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INVERTEBRATE MICROBIOLOGY

Spotted Fever Group *Rickettsiae* in Ticks in Cyprus

Dimosthenis Chochlakis · Ioannis Ioannou · Vassilios Sandalakis · Theodoros Dimitriou · Nikolaos Kassinis · Byron Papadopoulos · Yannis Tselentis · Anna Psaroulaki

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Abstract In two surveys conducted from March 1999 to March 2001 and from January 2004 to December 2006, a total of 3,950 ticks (belonging to ten different species) were collected from seven domestic and wild animals (goat, sheep, cattle, dog, fox, hare, and mouflon) from different localities throughout Cyprus. In order to establish their infection rate with Spotted Fever *Rickettsiae* (SFG), ticks were pooled and tested by polymerase chain reaction targeting *gltA* and *ompA* genes, followed by sequencing analysis. When tick pools tested positive, individual ticks were then tested one by one, and of the 3,950 ticks screened, rickettsial DNA was identified in 315 ticks (infection rate, 8%). Five SFG *Rickettsiae* were identified: *Rickettsia aeschlimannii* in *Hyalomma marginatum marginatum*, *Rickettsia massiliae* in *Rhipicephalus turanicus* and *Rhipicephalus sanguineus*, *Rickettsia sibirica mongolotimonae* in *Hyalomma anatolicum excavatum*, and a Rickettsia endosymbiont of *Haemaphysalis sulcata* (later described as *Rickettsia hoogstraalii*) in *Haemaphysalis punctata*. Two additional genes, *17 kDa* and *ompB*, were targeted to characterize a new genotype of "*Candidatus Rickettsia barbariae*" genotype in *R. turanicus*, designated here as "*Candidatus Rickettsia barbariae*" Cretocypriensis. These results confirm the presence of a spectrum of SFG *Rickettsiae* on the island. Further studies are necessary to gain better knowledge on the epidemiology of SFG *Rickettsiae* in Cyprus.

D. Chochlakis · V. Sandalakis · B. Papadopoulos · A. Psaroulaki (✉)
Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine (WHO Collaborating Center), Medical School, University of Crete, Voutes, Heraklion, Crete 71100, Greece
e-mail: annapsa@med.uoc.gr

N. Kassinis
Veterinary Services, Nicosia, Cyprus

I. Ioannou · T. Dimitriou
Game Fund, Ministry of the Interior, Nicosia, Cyprus

Y. Tselentis
Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine (WHO Collaborating Center), Medical School, University of Crete, Voutes, Heraklion, Crete 71100, Greece

Introduction

Spotted fever group (SFG) *Rickettsiae* are obligate intracellular, Gram-negative bacteria. They belong to the genus *Rickettsia* within the family *Rickettsiaceae* in the order *Rickettsiales* [52]. *Rickettsiae* are subdivided into the typhus and the SFG group; SFG are mainly associated with hard ticks (Ixodidae), some of which can transmit them transstadially and transovarially and serve both as vectors and reservoirs for these pathogens [59]. Vertebrates (small mammals, rodents, and lagomorphs) are suspected to serve as reservoirs for *Rickettsiae*; however, they may also be accidental hosts and acquire infection by a tick bite [52]. Human infection can cause spotted fever, with clinical features including fever, headache, rash, and occasional eschar formation at the site of the tick bite [40]. The number of representatives of the genus *Rickettsia* and the number of newly described rickettsioses have increased in recent decades because of improved cell culture isolation techniques and extensive use of bacterial detection and identification by molecular techniques, and these zoonoses are now recognized as emerging vector-borne infections worldwide [40].

Old rickettsioses include epidemic typhus (*Rickettsia prowazekii*) [7], Rocky Mountain spotted fever (*Rickettsia rickettsii*) [52], Mediterranean spotted fever (*Rickettsia*

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conorii) [55], and murine typhus (*Rickettsia typhi*) [11]. However, the number of bacteria within the genus *Rickettsia* has increased in recent years, and recently described rickettsioses are recognized as emerging vector-borne infections worldwide. A number of pathogenic *Rickettsiae* (e.g., *Rickettsia africae* [26], *Rickettsia honei* [3], *Rickettsia felis* [42], *Rickettsia akari* [48], *Rickettsia aeschlimannii* [5], *Rickettsia helvetica* [4], *Rickettsia parkeri* [49], *Rickettsia slovaca* [12], *Rickettsia sibirica mongolotimonae* [12], *Rickettsia raoultii* [41], *Rickettsia massiliae* [6], and *Rickettsia monacensis* [57]) have been isolated from ticks or humans in recent decades. Furthermore, a large number of rickettsial species (most of them detected in arthropods and not yet associated with disease) have been recognized or are partially characterized as *Rickettsia* spp. [33].

The SFG constitutes a phylogenetically distinct clade of *Rickettsiae*, which includes species predominantly transmitted by ticks [40]. The group consists of a number of pathogenic *Rickettsiae* that cause the so-called tick-borne (TB) rickettsioses in humans, with clinical features, including fever, headache, rash, and occasional eschar formation at the site of the tick bite [52].

Until recently, *R. conorii* and *R. typhi* have been regarded as being the only existing rickettsial species on the island of Cyprus. A number of clinical cases of SFG rickettsioses are annually reported by clinicians as *R. conorii* or *R. typhi* infections (data collected from the public health division of the ministry of health). Since the 1980s, a number of studies conducted in Cyprus have documented the presence of *R. conorii* and *Rickettsia* spp. in human populations and ticks [24, 25, 28, 29, 46].

The aim of the present study was to detect and characterize *Rickettsiae* in naturally infected hard ticks collected in Cyprus from domestic and wild animals using polymerase chain reaction (PCR) and sequence analysis.

Materials and Methods

Sampling and Identification of Ticks

Ticks were collected during two time periods in Cyprus: From March 1999 through March 2001, 870 ticks were collected from ruminants (goats, sheep, and bovine) and dogs. From January 2004 to December 2006, a total of 3,080 ticks were collected from ruminants, dogs, and wild mammals (foxes, wild rabbits, and mouflons) from different sites, around the island of Cyprus. A total of 98 breeding units were visited throughout the island, with each site being visited eight times to cover each season. Samples collected from the breeding units were done so by the authors, whereas ticks from wild mammals were collected by staff at local veterinarian stations after they were brought in by hunters. Ticks were kept in ethanol until they were sent at the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses, and Geographical Medicine (Crete, Greece), where all tests took place. Before treatment, ticks were morphologically identified to the species level using standard taxonomic keys [37] and split into different Eppendorfs according to species, gender, and animal host from which they were collected. Subsequently, they were disinfected using 70% ethanol, rinsed in distilled water for 10 min, dried on sterile filter paper, and triturated individually into sterile tubes along with 200 μl sterile phosphate buffered saline, in a laminar flow hood. Pools (of 200 μl in final volume) were created based on tick species and host origin. Each pool consisted of up to ten ticks. Individual ticks and pools were stored at −80°C until DNA extraction.

DNA Extraction and Detection of *Rickettsia* spp.

DNA was extracted using the QIAampTissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA extracts were stored at −20°C.

Initially, a first screening for the presence of rickettsial DNA was performed by testing pools using primers RpCS 877p-RpCS 1258n targeting a 381-bp portion of the rickettsial citrate synthase (*glta*) gene [54]. Each tick specimen of each positive pool was further subjected to PCR using the same primers, as described above. The positive ticks were subsequently subjected to a second PCR using primers Rr19070p–Rr190602n, targeting a 532-bp portion of the *ompA* gene of *Rickettsia* spp. [53]. To further characterize *Rickettsia* spp., primers 17kdf–17kdr targeting a 434-bp portion of the 17-kDa protein gene, and primers BG1-21–BG2-20 targeting a 650-bp portion of the *ompB* gene were used, as previously described [13, 16]. DNA extracts of *R. conorii* and *R. typhi* were used as positive controls, and ultra pure water was used as a negative control in each amplification.

All amplifications were conducted using a MyCycler (Bio-Rad) DNA thermal cycler. PCR products were electrophoresed on 1.5% agarose gel and visualized following 30-min incubation in GelStar Nucleic Acid Gel Stain (Lonza, USA) according to the manufacturer's instructions. Positive PCR products were purified using the QIAquick PCR purification spin kit (Qiagen, Germany) and were directly sequenced with the above described primers using the sequencer CEQ 8000 Beckman Coulter (Bioanalytica–Genotype, Athens). All sequences were processed using nucleotide Blast (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/BLAST) and were aligned using ClustalW [60]. Sequences were submitted at GenBank to obtain the corresponding accession numbers. The phylogenetic trees were constructed using the MEGA 4.1 beta software.

Phylogenetic Analysis

Phylograms were constructed in MEGA v.4 software. The neighbor-joining method was used to build the trees, while the evolutionary distances were computed using the Kimura two-parameter method.

Results

During the first study period (1999–2001), a total of 870 ticks, representing seven species (*Rhipicephalus turanicus*, *Rhipicephalus sanguineus*, *Rhipicephalus bursa*, *Hyalomma anatolicum excavatum*, *Hyalomma marginatum marginatum*, *Haemaphysalis sulcata*, and *Haemaphysalis punctata*), were collected from goats, sheep, and dogs. During the second study period (2004–2006), 3,080 ticks, representing ten species (*R. turanicus*, *R. sanguineus*, *R. bursa*, *H. anatolicum excavatum*, *H. sulcata*, *H. punctata*, *Hyalomma marginatum rufipes*, *H. marginatum*, *Ixodes ventalloi*, and *Ixodes gibbosus*), were collected from ruminants (goats, sheep, and bovine), dogs, and wild (mouflon, fox, and hare) animals (Table 1).

Rickettsial DNA was detected in 315 of 3,950 ticks [infection rate (IR), 8%] using the PCR method targeting two genes, *gltA* and *ompA*. The primers used were specific for both of the rickettsial genes. All samples tested positive for rickettsial DNA were sequenced, and sequences were analyzed using the BLAST algorithm and the DNAStar Lasergene software package. Rickettsial DNA was detected in the following seven tick species: *R. sanguineus* (in 130 out of 1,606 ticks, IR of 8.1%), *R. turanicus* (125/805, IR of 15.5%), *R. bursa* (20/734, IR of 2.7%), *H. marginatum* (10/83, IR of 12%), *H. anatolicum excavatum* (20/301, IR of 6.6%), and *H. punctata* (10/33, IR of 30.3%). The highest infection rate was calculated in *H. punctata* (IR, 30.3%), where *R. hoogstraalii* was identified, while the lowest infection rate was observed in *R. bursa* (IR of 2.7%), where *R. massiliae* was identified (Table 2).

Ten of 33 (30.3%) *H. punctata* ticks, collected from mouflons, were found to be positive for rickettsial *gltA* gene. The *gltA* sequence (EU448154) was 100% similar to *R. hoogstraalii* (FJ767737.1). In these samples, we could not obtain a PCR product of the *ompA*. Ten of 83 (12%) *H. marginatum* ticks, collected from goat and sheep, were found to be positive for rickettsial *gltA* and *ompA* genes. The *gltA* gene (JF803904 and JF803905) was found to share 100% sequence homology with the *R. aeschlimannii* strain Stavropol (DQ235776), while the *ompA* gene sequence (JF803906 and JF803907) shares 99.71% (518/520b) sequence similarity to the *R. aeschlimannii* strain Stavropol (DQ235777). Two nucleotide mutations were detected in the *ompA* partial gene sequence, 112T>G and 143T>A, which resulted in two amino acid alterations, S38A and L48H (DQ235777 numbering). Five of 805 (0.6%) *R. turanicus* ticks, collected from dogs, were found to be positive for rickettsial *gltA* (JF803910) and *ompA* genes with the sequences sharing 100% homology with "*Candidatus Rickettsia barbariae*" (EU272185 and EU272186 respectively). In this case, we performed another PCR for *ompB* to verify the detected rickettsial species. Once again, the *ompB* sequence (GU353186) was 100% similar to "*Candidatus Rickettsia barbariae*" (EU272187). Eighty-three of 805 (10.3%) *R. turanicus*, 130 of 160 (8.1%) *R. sanguineus*, and 20 of 734 (2.7%) *R. bursa* ticks collected from fox, hare, dog, and goat tested positive for the *gltA* and *ompA* genes with the product sequences being quite similar to *R. massiliae* strain MTU5 (CP000683). The *gltA* sequences (GU353181, JF803908, and JF803909) were 99.71% (344/345b) similar to CP000683 with the difference lying on one nucleotide change (822A>G), which leads to a silent mutation. Sequences of the *ompA* (EU448161, EU448162, EU448163, EU448164, and EU448165) were 99.38% (488/491b) similar to the *R.*

Table 1 The number of ticks collected from wild, domestic and animals of veterinary importance are shown

Animal species	Ectoparasite species											Total ticks
	H. sulcata	*H. anatolicum excavatum*	*R. sanguineus*	*R. bursa*	*R. turanicus*	*H. marginatum marginatum*	*I. ventalloi*	*H. marginatum rufipes*	*I. gibbosus*	*H. punctata*	*R. pusillus*	
Goat		32	40	413	112	50			1			648
Sheep		144	73	99	40	32						388
Bovine		37		2	4			1				44
Dog		5	1,383		163							1,551
Mouflon	258	83		216	46	1			23	33		660
Fox			4		175		21		19			219
Hare			106	4	265		45		1		19	440
Total ticks	258	301	1,606	734	805	83	66	1	44	33	19	3,950

Table 2 Molecular positivity and infection rate are shown for the collected ticks: *Rickettsia* species detected in positive tics and their associated host animal species are shown

Ectoparasites		PCR		Infection Rate (%)		Sequencing analysis			
Species	*No*	*Positive*		-		*Rickettsia* species detected	*Genbank accession No*	*Gene*	*Animal origin*
H. punctata	33	10		30.3		***R. hoogstraalii***	EU448154	*glta*	Mouflon
R. bursa	734	20		2.7		***R. massiliae***	EU448161	*ompA*	Goat
R. turanicus	805	***Total number of positive ticks*** = 125	83	***Total Infection rate*** = 15.5	10.3	***R. massiliae***	GU353181 JF803908 JF803909	*gltA*	Dog Fox Hare
							EU448162 EU448163 EU448164	*ompA*	
			5		0.6	"***Candidatus* R. barbariae**:"	JF803910	*gltA*	Dog
							GU353186	*ompB*	
			11		1.4	***Rickettsia* sp.**	EU448155 EU448156 JF803898	*gltA*	Sheep Hare Goat
							EU448158 EU448159 EU448160 JF803899	*ompA*	Dog Sheep Goat Hare
			26		3.2	"*Candidatus* R. barbariae" genotype Cretocypriensis	GU353183 JF803886 JF803887 JF803888	*gltA*	Fox Goat Hare Mouflon
							EU194445 JF803889 JF803890 JF803891	*ompA*	
							GU353187 JF803892 JF803893 JF803894	*ompB*	
							GU353184 JF803895 JF803896 JF803897	*17kDa*	
H.a.excavatum	301	***Total number of positive ticks*** = 20	15	***Total Infection rate*** = 6.6	5	***R. sibirica mongolotimonae***	JF803902 JF803903	*gltA*	Goat, Sheep
							JF803900 JF803901	*ompA*	
			5		1.6	***Rickettsia* sp.**	EU448157	*gltA*	Sheep
R. sanguineus	1606	130		8.1		***R. massiliae***	EU448165	*ompA*	Dog
H. m. marginatum	83	10		12		***R. aeschlimannii***	JF803904 JF803905	*gltA*	Goat, Sheep
							JF803906 JF803907	*ompA*	

massiliae strain MTU5 (CP000683). The differences were four nucleotides, which resulted in two amino acid alterations A2F and T4A (CP000683 numbering). Three hundred one *H. anatolicum excavatum* ticks were collected from sheep and goats and 20 (6.6%) of them were positive for rickettsial DNA. Fifteen of the samples (5%) were identified as *R. sibirica mongolotimonae* (100% similarity), based on both *gltA* and *ompA* genes (accession numbers: *gltA*, JF803902 and JF803903; *ompA*, JF803900 and JF803901), and five samples (1.6%) were identified to only *Rickettsia* species sharing only 99.72% (357/358b) sequence homology with *Rickettsia* sp. RR01 (GU056205), *R. rhipicephali* strain HJ5 (DQ865206), "*Candidatus R. kulagini*" strain Kertch (DQ365806), and *R. rhipicephali* 3-7-6 (U59721) for the *gltA* gene (JF803898, EU448157, EU448156, and EU448155), whereas for the *ompA* gene (JF803899, EU448160, EU448159, and EU448158), the best sequence homology, 99.21% (506/510b) was seen in *Rickettsia* sp. ZJ43/2007. This last rickettsial species was also found in 11 of 805 (1.3%) *R. turanicus* ticks collected from hare, sheep and goat, and dog. The amplicon sequence obtained for the *gltA* gene was altered by one amino acid when compared to *Rickettsia* sp. RR01 (GU056205), where there was a nucleotide alteration 844C>T that led to an amino acid change H282Y (DQ365806 numbering). The same sequence aligned against three additional homologous sequences obtained from *R. turanicus* ticks (DQ865206, DQ365806, and U59721) revealed a nucleotide alteration, 878A>G, which, in the amino acid sequence, results to K293R (DQ365806 numbering). Finally, in 26 of 805 (3.2%) *R. turanicus* ticks collected from hare, goat, mouflon, and fox, rickettsial DNA was detected for the *gltA* and *ompA* genes. The partial gene sequences retrieved, shared closest similarity to "*Candidatus R. barbariae*." The *gltA* gene sequence (GU353183, JF803886, JF803887, and JF803888) shared 99.70% (344/345b) similarity with "*Candidatus R. barbariae*" (EU272185), with the difference being a silent mutation 354A>G (EU272185 numbering). Similarly, the *ompA* gene (EU194445, JF803889, JF803890, and JF803891) shared 99.80% (505/506b) sequence similarity to "*Candidatus R. barbariae*" (EU272186) due to a silent mutation, 45T>C (EU272186 numbering). In this case, two additional genes, *ompB* and *17 kDa*, were tested using the same methodology. Once again, the partial *ompB* gene product sequence (GU353187, JF803892, JF803893, and JF803894) revealed a similarity of 99.83% (615/616b) to the "*Candidatus R. barbariae*" (EU272187). In contrast to the first two genes, the *ompB* amplicon revealed a nucleotide mutation (1229A>G) resulting to an amino acid substitution, N410S (EU272187 numbering). The *17 kDa* partial gene product (GU353184, JF803895, JF803896, and JF803897) was sequenced, and its best BLAST match were *R. rickettsii* strain Iowa (CP000766) and *R. rickettsii* strain ai103.1 (GU723477). The similarity of the sequence with these best matches was 99.75% (398/399b). When the Query sequence was aligned to *R. rickettsii* strain Iowa, there was a nucleic acid mutation (458G>A), which resulted in an amino acid alteration (G153E) (CP000766 numbering). The same sequence when aligned to *R. rickettsii* strain ai103.1 reveals a nucleic acid mutation in position 410b (410A>G), leading to an amino acid mutation (D137G). In an attempt to characterize this *Rickettsia* species, amplicon sequences were used to construct four phylogenetic trees (Fig. 1). The phylogenetic relationship of this *Rickettsia* species with validly *Rickettsia* species published names, for which *gltA*, *ompA*, *ompB*, and *17 kDa* gene sequences are available, was evaluated for each gene, and the evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in <50% bootstrap replicates were collapsed. The evolutionary distances were computed using the Kimura two-parameter method. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). All four genes revealed similarities of >99% with validly published *Rickettsia* species (*gltA*, 99.70%; *ompA*, 99.80%; *ompB*, 99.83%; and *17 kDa*, 99.75%). Therefore, on the basis of genotypic criteria and on the *Rickettsia* species definition [20], our initially unidentified *Rickettsia* sp. strain belonged to an existing species and is classified as a new genotype; thereafter, it was given the name of "*Candidatus Rickettsia barbariae*" genotype Cretocypriensis.

Sequences were deposited in GenBank, and the accession numbers are displayed in Fig. 1.

Discussion

The most well-known SFG *Rickettsia* in Cyprus is *R. conorii* [46]. It is transmitted by *R. sanguineus* and causes "boutonneuse" or Mediterranean spotted fever [40]. Prior to this study, *R. conorii* was the only tick-borne SFG *Rickettsia* pathogenic to humans described in Cyprus [46].

In the present study, a high diversity of *Rickettsia* species were detected within the ticks collected throughout the island of Cyprus. Included among these were seven *Rickettsia* species, in particular, *R. massiliae*, *R. aeschlimannii*, *R. sibirica mongolotimonae*, "*Candidatus Rickettsia barbariae*," *Rickettsia hoogstraalii*, a new genotype of a known *Rickettsia* sp. assigned as "*Candidatus Rickettsia barbariae*" genotype Cretocypriensis and a partially characterized *Rickettsia* sp. closely related but distinct from the *R. rhipicephali–R. massiliae* lineage. Surprisingly, *R. conorii*

was not detected. Although not all *Rickettsia* spp. detected in the present study are pathogenic, numerous *Rickettsia* spp., first described in ticks and initially characterized of "unknown pathogenicity," have later been proven to be pathogenic to humans.

This is the first report describing the presence of *R. aeschlimannii* and *R. sibirica mongolotimonae* in Cyprus. These *Rickettsiae* were detected in *Hyalomma* spp. (*H. marginatum* and *H. anatolicum excavatum*, respectively). These ticks are believed to parasitize migratory birds during their immature life stages and can be transported to Europe from Africa where they reside from October to April [62]. Migratory birds carrying ticks infected with *R. sibirica mongolotimonae* have been considered a source of human infection [52].

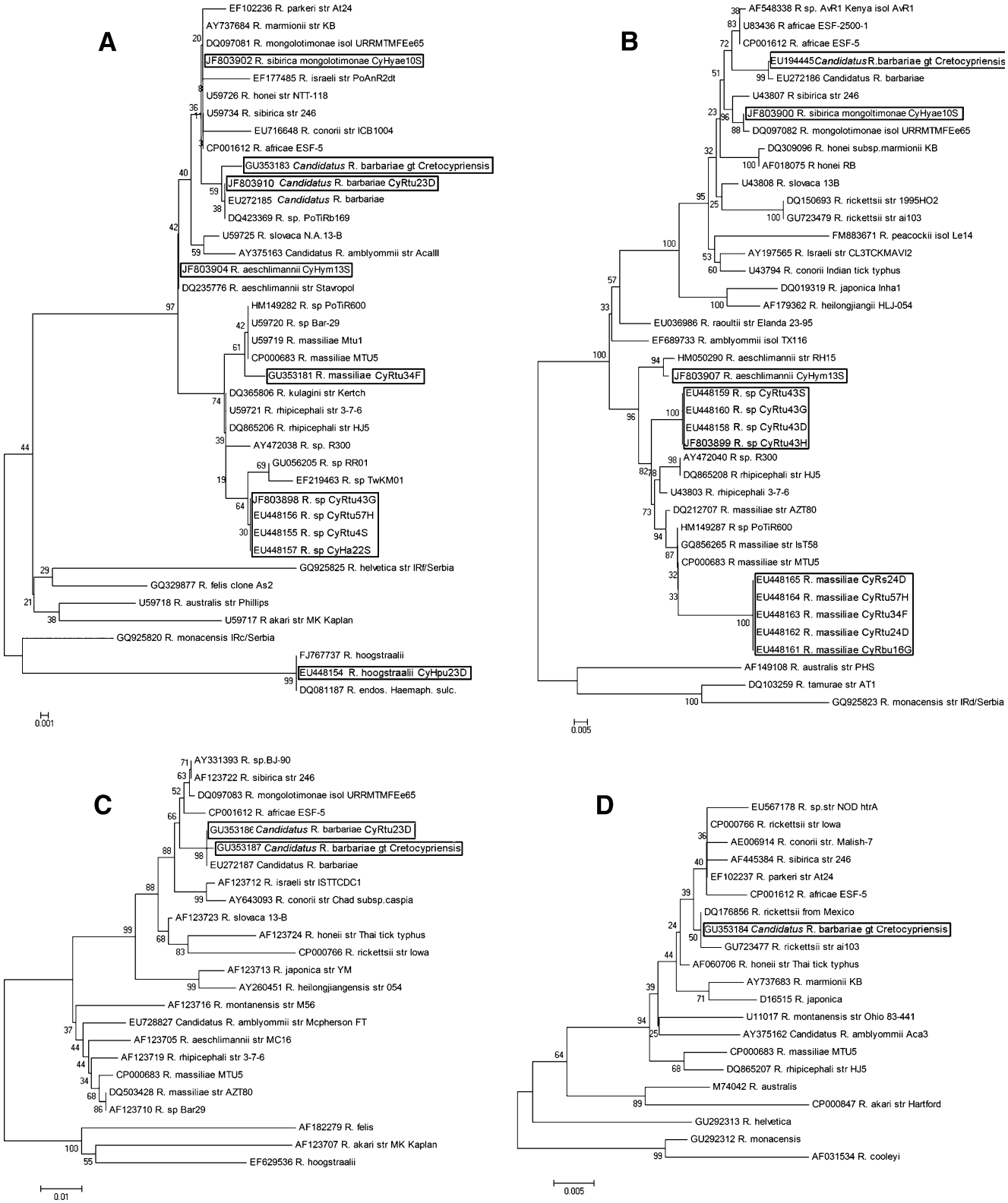
Hyalomma were present on several hosts in different parts of the island, meaning that Cyprus may be regarded as an entrance gate and that these ticks may well adapt in new environments. Birds constitute the main hosts of larvae and nymphs of *H. marginatum marginatum* and play an important role in the dissemination of this tick species. The changing climate and environment in central Europe may facilitate the establishment of pathogen-carrying tick species transported by birds.

The present study reports the first detection of *R. aeschlimannii* in ticks collected from sheep and goats in Cyprus. *R. aeschlimannii* was first characterized as a new SFG *Rickettsia* following its isolation from *H. m. marginatum* in 1997 in Morocco [5]. It was demonstrated as a human pathogen causing disease in two patients returning from Morocco to France [51] and in a patient in South Africa [43]; recently, two more cases were described in Algeria [34]. This *Rickettsia* was shown to be transstadially and transovarially transmitted in *Hyalomma* spp., indicating that these ticks may act both as vectors and as reservoirs of the pathogen [31]. The pathogen has been detected in this tick species (*H. marginatum rufipes*) in various African countries (Zimbabwe, Niger, and Mali) [38] and within the Mediterranean region (Portugal, Croatia, Spain, Algeria, Egypt, and Corsica), either in *H. marginatum marginatum* or in *H. marginatum rufipes* [27, 31, 40]. This *Rickettsia* species has been detected in its vector in sheep in Greece [47], as well as in other *Hyalomma* spp. including *H. aegyptium* in Algeria [9], *H. dromedarii* and *H. impeltatum* in Egypt [27], and in *H. truncatum* in Senegal [32]. Given the detection of this pathogen in *H. marginatum marginatum* ticks in this study, greater effort is required to assess the risk to farm owners who have frequent contact with this tick species and their hosts.

Rickettsia sibirica mongolitimonae was also detected in *Hyalomma* (*H. anatolicum excavatum*) removed from dogs, sheep, and goats. This pathogen was first isolated from *Hyalomma asiaticum* in inner Mongolia in 1991 [63]. It has been associated with human infections in France, Algeria, South Africa, Greece, Portugal, and Spain [1, 21, 22, 44, 45, 50]. The most recent case was reported in a French traveler who returned from Egypt [58]. Tick vectors of this pathogen are thought to include species of *Hyalomma*, such as *H. asiaticum* in inner Mongolia [63], *H. anatolicum excavatum* and the patient from whom the tick was removed [45], *H. truncatum* in Niger [39] and Senegal [32], and several *Hyalomma* species in Israel [23]. Recently, *Rhipicephalus pusillus* removed from an Egyptian mongoose were found to be positive for *R. sibirica mongolotimonae* in south Portugal [14]. As with *R. aeschlimannii*, it seems that the distribution of *R. sibirica mongolitimonae* corresponds to that of *Hyalomma* spp. [35].

R. massiliae was the most prevalent rickettsial species occurring all over the island since it was detected in *R. bursa*, *R. sanguineus*, and *R. turanicus*. The main reservoirs for *R. massiliae* are ticks within the genus *Rhipicephalus* (*R. sanguineus* and *R. turanicus*), in which transstadial and transovarial transmission occur [30]. It was first isolated from *R. sanguineus* in Marseille in 1992 [6], while its genomic variant, strain GS, has been isolated from *R. sanguineus* collected in Fokida, Greece [2]. Since then, it has been detected in both *R. sanguineus* and *R. turanicus* in Greece, Spain, Portugal, Switzerland, France, Algeria, Morocco, Israel [23], and in Sardinia [6, 10, 13, 36, 40, 56]. A strain close to Bar29 has been isolated from *R. sanguineus* ticks collected in Arizona (North America) [18]. The pathogen has also been detected in *Rhipicephalus* spp. ticks (*Rhipicephalus mushamae*, *Rhipicephalus lunulatus*, *Rhipicephalus sulcatus*, and *Rhipicephalus guilhoni*) in various African countries including Central African Republic, Mali, Algeria, Morocco [8, 10, 17, 30, 32, 39, 56]. The pathogenicity of *R. massiliae* was unknown for several years after it was detected; however, recent findings have proven otherwise [61]. In the current study, ticks infected with this pathogen were predominantly removed from goats, sheep, and dogs and, to a lesser extent, from

Figure 1 Phylogenetic analysis of *gltA* (345 bp) (**a**), *ompA* (506 bp) (**b**), *ompB* (616 bp) (**c**) and *17 kDa* (399 bp) (**d**) partial genes of the current survey including the initially uncharacterized and further named after "*Candidatus R. barbariae*" genotype Cretocypriensis sequences. Accession numbers of the submitted sequences (*framed*) and the sequences used for the construction of the phylogenetic trees are all shown at the equivalent branch. *Rickettsia sibirica monglolotimonae* CyHae10S sequences are identical to *R. sibirica monglolotimonae* CyHae5G. *Rickettsia aeschlimanii* CyHym13S sequences are identical to *R. aeschlimanii* CyHym8G. All sequences of "*Candidatus R. barbariae*" genotype Cretocypriensis were identical to each other; thus, only one sequence (accession number) is shown in the trees. *Rickettsia massiliae* strain *psaroulakii* represents all three sequences (GU353181, JF803908, and JF803909). All groups of sequences framed together are identical to each other



foxes and hares. The detection of this *Rickettsia* within several *Rhipicephalus* ticks highlights a broad level of exposure, given the variety of species they parasitize. Furthermore, the infection rate observed in *R. sanguineus* (8.1%), the principal tick found parasitizing dogs, increases the risk of contact with the pathogen for human owners. For this reason, the pathogen and its vector may be of concern to both human and animal health.

In the current study, the presence of *R. hoogstraalii* was demonstrated in *H. punctata*. This *Rickettsia* was first detected in Croatia in 2006 [16] and named "*Candidatus R. hoogstraalii*" before being elevated to *R. hoogstraalii* sp. nov. after further molecular characterization [15]. It has been detected in Spain and Georgia (USA); however, this is the first documentation of its presence in southeast Europe. In Croatia, the pathogen was detected in *H. sulcata* collected from sheep and goats, while in the present study, *H. punctata* were found feeding habitually on Mouflons (wild sheep).

This study identified a new "*Candidatus R. barbariae*" genotype by sequence analysis of partial sequences of the *17 kDa*, *ompA*, *ompB*, and *gltA* genes. Sequence homology with recognized members of the genus suggests that this may be a new genotype and, as a result, was named after "*Candidatus R. barbariae*" genotype Cretocypriensis. A 99% sequence similarity was achieved when comparing our results with published information (*gltA*, 344/345b; *ompA*, 505/506b; *17 kDa*, 398/399b). With the exception of *17 kDa*, which clustered closer to a *R. rickettsii* strain, the rest of the genes clustered closer to a "*Candidatus R. barbariae*" strain along with the *Rickettsia* sp. PoAnR2dt strain (in the case of *gltA* gene). What is worth noting is that this new genotype was only detected in *R. turanicus* despite the difference in host (dog, sheep, and goat). Our findings suggest that *R. turanicus* may play an important role in the life cycle of "*Candidatus R. barbariae*" genotype Cretocypriensis.

During the present study, *R. massiliae*, "*Candidatus R. barbariae*," and its new genotype, Cretocypriensis, were detected in *R. turanicus* and *R. sanguineus*. In a past study, "*Candidatus R. siciliensis*" was detected in *R. turanicus* removed from an asymptomatic 22-month-old woman in Sicily [19]. Our results suggest that *R. turanicus* frequently parasitized a range of host animals, both wild and domestic, spanning a wide distribution that covered almost all localities sampled. Changes in climate and human habitat modification that may result in lowered landscape diversity could provide an opportunity for *R. turanicus* to increase its current range, given the tick's broad host specificity and existing wide distribution. If the tick were a vector of disease, adaptation to and expansion of its distribution could expose the tick to new hosts. However, further studies are needed to investigate its role in disease transmission and pathogenicity of the *Rickettsiae* detected in this study and the role of both *R. turanicus* and *Candidatus Rickettsiae*.

The findings of this current study have extended the knowledge of the distribution of ticks and their associated SFG *Rickettsiae* in Cyprus. According to these findings, in addition to *Rhipicephalus* species, members of the genus *Hyalomma* and *Haemaphysalis* may also play an important role in the epidemiology of SFG *Rickettsiae*. Clinicians in Cyprus should consider a range of SFG diseases in the differential diagnosis of patients with febrile illnesses. Moreover, atypical cases of rickettsioses may exist, due to agents other than *R. conorii*, and may escape diagnosis. Additional studies are needed to determine the epidemiological and clinical relevance of different rickettsioses detected in Cyprus. Furthermore, sequencing of the full length of 16S rRNA, *ompA*, *ompB*, *gltA*, and *sca4* genes would enable better characterisation of the genotypes detected. A comparison of rickettsial strains isolated or detected from other regional countries would provide a better understanding of the epidemiological situation of SFG *Rickettsiae* in the Mediterranean region. In the near future, with unpredictable changes to climate, ticks may be considered as epidemiological markers for the number of infectious agents they are capable of transmitting.

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