Anaplasma capra in sheep and goats on Corsica Island, France: A European lineage within A. capra clade II?

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Abstract: Anaplasmosis is a tick-transmitted disease due to several species of the genus *Anaplasma*. In 2019, we demonstrated the presence of *Anaplasma capra* in two deer species at a zoological park in mainland France. As we suspected its presence in Corsica, we surveyed 11 geographically distant sheep or goat farms. Using molecular tools such as nested PCR targeting 16S ribosomal RNA (rRNA), citrate synthase (*gltA*) and heat-shock protein (*groEL*) genes, we detected the presence of *A. capra* on 5/11 farms, in 26/108 blood samples (24%), in sheep as well as in goats. Genotyping and phylogenetic analysis of *A. capra* revealed that isolates from Corsica island grouped closely with *A. capra* isolates reported in red deer and swamp deer from a zoological reserve in mainland France, as well as in roe deer from Spain, in a separate and well supported clade within *A. capra* clade II. This third report of the tick-borne bacterium *A. capra* in Europe suggests a potentially larger presence of this pathogen on the European continent, on domestic, native as well as wild ruminants, a broad host range already described in Asian countries for this species.

Keywords: *Anaplasma capra*, sheep, goat, phylogeny, Corsica, Europe

1. Introduction

The order Rickettsiales was reorganized in 2001 based on 16S rRNA and *groEL* sequence analyses and six *Anaplasma* species have been recognized in this genus: *Anaplasma bovis, Anaplasma centrale, Anaplasma marginale, Anaplasma ovis, Anaplasma phagocytophilum and Anaplasma platys* (Dumler et al., 2001). Since this last reclassification of Anaplasmataceae twenty years ago, two new *Anaplasma* species have been identified, *Anaplasma odocoilei* and *Anaplasma capra,* as well as a large number of unclassified genovariants that cannot be assigned to known species (Rar et al., 2021). *Anaplasma* spp. are Gram-negative obligate intracellular bacteria transmitted by ticks to vertebrate hosts, which may include humans. In wild and domestic animals, bacteria infect hematopoietic cells and can cause a persistent infection, which means that these hosts represent important long-term reservoirs (Rar et al., 2021).

Anaplasma capra has been recently described in several Asian countries (China, Japan, Malaysia and Korea), in domestic as well as wild animals. *Anaplasma capra* was named for the first time in northern China following the isolation and characterization of this pathogen in 120 samples from asymptomatic goats in 2012 (Li et al., 2015). It was then reported in sheep from 12 provinces in China (Yang et al., 2017), in sheep and goats in China at 52 sites from 8 provinces (Peng et al., 2018) as well as in bovines from south Korea (Seo et al., 2018). In Japan, *A. capra*-related infection of wild ruminants was detected a few years earlier (Kawahara et al., 2006; Sato et al., 2009), and more recently also in Malaysia (Koh et al., 2018), China (Yang et al., 2018) and South Korea (Amer et al., 2019). The host range of this species is not limited to ruminants, as *A. capra* also infects dogs (Shi et al., 2019) and humans (Li et al., 2015), suggesting a wide host range panel for this species. *Anaplasma capra* was indeed detected in 28 patients in northern China, in a survey of 477 patients suffering from non-specific febrile

manifestations, including fever, headache, malaise, myalgia, chills and report of a tick bite within past 2 months (Li et al., 2015).

Based on *gltA* or *GroEL* phylogenies, two separate clades, I and II (thus named in Miranda et al., 2021, also previously referred to as genotypes 1 and 2) are consistently delineated within *A. capra* (Yang et al., 2018; Peng et al., 2018; Jouglin et al., 2019; Miranda et al., 2021). Clade I gathers *A. capra* sequences from sheep, goats, cattle, dogs, humans and ticks. Clade II also contains *A. capra* sequences from sheep and goats, and also from a variety of wild ruminants. The geographical distribution of *A. capra* is not restricted to Asian countries, as our recent study demonstrated its presence in red deer and swamp deer at a zoological park in France, the first report of *A. capra* in Europe (Jouglin et al., 2019). The occurrence of *A. capra* on roe deer was later reported in Spain, from several and geographically distant areas (Remesar et al., 2021). This recent finding confirms the presence of *A. capra* on deer species in non-captive conditions, and raises the question of its geographical distribution in Europe.

In this previous study (Jouglin et al., 2019), we discovered that the 23S rRNA deer sequences of *A. capra* aligned closely with a sequence from an unknown *Anaplasma* species provisionally named "Candidatus *Anaplasma mediterraneum*", detected from sheep in Corsica (Dahmani et al., 2017). Corsica is a French island located in the Mediterranean Sea, south east of the French mainland (Figure 1). Corsica consists of two administrative departments (Corse - du - Sud and Haute - Corse) and 360 communes (the smallest administrative unit in France) (Figure 1).

Animal husbandry represents an important economic activity (sheep, goats, cattle and pigs) on the island. Various species of *Anaplasma* have been detected in Corsica: *A. ovis, A. bovis, A. phagocytophilum* and *A. marginale* (Grech-Angelini et al, 2019; Dahmani et al., 2017) but the occurrence of *A. capra* was never reported. We decided to conduct the present survey to

specifically search for *A. capra* in sheep and goats on this island, and thus to eventually confirm autochthonous presence of *A. capra* in Europe.

2. Material and methods

2.1. Animal sampling

Between February and August 2020, we monitored infections on sheep and goat farms by *Anaplasma* spp. in collaboration with the Corsican Livestock Health Defense Group (GDS). As part of national surveillance for animal diseases, veterinarians collected goat and sheep blood samples for this study. The manipulation and blood sampling of animals was approved by the local animal ethical committee (CERVO request CERVO-2019-7-V). A total of 108 heparin blood samples were obtained from domestic animals. On each farm, up to ten animals were selected. In order to ensure that the animals had been in contact with ticks previously, the sampled goats and sheep were at least 2 years old. Eleven farms were included in this study, 7 sheep farms and 4 goat farms, 7 from Corse-du-Sud and 4 from Haute-Corse (Figure 1). They were localized in 9 geographically distant municipalities.

2.2. Molecular detection and characterization of Anaplasma spp.

Genomic DNA was extracted from blood following previously described protocols (Jouglin et al., 2017). DNA was stored at -20°C until further use. Firstly, all samples were tested by a PCR targeting the 16S RNA ribosomal gene: infection by *Anaplasma* spp. was screened using the Ana16Sup1/Ana16Sdo3 primer pair which produces an amplicon of 1089 bp. Then a nested PCR using the Ana16SIntup1/Ana16sdo1 primer pair was performed which produced a 581 bp amplicon (Jouglin et al., 2019). All positive amplicons were sequenced to identify the detected *Anaplasma* species. In order to further characterize the *Anaplasma* species, all samples were also subjected to the amplification of heat-shock protein (*groEL*) and citrate synthase (*gltA*) gene fragments, by nested PCR using *A. capra* specific primer sets (Li et al., 2015; Jouglin et al., 2019).

To prevent cross contamination, DNA extraction, amplification and detection of PCR products were done in separate rooms and even different buildings. A negative control (extraction and amplification) was included to control potential contamination in each of these two procedures. PCR products were analyzed on 1% agarose gels supplemented with ethidium bromide, and visualized under UV light. A 100-bp DNA ladder (Solis BioDyne, Estonia) was used. The amplicons were purified using ExoSAP-IT (Ozyme, France) and sequenced bi-directionally (Sanger conventional sequencing, GATC, Germany).

2.3. Sequence alignment and phylogenetic analysis

After the sequencing, chromatograms were analyzed with 4peaks (https://nucleobytes.com/4peaks) and cleaned sequences were identified using the BLASTn program (https://www.ncbi.nlm.nih.gov/Blast). All sequences were aligned together to identify potential nucleotide polymorphisms.

The sequences obtained in our study were first aligned with Muscle (Edgar, 2004) to determine redundancy and to keep only unique sequences in the phylogenetic analyses. For phylogenies, we built sequence collections of *Anaplasma* spp., choosing, when possible, several representative sequences from each species of *Anaplasma* described so far. This collection was completed with a BLASTn search against the nucleotide non-redundant (nr) database from Genbank (http://www.ncbi.nlm.nih.gov/genbank). Then the unique sequences obtained from this study were included. All sequences were aligned with Muscle (with the "codon" option for *groEL* and *gltA*) and the resulting alignments were trimmed manually to contain only the region corresponding to the longest unique sequences produced in our study. Then a phylogenetic tree was obtained with the maximum likelihood method (ML), with the partial deletion option, choosing the Tamura-Nei model of substitution with uniform rates. Phylogenies were tested by bootstrap (n=100). All analyses (alignments and phylogenies) were performed using MegaX (Kumar et al., 2018). Phylogenetic trees were edited graphically with Iroki (Moore et al., 2020).

3. Results

Out of 108 heparinized blood samples from sheep and goats, 26 (24%) were positive for *A. capra* on the basis of cumulative 16S rRNA PCR amplification, sequencing and Blastn results. The positivity rate was 30% (21/70) and 13% (5/38) in sheep and goats respectively. *A. capra* was detected on four sheep farms and one goat farm, in both Haute-Corse and Corse du Sud (Figure 1). The number of infected animals in each positive herd varied between 1 and 10, on 10 sampled animals. *Anaplasma capra* was not detected in 6 farms (3 sheep farms and 3 goat farms), five in Corse du Sud and one in Haute-Corse.

Further analyses of the 108 blood samples yielded 20 partial *groEL* gene sequences and 18 partial *gltA* sequences from blood samples already found positive using 16S rRNA nPCR. Sequence lengths ranged between 263-526 bp for the 16S rRNA gene, 420-735 bp for *gltA*, and 418-967 bp for *groEL*. All 16S rRNA (n=26) and *gltA* (n=18) sequences obtained in this study, from either goat or sheep, were identical, and showed 100% identity with *A. capra* sequences reported in red deer and swamp deer from the Haute Touche zoological park in France (Genbank accession numbers MH084719-22). In Corsica, two different partial *A. capra groEL* sequences were obtained, differing by a single SNP (G/A at position 623 when compared to MH084717 from previous study), a substitution found in *A. capra* from only two sheep from the same farm (OVI20-01-E10 and OVI20-05-E10). Otherwise, all Corsican sheep and goat *A. capra groEL* partial sequences differed from continental deer sequences by another SNP (C/A at position 425 when compared to MH084717). Identities between the *A. capra groEL* sequences from Corsican sheep and goats and the *A. capra groEL* sequences from deer in France ranged between 99.78 and 99.89%.

The *A. capra* partial and representative sequences obtained in this study were deposited in the Genbank database under the following accession numbers: *groEL* (MW930530-32), *gltA* (MW930533-35), and 16S rRNA (MW930536-38).

The phylogenetic study of 16S rRNA sequences showed that isolates from Corsica (this study) grouped with other sequences assigned to *A. capra,* from different geographic origins (mostly Asian countries), supported by a high bootstrap value (Figure 2). Of note, within this group, the Corsican isolates grouped more closely with the two isolates from mainland France (Jouglin et al., 2019) and with three isolates from Spain. However, as the 16S rRNA sequence is partial, short, and highly conserved, this grouping of the isolates from mainland France, Corsica and Spain was not strongly supported. The phylogenies of two coding genes, *gltA* and *groEL*, also confirmed that the Corsican isolates belong to *A. capra* (Figures 3 and 4). Besides, sequences from these two genes, more variable than the 16S rRNA, allowed to distinguish two well differentiated clades within the *A. capra* species, also referred to as clades I and II in several previous studies (Yang et al., 2017, 2018; Peng et al., 2018; Miranda et al., 2021). For both *gltA* and *groEL*, we found that the Corsican isolates grouped in one of these clusters (clade II), and that within this clade, they grouped with strong bootstrap support with the mainland France isolates discovered in our previous study. The longest *A. capra groEL* sequence from roe deer in Spain was included and also grouped with the sequences from the French isolates, forming a potential European lineage.

4. Discussion

In this study we confirm the presence of *A. capra* in Corsica, both in goats and sheep, in about half of the tested flocks, spatially well distributed on the island. The number of infected animals was also quite high on some farms, with overall 24% of the small ruminants tested being infected, indicating an important circulation of the bacteria and/or its maintenance in the host once transmitted by ticks. This is the third report of *A. capra* in Europe, the second in France, but this time on two different domestic animal species, and on several different locations in Corsica, indicating the endemicity of this bacteria in this island.

The impact of *A. capra* on animal health has not been evaluated yet, but it was suspected to be responsible of non-specific febrile manifestations (fever, headache, malaise, myalgia, chills) in several tick-bitten patients in China (Li et al., 2015). In a future study, we will be evaluate a potential role of *A. capra* on abortion by comparing *A. capra* prevalences in herds with history of abortions and herds without.

Anaplasma capra exhibits at least two markedly divergent clades (named clades I and II or genotypes 1 and 2 in Yang et al., 2017, 2018; Peng et al., 2018; Miranda et al., 2021). Phylogenetic analysis of Corsican *A. capra*, especially thanks to the use of resolutive markers like the partial sequences of two coding genes, *gltA* and *groEL*, shows that all isolates from sheep and goats in Corsica belong to clade II, which is relatively divergent from clade I. Human isolates of *A. capra* belong to clade I, sheep and goat isolates belong to either clade I or II, while most genotypes identified in wild ruminants (Asian or European) cluster within clade II (Yang et al. 2018; Jouglin et al., 2019). Finally, we stress that the sequences described in this study from Corsica showed a low level of genetic variation (all sequences for the three genes were identical, with the exception of a single substitution for *groEL*) suggesting a possible single and recent event of introduction of *A. capra*. Furthermore, the sequences from Corsica, mainland France and Spain are genetically very close, although not identical. They indeed form a well-supported clade (bootstrap value > 90 for *groEL*) within clade II. For *glta*, we also found a robust grouping (bootstrap value > 90) of the sequences from Corsica and mainland France (no Spanish sequences were available for this gene). Overall, this suggests a common ancestry and a relatively recent divergence on the European continent. New samples that could be discovered elsewhere in Europe would greatly help to clarify the history of this pathogen on this continent.

Anaplasma capra was detected in several tick species in China (Li et al., 2015; Guo et al., 2019), but the vectorial competence of these vectors for *A. capra* transmission has not yet been evaluated. Nine different tick species have been identified on Corsican animals: *Rhipicephalus*

bursa, *Rhipicephalus sanguineus sensu lato*, *Rhipicephalus* (*Boophilus*) *annulatus*, *Hyalomma marginatum*, *Hyalomma scupense*, *Dermacentor marginatus*, *Ixodes ricinus*, *Haemaphysalis punctata* and *Haemaphysalis sulcata* (Grech-Angelini et al., 2016; 2020), but the almost sole tick species found on sheep and goats in Corsica was *R. bursa* (Grech-Angelini et al., 2016; Cabezas-Cruz.et al., 2019), therefore a good candidate to be an *A. capra* vector.

5. Conclusion

The presence of *A. capra* was confirmed in 26 blood samples from five different farms from Corsica, both in goats and sheep. Furthermore, our phylogenetic analyses indicate that the newly identified *A. capra* isolates from Corsica are genetically very close to the *A. capra* recently identified on the French mainland and to isolates from Spain. Corsica, mainland France and Spanish isolates, despite host and geographical origin differences, cluster together in one clade within clade II of *A. capra*, suggesting a relatively recent common ancestor of European isolates.

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Conflict of interest: none.

Authors' contribution

Maggy Jouglin: Investigation, Formal analysis, Methodology, Visualization, Writing-Original draft. **Claude Rispe:** Formal analysis, Visualization, Reviewing and Editing. **Sébastien Grech-Angelini:** Resources, Reviewing and Editing. **Mélanie Gallois:** Resources, Conceptualization, Supervision. **Laurence Malandrin:** Funding acquisition, Conceptualization, Supervision, Writing-Original draft, Reviewing and Editing.

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Legends of figures

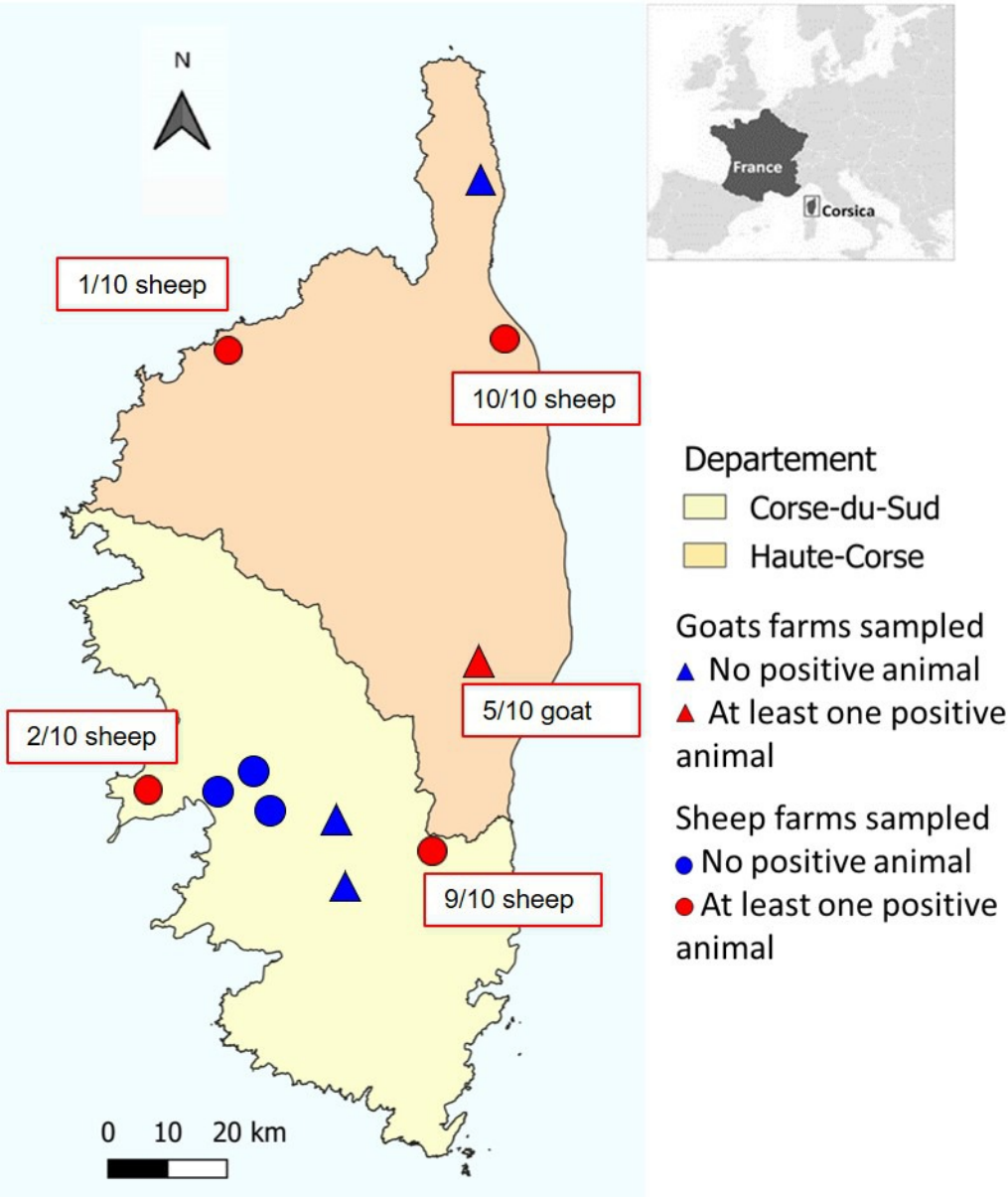
Figure 1: Map of Corsica (France) showing the location of goat and sheep farms and the number of *A. capra* positive animals when detected in a farm.

Figure 2: Maximum likelihood phylogeny of partial 16S rRNA sequences of *Anaplasma* isolates. Alignment length was 582 base pairs. Sequences of *Ehrlichia* and *Rickettsia* were used as outgroups. Sequence labels are composed of the species names (between inverted commas if needing re-assignation), accession number, origin (vertebrate host, or other), country. Label of sequences obtained in this study is in blue. Representative sequences from both goat and sheep samples obtained in this study were used in the phylogeny. Branches corresponding to *A. capra* are in purple. High bootstrap support for nodes is shown by filled circles (bootstrap > 90) or open circles (bootstrap > 80).

Figure 3: Maximum likelihood phylogeny of partial *gltA* sequences of *Anaplasma* isolates. Alignment length was 762 base pairs. Sequences of *Ehrlichia* and *Rickettsia* were used as outgroups. Sequence labels are composed of the species names (between inverted commas if needing reassignation), accession number, origin (vertebrate host, or other), country. Label of sequences obtained in this study is in blue. Representative sequences from both goat and sheep

samples obtained in this study were used in the phylogeny. Branches corresponding to *A. capra* are in purple. High bootstrap support for nodes is shown by filled circles (bootstrap > 90) or open circles (bootstrap > 80). Clades I and II are indicated as well as the European lineage (green).

Figure 4: Maximum likelihood phylogeny of partial *groEL* sequences of *Anaplasma* isolates. Alignment length was 987 base pairs. Sequences of *Ehrlichia* and *Rickettsia* were used as outgroups. Sequence labels are composed of the species names (between inverted commas if needing re-assignation), accession number, origin (vertebrate host, or other), country. Labels of sequences obtained in this study are in blue. Representative sequences from both goat and sheep samples obtained in this study were used in the phylogeny. Branches corresponding to *A. capra* are in purple. High bootstrap support for nodes is shown by filled circles (bootstrap > 90) or open circles (bootstrap > 80). Clades I and II are indicated as well as the European lineage (green).


N
France
Corsica
1/10 sheep
10/10 sheep
5/10 goat
2/10 sheep
9/10 sheep
0 10 20 km
Departement
Corse-du-Sud
Haute-Corse
Goats farms sampled
No positive animal
At least one positive animal
Sheep farms sampled
No positive animal
At least one positive animal

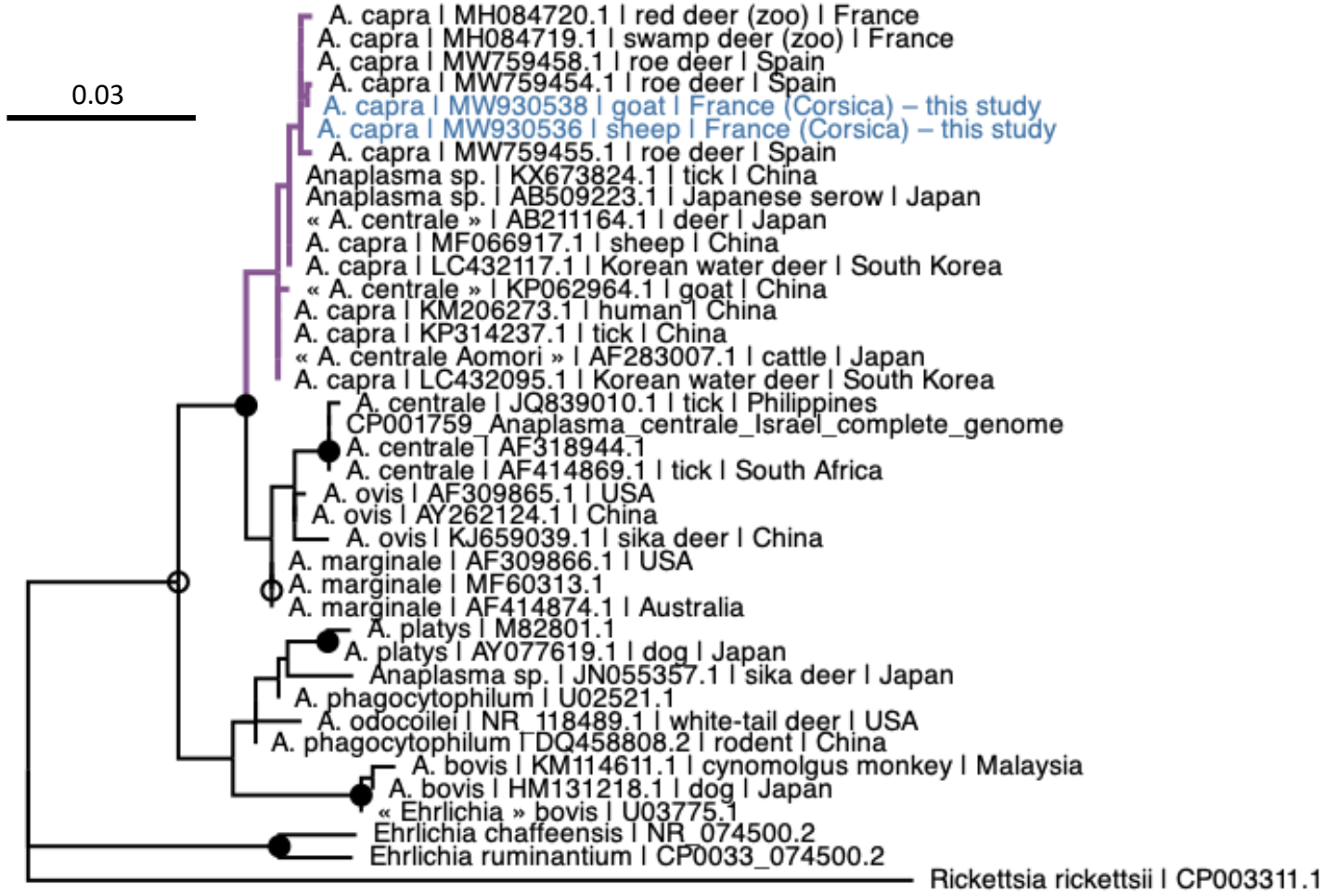


Figure 2: Maximum likelihood phylogeny of partial 16S rRNA sequences of *Anaplasma* isolates. Alignment length was 582 base pairs. Sequences of *Ehrlichia* and *Rickettsia* were used as outgroups. Sequence labels are composed of the species names (between inverted commas if needing re-assignation), accession number, origin (vertebrate host, or other), country. Label of sequences obtained in this study is in blue. Representative sequences from both goat and sheep samples obtained in this study were used in the phylogeny. Branches corresponding to *A. capra* are in purple. High bootstrap support for nodes is shown by filled circles (bootstrap > 90) or open circles (bootstrap > 80).

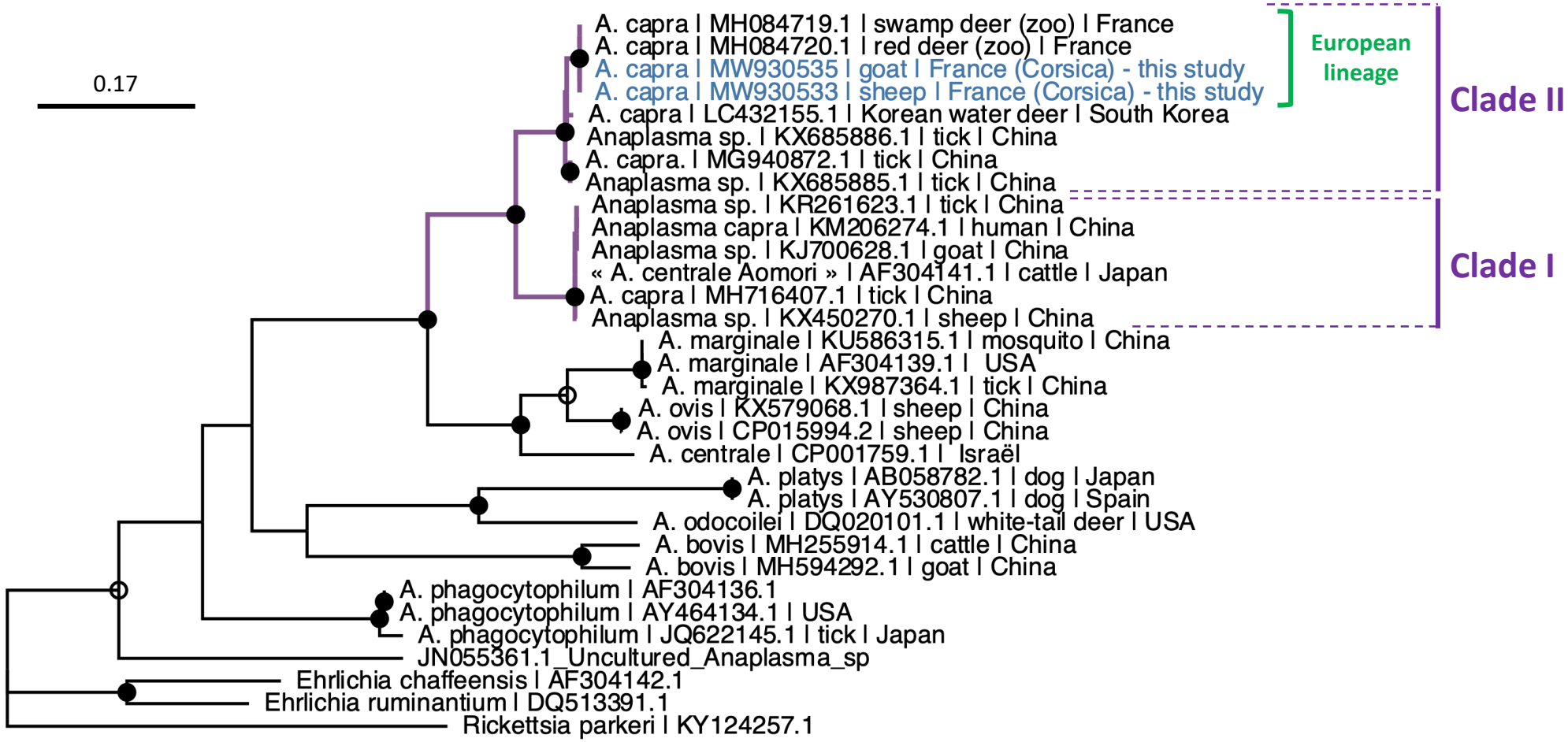


Figure 3: Maximum likelihood phylogeny of partial *gltA* sequences of *Anaplasma* isolates. Alignment length was 762 base pairs. Sequences of *Ehrlichia* and *Rickettsia* were used as outgroups. Sequence labels are composed of the species names (between inverted commas if needing reassignation), accession number, origin (vertebrate host, or other), country. Label of sequences obtained in this study is in blue. Representative sequences from both goat and sheep samples obtained in this study were used in the phylogeny. Branches corresponding to *A. capra* are in purple. High bootstrap support for nodes is shown by filled circles (bootstrap > 90) or open circles (bootstrap > 80). Clades I and II are indicated as well as the European lineage (green).

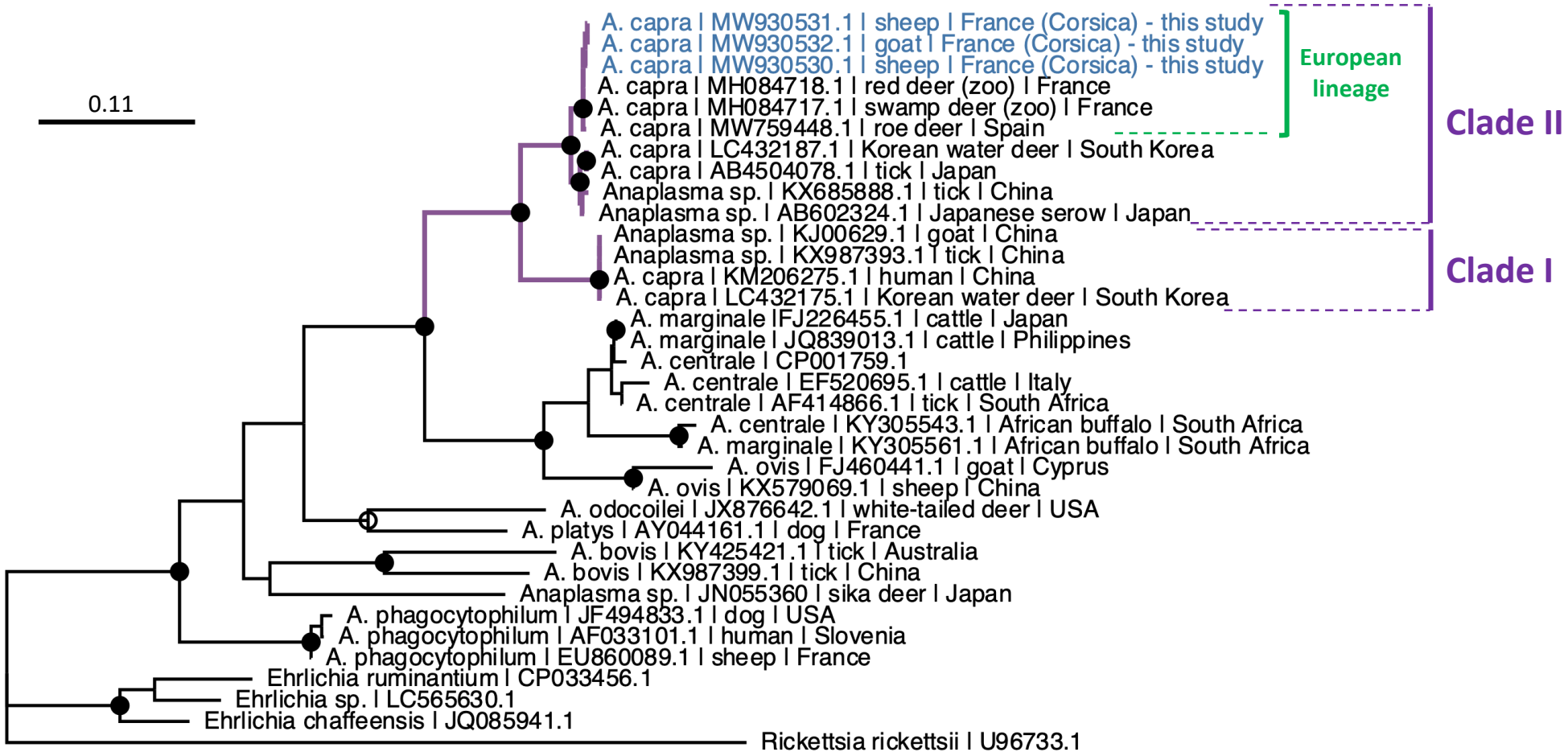


Figure 4: Maximum likelihood phylogeny of partial *groEL* sequences of *Anaplasma* isolates. Alignment length was 987 base pairs. Sequences of *Ehrlichia* and *Rickettsia* were used as outgroups. Sequence labels are composed of the species names (between inverted commas if needing re-assignation), accession number, origin (vertebrate host, or other), country. Labels of sequences obtained in this study are in blue. Representative sequences from both goat and sheep samples obtained in this study were used in the phylogeny. Branches corresponding to *A. capra* are in purple. High bootstrap support for nodes is shown by filled circles (bootstrap > 90) or open circles (bootstrap > 80). Clades I and II are indicated as well as the European lineage (green).