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Anaplasma phagocytophilum in ticks from parks in the Emilia-Romagna region of northern Italy

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Summary

The aim of this study was to assess the prevalence and phylogenetic characterisation of *Anaplasma phagocytophilum* in ticks in three parks of the Emilia-Romagna region of northern Italy, an area in which no survey of this agent had previously been conducted. A total of 360 tick samples were analysed; 292 were sourced from the environment and 68 from animals and humans. Real-time polymerase chain reaction revealed that 33 tick samples (9.2%) were positive for *A. phagocytophilum*. *Ixodes ricinus* was the only species found positive from the samples retrieved from the environment and was the most commonly infected among ticks removed from hosts. Sequence analysis of the 23S-5S rRNA gene performed on 23 samples revealed six variant sequences that differed by only a few nucleotides when compared to the GenBank sequences from humans, horse and small mammals. *Msp4* gene sequences obtained from 7 samples were compared to those described in ruminants, especially roe deer (*Capreolus capreolus*) and goat isolates from different countries. The results of this study provided evidence of the circulation of *A. phagocytophilum* in the sites studied and indicated the possible involvement of wild ruminants. Additional studies that extend the sampling areas, or cover different sites, would contribute to a better understanding of the ecology and disease dynamics of *A. phagocytophilum* in northern Italy and would provide valuable information on zoonotic risks.

Keywords

Anaplasma phagocytophilum, Anaplasmosis, Emilia-Romagna, Italy, *Ixodes ricinus*, *Msp4*, Polymerase chain reaction, rRNA, Tick.

Anaplasma phagocytophilum in zecche raccolte in parchi della regione Emilia-Romagna (Italia)

Riassunto

La ricerca ha avuto l'obiettivo, in assenza di precedenti indagini, di studiare la prevalenza e la filogenesi di Anaplasma phagocytophilum, *isolato da zecche raccolte in 3 parchi della regione Emilia-Romagna, in Nord Italia. Sono stati analizzati 360 campioni di zecche, 292 raccolti in ambiente e 68 da animali e uomo. Trentatrè campioni di zecche (9,2%) sono risultati positivi ad* A. phagocitophylum *mediante real-time PCR.* Ixodes ricinus *è stata la specie trovata con maggior frequenza infetta tra quelle raccolte dall'ospite e la sola risultata positiva tra quelle raccolte nell'ambiente. L'analisi delle sequenze del gene 23S-5S rRNA, effettuata su 23 campioni, ha evidenziato 6 varianti differenti che differiscono per pochi nucleotidi dalle sequenze di ceppi presenti in GenBank isolati da uomo, cavallo e micromammiferi. Le sequenze del gene* Msp4, *ottenute da 7 campioni,*

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sono risultate correlate con isolati descritti nei ruminanti, in particolare caprioli e capre, in differenti paesi. I risultati ottenuti hanno evidenziato la presenza nelle aree studiate di A. phagocitophylum *che, presumibilmente, circola nei ruminanti selvatici. Ulteriori indagini, ampliando le aree di studio o scegliendo differenti siti di campionamento, potrebbero essere utili per approfondire la conoscenza sull'ecologia e la dinamica dell'infezione nel Nord Italia, al fine di valutare il rischio zoonosico.*

Parole chiave
Anaplasma phagocytophilum, Anaplasmosi, Emilia-Romagna, *Msp4*, *Ixodes ricinus*, rRNA, Italia, Zecche.

Introduction

Anaplasma phagocytophilum is an obligate, intracellular bacterium transmitted by ticks. It is the agent of tick-borne fever in ruminants and granulocytic anaplasmosis in dogs, cats and horses. In humans, *A. phagocytophilum* can cause human granulocytic anaplasmosis which results in different clinical manifestations, from asymptomatic cases to febrile episodes and rare mortality (3). This is the second most common tick-transmitted infection in the United States and it is also endemic in some countries of Europe and Asia. In Europe, human cases have increased in Austria, Estonia, the Netherlands, Poland, Slovenia, Spain, Sweden and the far eastern areas of Russia (10). In Italy, two clinical cases of human granulocytic anaplasmosis (called ehrlichiosis) were described in the north-eastern region of the country (31) where a rate of 6% human seroprevalence was observed (4).

Various *Ixodes* species can transmit *A. phagocytophilum*, such as *Ixodes ricinus* in Europe, *I. persulcatus* in Asia, *I. scapularis* in the eastern areas of the United States and *I. pacificus* in California (16). Different wild animals serve as reservoirs, such as rodents and cervids (12, 15). Although humans can rarely be infected by direct contact with infected animal blood (3), tick bites are the most common route of transmission. The life-cycle of the tick includes three feeding stages, namely: larva and nymphs which usually feed on small mammals, and adults which tend to feed on larger mammals (23). Both larval and nymphal ticks acquire *A. phagocytophilum* from infected hosts and then trans-stadially transmit the pathogen during molting. There are no reports of trans-ovarial transmission and *A. phagocytophilum* has not been reported in unfed larvae (25).

In Italy, anaplasma infections in animals are mostly reported in Sicily (35) and southern Italy (24); there are serological and molecular reports from different mammals in central Italy (13, 14). Roe deer (*Capreolus capreolus*) have been shown to be a potential reservoir in the northern-eastern areas of Italy (6) and a recent study documents infection in fallow deer (*Dama dama*) and feeding ticks from an Italian nature reserve (Mesola wood) in the Emilia-Romagna Region of northern Italy (37). The aim of this study was to assess the prevalence of *A. phagocytophilum* in ticks in some parks of the provinces of Bologna and Ravenna in the Emilia-Romagna Region, where no studies on the agent had previously been performed, and to phylogenetically characterise any unique strains.

Material and methods

Tick sampling

Environmental tick sampling was performed every two weeks during the period from April to October 2010 in four sites of the Emilia-Romagna Region as follows:

- Cà de Mandorli (44°26′32″N-11°26′30″E) (site CM) in the Gessi Bolognesi e Calanchi dell'Abbadessa Regional Park
- Ciagnano (44°24′57″N-11°27′16″E) (site CG), in the Gessi Bolognesi e Calanchi dell'Abbadessa Regional Park
- Monteveglio Abbey Park (44°28′11″N-11°5′14″E) (site MV)
- Carnè Park (44°13′34″N-11°44′22″E) (site CR).

The latter site was only sampled during the period from May to October due to closure of the park. The environment was characterised by scrub, undergrowth and dry leaves (on the trails) or covered by grass (grass-covered areas). The presence of wild ungulates was reported in all these sites; there were signs of

wild boar activity and some deer were observed during sampling. For each site, trails or areas of different width were identified as follows:

- CG and MV were characterised by trails bordering wooded areas
- in CM two areas were sampled near the trails
- in CR the sampling was performed in two picnic areas and on a trail.

Ticks were collected by continuous flagging with a 1 m × 1 m white cotton cloth from transects of 20 m along the uphill side of the trails which typically have higher tick densities than the downhill sides (19); the totality of grass-covered areas were flagged. Ticks were removed from the flag every 2 m (subtransect) according to the study by Li and Dunley (20) to reduce the effect of tick drop-off. The total surface flagged was 95 m² at CM, 120 m² at CG, 180 m² at MV and 356 m² at CR. Adult ticks were also collected from different domestic animals (dogs, horses, a cat and a bovine) by some private veterinarians and from humans in the same zones ('AN' samples or 'feeding ticks from animals'). All ticks were collected in tubes with 70% alcohol and were identified by taxonomic keys (18, 23). They were stored until use for molecular biology.

DNA analyses

DNA was extracted with Nucleo Spin Extract II (Macherey-Nagel GmbH & Co. KG Düren), according to the instructions of the manufacturer, except for an increased period of lysis of ticks from 10 min to 30 min. Ticks were processed individually in the case of adults or in pools of five in the case of nymphs. A total of 360 samples were screened for the presence of *A. phagocytophilum* DNA with the highly sensitive real-time Taqman polymerase chain reaction (PCR) (StepOnePlus, Applied Biosystem, Life Technologies Corporation, Carlsbad, California) assay, targeting the multiple-copy *msp2/p44* gene (11). Samples with a cycle threshold (Ct) value of <40 and a characteristic amplification curve were considered positive. A nested PCR protocol was performed on samples with a Ct value <38. Two genes were analysed, namely: the 23S-5S rRNA intergenic spacer and major surface protein 4 (*msp4*). All PCR reactions were conducted in an Eppendorf Mastercycler® ep thermal cycler (Eppendorf AG, Hamburg). The 23S-5S rRNA region was amplified with external primers ITS2F (5′-AGGATCTGACTCTAGTACGAG-3′) and ITS2R (5′-CTCCCATGTCTTAAGACAAAG-3′), and internal primers ITS2iF (5′-ATACCTCTGG TGTACCAGTTG-3′) and ITS2iR (5′-TTAACTT CCGGGTTCGGAATG-3′) at the following thermocycler conditions: 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min, followed by a 4 min extension at 72°C (29). For *msp4*, the external primers were MSP4P3 (5′-TG AAT TAC AGA GAA TTG CTT GTA GG-3′) and MSP4P5 (5′-TTA ATT GAA AGC AAT CTT GCT CCT ATG-3′) and the internals were MSPF1(5′-CTA TTG G(C+T)G G(A+G+C+T)G C(C+T)A GAG T-3′) and MSPR2 (5′-GTT CAT CGA AAA TTC CGT GGT A-3′) (8).

The thermocycling conditions were as follows: 94°C for 5 min, 35 cycles at 94°C for 20 sec, 58°C for 20 sec and 72°C for 50 sec, followed by an extension of 5 min at 72°C. All reactions were performed in 25 µl volumes with 2.5 µl DNA for the first round and 1 µl for the second, 2.5 µl of each primer (concentration: 5 µM), and 12.5 µl of GoTaq Green Master mix (Promega, Madison, Wisconsin). Amplified DNA was observed on a 1% agarose gel stained with GelStar nucleic acid stain (Lonza, Rockland, Maine). Appropriate sized bands were excised and cleaned with a Qiagen gel extraction kit (Qiagen, Valencia, California).

Sequence analyses

Gel-extracted PCR products were sequenced on an ABI 3730 sequencer (Davis Sequencing, Davis, California). The sequences were corrected by visual analysis and trimmed to a final length of 248 bp for the 23S-5S rRNA fragment and 331 bp for the *msp4* gene through BioEdit 7.0.9 and subjected to database search by BLAST (2). Phylogenetic analyses were performed for both genes with the Mega 5 programme (34) using a maximum likelihood method under the Kimura 2-parameter model and bootstrap resampling 1 000 times.

Statistical analyses

A χ^2 test was performed using the program EpiInfo 3.5.1 to evaluate different positive rates among sampling sites, month of sampling and among nymphs and adults of *Ixodes ricinus*. A value of $p<0.05$ was considered statistically significant.

Results

A total of 360 samples obtained from 1 165 nymphs from the environment (233 samples) and 127 adults (59 from the environment and 68 from animals and humans) were tested for the presence of *A. phagocytophilum* DNA. The most common species sampled was *I. ricinus*, from both the environment and from animals (dogs, cats and horses) or humans (Table I). A total of 33 samples (9.2%) were positive for *A. phagocytophilum* infection by real-time PCR. Positive samples were found in three sampling sites (CM, CG and MV) and among feeding ticks. *I. ricinus* was the only species found positive from the environment and was the most commonly infected species among ticks feeding on humans, dogs and cats. The other Ixodidae found positive from animals were *Rhipicephalus turanicus* from a dog and a horse, *R. sanguineus*, *R. bursa* from a dog and *I. acuminatus* also from a dog. No statistically significant difference was found in infection prevalence between nymphs and adults of *I. ricinus* ($p = 0.429$). The prevalence of *A. phagocytophilum* in questing ticks was significantly higher in April (15.8%) than in May (5.7%; $p = 0.03$) and June (4.2%; $p = 0.037$); no positive ticks were found during the other months. Significant differences of prevalence were found in site CR, where no infected ticks were found, compared to site CM ($p = 0.046$) and site MV ($p = 0.008$).

Of 26 samples with Ct values <38, the amplification of the 23S-5S rRNA gene was successful for 23 samples. Among these, only three strains were obtained from *I. ricinus* attached to animals: one from a dog and two from the same cat. We obtained six variants of the 23S-5S rRNA sequences (248 bp) differing by only a few nucleotides in comparison to the GenBank sequences from humans, horses and small mammals. In particular, a comparison of our sequences to the human strain HZ (Genbank accession # CP000235) revealed a group of seven strains with three single nucleotide polymorphisms (SNPs) (group A: two samples from site MV, three from site CG and two from feeding ticks) and another group of seven strains with two SNPs (group B: three samples from site CM, two from MV, one from CG and one from feeding ticks). In addition, we identified another group of two strains (CG39 and MV13) with three different SNPs and three single strains differing from one (CG26), two (MV19) and three (MV20) SNPs. (Table II).

Each different sequence type was submitted to the GenBank database (accession numbers: JQ040534-JQ040539). The phylogram obtained by ML analyses of the 23S-5S rRNA gene showed that our strains clustered together with the samples of mammalian origin from the United States available in GenBank and they were clearly separate from a lizard strain (JF487929) that was more closely related to *A. marginale* (AY048815), used as out-group. The clade that included our samples was further partitioned, with low bootstrap support, based on the SNPs described above (Fig. 1). The *msp4* gene was amplified from eight samples, as follows: AN10 and CG104 from group A, CM38 and CG110 from group B, MV13, CG26, MV19 and MV20, seven of which provided suitable sequences. Two variants that differed only by a single nucleotide substitution and that retained the same protein sequence were identified. The two sequence types were submitted to GenBank (accession numbers JQ068824 and JQ178338). The sequences were aligned with related sequences available from GenBank and the phylogenetic analysis showed that our isolates lay within a cluster including sequences from ruminants, especially roe deer and goat isolates from different countries (Fig. 2).

Table I
Prevalence of *Anaplasma phagocytophilum* in tick samples

Tick species	Site CM	Site CG	Site MV	Site CR	Animals (AN)	Total
Ixodes ricinus						
Nymphs	5/43 (11.6%)*	6/89 (6.7%)	8/56 (14.3%)	0/44 (0%)	0/0	19/232 (8.2%)
Adults	0/4 (0%)	2/27 (7.4%)	2/11 (18.2%)	0/10 (0%)	6/32 (18.7%)	10/84 (11.9%)
Ixodes acuminatus						
Nymphs	0/0	0/0)	0/0	0/0	0/0	0/0
Adults	0/0	0/2 (0%)	0/0	0/1 (0%)	1/1 (100%)	1/4 (25%)
Dermacentor marginatus						
Nymphs	0/0	0/0	0/0	0/0	0/0	0/0
Adults	0/1 (0%)	0/0	0/0	0/0	0/0	0/1 (0%)
Hyalomma marginatum						
Nymphs	0/0	0/0	0/0	0/0	0/0	0/0
Adults	0/0	0/3 (0%)	0/0	0/0	0/3 (0%)	0/6 (0%)
Hyalomma detritum scupense						
Nymphs	0/0	0/0	0/0	0/0	0/0	0/0
Adults	0/0	0/0	0/0	0/0	0/1 (0%)	0/1 (0%)
Hyalomma detritum detritum						
Nymphs	0/0	0/0	0/0	0/0	0/0	0/0
Adults	0/0	0/0	0/0	0/0	0/1 (0%)	0/1 (0%)
Scaphixodes frontalis						
Nymphs	0/0	0/1 (0%)	0/0	0/0	0/0	0/1 (0%)
Adults	0/0	0/0	0/0	0/0	0/0	0/0
Rhipicephalus bursa						
Nymphs	0/0	0/0	0/0	0/0	0/0	0/0
Adults	0/0	0/0	0/0	0/0	1/2 (50%)	1/2 (50%)
Rhipicephalus sanguineus						
Nymphs	0/0	0/0	0/0	0/0	0/0	0/0
Adults	0/0	0/0	0/0	0/0	1/7 (14.3%)	1/7(14.3%)
Rhipicephalus turanicus						
Nymphs	0/0	0/0	0/0	0/0	0/0	0/0
Adults	0/0	0/0	0/0	0/0	1/21 (4.7%)	1/21 (4.7%)
Total	5/48 (10.4%)	8/122 (6.6%)	10/67 (14.9%)	0/55 (0%)	10/68 (14.7%)	33/360 (9.2%)

* no. positive/no. examined (%) samples

Site CM questing ticks from Cà de Mandorli (44°26′32″N-11°26′30″E)
Site CG questing ticks from Ciagnano (44°24′57″N-11°27′16″E)
Site MV questing ticks from Monteveglio Abbey Park (44°28′11″N-11°5′14″E)
Site CR questing ticks from Carnè Park (44°13′34″N-11°44′22″E)

AN feeding ticks from animals

Table II
Differences in 23S-5S rRNA nucleotide sequences among *Anaplasma phagocytophilum* strains

Strain (accession number)	Host	Nucleotide position 2787	2820	2842	2847	2853	2854	2861	2881
HZ (CP000235)	*Homo sapiens*	C	A	G	A	C	G	A	A
MRK (JF451139)	*Equus caballus*	*	*	A	*	*	*	*	G
Group A (JQ040534)	*Ixodes ricinus*	*	*	A	*	*	A	G	*
Group B (JQ040535)	*Ixodes ricinus*	*	*	A	*	*	A	*	*
CG39/MV13 (JQ040536)	*Ixodes ricinus*	T	*	A	*	*	A	*	*
CG26 (JQ040537)	*Ixodes ricinus*	*	*	*	*	*	A	*	*
MV19 (JQ040538)	*Ixodes ricinus*	*	*	A	*	T	*	*	*
MV20 (JQ040539)	*Ixodes ricinus*	*	*	A	*	*	A	*	G
Nf (JF451141)	*Neotoma fuscipes*	*	*	A	*	*	*	*	*
Sg (JF451142)	*Sciurus griseus*	*	G	A	*	*	*	*	G

* nucleotides identical to the reference HZ isolate sequence

Group A MV11, MV21, CM11, AN10, AN12, CG104, CG56, CG21
Group B CM38, CG93, MV3, AN28, CG110, CM45, CM14, MV41

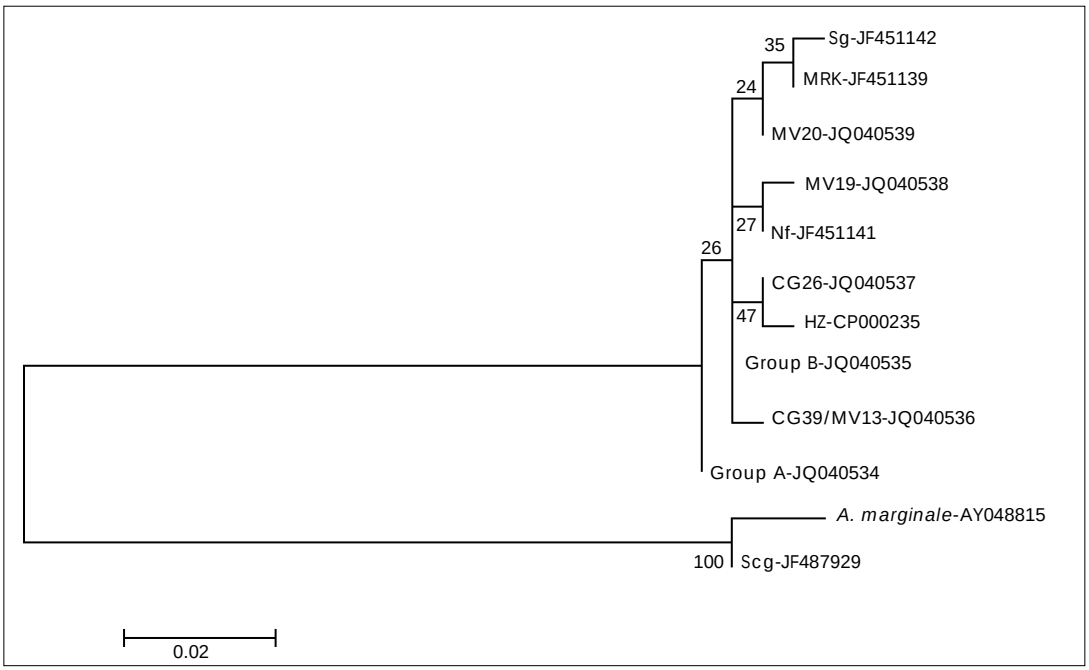

Figure 1
Phylogenetic analyses of *Anaplasma phagocytophilum* strains based on the 23S-5S rRNA using the ML method Kimura 2 parameter model and bootstrap analysis of 1 000 replicates
Group A: MV11, MV21, CM11, AN10, AN12, CG104, CG56, CG21
Group B: CM38, CG93, MV3, AN28, CG110, CM45, CM14, MV41

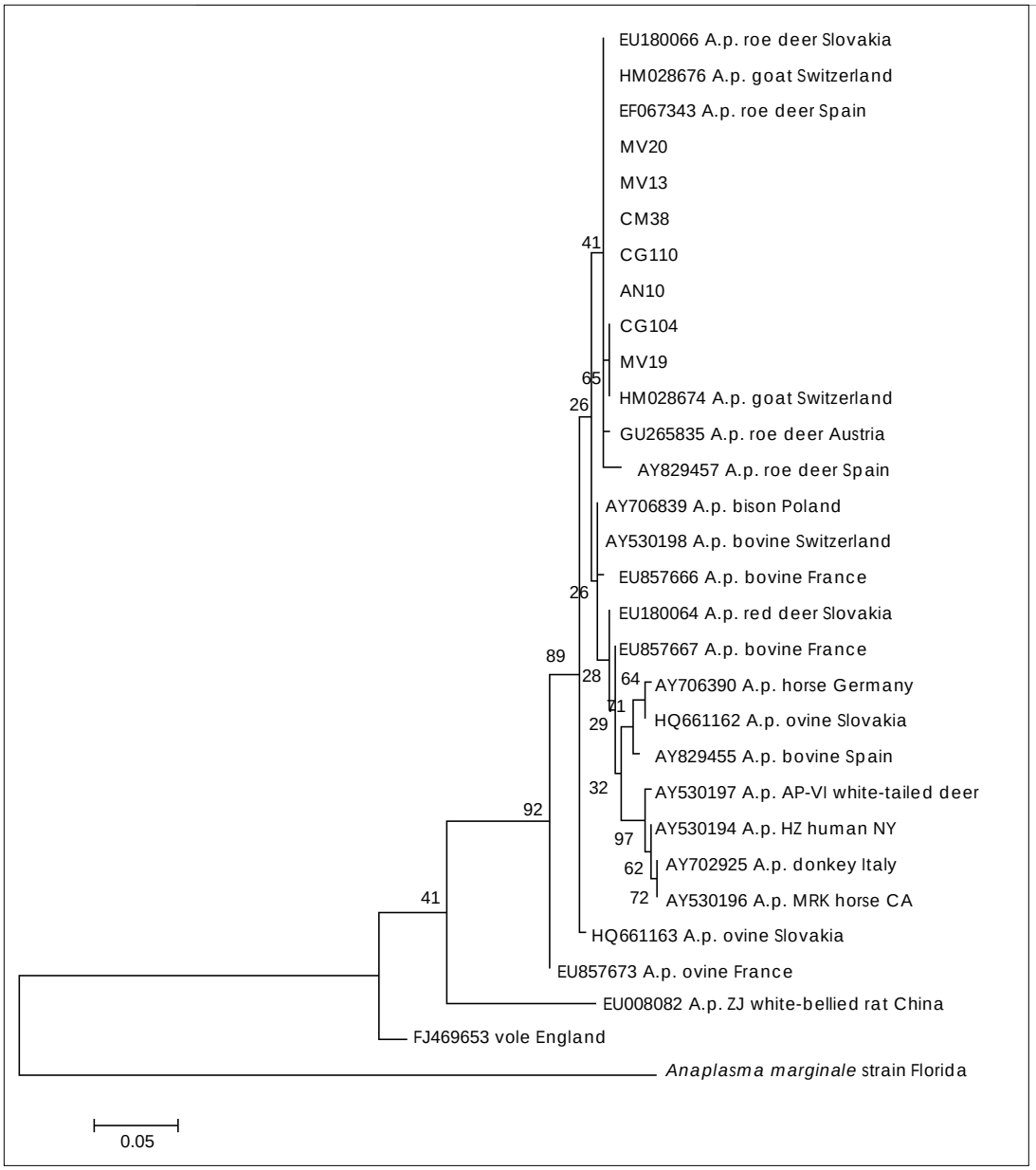

Figure 2
Phylogenetic analyses of *Anaplasma phagocytophilum* strains based on the *msp4* gene using the ML method Kimura 2 parameter model and bootstrap analysis of 1 000 replicates
Sequences submitted to GenBank: CM38 (JQ068824) identical to MV13, MV20, CG110, AN10 and MV19 (JQ178338) identical to CG104

Discussion

To date, the presence of *A. phagocytophilum* in Italy has been investigated primarily in the central and southern regions from a variety of animals including horses, cattle, porcupine, sheep, dogs, cats, fallow deer, fox and mice (13, 14, 24, 26, 35, 36). Veronesi *et al.* (37) reported the prevalence of *A. phagocytophilum* in fallow deer to be confined to a nature reserve in northern/central Italy and Carpi *et al.* (6) reported prevalence in roe deer in north-eastern Italy. These studies also analysed ticks that had been recovered from animals but only a few questing ticks were analysed in Sicily (36).

In a recent survey conducted in northern-eastern Italy revealed a prevalence rate of 1.5% in adult questing ticks (5). Conversely, surveys concerning the presence of *A. phagocytophilum* in ticks from the environment in the Emilia-

Romagna Region have not been reported previously. In our survey, the prevalence rate obtained was 7.9% in questing ticks and 14.7% in feeding ticks. Surveys conducted in other European countries (Norway, Poland and Switzerland) reported an infection prevalence of *A. phagocytophilum* in questing ticks that ranged from 1.4% to 17.5% (17, 22, 30) with the exception of Bulgaria where a prevalence rate of 33.9% was reported (7).

Among questing ticks, we detected *A. phagocytophilum* only in *I. ricinus*. Based on the literature, in Europe, *I. ricinus* is the only tick proven to be a vector of *A. phagocytophilum*, both in natural and experimental conditions (26). In its immature stages, this tick tends to infect common reservoir hosts, such as small mammals, rodents and insectivores (23). Among feeding ticks observed in our study, some of which were engorged, we found *I. ricinus*, *R. turanicus*, *R. sanguineus*, *R. bursa* and *I. acuminatus* to be positive for *A. phagocytophilum* by real-time PCR. Despite this finding, we cannot determine whether they play a vector role in the epidemiology of this disease or if they were positive only because the host was infected. Unfortunately it was not possible to analyse the blood of the animals from which ticks were collected.

A comparison of 23s-5s rRNA gene sequences from our strains with the few isolates present in GenBank from humans, horses and small rodents from the United States indicates that our strains are related to strains collected from these animals, but they are considerably different from a lizard strain also from United States that was hypothesised to be a different *Anaplasma* species (29). The few SNPs found among the mammal isolates did not result in distinct phylogenetic clustering even though the samples came from different hosts and geographic origins.

The *msp4* gene has been widely used to characterise *A. phagocytophilum* strains. Different authors have used this gene to delineate the host tropism of the various strains isolated (8, 9, 32). The polymorphic multiple-gene *pfam01617* family encoding the immunodominant major surface proteins (msp) is likely to be important in the pathogenesis in the mammalian host and, therefore, variation in the coding region could have an effect on host tropism, pathogenicity and persistence in animals (21). For phylogenetic analyses, a genetic marker which is conserved through the life-cycle of the bacterium is preferable. Previous *in vitro* studies assessed a good genetic stability of the *msp4* gene both in human HL-60 and tick ISE6 cells, thereby making it suitable for phylogenetic analyses concerning the host tropism (8). The analysis of this gene showed that our strains were related to isolates from ruminants, in particular to those from roe deer and goats. This suggests that these strains could be of wild ruminant origin, most likely roe deer, which are widely reported in the areas sampled. The presence of *A. phagocytophilum* in the wild ruminant population, mainly roe deer and red deer, and in ticks associated with these ruminants, has been observed in several areas of Europe (6, 27, 33). Our isolates from feeding ticks from the cat and the dog were also included in this clade. There are no *A. phagocytophilum msp4* sequences from dogs or cats in GenBank so, using this genetic marker, we cannot determine if these strains were of ruminant origin and therefore already present in the vector before the blood-meal, or if they originated from the blood of these hosts.

Using other genetic marker (i.e. 16S rRNA gene) Alberti *et al.* (1) highlighted that equine and canine *A. phagocytophilum* strains present in Sardinia were phylogenetically related to pathogenic strains from the United States. On the other hand, strains causing human granulocytic anaplasmosis in the United States and in parts of Europe, were different from those that caused infection in ruminants. Furthermore, experimental infections of ruminants with a human strain were unsuccessful (28). Consequently, whether the strains distributed in the area of study could be pathogenic for humans needs to be elucidated.

Conclusions

The results of this study provide evidence of the circulation of *A. phagocytophilum* in two out of three parks studied in the Emilia-Romagna Region. The strains found in the tick populations of these sites are related to strains of ruminant origin. Further studies, extending the sampling areas or selecting different sites, could be useful to better delineate the ecology and disease dynamics of *A. phagocytophilum* in northern Italy and to clarify the inherent zoonotic risk.

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Conflict of interest

All authors declare that they or their institutions have no financial and personal relationship with other people or organisations that may compromise or inappropriately influence or bias their work.

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