Article

Rickettsial Agents Associated with Ectoparasites in Attica, Greece

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Simple Summary: Rickettsial pathogens, harbored in ectoparasites such as fleas and ticks found on cats and dogs, may cause vector-borne diseases. The prefecture of Attica in Greece, where the greater Athens metropolitan area is located and a large part of the Greek population resides, is poorly studied regarding these pathogens. To investigate their presence, we screened fleas and ticks collected from cats and dogs in veterinary clinics over 13 months. Using molecular methods, we tested the specimens for rickettsial organisms, such as species of *Rickettsia*, *Wolbachia*, and *Ehrlichia*. *Rickettsia felis* and *Wolbachia* were found in the examined fleas. *Rickettsia conorii* and *Ehrlichia canis* were found in the examined ticks, as well as *Candidatus* Midichloria mitochondrii, a little-studied symbiotic organism of ticks, which can be transmitted to humans. Our study shows that in the Attica region, fleas and ticks infesting dogs and cats harbor rickettsial pathogens that may pose a risk to human health. In this context, it contributes to the study of their epidemiology in the area. Additionally, it highlights the need for increased vigilance in the surveillance of vector-borne diseases and continued research to assess their potential implications for public health in this metropolitan area.

Abstract: The bacteria of the families *Rickettsiaceae* and *Anaplasmataceae*, harbored by arthropod vectors, may cause disease in animals and humans. The aim of this study was to screen ectoparasites collected from cats and dogs in Attica, Greece for the bacteria of the Rickettsiales group, by molecular methods. The ectoparasites examined were *Ctenocephalides felis* fleas and *Rhipicephalus sanguineus* s.l., *Rhipicephalus* sp., and *Ixodes* sp. ticks. *Rickettsia felis* was detected in 4.8% of *C. felis* fleas, and *Rickettsia conorii* was detected in 7.3% of *R. sanguineus* s.l. ticks. *Ehrlichia canis* was found in one *R. sanguineus* s.l. tick, and *Wolbachia pipientis* was detected in the majority of fleas. Another endosymbiont, *Cancidatus* Midichloria mitochondrii (*Cancidatus* Midichloriaceae), was detected in one *Ixodes* sp.. This is the first report of *R. conorii* and *E. canis* in *R. sanguineus* s.l. ticks in this study area. Given the fact that Greece is considered endemic for spotted fever group rickettsioses, further investigation of these rickettsial pathogens' distribution in their vectors and hosts could enhance our knowledge of their epidemiology, in order to assess their potential implications for public health in this metropolitan area.

Keywords: *Rickettsia* sp.; *Ehrlichia* sp.; *Wolbachia* sp.; *Ca.* Midichloria mitochondrii; fleas; ticks; PCR; Greece

1. Introduction

According to recent molecular evidence and phylogenetic data, intracellular bacteria of the order Rickettsiales are classified into three families: *Rickettsiaceae*, *Anaplasmataceae*, and

"*Candidatus* Midichloriaceae" [1]. The family *Rickettsiaceae* includes the genera *Rickettsia* and *Orientia,* and the family *Anaplasmataceae* includes the genera *Anaplasma*, *Ehrlichia*, *Wolbachia*, and *Neorickettsia*. Bacteria of these genera are associated with hematophagous arthropods and—if implicated in animal and human diseases—are characterized as pathogens, whereas if not, as endosymbionts. The third family comprises nine genera and includes the species "*Ca*. Midichloria mitochondrii", which is the first representative of this family, an endosymbiont of the tick *Ixodes ricinus* and other arthropods [1].

Rickettsial diseases are prevalent and important tick- and flea-borne zoonoses. *Rickettsia* spp. that have been implicated in animal and human diseases are classified into the spotted fever group (SFG), the typhus group, and the *R. belli* group [2]. Ixodid ticks transmit the majority of *Rickettsiae,* but recently many argasid ticks have also been identified as carriers [3]. *Rickettsia felis* and *Rickettsia conorii* of the SFG are mainly transmitted by *Ctenocephalides felis* fleas and *Rhipicephalus sanguineus* ticks, respectively [4]. In the family *Anaplasmataceae, Ehrlichia chaffeensis* causes human monocytic ehrlichiosis and *Anaplasma phagocytophilum* (previously known as *Ehrlichia phagocytophila*) causes human granulocytic anaplasmosis (previously known as human granulocytic ehrlichiosis) [4]. *Ehrlichia canis*, the etiological agent of canine monocytic ehrlichiosis, has also been isolated from human blood specimens [5]. In the same family, the genus *Wolbachia* is found mainly in arthropods and nematodes and includes a single formally recognized species, *Wolbachia pipientis* [6].

Several rickettsial pathogens have been identified in humans, animal hosts, and vectors in serological or molecular studies in Greece. Regarding ticks, studies have focused mainly on Northern and Central Greece [7–11] and the island of Cephalonia [12]. Although the Attica prefecture was included among others areas in several studies, there are no data on rickettsial pathogens in ticks in this area. Attica is the most densely populated part of the country, with high numbers of stray and free roaming cats and dogs in urban and suburban areas. The aim of this study was to investigate the presence of rickettsial agents in ectoparasites of cats and dogs in the Attica region, in order to assess the possible risk they may pose to human health in the greater Athens metropolitan area.

2. Materials and Methods

Fleas and ticks from cats and dogs were collected from September 2016 to October 2017 from five veterinary clinics. The animals came from 39 areas in the Attica region (Figure 1) and were mostly stray cats and dogs. Only parasitized animals for ticks and fleas were accessed. No multiparasitism was recorded. From each animal, either fleas or ticks were collected. The collection was implemented in the context of a non-experimental clinical veterinary practice, and in the case of owned animals, the owners were informed and oral consent was obtained. Collected ectoparasites were placed in tubes containing 95% ethanol and stored at 4 °C until DNA extraction. The arthropods were photographed and identified using standard morphological identification keys and relevant publications [13–15].

Ectoparasites were washed twice for 10 min in sterile distilled water and dried on sterile filter paper. DNA extraction was performed using the NucleoSpin Tissue Kit (Macherey-Nagel GmbH, Düren, Germany) with the following modification: following placement in a microcentrifuge tube containing the T1 buffer and cutting gently into pieces with a sterile scalpel, proteinase K was added and the tube was incubated overnight at 56 °C. The remaining procedure was carried out according to the manufacturer's instructions. Extracted DNA was stored at −20 °C until testing. All flea and tick samples were tested successfully for the absence of PCR inhibitors by the amplification of a fragment of the ectoparasites' cytochrome oxidase subunit I (COI) gene using the previously described primers [16].

The ectoparasites were screened for *Anaplasmataceae* DNA, using the primer set EHR16SR–EHR16SD, which amplifies a 345-bp fragment of the 16S rRNA gene. Conventional PCR was carried out in a total volume of 25 μL containing 3 μL DNA, as previously described [17]. A BioRad S1000 thermocycler was used, and PCR products were visualized using the Bio-Rad GelDoc XR+ Gel Documentation System (Chemidoc XRS+

Gel Imaging System) after electrophoresis on a 2% agarose gel, stained with ethidium bromide. Experiments were performed twice, and negative controls containing sterile water were included.
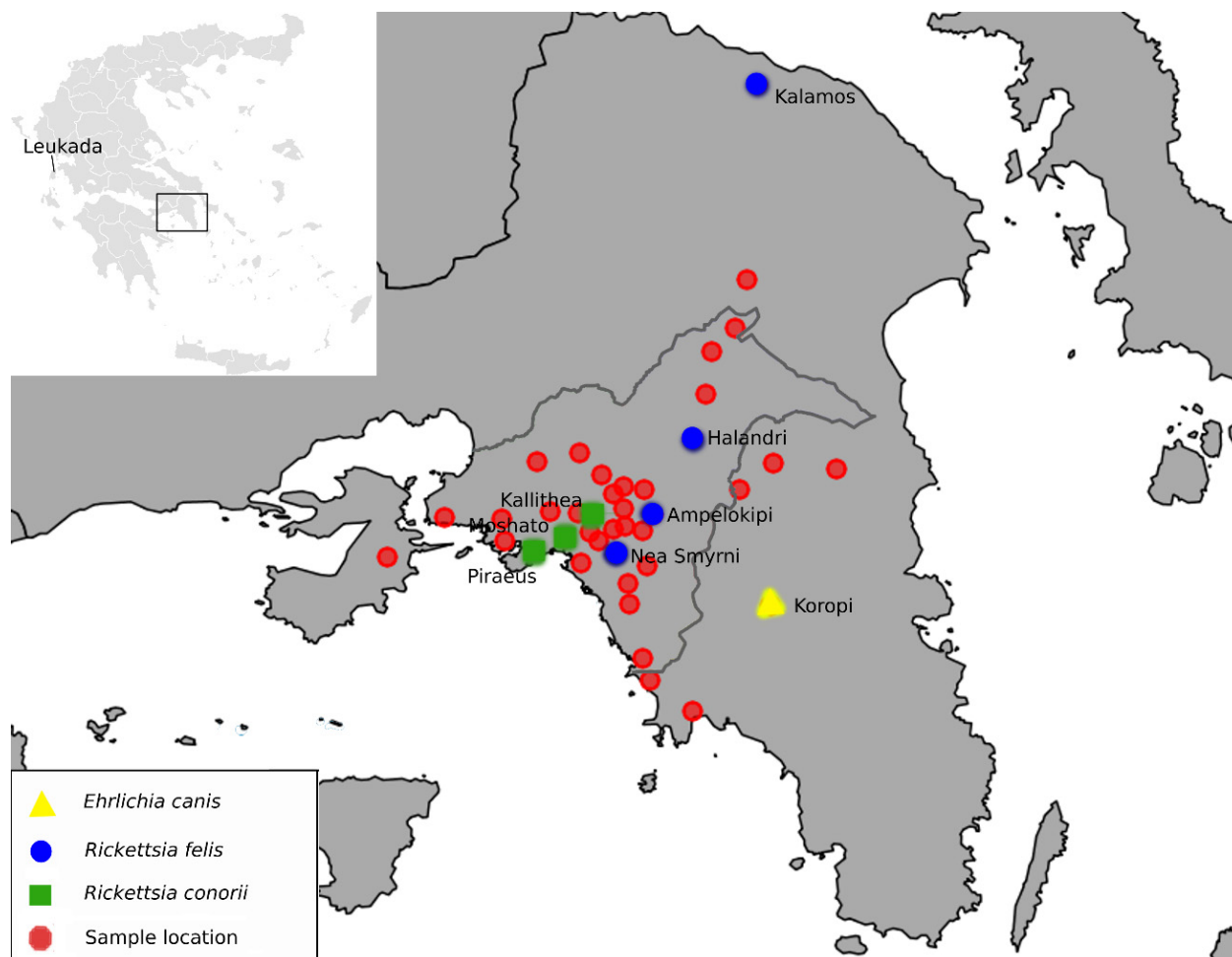

Figure 1. Sampling sites in Attica region are represented by red circles. Colored shapes indicate locations where ectoparasites positive for *R. felis* (blue), *R. conorii* (green), and *E.canis* (yellow) were collected. (Athens urban area is outlined by a gray line. Leukada island is indicated in the upper left corner of the map).

All positive PCR products from ticks and nine positives from fleas were purified, using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's instructions and sequenced in both directions using the PCR primers (CeMIA, Larissa, Greece). 16S rRNA sequences were aligned manually using CLUSTAL X, and phylogenetic analysis was performed using PHYLIP [18] by applying the maximum likelihood analysis.

Additionally, all samples from fleas that tested positive for *Anaplasmataceae* 16S rRNA were screened for *Wolbachia* using a *Wolbachia* spp. (panWolbachia) qPCR targeting the 23S rRNA gene, as previously described [19]. Samples that tested positive were then further examined for *Wolbachia pipientis* by the qPCR targeting *rpoB* gene, as previously described [20].

All flea DNA samples were also screened for *Rickettsia felis* by qPCR targeting the biotin synthase gene as previously described [21]. As cat fleas have been implicated as the most probable vectors of this rickettsial pathogen [22], the fleas in our study were tested directly for *R. felis* and not for the presence of other SFG rickettsial agents.

All tick samples were initially screened for the presence of SFG rickettsial DNA by qPCR targeting a fragment of the *gltA* gene as previously described [23]. SFG *Rickettsia* positive DNA samples were further tested for *Rickettsia conorii* by qPCR targeting a putative acetyltransferase gene, as previously described [23]. The PCR assays used are presented in Table 1.

Table 1. PCR assays used for the screening for rickettsial agents.

Target Species	Target Gene	PCR Primer and Probe Sequences (5′–3′)	Product Size (bp)	Reference
SFG *Rickettsia* genus specific	*gltA*	F: GTG AAT GAA AGA TTA CAC TAT TTAT R: GTA TCT TAG CAA TCA TTC TAA TAG C FAM-CTA TTA TGC TTG CGG CTG TCG GTT C-TAMRA	166	[23]
Rickettsia conorii	Putative acetyltransferase	F: TTG GTA GGC AAG TAG CTA AGC AAA R: GGA AGT ATA TGG GAA TGC TTT GAA FAM-GCG GTT ATT CCT GAA AAT AAG CCG GCA-TAMRA	116	[23]
Rickettsia felis specific	Biotin synthase	F: ATG-TTC-GGG-CTTCCG-GTA-TG R: CCG-ATT-CAG-CAGGTT-CTT-CAA 6-FAM-GCT-GCG-GCGGTA-TTT-TAG-GAA-TGGG-TAMRA	120	[21]
Anaplasmataceae	16S rRNA	EHR16SD: GGT ACC YAC AGA AGA AGTCC EHR16SR: TAG CAC TCA TCG TTT ACAGC	345	[17]
PanWolbachia	23S rRNA	F: CCA AAA TTA CAG CTA AGT GG R: AGT GAG CTG TTA CGC TTT CT 6FAM-TACAGCTAGGAGGTTGGCTT-TAMRA	100	[19]
Wolbachia pipientis	*rpoB*	F: TCATGCGCGTTCAGTAG-GAC R: TGCCCAACATTC-CATTTCAC 6FAM-AGGAAAGTCTCATTTTGGTGGTAGCG-TAMRA	103	Unpublished

3. Results

A total of 145 animals were examined, 92 cats and 53 dogs, from 39 localities in Attica distributed in 7 regional units. The numbers of collected ectoparasites per regional unit were as follows: 44 from South Athens, 37 from Central Athens, 25 from North Athens, 19 from East Attica, 17 from Piraeus, 2 from West Athens, and 1 from Salamina (an island in the Attica area). One ectoparasite from each animal was used: 104 fleas (86 from cats (13 owned/73 strays)/18 from dogs (12 owned/6 strays)) and 41 ticks (6 from cats (1 owned/5 strays)/35 from dogs (16 owned/19 strays)). In the case of many fleas/ticks, we chose to examine only one ectoparasite per animal because of financial issues with the processing of the ectoparasites. All fleas were identified as *Ctenocephalides felis* (97♀/7♂), and

ticks were identified as *Rhipicephalus sanguineus* s.l. (34♀/4♂), *Rhipicephalus* sp. (2 nymphs), and *Ixodes* sp. (1♀).

Conventional PCR was positive for the *Anaplasmataceae* 16S rRNA gene in 61/104 (58.7%) fleas and 2/41 (4.9%) ticks. The sequencing of PCR products from 9 of the fleas yielded *Wolbachia pipientis*. A blast analysis of these PCR sequences revealed 100% homology with various *W. pipientis* strains from Spain (GenBank: LN864488) and USA (GenBank: CP051156, MF944223). The presence of *W. pipientis* in a total of 64/104 (61.5%) fleas was consequently confirmed using qPCRs for panWolbachia and for *W. pipientis* targeting *rpoB* (Table 2).

Sequencing of the two PCR products obtained from the ticks, both from dogs, yielded *Ehrlichia canis* in one *Rhipicephalus sanguineus* s.l. and *Candidatus* Midichloria mitochondrii in the *Ixodes* sp. (Table 2). The *E. canis* sequence had 100% homology with strains from Greece (GenBank: MN922610), USA (NCBI Reference Sequence: NR_118741) and China (GenBank: CP025749). The *Ca.* Midichloria mitochondrii sequence from the *Ixodes* sp. was 100% homologous with strains from Portugal (GenBank: KX359181), France (GenBank: KU559921), and Italy (GenBank: OM982404, HF568841, CP002130). Sequences derived from this study were deposited in GenBank with Accession Numbers ON678202-ON678207 and OP490629-OP490633.

All fleas and ticks were also tested to assess the prevalence of *Rickettsia* bacteria using qPCR. *R. felis* qPCR was positive in 5/104 (4.8%) fleas. SFG Rickettsia qPCR was positive in 3/41 (7.3%) ticks, two from cats and one from a dog. Consequent qPCR identified them all as *R. conorii* (Table 2).

Table 2. Rickettsial agents detected by PCR in fleas and ticks collected from dogs and cats in Attica, Greece.

Rickettsial Agent	Pathogen/ Endosymbiont	Prevalence (%)	Examined Ectoparasite	Method of Detection	Sequence Identity (%)	Query Cover (%)	GenBank ID
Anaplasmataceae	*Ehrlichia canis*	2.4%	*R. sanguineus* s.l.	PCR	100% (305/305 bp)	100%	MN922610
	Ca. Midichloria mitochondrii	2.4%	*Ixodes* sp.	PCR	100% (305/305 bp)	100%	KX359181
	Wolbachia pipientis	58.7% [a]	*C. felis*	PCR	100% (305/305 bp)	100%	CP051156
	Wolbachia pipientis	61.5%	*C. felis*	qPCR	na [b]		na [b]
SFG Rickettsiae	*Rickettsia conorii*	7.3%	*R. sanguineus* s.l.	qPCR	na [b]		na [b]
	Rickettsia felis	4.8%	*C. felis*	qPCR	na [b]		na [b]

[a] A subgroup of *Anaplasmataceae* PCR-positives was sequenced. [b] Not applicable.

4. Discussion

Data on rickettsial zoonotic agents in Greece derive mainly from human cases and animal studies, in certain areas of the country. Ectoparasite studies are few and have been mainly conducted in Northern Greece [7–11] and certain islands [12]. There are no studies of arthropod-borne (particularly tick-borne) zoonotic infections in Attica, although a very large proportion of the entire Greek population resides in this prefecture [24]. In the present study, all fleas collected from cats and dogs were identified as *C. felis*, confirming the wide distribution of cat fleas on dogs, observed in other ectoparasite studies [25]. *C. felis*, the cat flea, is the commonest flea found on cats and dogs and can inhabit human dwellings [26], where it frequently bites humans. *C. felis* transmits *R. felis* and *R. typhi* and can serve as a vector of *R. typhi* to humans [27]. Although *R. felis* is harbored by various hematophagous arthropods, including fleas, ticks, mites, and mosquitoes, its only known biological vector and main reservoir is *C. felis* [28].

4.1. Rickettsia Felis

In the present study, *R. felis* was detected in five *C. felis* fleas from four different localities Figure 1, four from stray cats and dogs and one from an owned dog.

Our results are supported by the findings of a previous study that detected *R. felis* and *R. typhi* in cat blood from stray and free-roaming cats in four areas of Greece, including

Athens [29]. In another Greek study, *R. felis* was also found in *C. felis* fleas [30]. There is serological evidence of the mostly subclinical infection of cats, dogs and other mammals; however, their role as *R. felis* reservoirs has not been fully elucidated. *R. felis* causes flea-borne spotted fever (or cat flea fever) in humans, an emerging rickettsial infection that presents with an acute febrile syndrome, similar to murine typhus, with which it may have previously been confused, based on serological diagnosis alone [31]. Many cases have been reported from countries around the Mediterranean [31] and sub-Saharan Africa, where asymptomatic infection in humans has also been described [22]. Our detection of *R.felis* in *C. felis* from owned animals indicates a possible route of *R. felis* transmission to human hosts and merits further investigation.

4.2. Rickettsia Conorii

R. conorii was detected in three *Rhipicephalus sanguineus* s.l. ticks from three localities Figure 1, two of which were from an owned cat and an owned dog and one from a stray cat. *R. conorii* has been previously detected in *R. sanguineus* and *R. turanicus* ticks from dogs, in different areas of Greece [7,12]. It has also been detected in the blood of dogs from four Greek islands, in the population of Northern Greece, and in a patient from Crete, by serological and molecular methods [32–34].

To our knowledge, this is the first detection of *R. conorii* in *R. sanguineus* s.l. ticks in the Attica region, notably, from dog ticks found on cats. *R. conorii* causes Mediterranean Spotted fever (MSF) syndrome. It is an epidemiologically complex disease, affected by numerous factors, including human behavior and vector interaction with the host. Its control, particularly in an epidemic setting, is a challenging feat and requires a better understanding of the reservoirs of the disease and their relationship to human infection [35]. In this context, the detection of *R. conorii* in cat ectoparasites in our study suggests a potential role for cats, as well as dogs, in the transmission of the pathogen to humans.

4.3. Ehrlichia canis

In the present study, *E. canis* was detected in one *R. sanguineus* s.l. tick from an owned dog, Figure 1. Although many reports from Greece have been recorded, they mainly concern dog epizootology [36–38]. Its presence in dog and cat blood in various areas of Greece has been documented by several studies, using molecular [39] and serological [29,32,40] methods. Our 16S rRNA sequence was found to be 100% homologous to one isolated from dog blood in Crete [39], thus supporting a possible role of *R. sanguineus* s.l. as vector of *E. canis* in this area. This is also supported by phylogenetic analysis based on 16S rRNA, which clusters our sequence (ON678206) with *E. canis* from Greece and relates it with other *E. canis* isolates (Figure 2). To the best of our knowledge, this is the first detection of *E. canis* in this vector in Greece.
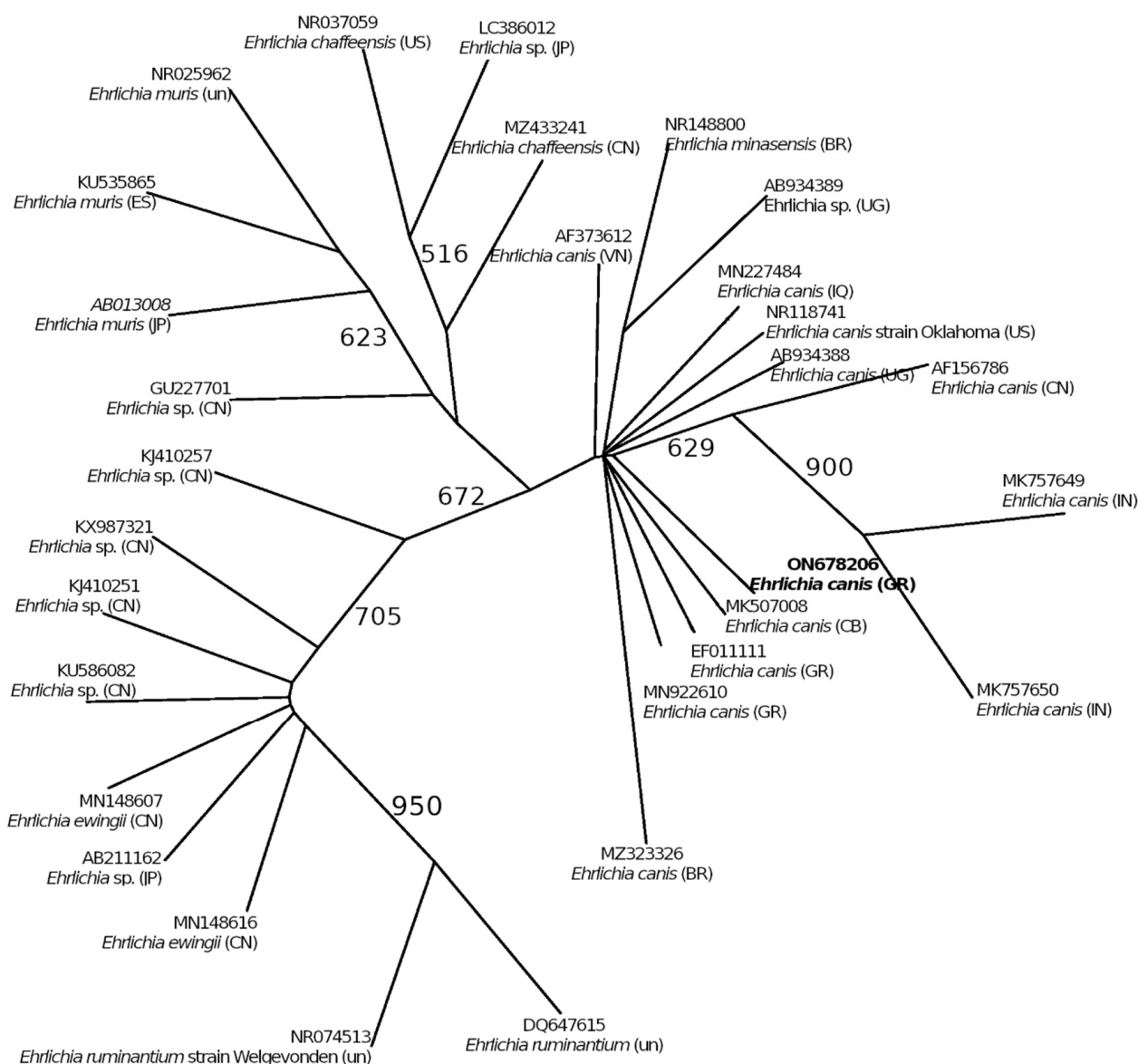
Figure 2. Maximum likelihood phylogenetic tree of *Ehrlichia canis* based on a 305 bp fragment of 16S rRNA. The numbers at the nodes indicate percentage bootstrap replicates of 1000. The sequence obtained in the present study is indicated in bold type.

E. canis, the causative agent of canine monocytic ehrlichiosis, is closely related to *E. chaffeensis*, the agent of human monocytic ehrlichiosis; although chiefly a canine pathogen, it occasionally infects humans and felines [41,42]. It has been isolated from human blood [43,44], and it has been proposed that it can cause both asymptomatic and symptomatic human infection [45].

4.4. Wolbachia pipientis

W. pipientis was detected in 61.5% of our *C. felis* samples. *W. pipientis* is found in arthropods as well as filarial nematodes populations [46]. Its presence may affect the host in various ways, including cytoplasmic incompatibility, male killing, induced parthenogenesis, and feminization [46,47]. *Wolbachia* is frequently found within insects, including potential human disease vectors such as fleas of veterinary importance, e.g., *C.felis* [48,49]. Our findings are in accordance with surveys from France (17.8%) [50] and Spain (69.4%) [51], more so with the latter, because the majority of fleas in both studies were female.

4.5. *Candidatus Midichloria mitochondrii*

Candidatus Midichloria mitochondrii was found in an engorged female Ixodes sp. tick from an owned dog. The tick may have been brought to Athens from the island of Leukada Figure 1, where the dog owner was on a trip before visiting the veterinary clinic. This is the first report of the detection of both an *Ixodes* sp. and *Ca.* M. mitochondrii in the greater Athens area. In Northern Greece, *Ca.* M. mitochondrii has been detected in *Ixodes ricinus* tick pools from goats [10]. Our sequence was 100% homologous to sequences isolated from *I. ricinus* from Portugal (GenBank: KX359181), from France (GenBank: KU559921), Italy (GenBank: CP002130) and from sequences isolated from human (GenBank: OM982404) and horse blood (GenBank: HF568841) in Italy. *Ca.* Midichloria mitochondrii is a Gram(-) intracellular bacterium that colonizes the mitochondria of host cells. It is mainly found in *I. ricinus*, with nearly 100% prevalence reported in females [52]. Although the mechanisms underlying this symbiotic interaction have not been elucidated, in silico metabolic reconstruction suggests it may play a nutrient-provisional role (B complex vitamin biosynthesis) that may enhance host fitness [52]. It has been shown that some patients exposed to *I. ricinus* bites are seropositive for *Ca.* M. mitochondrii antigens, suggesting that these antigens may be secreted together with tick saliva, thus triggering an immune response [53]. Alternatively, it is possible that ticks could transmit this endosymbiont to humans and consequently to other ticks, completing a tick-to-human-to tick transmission route, similar to that described for tick-borne pathogens. Indeed, this endosymbiont has been detected in the blood of humans and mammals bitten by ticks [54].

Many endosymbionts have been reported in arthropods. Particularly in ticks, their co-existence with pathogens is the subject of investigation since ticks are major vectors of pathogens affecting humans and animals. According to Bonnet et al. ''Ticks represent a compelling yet challenging system in which to study microbiomes and microbial interactions'' [55]. The microbiota of important tick species needs to be explored, in view of its potential role in disease ecology and epidemiology and thus of its public health implications. Moreover, given the experimental evidence that the antibiotic cleansing of bacterial endosymbionts had a negative impact on tick fitness, survival, and competence, the development of anti-microbiota vaccines presents an intriguing environmentally friendly alternative for the control of ticks and tick-borne pathogens [52].

In conclusion, the present study provides epidemiological data for the presence of various rickettsial species in the ectoparasites of cats and dogs in a little studied area, the greater Athens urban and peri-urban region, most notably *R. conorii* and *E. canis* in *R. sanguineus* s.l ticks. Certain epidemiological findings are described for the first time in this particular area, an area that is heavily populated, while also housing great numbers of stray cats and dogs, which frequently interact with humans. Moreover, the ectoparasites—cat fleas and brown dog ticks—found on the animals belong to widely distributed species that often enter human settlements in urban and peri-urban settings. All of the above underline a growing need for increased vigilance in the surveillance of vector-borne diseases in the area, as well as for further studies of vector and pathogen distribution to assess their potential implications for public health in this metropolitan area.

Data Availability Statement: All data used and presented in this study are available in public repositories as described in the results section.

Conflicts of Interest: The authors declare no conflict of interest.

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