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Molecular detection of *Coxiella* (Gammaproteobacteria: Coxiellaceae) in *Argas persicus* and *Alveonasus canestrinii* (Acari: Argasidae) from Iran

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ABSTRACT

Background: *Coxiella burnetii* and non-*C. burnetii* bacteria or endosymbiotic *Coxiella*-like were reported in various tick species. We aimed to detect *C. burnetii* within soft tick species, *Argas persicus* and *Alveonasus canestrinii*.
Methods: Argasid ticks were collected from different counties of Lorestan province, west of Iran. Partial fragments of 16S rRNA, *IS1111* insertion sequence, *com1*, *htpB*, and *icd* genes related to *Coxiella* genus were sequenced.
Results: A partial 16S rRNA and *com1* gene fragment as well as *IS1111* was detected in four *Ar. persicus* and twelve *Al. canestrinii* pools. Moreover, partial *htpB* and *icd* gene was only detected in one pool of *Ar. persicus*.
Conclusions: Detection of *C. burnetii* in tick samples was failed due to the occurrence of *Coxiella*-like endosymbionts and leads to misidentification. Thus, the house-keeping genes should be designated to distinguish *C. burnetii* within *Coxiella*-like endosymbionts.

1. Introduction

Coxiella burnetii (Gammaproteobacteria: Legionellales: Coxiellaceae), the causative agent of Q fever, is an intracellular Gram-negative bacterium infecting humans and animals [1]. Q fever is a global zoonosis with a wide range of reservoirs such as mammals, birds, and arthropods. In humans, *C. burnetii* is considered mainly as an airborne infection [2]. It has been reported that 49 tick species could be naturally infected by *C. burnetii* and species of the genus *Coxiella* [3,4]. In this regard, ticks act as vectors of the bacterium across wild vertebrates; particularly rodents, lagomorphs, and birds [4,5]. Although ticks do not play an essential role in the transmission of Q fever between humans and animals, they are essential for the maintenance of bacterial agents in the natural transmission cycle [4,6]. Moreover, the highly virulent *C. burnetii*, Nine Mile isolate, was first detected in a guinea pig parasitized by a *Dermacentor* tick [7]. The molecular detection of *C. burnetii* depends on the most frequently used targets , including single chromosomal genes such as *com1*, *htpB*; plasmids (*QpH1*, *QpRS*); an insertion sequence (*IS1111*), and *icd* gene encoding isocitrate dehydrogenase [5,8]. Some of the target genes used to detect *C. burnetii* yielded *Coxiella*-like endosymbionts (CLEs), which are present in the tick's body and lead to misidentification [9]. According to different hypotheses, CLEs in ticks are derived from the *C. burnetii* ingested by ticks which feed on an infected vertebrate host. According to the another scenario, the virulent *C. burnetii* might have also evolved from vertically transmitted CLEs [10]. Cultivation of CLEs has failed till today, and their genetic diversity and biological similarity to *C. burnetii* have been poorly defined [11]. CLEs are widely occurred among different tick species, suggesting a long coevolution relationship of *Coxiella* and ticks [12]. CLEs infect different organs of ticks including midgut, Malpighian tubules, ovaries, and salivary glands [13]. It is believed that CLEs in tick organs such as salivary glands are a kind of nutrient provisioning bacterial resource supporting the biosynthesis of several cofactors essential for the survival of ticks [14].

Recently, Seo et al. [15], detected some CLEs isolated from the mammal's blood based on 16S rRNA-sequencing. CLEs were detected in 52.4% of *Haemaphysalis longicornis* ticks [15], 2.2% of *I. ricinus* and *D. reticulatus* ticks [16]. The prevalence of CLEs widely varies among tick species, ranging from 6.25% in *R. sanguineus* sensu lato to 100% in *Amblyomma americanum* [13].

Q fever is an emerging public health concern in Iran [17,18]. However, there are few studies targeting characterization of *C. burnetii* agent in Iran [19,20] focusing on human and animal hosts of Q fever agent rather than tick vectors. Owing to longer longevity in nature, argasid ticks may be more important than ixodid species in the survival and transovarial transmission of *Coxiella* bacteria. The soft ticks

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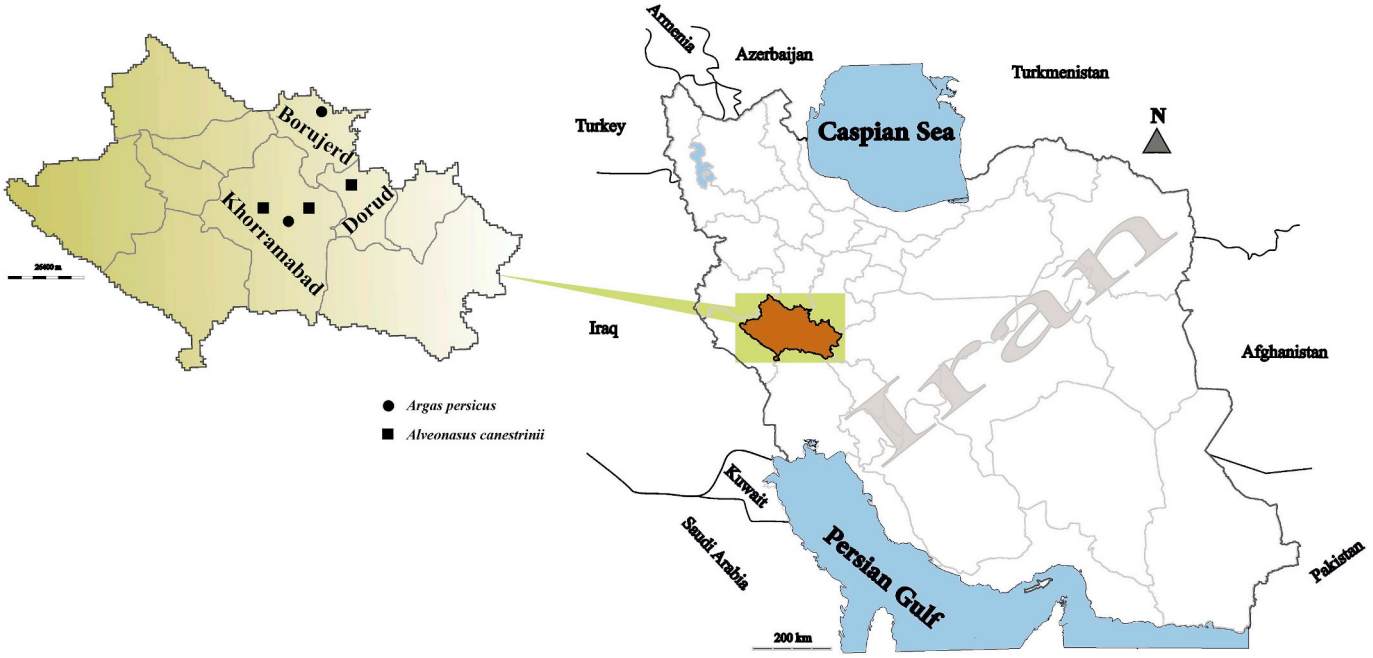

Fig. 1. Tick collection sites in Lorestan Province (western Iran) with three counties as the study area.

harbour many *Coxiella* sp., including some novel species [21][22]. Detection of *Coxiella* DNA from all stages of the argasid tick, *Carios capensis*, in its egg masses and unfed larvae provides evidence of transovarial and transstadial survival of *Coxiella* [23].

We aimed to detect *C. burnetii* in ticks using PCR with newly-designed and genus specific primers, but during the investigation, we did not find any *C. burnetii*, but CLEs.

2. Materials and methods

2.1. Tick collection and identification

A total of 750 argasid ticks were collected from 30 villages in three counties of Lorestan province in the west of Iran, called Borujerd, Dorud and Khorramabad (Fig. 1) over the springs and summers of 2016 and 2017. Different life stages of unfed and partially engorged argasid ticks were studied. Ticks were collected by forceps from wall cracks of thatched pens for sheep, goats, and aviary. The ticks were identified to species level under a stereomicroscope based on the taxonomical keys of Hosseini-Chegeni and Tavakoli [24] and Hosseini-Chegeni et al., [25].

2.2. Molecular assay and phylogenetic analysis

A total of 32 tick pools of different species and from different geographical regions were selected for molecular assays. Table 1 presents partial gene fragments and designed primers used in the study. PCR reactions were carried out in 25 μl final reaction volume (12.5 μl RedMaster PCR (Ampliqon®, Denmark), 1 μl of each primer (10 pm), 4 μl gDNA template, 6.5 μl double distilled water) under a temperature profile as follows: 3 min at 94 °C, 11 times [45 s at 94 °C, 50 s at 60 °C, 60 s at 72 °C], followed by 24 times [45 s at 94 °C, 50 s at 50 °C, 60 s at 72 °C], 3 min at 72 °C. Negative and positive PCR controls were sample-less (H_2O) and *C. burnetii* RSA-493 standard (Nine Mile phase I) DNA, respectively. PCR products were checked for their expected size before sequencing. The purified PCR products were submitted to a third party service provider for sequencing (FAZA Biotech. Co.). Homologies with available sequence data in GenBank were checked using BLAST analysis. The sequences were submitted to GenBank. Next, sequences were aligned using SeaView4 software (PRABI Rhone-Alpes Bioinformatics Center) [26]. Genetic distance between sequences was calculated using Maximum Composite Likelihood (MCL) model in the MEGA7 software [27]. To construct phylogenetic trees of *Coxiella*, aligned sequences for each 16S rRNA, *com1*, *htpB,* and *icd* genes as well as *IS1111* insertion sequence were analysed using Bayesian Inference (BI) method in BEAST® software (version 1.8.4) [28]. The output clades of phylogenetic trees were arranged and labelled based on the two criteria: posterior probability of greater than 98% support value and a reasonable genetic distance within and between the clade members.

Table 1
Gene fragments and PCR primers of *Coxiella* used in this study. Positions were matched to the complete genome sequence of *C. burnetii* RSA 493, deposited in GenBank (acc.no. AE016828).

PCR target	Size (bp)	Forward primer		Reverse primer	
		Sequence (5′ → 3′)	Position (bp-bp)	Sequence (5′ → 3′)	Position (bp-bp)
16S rRNA	577	GCA AAC AGG ATT AGA GAC C	166352–70	GCG ATT ACT AGC GAT TCC	166911–28
IS1111	598	CAA CTG TGT GGA ATT GAT GAG TGG	466800–23	GTA CCC GTT GAC AAT CCT CAT TG	466226–48
com1	697	ATC AGC GTT GGT TAA GTG A	1829428–46	CGG TTT GAA GGG TGA TTT G	1830106–24
htpB	620	CCA ACC ATT ACT AAA GAC GG	1649321–40	TCT TCG GCA ATC ACC A	1648721–36
icd	692	GAG TCT CTT AGT GAT GAC GGA	1144424–41	TGG CAT ATT GAA TCG CTC G	1143750–68

Table 2
Data related to study areas and ticks species used in this study.

Soft tick species	Host/collection place	Region	GPS coordinates (NE)	No. of collected ticks	No. of positive/examined tick pools*	Sequences (No.)
Ar. persicus	Aviary	Kah.	33°57′09.12″ 48°57′41.56″	50	2/4	16S rRNA (1), *com1* (1), *htpB* (1), *icd* (1)
A. persicus	On poultries in Aviary	Bad.	33°28′22.03″ 48°35′51.86″	50	2/3	*IS1111* (1), *com1* (1)
Al. canestrinii	Sheep, Goats/thatched barns	Sang.	33°13′55.41″ 48°33′36.85″	200	5/10	16S rRNA (1), *IS1111* (2)
Al. canestrinii	Sheep, Goats/thatched barns	Chesh.	33°19′54.45″ 48°54′09.40″	250	5/10	16S rRNA**, *IS1111***, *com1***
Al. canestrinii	Sheep, Goats/thatched barns	Lenj.	33°26′35.83″ 49°00′41.59″	200	2/5	16S rRNA**, *IS1111***, *com1***

***Ar*:** *Argas*, ***Al*:** *Alveonasus*, **Kah:** Borujerd city, Kahleh village; **Bad:** Khorramabad city, Badeh village; **Sang:** Khorramabad city, Nojian waterfall Rd., Sang Tarashan village; **Chesh:** Khorramabad city, Bisheh waterfall Rd., Cheshm-e Paryan village; **Lenj:** Dorud city, Lenjabad village. * Each tick pool contains 3–5 individual tick according to species, sex, life cycle, engorgement status. ** Sequencing was failed.

3. Results

3.1. Tick species

Argas persicus and *Alveonasus canestrinii* were identified from different geographical regions (Table 2). Data of collected ticks, positive, examined tick pools, and sequencing results are summarized in Table 2.

3.2. Molecular assay and phylogenetic analysis

A partial 16S rRNA and *com1* gene fragment as well as *IS1111* was amplified in four *Argas persicus* and twelve *Alveonasus canestrinii* pools. Moreover, partial *htpB* and *icd* gene was only detected in one pool of *Ar. persicus*. Nine sequences with GenBank accession numbers KX611879, MF359275 (16S rRNA), MF359272-4 (*IS1111*), MF370852-3 (*com1*), MF370854 (*htpB*), and MG387134 (*icd*) were obtained. Phylogenetic trees were constructed including in- and out-group taxa (Fig. 2). The constructed 16S rRNA phylogeny indicated that a *Coxiella* sp. detected in this study was an exception. The genetic distance between *Coxiella* 16S rRNA clade with a single sequence of *Coxiella* (KX611879) was found to be 9%. Also, 27% genetic distance was found between *IS1111 C. burnetii* sequences in GenBank and *Coxiella* spp. from this study. According to *com1* sequence data, 9% genetic distance was discovered between *C. burnetii* clade in GenBank and *Coxiella* clade from the present study. A single *htpB* sequence from the present study isolated from *Ar. persicus* revealed 3% genetic distance only with *C. burnetii* clade and 2–6% with other CLEs sequences isolated from soft ticks. Based on the 16S rRNA analysis described above, all *Coxiella* in this study can be grouped as a single species. However, *C. burnetii* and CLEs showed two distinct species based on *IS1111*, *com1*, *htpB*, and *icd* amplicons.

4. Discussion

The 16S rRNA of *Coxiella* was detected in *Argas persicus* and *Alveonasus canestrinii*. The detection of 16S rRNA of *Coxiella* sp., CLEs, and *C. burnetii* was reported across various tick species such as *Amblyomma*, *Rhipicephalus*, *Haemaphysalis*, *Dermacentor*, *Ixodes* and *Ornithodoros* [9], *Bothriocroton* [29], and *Argas* [30]. All *Coxiella* 16S rRNAs were observed as a single clade in the 16S rRNA phylogenetic tree except a highly divergent *Coxiella* (KX611879) of *Al. canestrinii* tick specimen. In the phylogenetic tree, 98–99% sequence identity was found within the single clade of 16S rRNA. Zhong [13] reported 91–98% sequence identity in 16S rRNA among CLEs in GenBank. Based on 16S rRNA, different strains of *C. burnetii* are highly related (99%), suggesting the homogeneity of the *C. burnetii* as a single species [31]. We observed that *Ar. persicus* and *Al. canestrinii* were infected by a *Coxiella* sp. based on *IS1111* insertion element sequence data. The *IS1111* sequences of this study were clustered together as a single clade. On the other hand, *IS1111* sequences of *C. burnetii* appeared as a separate clade. Identification of *Coxiella* sp., CLEs and *C. burnetii* using *IS1111* insertion element was reported from various isolation sources, including human blood [32], vaginal discharge from goats [33], cow's milk [34], and several tick species including *Bothriocroton* [29], *Rhipicephalus*, *Haemaphysalis*, *Ornithodoros*, *Argas* [9], *Amblyomma*, *Hyalomma* [35] along with unknown tick species [36]. The member of *Coxiella* clade of this study indicated 83% sequence identity with *C. burnetii* based on GenBank sequence analysis. However, the upstream of the *IS1111* insertion element was specifically reported to differentiate *C. burnetii* isolates across animal samples [34]. Interestingly, in contrast to the results obtained from this study, *IS1111* has proved specific to *C. burnetii* in tick samples (according to GenBank sequence data) such as *Hyalomma* [Acc. No. KX852467-71], *Rhipicephalus* [Acc. No. KU994893] (unpublished data), *Rhipicephalus* [KT345175] [37], unknown tick species [Acc. No. KT956189-91] [36], and other ixodid species [Acc. No. KP878696]. Furthermore and in line with the present study findings, *IS1111* was unable to detect *C. burnetii* in *Bothriocroton* spp. [Acc. No. EU430257] [29], *Rhipicephalus* spp. [Acc. No. KT345184-5], *Haemaphysalis* spp. [Acc. No. KT345183], *Ornithodoros* spp. [Acc. No. KT345176-181] and *Argas* spp. [Acc. No. KT345182] (Duron 2015). The intrinsic transposition activity of *IS1111*, the presence of numerous *IS1111* copies in *C. burnetii* genomes, and the lateral transfer of *C. burnetii IS1111* to non-*Coxiella* bacteria may occur in ticks [37]. Therefore, detection of *IS1111* in ticks may not be sufficient to definitely conclude the presence of *Coxiella* in the tick's body. The detection of *IS1111* in ticks confirms the infection by CLEs rather than by *C. burnetii*. This may lead to an overestimation of pathogen prevalence [37]. In the present study, a fragment of the *com1* gene was detected in two *Ar. persicus* tick pools. The sequences showed 91% identity with *C. burnetii* sequences submitted in GenBank. Therefore, two clades with 9% genetic distance were constructed in the *com1* phylogenetic tree. A single *com1* sequence from an *Ar. reflexus*, earlier submitted from Iran by the authors [Acc. No. MF359024], showed 90–91% sequence identity with GenBank *C. burnetii* sequences, and 4% genetic distance with *Coxiella* clade of the present study. Numerous studies have reported *Coxiella* based on the sequence analysis of *com1* gene [38][39][40][41]. Furthermore, some unpublished *C. burnetii* sequences [Acc. No. KU170960-4, KT071010] were deposited in GenBank detected from *Hyalomma* and *Rhipicephalus* ticks, supposing that these tick species could act as disease vectors. Due to the presence of conserved gene loci among both CLEs and *C. burnetii*, the amplicons should be sequenced for verification. In this regard, caution is needed when interpreting the detection of *C. burnetii* in tick samples without the *com1* sequencing[]. For instance, PCR-RFLP primers used by Špitalská and Kocianová [42] showed 100% sequence identity with sequences of CLEs deposited in GenBank including sequences of the present study [Acc. No: MF370852–3 and MF359024]. A sensitive Q-PCR detection system

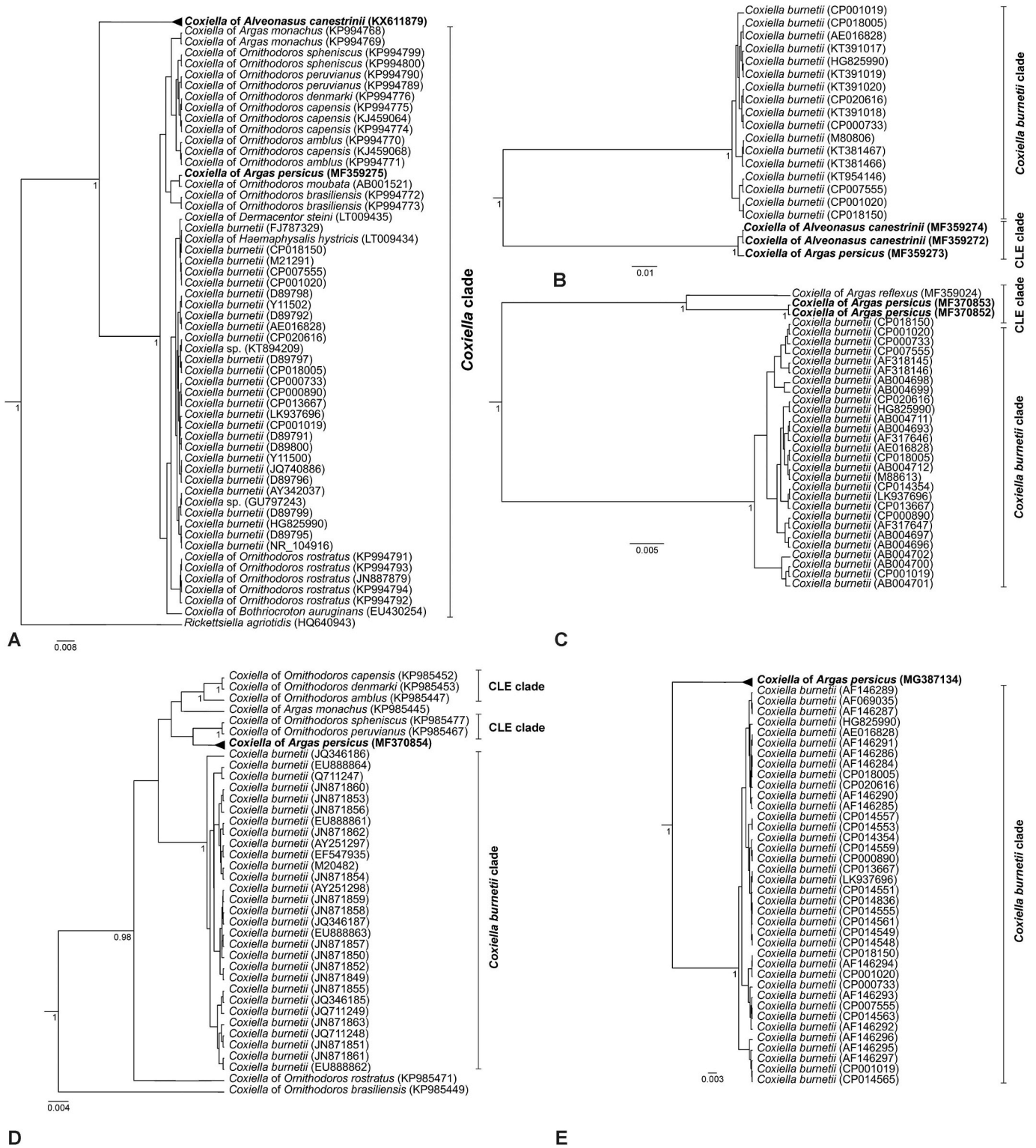

Fig. 2. The phylogenetic relationship of *Coxiella* taxa derived from analysing 16S rRNA (A), *IS1111* (B), *com1* (C), *htpB* (D) and *icd* (E) sequence data of GenBank and this study based on Bayesian Inference (BI). Phylogenetic trees indicating a single clade consists of all *Coxiella* species taxa (A), *Coxiella burnetii* species (B–E) and *Coxiella*-like endosymbionts (CLE) clades as well as many single branches. Only a part of 16S rRNA GenBank sequence data (A) was analysed due to an irregular phylogenetic tree with low posterior probability values. Numbers below nodes indicate the posterior probability value in BI analyses (10 million reiterations). Taxa are presented as genus and /or species name following GenBank accession number are given between parentheses, taxon of the present study indicated as bold by genus name. *Rickettsiella* and *Coxiella*-such as endosymbionts were examined as an out-group in the phylogenetic tree.

based on *com1* gene target was designed to avoid amplification of CLEs instead of *C. burnetii* [43]. On the other hand, the mentioned references and accession numbers in GenBank reported *com1* as a specific target to detect *C. burnetii* in human, animal, and tick samples. The present study reports the occurrence of non-*C. burnetii com1* partial gene in soft ticks for the first time. The amplification of the *com1* gene in ticks does not necessarily imply the presence of Q fever agent. In the present work, a single *htpB* sequence of *Coxiella* detected from *Ar. persicus* revealed 3%

genetic distance from the *C. burnetii* clade in the phylogenetic tree. There are studies which have detected *htpB* gene target of *C. burnetii* or CLEs in tick or non-tick samples [11][44][45][46]. However, some studies such as those published by Reeves et al. [11], are doubtful, since they did not confirm PCR results by DNA sequencing. Reye et al. [44], found 1% infection rate of *C. burnetii* in *Ixodes ricinus* ticks through examining *htpB* as the target gene. The GenBank *htpB* sequences of CLEs isolated from *Dermacentor silvarum* [KP985490]), *D. marginatus* [KP985488]), *Haemaphysalis punctata* [KP985492], *Amblyomma cajennense* [KP985482], *Am. monachus* [KP985445], *Ornithodoros rostratus* [KP985468], *O. brasiliensis* [KP985449], *O. capensis* [Acc. No: KP985451], *O. amblus* [Acc. No: KP985447] showed 76–95% genetic identity with a single *htpB* sequence of the present study. According to these data, CLEs might consist of several species. Finally, a single *icd* sequence of the present study isolated from *Ar. persicus* revealed only 6–7% genetic distance with *C. burnetii* from GenBank. The *icd* gene was reported as a good genetic marker to differentiate various *C. burnetii* isolates by PCR-RFLP [47,48]. Cooper et al. [49], reported the *icd* gene of *C. burnetii* in tick species, including *Ix. holocyclus* and *Am. triguttatum* along with the whole blood of various native Australian marsupials. The *icd* gene was introduced as a specific gene for detection of *C. burnetii* in *Ha. longicornis* [38]. However, due to the presence of other *Coxiella* species (including CLEs) in the tick's body, the *icd* gene cannot determine pathogenic *C. burnetii* at least within soft ticks [50].

5. Conclusions

The molecular identification of *Coxiella* sequences based on 16S rRNA, *com1*, *htpB,* and *icd* gene sequences as well as *IS1111* insertion sequence revealed that it could be classified as CLEs. Thus, the housekeeping genes should be designated to distinguish *C. burnetii* within *Coxiella*-like endosymbionts.

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Ethics approval and consent to participate

Not applicable.

Declaration of competing interest

The authors declare that they have no competing interests.

Author statement

Asadollah Hosseini-Chegeni designed the study. Asadollah Hosseini-Chegeni and Mohammad Hassan Kayedi collaborated to the sample collection. Asadollah Hosseini-Chegeni done laboratory assays. Asadollah Hosseini-Chegeni and Mohammad Hassan Kayedi contributed to the manuscript writing.

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