphysalis* (Doan et al., 2013; Kawahara et al., 2006; Malaisri et al., 2015), *Dermacentor* (Dergousoff and Chilton, 2011), *Ixodes* (Kang et al., 2016). In China, *A. bovis* was identified in several tick species, mainly including *De. abaensis*, *De. nuttalli*, *Ha. concinna*, *Ha. danieli*, *Ha. longicornis*, *Ha. qinghaiensis*, *Hy. anatolicum*, *Ixode crenulatus*, and the infection rate was significantly different, varing from 0.1% to 11.1% (Han et al., 2019; Kang et al., 2014; Qin et al., 2018; Wei et al., 2016; Yu et al., 2017; Zhuang et al., 2018). In addition, this organism was also found in sheep, goat, cattle, dog, deer, with the prevalence from 4.1% to 24.4% (Cui et al., 2017; Li et al., 2016; Liu et al., 2012; Yang et al., 2015; Yang et al., 2018; Zhang et al., 2016; Zhou et al., 2018), and even 78.3% and 47.1% in wild Mongolian gazelle (Li et al., 2014) and Reeves' muntjac (Yang et al., 2014), respectively.

A better understanding of the geographical distribution of ticks and associated pathogens is important from a public health perspective for humans and animals. In this study, ticks were collected from the bodies of livestock in Shaanxi, and *A. bovis* was detected by *groEL* gene to reveal its genetic diversity and phylogenetic relationship within it.

2. Materials and methods

2.1. Ticks collection and identification

From April 2017 to August 2018, each unfed adult tick (walking on the animal) per animal was collected directly from the surface of livestock (cattle, goat or sheep) from 16 sites in Shaanxi Province, China (Fig. 1). All the ticks were firstly identified based on the morphological characters (Chen et al., 2011; Lu and Wu, 2003). Further, one quarter of collected ticks randomly selected were molecularly confirmed by amplifying, sequencing and analyzing of 710-bp of the *COI* gene using LCO1490 and HCO2198 primers the *COI* gene (Folmer et al., 1994).

2.2. DNA extraction

All collected tick samples were firstly individually washed with 70% ethanol for five minutes and then with phosphate-buffered saline (PBS) for three times. The total DNA was extracted from each tick individually using Universal Genomic DNA Kit (CWBIO, Beijing, China) following the manufacturer's instructions. DNA was diluted into 50 μL double distilled water and stored in −20 °C for further molecular identification of ticks and detection of *A. bovis*.

2.3. Detection of A. bovis with nested PCR

Firstly, *A. bovis* was identified using nested PCR with primer pairs EC9/EC12A and AB1f/AB1r targeting the *rrs* gene described by Kawahara et al. (2006).

All the *groEL* gene sequences of *A. bovis* were downloaded from GenBank database, and novel primer pairs were designed based on the conversed regions. *A. bovis* was identified using semi-nested PCR: the primers bovis-groEL-F1 (5′-GTTCGCAGTATTTTGCCAGT-3′) and bovis-groEL-R (5′-CTGCRTTCAGAGTCATAAATAC-3′) for the primary round, and bovis-groEL-F2 (5′-ATCTGGAAGRCCACTATTGAT-3′) and bovis-groEL-R for the second. A DNA sample of *An. bovis* from positive tick and distilled water were used as positive and negative controls, respectively.

PCR reactions were performed in 50 μL volumes. The PCR mixture contained 25 μL *Premix Taq* (Takara, Dalian, China), 3 μL extracted total DNA as template, 2 μL for each primer (10 pmol) and 18 μL water. Thermal cycling was performed with an initial denaturation at 94 °C for 5 min then 30 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 40 s, and elongation at 72 °C for 1 min, and a final extension at 72 °C for 7 min for the both rounds of nested PCR.

In order to avoid contamination, each operation of DNA extraction, PCR mixture preparation, DNA template addition, and agarose gel electrophoresis was performed in a fume hood in separate room. In addition, filter tips were also used to prevent contamination in each assay.

2.4. Sequencing of PCR products

The PCR amplicon with expected size was purified using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (TaKaRa, Dalian, China). The purified DNA was inserted into pMD19-T vector (TaKaRa, Dalian, China), and subsequently the ligated products were transformed into *E.coli* DH5α competent cells. The positive insert was confirmed by PCR and sequenced using the ABI-PRISM Dye Termination Sequencing kit and the ABI 3730 genetic analyzer with universal M13 forward and M13 reverse primers.

2.5. Sequences analysis

The newly generated nucleotide sequences were assembled, edited and aligned and the identities were calculated using the Lasergene program, version 5 (DNASTAR, Inc., Madison, WI). The phylogenetic tree was reconstructed using software PhyML v3.2 (Guindon et al., 2010) with the best-fit nucleotide substitution model determined by MEGA 7.0 (Kumar et al., 2016). Meanwhile, Bayesian method was also used to evaluate the phylogenetic tree of *groEL* gene using by MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). Four independent Metropolis-coupled Markov Chain Monte Carlo (MCMC) chains (three hot and one cold MCC chains) were used and sampled every 100 generations in the Bayesian analysis. The threshold for average standard deviation of split frequencies is < 0.01 and Effective Sample Size (ESS) of all parameters is > 100. Finally, the initial 25% of trees were excluded as burnin.

2.6. Sequence accession numbers

All *groEL* gene sequences obtained here have been submitted to GenBank and assigned accession numbers MK340764-MK340818. In addition, 10 representative *COI* gene sequences of ticks and 6 representative *rrs* gene sequences of *A. bovis* selected from each group sharing 100% identities were submitted to GenBank as MN045871-MN045880 and MN044712-MN044717.

3. Results

3.1. Collection and identification of ticks

Nine hundred and eighty-seven ticks (412 from cattle, 274 from sheep, and 301 from goats) were collected from 16 sites of four cities in Shaanxi Province, China. Morphological identification showed that they belonged to two tick species: *Rh. microplus* and *Ha. longicornis*. Two hundred and sixty-four ticks were selected to be confirmed by molecular method, and the *COI* gene sequences were obtained from all these ticks. All the *COI* gene sequences obtained in this study were sequenced

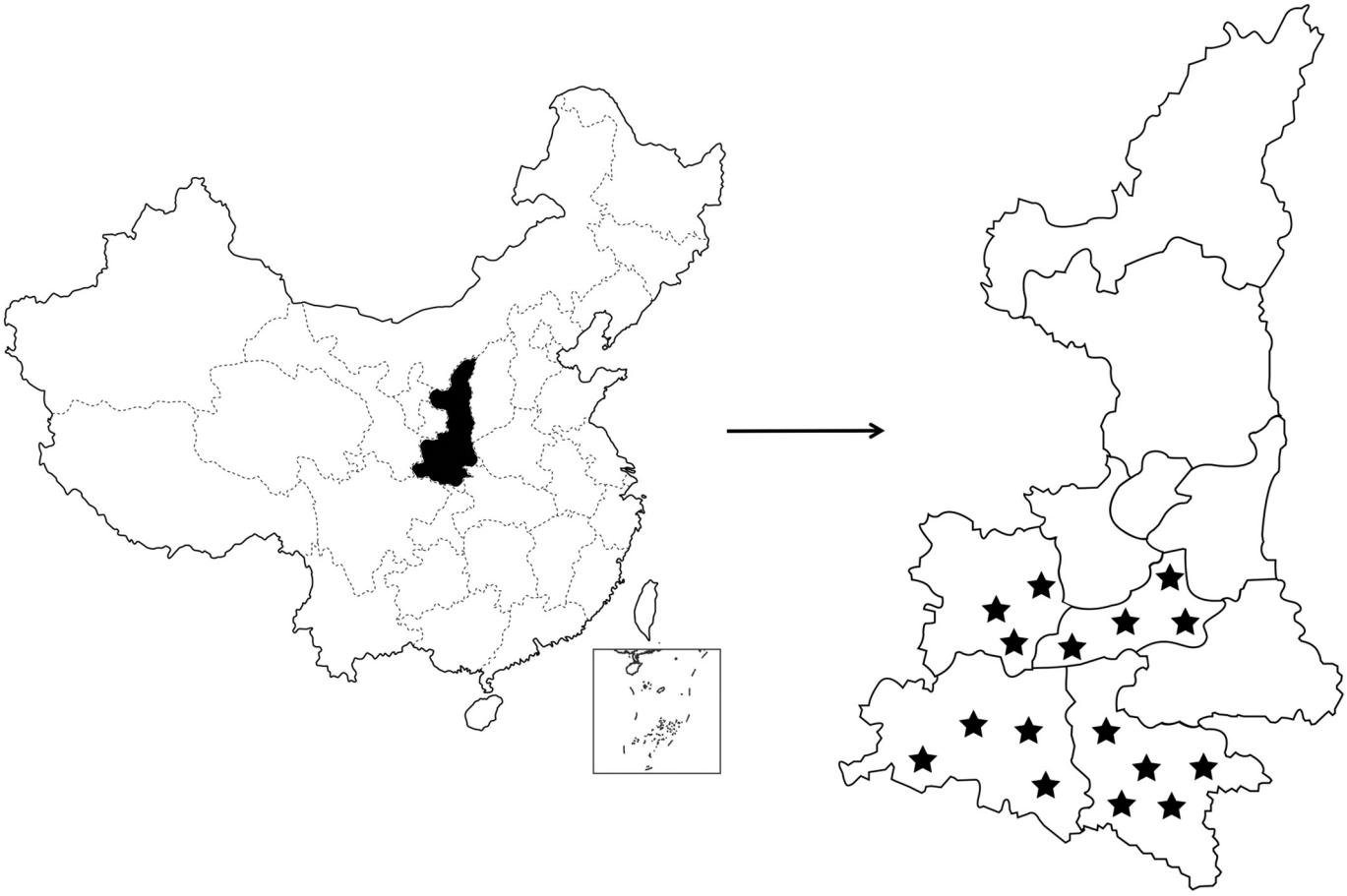

Fig. 1. The location of collection sites of hard ticks (★) in Shaanxi Province, China.

and blasted in GenBank database. They shared the highest nucleotide identities, 99.4–99.8% and 99.5–99.9%, with *COI* gene sequences of *Rh. microplus* and *Ha. longicornis*, respectively. Four and six sequences selected from each group sharing 100% identities in *Rh. microplus* and *Ha. longicornis* represent the diversity of the sequences obtained in our study, respectively. These ten *COI* gene sequences of ticks also corresponded to *Rh. microplus* and *Ha. longicornis* on the ML phylogenetic tree (Fig. 2). Hence, all ticks belonged two species based on the morphological classification, with 471 *Rh. microplus* and 516 *Ha. longicornis*, and 246 ticks were further confirmed by nucleotide identity and phylogenic analysis.

3.2. Screening of *A. bovis* in ticks

For the *rrs* gene, expected size of PCR products were amplified from 72 ticks, and all the amplicons were sequenced. BLAST research showed that 43 had > 99.0% identities with the *rrs* gene of *A. bovis*, 29 were closely related to other Alphaproteobacteria (including *Rhizobium* sp. and *Sphingomonas* sp.) rather than Rickettsiales bacteria.

Using semi-nested PCR amplifying the *groEL* gene, PCR products with expected band sizes (845 bp) were obtained from 55 tick samples and sequenced (Table 1). BLAST analysis showed that all sequences generated in this study shared the highest nucleotide identity of 80.9–100% with those of *A. bovis* registered in GenBank. All the samples positive for the *rrs* gene were also positive for *groEL* gene. In addition, the nucleotide identity of the new *groEL* gene sequences ranged from 90.9% to100%. The sequences from Ankang, Hanzhong, Baoji and Xi'an shared 92.9–100%, 99.7–100%, 91.0–100% and 92.8–99.6% identities, respectively. Furthermore, this inconsistency was verified by that samples were positive for *groEL* but negative for the *rrs* gene were retested by amplifying the *rrs* and *groEL* genes simultaneously.

3.3. Phylogenetic analysis of groEL gene

The phylogenetic trees based on ML and Bayesian methods had the similar topology. On the phylogenetic tree (Fig. 3), all sequences, including those identified in the current study and previous studies were classified into four well-supported lineages (bootstrap values > 90 and posterior node probabilities > 0.9). Fifty-five *groEL* gene sequences obtained in this study were located into the first and second lineages. The sequences identified in ticks from Austria formed the third lineage, and uncultured *Anaplasma* sp. clone 499 (JN588562), Kh-Hc215 (JX092095) and Am-vole57 (JX092099) identified in tick and wild animals formed the fourth.

Forty-five sequences, including 32 from Ankang, 3 from Hanzhong, 8 from Baoji and 2 from Xi'an were classified into lineage 1. In addition, this lineage also contained two sequences of *Anaplasma* sp. BL126–13 and *Anaplasma* sp. TC250–2 from Xinjiang Autonomous Region (Kang et al., 2014), and uncultured *Anaplasma* sp. clone SY49 from Shenyang city, China (Dong et al., 2014). As a whole, these sequences belonging to the first lineage shared 95.0–100.0% nucleotide identities. In this lineage, most sequences from Ankang had a close genetic relationship with those from Hanzhong city. However, Ankang-Rm-289 was closely related to variants obtained from Baoji and Xi'an cities found in this study, as well as uncultured *Anaplasma* sp. clone SY49. Furthermore, Ankang-Rm-1, Ankang-Rm-126 and Ankang-Rm-278 formed distinct clades, respectively.

Ten strains recovered herein, including 2 from Ankang, 6 from Baoji and 2 from Xi'an formed the second lineage. Ankang-Rm-22, Xi'an-Hl-113, Xi'an-Rm-65, Baoji-Rl-25 and Baoji-Rl-33 clustered with

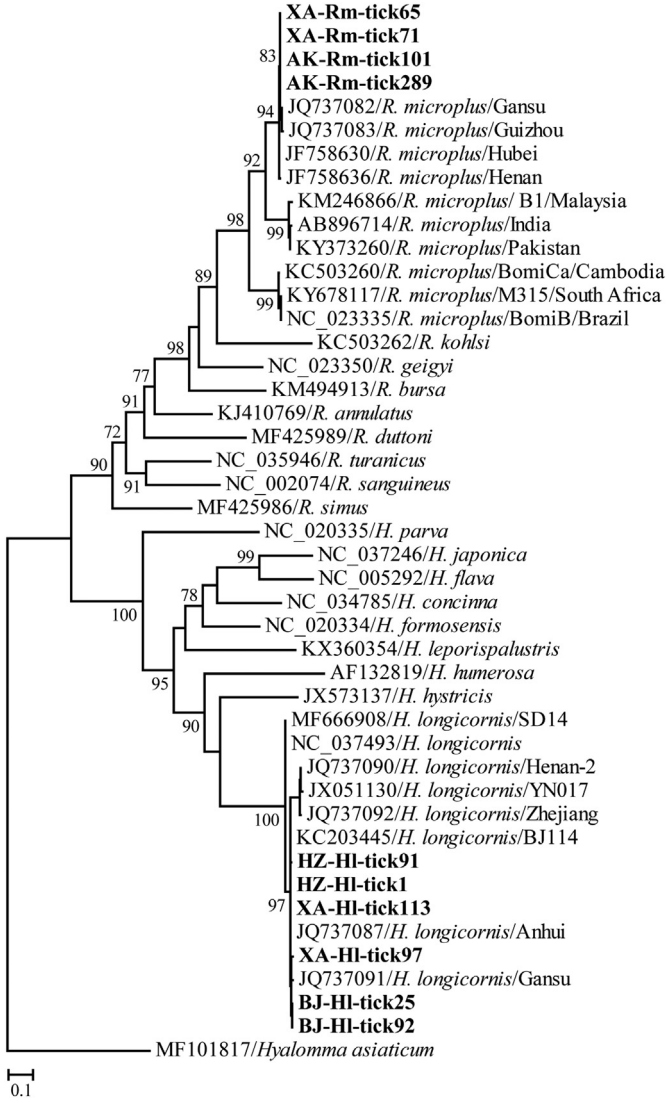

Fig. 2. Molecular identification of hard ticks based on the phylogenetic analysis of *COI* gene. *Hy. asiaticum* was used as an outgroup. Numbers at each node indicated bootstrap values. The *COI* gene sequences obtained in this study were shown in bold.

Table 1
Detection of *A. bovis* in ticks in Shaanxi Province, China.

Areas	Sites	*R. microplus*			*H. longicornis*		
		Cattle	Sheep	Goats	Cattle	Sheep	Goats
Ankang	Site 1	3/24	1/19	2/28	–	–	–
	Site 2	1/28	1/25	2/37	–	–	–
	Site 3	3/18	4/31	2/12	–	–	–
	Site 4	1/29	3/53	1/17	–	–	–
	Site 5	3/28	2/24	5/44	–	–	–
Hanzhong	Site 6	–	–	–	1/8	0/9	0/11
	Site 7	–	–	–	1/29	0/20	1/19
	Site 8	–	–	–	0/8	0/14	0/11
	Site 9	–	–	–	0/14	0/21	0/13
Baoji	Site 10	–	–	–	7/74	1/11	1/9
	Site 11	–	–	–	0/32	1/24	1/15
	Site 12	–	–	–	1/18	1/17	1/23
Xi'an	Site 13	0/0	0/3	0/5	0/9	0/10	2/15
	Site 14	0/3	1/9	0/5	0/12	0/7	0/9
	Site 15	0/6	0/7	0/3	0/11	1/7	0/23
	Site 16	0/7	0/8	0/5	0/11	0/4	0/8
Total		35/471			20/516		

WHANASN-6-1 and WHARSL-38-2 detected in mosquitoes from Wuhan (Guo et al., 2016) and ApCQ5a (KX276166) (previously identified as *A. phagocytophilum*) in goat from Chongqing, China. Another four strains (Baoji-Rl-2, Baoji-Rl-12, Baoji-Rl-41 and Baoji-Rl-87) clustered with WHHLHP-119 in ticks from Wuhan (Lu et al., 2017), WHANSA-24-1 and WHARSA-40-2 in mosquitoes also from Wuhan (Guo et al., 2016), and HB-MC-A25 (KF569919) and HB-MC-M3 (KF569924) (previously identified as *A. phagocytophilum*). In this lineage, Ankang-Rm-312 formed a distinct clade. All these strains belonging to the second lineage shared 94.4–100.0% nucleotide identities.

4. Discussion

Rapid and accurate diagnosis of pathogens of infectious diseases plays a vital role in disease diagnose, control and prevention. For Rickettsiales bacteria, the *rrs* gene is often used and invaluable for the identification and phylogenetic analysis (Liu et al., 2005; Roux and Raoult, 1995; Stothard et al., 1994; Zhou et al., 2010; Weisburg et al., 1991). However, it's difficult to well infer the genetic diversity and evolutionary relationship of intraspecies for Rickettsiales bacteria using *rrs* gene. Meanwhile, several studies showed that the *groEL* and *gltA* genes exhibited higher variation and were more useful for molecular identification and phylogenetic analysis of *Rickettsia*, *Ehrlichia* and *Anaplasma* species (Chisu et al., 2018; Dumler et al., 2001; Inokuma et al., 2001; Lee et al., 2003; Park et al., 2005; Roux et al., 1997). As for as *A. bovis*, its MSP genes were unavailable although these genes were effective for molecular detection and genetic diversity analysis of other bacteria in Anaplasmataceae (Rar and Golovljova, 2011).

In this study, *A. bovis* was initially identified by amplifying the *rrs* gene (Kawahara et al., 2006). However, BLAST research showed that not all the sequences belonged to the *rrs* gene of *A. bovis*. In addition, we only obtained seven nearly complete *rrs* gene sequences from thirty-one positive samples using the published protocol (Ge et al., 2016; Goethert and Telford 3rd., 2003; Guo et al., 2016; Kawahara et al., 2006; Liu et al., 2012; Lu et al., 2017; Sashika et al., 2011; Tay et al., 2015) (data not shown). For *A. bovis*, it's difficult to design the primers targeting *gltA* gene because of only four *gltA* gene sequences deposited in GenBank database. Therefore, novel primers were designed to amplify the *groEL* gene of *A. bovis* in the current study, and the results of detection showed that the partial *groEL* gene was more useful than *rrs* gene. In addition, our results also suggested that the partial *groEL* gene sequence contained more phylogenetic information and was suitable for the phylogenetic analysis. Hence, *groEL* gene is invaluable for the identification and phylogenetic analysis of *A. bovis*.

A. bovis presents worldwide distribution, and its DNA has been detected in ticks, mosquitoes, and diverse vertebrate animals (Battilani et al., 2017; Guo et al., 2016; Rar and Golovljova, 2011). Our phylogenetic analysis revealed that all the *groEL* gene sequences, including those obtained in this study and known sequences in GenBank database, were divided into four lineages. In addition, the sequences obtained in our study and other known sequences in China were classified into three lineages. All these suggested great diversity of *A. bovis* in China and outside China. Further, our phylogenetic analysis revealed the co-circulation of at least two lineages of *A. bovis* in Ankang, Baoji and Xi'an in *R. microplus* and *H. longicornis*, two common hard ticks carried *A. bovis* in China, revealing genetic diversity of *A. bovis* in local. In addition, genetic diversity of *A. bovis* may be underestimated because it has been identified in diverse vertebrates with approximately 1% divergence of *rrs* gene.

In conclusion, *groEL* gene is of great value to molecular detection and phylogenetic analysis of *A. bovis*. Furthermore, *A. bovis* identified in and outside China exhibited great genetic diversity based on the *groEL* gene.

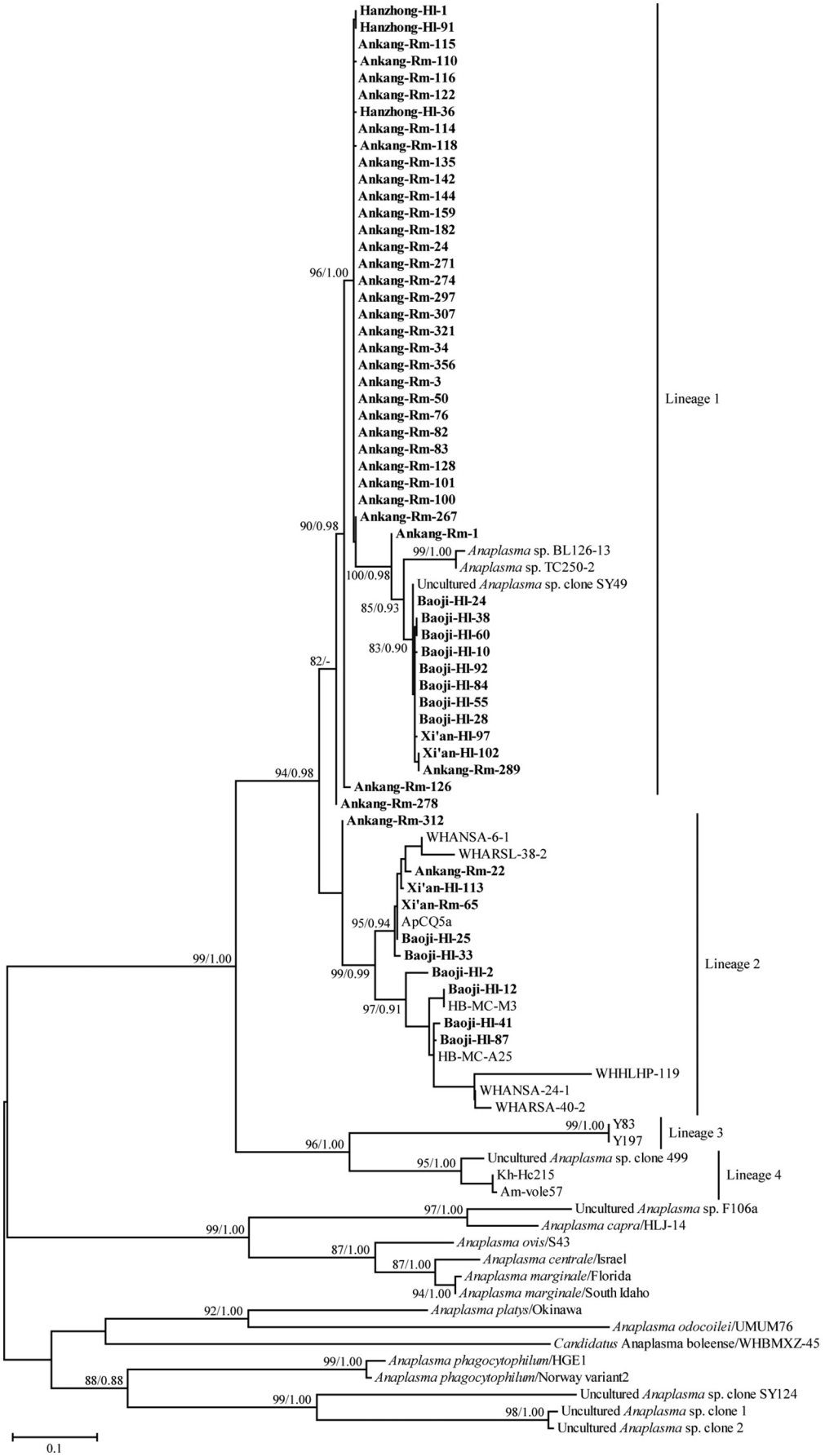

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Fig. 3. Phylogenetic tree based on the *groEL* gene sequences of *A. bovis* including those obtained in this study and known sequences in and out of China inferred using the ML and Bayesian methods. Numbers (> 70/ > 0.7) indicated bootstrap values or posterior node probabilities. The tree was mid-point rooted for clarity and the scale bar represents the number of nucleotide substitutions per site. The *groEL* gene sequences obtained in this study were shown in bold.

Funding

This work was supported by the National Natural Science Foundation of China [Grant number 31700159].

Declaration of Competing Interest

None.

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