Novel Genotypes of Nidicolous Argas Ticks and Their Associated Microorganisms From Spain

Ana M. Palomar 1*, Jesús Veiga 2, Aránzazu Portillo 1, Sonia Santibáñez 1, Radovan Václav 3, Paula Santibáñez 1, José A. Oteo 1 and Francisco Valera 2

1 Centre of Rickettsiosis and Arthropod-Borne Diseases, Hospital Universitario San Pedro-Center for Biomedical Research of La Rioja (CIBIR), Logroño, Spain. 2 Departamento de Ecología Funcional y Evolutiva, Estación Experimental de Zonas Áridas -Consejo Superior de Investigaciones Científicas (EEZA-CSIC), Ctra. de Sacramento s/n, La Cañada de San Urbano, Almería, Spain. 3 Institute of Zoology, Slovak Academy of Sciences, Bratislava, Slovakia

The knowledge of the distribution, richness and epidemiological importance of soft ticks of the genus Argas is incomplete. In Spain, five Argas species have been recorded, including three ornithophilic nidicolous ticks, but their associated microorganisms remain unknown. This study aimed to investigate ticks from bird nests and their microorganisms. Ticks were collected extensively from natural cavities and nest-boxes used by European rollers (Coracias garrulus) and little owls (Athene noctua) in Southeastern and Central Spain. Ticks were morphologically and genetically identified and corresponding DNA/RNA tick extracts were analyzed [individually (n = 150) or pooled (n = 43)] using specific PCR assays for bacteria (Anaplasmataceae, Bartonella, Borrelia, Coxiella/Rickettsiella, and Rickettsia spp.), viruses (Flaviviruses, Orthonairoviruses, and Phenuiviruses), and protozoa (Babesia/Theileria spp.). Six Argas genotypes were identified, of which only those of Argas reflexus (n = 8) were identified to the species level. Two other genotypes were closely related to each other and to Argas vulgaris (n = 83) and Argas polonicus (n = 33), respectively. These two species have not been previously reported from Western Europe. Two additional genotypes (n = 4) clustered with Argas persicus, previously reported in Spain. The remaining genotype (n = 22) showed low sequence identity with any Argas species, being most similar to the African Argas africolumbae. The microbiological screening revealed infection with a rickettsial strain belonging to Rickettsia fournieri and Candidatus Rickettsia vini group in 74.7% of ticks, mainly comprising ticks genetically related to A. vulgaris and A. polonicus. Other tick endosymbionts belonging to Coxiella, Francisella and Rickettsiella species were detected in ten, one and one tick pools, respectively. In addition, one Babesia genotype, closely related to avian Babesia species, was found in one tick pool. Lastly, Anaplasmataceae, Bartonella, Borrelia, and viruses were not detected. In conclusion, five novel Argas genotypes and their associated microorganisms with unproven pathogenicity are reported for Spain. The re-use of nests between and within years by different bird species appears to be ideal for the transmission of tick-borne microorganisms in cavity-nesting birds of semiarid areas. Further work should be performed to clarify the taxonomy and the potential role of soft Argas ticks and their microorganisms in the epidemiology of zoonoses.

Keywords: soft ticks, Argas spp., nidicolous, cavity-nesting birds, tick-borne bacteria, tick-borne viruses, tick-borne protozoa, Spain
INTRODUCTION

Soft ticks of the genus *Argas* Latreille, 1795 (Ixodida; Argasidae) are distributed worldwide and include around 60 species (1). Of them, only eight species have been described in the Western Palearctic region, specifically, *Argas gilcolladoi*, *Argas persicus*, *Argas reflexus*, *Argas transgariepinus*, *Argas vespertilionis*, *Argas macrostigmatus*, *Argas vulgaris*, and *Argas polonicus* (2–5). All but the latter three species have been reported in Spain (Southwestern Europe) as parasites of birds or bats (6). The majority of *Argas* spp. are nidicolous and birds are exclusive vertebrate hosts for several species, mainly those of *Persicargas* subgenera, while humans are accidental hosts (7, 8). The genus *Argas* includes species responsible for the transmission of pathogens of medical and veterinary interest. Apart from conditions caused directly by soft ticks, such as toxicosis and anaphylaxis (9, 10), these ticks carry microorganisms that could be agents of infectious diseases. Specifically, *Argas* species can vector bacterial pathogens such as *Borrelia anserina* and *Aegyptianella* spp. and viruses such as *Isslk-kul virus* (11, 12). Other microorganisms with unproved pathogenicity have been detected in *Argas* ticks: bacteria from genera *Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Francisella*, *Rickettsia*, and *Rickettsiella*, viruses belonging to *Flaviviridae*, *Orthomyxoviridae*, *Orthonairovirdae*, *Phenuiviridae*, and *Reoviridae* families, and protozoans such as *Babesia* and *Hemolivia* spp. (7, 13–17).

The lack of information on the natural history and distribution of various *Argas* species, their incorrect or incomplete taxonomic description, and the fact that some species share morphological features but have not been molecularly examined, are responsible for the poor knowledge of *Argas* ticks in Spain. Moreover, their role in the epidemiology of tick-borne microorganisms has not been studied in this country. Here, we aim at describing soft ticks from natural and artificial nests occupied by different cavity-nesting birds in Spain and the prevalence of selected tick-borne microorganisms.

MATERIALS AND METHODS

Study Area and Study System

The main study area (~50 km²) lies in the Desert of Tabernas (Almeria province, SE Spain, 37.08°N, 2.35°W). The landscape mostly consists of open shrubland with olive and almond groves interspersed among numerous dry riverbeds—ramblas. The climate in this area is semi-arid Mediterranean with a marked water deficit during long, hot summer months. The mean annual rainfall is ~230 mm, with high inter- and intra-annual variability (18). Tick samples also were collected in Segovia and Guadalajara provinces (both in the interior of the Iberian Peninsula), whose climate is Mediterranean with some continental characteristics.

In the main study area in Almeria, natural cavities in sandstone cliffs, seminatural cavities in stone bridges and abandoned farmhouses and nest boxes provide nest sites for cavity-nesting birds, namely, the European roller (*Coracias garrulus*, hereafter roller), the little owl (*Athene noctua*) and the rock/feral pigeon (*Columba livia*, hereafter pigeon). In this study, we sampled ticks in cavities occupied by rollers and little owls. The roller is a migratory bird that arrives at its breeding grounds in the study area during the second fortnight of April whereas the little owl is a resident bird. Both species rear a single brood per year (19). In contrast to these species, the pigeon is a resident bird that breeds at any time of the year in our study area and does not use nest boxes. Other species breeding in natural and seminatural cavities mainly include jackdaws (*Corvus monedula*), and common kestrels (*Falco tinnunculus*), whereas Scops owls (*Otus scops*), spotless starlings (*Sturnus unicolor*), and house sparrows (*Passer domesticus*) can breed in nest boxes.

Given nest-site limitation in the study area, both intra- and interspecific competition for suitable nesting holes occur and individual cavities can be re-used by different species both within and between years. This is frequently the case in Almeria, so that many samples were collected from natural and seminatural cavities of rollers and little owls previously used by pigeons. The samples from Segovia were collected from a natural tree hole occupied by rollers but excavated by the Iberian green woodpecker (*Picus sharpei*), whereas the samples from Guadalajara were taken from rollers breeding in nest boxes.

Tick Collection and Preservation

In the framework of a long-term project of cavity-nesting birds in the Desert of Tabernas, cavities and nest boxes have been routinely inspected during each breeding season since 2005 and both nestlings and nest material periodically examined for ectoparasites. Ticks were collected from cavities occupied by breeding rollers and little owls during 2009, 2012, 2015, and 2018–2020. Additionally, four tick individuals were obtained from roller nests in Central Spain (Guadalajara and Segovia) in 2004 (Table 1). Ticks collected until 2018 were preserved in ethanol, while ticks obtained in 2019 and 2020 were kept fresh upon delivery to the Centre of Rickettsiosis and Arthropod-borne Diseases (CRETAV). Before frozen at ~80°C until later analysis, fresh ticks were identified and a single leg of each specimen was dissected.

Tick Identification

The taxonomic identification of the ticks was carried out using morphological keys (20–23). Tick individuals were surface-sterilized and DNA was individually extracted from a single leg of each tick specimen using incubations with ammonium hydroxide (24). The obtained DNA templates were used for genetic characterization by the amplification and sequencing of the 16S rRNA fragment gene (25). Two other mitochondrial genes, 12S rRNA, and cytochrome oxidase subunit I (COI), were also used in analyses (Supplementary Table 1).

Microbial Screening

Ticks were pooled (from 1 to 7 specimens; whole larvae and body halves for the other life stages) according to tick species or genotype, origin and date of collection and, when possible, tick developmental stage. DNA extracts from pools of ticks preserved in ethanol were obtained using the Qiagen DNA DNeasy blood and tissue kit (Qiagen, Hilden, Germany), following the manufacturer’s recommendations. Ticks of each
| Province | Municipal boundary | Nest Coordinates | Date of collection | Host | Preservation method | Developmental stage/Gender | No. of specimens (No. of pools) | Tick species |
|----------|--------------------|------------------|-------------------|------|---------------------|---------------------------|-------------------------------|--------------|
| Almería | Tabernas | Diego tronco 37° 3′58.43″N;2° 21′19.38″W | 17/06/2018 | Coracias garrulus | Ethanol | Larvae | 2 (1) | Argas sp. EEZA-CRETAV3 |
|         |                    | Redondo Paloma 37° 3′58.39″N;2° 21′19.48″W | 27/06/2020 | C. garrulus | Fresh-Frozen | Larvae | 7 (1) | Argas sp. EEZA-CRETAV3 |
|         |                    |                  | 28/06/2020 | C. garrulus | Fresh-Frozen | Nymph | 1 (1) | Argas reflexus |
| RG 2N  |                    |                  | 17/06/2009 | C. garrulus | Ethanol | Nymphs | 2 (1) | Argas sp. EEZA-CRETAV3 |
| RH Grieta |                 | 37° 4′14.86″N;2° 20′27.65″W | 2012 | C. garrulus | Ethanol | Male | 1 (1) | Argas sp. EEZA-CRETAV2 |
| RH SV  |                    | 37° 3′55.46″N;2° 20′34.25″W | 31/05/2012 | C. garrulus | Ethanol | Nymphs | 1 (1) | Argas sp. EEZA-CRETAV1 |
| Tapadera alberca |     | 37° 3′54.53″N;2° 21′30.43″W | 01/06/2018 | Athene noctua | Ethanol | Nymphs | 7 (2) | A. reflexus |
| Tapadera cueva |              | 37° 3′56.71″N;2° 21′24.29″W | 08/06/2015 | C. garrulus | Ethanol | Nymphs | 1 (1) | Argas sp. EEZA-CRETAV3 |
|         |                    |                  | 09/05/2018 | A. noctua | Ethanol | Adults or nymphs (last stage) | 3 (1) | Argas sp. EEZA-CRETAV1 |
|         |                    |                  | 08/06/2018 | A. noctua | Ethanol | Nymphs | 6 (2) | Argas sp. EEZA-CRETAV1 |
|         |                    |                  | 08/06/2018 | A. noctua | Ethanol | Nymphs | 3 (1) | Argas sp. EEZA-CRETAV2 |
|         |                    |                  | 23/05/2019 | A. noctua | Fresh-Frozen | Nymphs | 12 (2) | Argas sp. EEZA-CRETAV1 |
|         |                    |                  | 30/05/2019 | A. noctua | Fresh-Frozen | Nymphs | 4 (1) | Argas sp. EEZA-CRETAV2 |
|         |                    |                  | 10/06/2019 | A. noctua | Fresh-Frozen | Male | 1 (1) | Argas sp. EEZA-CRETAV1 |
|         |                    |                  | 10/06/2019 | A. noctua | Fresh-Frozen | Nymph | 1 (1) | Argas sp. EEZA-CRETAV2 |
|         |                    |                  | 10/06/2019 | A. noctua | Fresh-Frozen | Nymph | 2 (1) | Argas sp. EEZA-CRETAV1 |
|         |                    |                  | 10/06/2019 | A. noctua | Fresh-Frozen | Nymph | 1 (1) | Argas sp. EEZA-CRETAV2 |
|         |                    |                  | 13/05/2020 | A. noctua | Fresh-Frozen | Male | 1 (1) | Argas sp. EEZA-CRETAV1 |
|         |                    |                  | 13/05/2020 | A. noctua | Fresh-Frozen | Nymphs | 5 (1) | Argas sp. EEZA-CRETAV2 |
|         |                    |                  | 13/05/2020 | A. noctua | Fresh-Frozen | Females | 2 | Argas sp. * |
|         |                    |                  | 13/05/2020 | A. noctua | Fresh-Frozen | Nymphs | 1 (1) | Argas sp. EEZA-CRETAV1 |
|         |                    |                  | 13/05/2020 | A. noctua | Fresh-Frozen | Nymphs | 1 (1) | Argas sp. EEZA-CRETAV2 |
|         |                    |                  | 11/06/2020 | A. noctua | Fresh-Frozen | Adult | 1 (1) | Argas sp. EEZA-CRETAV1 |
|         |                    |                  | 11/06/2020 | A. noctua | Fresh-Frozen | Nymphs | 5 (1) | Argas sp. EEZA-CRETAV2 |
|         |                    |                  | 11/06/2020 | A. noctua | Fresh-Frozen | Adult | 1 (1) | Argas sp. EEZA-CRETAV1 |
|         |                    |                  | 11/06/2020 | A. noctua | Fresh-Frozen | Nymphs | 12 (2) | Argas sp. EEZA-CRETAV1 |

(Continued)
### TABLE 1 | Continued

| Province | Date of collection | Host | Preserved method | Developmental stage/Gender | No. of specimens (No. of pools) | Tick species |
|----------|-------------------|------|------------------|-----------------------------|--------------------------------|--------------|
| Tahal | 15/06/2020 | A. noctua | Fresh-Frozen | Larva, 1 | 1 | Argas sp. |
| C. garrulus | 15/06/2018 | Fresh-Frozen | Nymphs, 25 (5) | Avgas sp. EEZA-CRETAV1 | Nymphs, 8 | Argas sp. |
| Tahal | 18/06/2018 | C. garrulus | Fresh-Frozen | Nymphs, 1 (1) | 1 | Argas sp. EEZA-CRETAV2 |
| C. garrulus | 05/07/2004 | Ethanol | Nymphs, 2 (2) | Avgas sp. EEZA-CRETAV5 | Nymphs, 1 (1) | Argas sp. |
| Segovia | 15/07/2004 | C. garrulus | Ethanol | Larvae, 2 (2) | 1 | Argas sp. EEZA-CRETAV4 |

Data about sample origin (province, municipality, coordinates of the nest where individuals were captured), collection date, avian host, method of tick preservation, developmental stage, and gender (when possible), No. of ticks and No. of pools formed, and tick taxon name are given. *not processed; ‡, †, ‡ same pool.

### Prevalence of Infection

The prevalence of infection (PI) was estimated by:

$$\text{Prevalence of Infection (PI)} = \frac{(\text{No. of positive ticks/total No. of ticks analyzed})}{100\%}$$

When microorganisms were amplified from pools of more than one tick, prevalence was calculated assuming that each positive pool contained only one positive tick. This estimate, known as minimum infectious rate (MIR), is calculated as follow:

$$\text{MIR} = \frac{(\text{No. of positive pools/total No. of individual ticks analyzed})}{100\%}$$

### Analysis of Nucleotide Sequences

Nucleotide sequences were analyzed using the BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the resulting sequences were submitted to GenBank. The Clustal Omega online software (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for multiple sequence alignment. Phylogenetic analyses were conducted with MEGA X (http://www.megasoftware.net) using the maximum likelihood method including all sites. The nucleotide substitution model was selected according to the Akaike information criterion implemented in MEGAX. Confidence values for individual branches of the resulting trees were determined by bootstrap analysis with 500 replicates.
Table 2 | Highest similarities of the Argas genotypes detected in this study reached with public sequences from GenBank.

| Fragment gene; GenBank accession No. | Identity (%) | Tick species (GenBank accession no.) |
|--------------------------------------|--------------|-------------------------------------|
| Argas reflexus                       |              |                                     |
| 16S rRNA; MW289075                   | 100          | A. reflexus (L34322)                |
| 12S rRNA; MW289084                   | 96.6         | A. reflexus (U958865)               |
| COI; MW288388                        | 81.5         | A. walkerae (KJ133584)              |
| Argas sp. EEZA-CRETAV1               |              |                                     |
| 16S rRNA; MW289069                   | 90.0         | A. vulgaris (AF001403)              |
| 12S rRNA; MW289077                   | 94.1         | A. lagenoplastis (KJ133580)         |
| COI; MW288380                        | 88.0         | A. lagenoplastis (KJ133581)         |
| Argas sp. EEZA-CRETAV2               |              |                                     |
| 16S rRNA; MW289070                   | 98.8         | A. polonicus (AF001403)             |
| 12S rRNA; MW289078                   | 93.8         | A. lagenoplastis (KJ133581)         |
| COI; MW288382                        | 88.3         | A. lagenoplastis (KJ133581)         |
| Argas sp. EEZA-CRETAV3               |              |                                     |
| 16S rRNA; MW289072                   | 92.1         | A. africolumbae (UQ665720)          |
| 12S rRNA; MW289081                   | 92.1         | A. africolumbae (KJ133580)          |
| COI; MW288385                        | 85.3         | A. africolumbae (KJ133581)          |
| Argas sp. EEZA-CRETAV4               |              |                                     |
| 16S rRNA; MW289073                   | 96.5         | A. persicus (MT012684)              |
| 12S rRNA; MW289083                   | 95.3         | A. persicus (MT012684)              |
| COI; MW288386                        | 90.1         | A. persicus (KJ133581)              |
| Argas sp. EEZA-CRETAV5               |              |                                     |
| 16S rRNA; MW289074                   | 97.3         | A. persicus (MT012684)              |
| 12S rRNA; Not obtained               |              | A. persicus (MT012684)              |
| COI; MW288387                        | 90.7         |                                     |

*One more sequence with a single nucleotide substitution was obtained (MW289076).*
*One more sequence with two nucleotide substitutions was obtained (MW288389).*
*One more sequence with four nucleotide substitutions was obtained (MW288381).*
*One more sequence with a single nucleotide substitution was obtained (MW289071).*
*Two more sequences with one and three nucleotide substitutions were obtained (MW289079; MW289080).*
*Two more sequences with 20 and 16 nucleotide substitutions were obtained (MW288383; MW288384).*
*One more sequence with two nucleotide substitutions was obtained (MW289082).*
*There are not public sequences for A. vulgaris and A. polonicus.*

**RESULTS**

**Tick Identification**

One hundred and sixty-three ticks, mainly nymphs, were collected from bird nests in Almería (n = 159), Guadalajara (n = 2), and Segovia (n = 2). Arthropods were obtained from nest material in cavities occupied by little owl (n = 129) and roller (n = 34) or, in few cases, from nestlings of these species (Table 1). All the specimens were morphologically identified as Argas spp. and 150 specimens were further studied molecularly. Examination of morphological characters enabled the identification of eight nymphs as *A. reflexus*, but the morphological identification of the remaining ticks could not be accurately performed with available keys. The *A. reflexus* identification was corroborated molecularly based on 16S rRNA fragment gene (Table 2). The molecular identification was not conclusive for the remaining 142 tick samples, which were grouped based on the 16S rRNA results into five different genotypes, designated as Argas spp. EEZA-CRETAV1–5 (Tables 1, 2). Based on 16S rRNA gene analyses, Argas sp. EEZA-CRETAV1 (n = 83) and Argas sp. EEZA-CRETAV2 (n = 33) were closest to *A. vulgaris* and *A. polonicus*, respectively (Table 2). In turn, the Argas sp. EEZA-CRETA3 genotype (n = 22) shared the highest identity (>92.2%) with *Argas africolumbae*. Lastly, Argas sp. EEZA-CRETA4 (n = 2) and Argas sp. EEZA-CRETA5 genotypes (n = 2) shared highest identities with *A. persicus* (Table 2). The phylogeny inferred from 16S rRNA analysis corroborates the BLAST results (Figure 1). Phylogenetic analyses based on 12S rRNA and COI fragment genes could not be performed because of the lack of homologue sequences for the majority of Argas spp.

Argas spp. EEZA-CRETAV1–3 specimens were collected in Almería, whereas those of Argas spp. EEZA-CRETAV4–5 were obtained in Segovia and Guadalajara. Also, some of the ticks of Argas spp. EEZA-CRETAV1–2 genotypes were collected from the same nests (Table 1).

**Bacterial Screening**

All the DNA extracts (individual samples and pools) gave positive results for the tick-16S rRNA PCR assay and, consequently, were screened for bacteria (Table 3). Amplicons for *ompA* gene were obtained from 112 DNA extracts from tick legs (PI = 74.7%). Specifically, *Rickettsia* was amplified from 81 and 31 samples belonging to Argas sp. EEZA-CRETA1 (PI = 97.6%) and Argas sp. EEZA-CRETA2 (PI = 93.9%) samples, respectively. DNA extracts corresponding to individual tick-leg samples from *A. reflexus* and Argas sp. EEZA-CRETA3–5 specimens were negative, but body-halve extracts of these specimens were further analyzed using pooled samples. The nucleotide extracts from these pools were negative for all A.
FIGURE 1 | Phylogenetic tree based on 16S rRNA analysis showing the relationships between tick species and genotypes identified in this study and published validated Argas species. The evolutionary analysis was inferred using the maximum likelihood method and general time reversible + G model with Mega X. The (Continued)
reflexus specimens, but yielded positive results for two out of six pools of Argas sp. EEZA-CRETAV3 specimens (MIR = 9.1%) (comprising a nymph collected from a roller nest in Almería in 2015 and larvae that were attached to a roller nestling in Almería in 2020), and all the pools of Argas sp. EEZA-CRETAV4–5 specimens (MIR = 100% for the two pools) (Table 3).

All the ompA gene sequences obtained were identical and showed the highest identity with Rickettsia fournieri (Table 4). Selected Rickettsia-positive samples were further genetically characterized by the amplification of five more rickettsial fragment genes (26). Nucleotide sequences for the respective genes were identical and showed highest identities with R. fournieri and Candidatus Rickettsia vini (Table 4). The phylogenetic tree based on the concatenated fragment genes of the Rickettsia strain detected, designated as Rickettsia sp. EEZA-CRETAV, corroborated the close relation with both R. fournieri and Ca. R. vini (Figure 2).

A total of 27 nucleotide sequences were obtained using the rpoB PCR assay selected for the Coxiiella/Rickettsiella detection but highest identities with validated bacterial species reached < 85% for 14 samples. The genetic analysis of the amplicon obtained from an Argas sp. EEZA-CRETAV1 nymph, collected in Almería in 2012, showed the highest identity with Francisella persicus (PI = 0.7% and PI = 1.2% for Argas sp. EEZA-CRETAV1) (Tables 3, 5). The novel Francisella strain molecularly described in this study was designated as Francisella sp. EEZA-CRETAV. Coxiiella-like strains were successfully amplified from 10 pools (MIR = 6.7%). Two out of three A. reflexus pools (with ticks collected in a little owl nest in Almería in 2018) showed infection with a Coxiiella strain previously detected in this tick species (MIR = 25%) (Table 5). A new strain of Coxiiella spp., designated as Coxiiella sp. EEZA-CRETAV1, was amplified in all the pools of Argas sp. EEZA-CRETAV3 specimens (n = 6, MIR = 27.3%). One more Coxiiella strain, Coxiiella sp. EEZA-CRETAV2, was detected in two pools of different tick genotypes, an Argas sp. EEZA-CRETAV2 (MIR = 3%) female collected in Almería in 2015 and an Argas sp. EEZA-CRETAV4 (MIR = 50%) larva collected in Segovia in 2004 (Table 3).

Nucleotide sequences corresponding to rpoB of groEL genes of these two novel strains shared 95.3 and 97% identity, respectively, and reached highest identities with Coxiiella strains detected in Ornithodoros ticks (Table 5). Moreover, pools integrated over a single specimen were also submitted for groEL analysis and a nymph belonging to Argas sp. EEZA-CRETAV2, collected in Almería in 2018, showed infection with Rickettsiella sp. (PI = 0.7% and PI for Argas sp. EEZA-CRETAV2 = 3%; Table 3). The corresponding amplicon, designated as Rickettsiella EEZA-CRETAV, showed highest identities with Rickettsiella species amplified from Ornithodoros normandi (Table 5).

All the tick pools were examined by PCR assays for the presence of Anaplasmataceae, Bartonella, and Borrelia species and all gave negative results.

**Viral Screening**

Twenty-one pools originated from fresh/frozen ticks [n = 111 ticks: 1 tick (1 pool) of A. reflexus, 67 (12) of Argas sp. EEZA-CRETAV1, 26 (5) of Argas sp. EEZA-CRETAV2 and 17 (3) of Argas sp. EEZA-CRETAV3 specimens; Table 1] were analyzed for the presence of viruses belonging to families Flaviviridae, Orthonairoviridae, and Phenuiviridae. No sequences were amplified using the selected PCR assays.

**Protozoan Screening**

Tick DNA from 43 pools was analyzed using a PCR assay that amplifies 18S rRNA gene of Babesia and Theileria spp. (Table 3, Supplementary Table 1). Babesia sp. was detected from a male Argas sp. EEZA-CRETAV1 collected in Almería in 2015 (MIR = 0.7%, MIR = 1.2% for Argas sp. EEZA-CRETAV1) (Table 3). Three more ticks (one Argas sp. EEZA-CRETAV1 and two Argas sp. EEZA-CRETAV2 ticks) collected simultaneously from the same nest gave negative results. Based on the analysis of 18S rRNA gene, the closets Babesia sp. from the detected strain, designated as Babesia sp. EEZA-CRETAV, was Babesia ardeae (KY436057; 95.8% identity) (Figure 3). Two more genes, ITS1 and ITS2, were also examined, but currently there are no B. ardeae sequences available for these markers. The analysis of these genes showed highest (<82%) identities with Babesia poelea (accession no. DQ200887). A pool formed by 4 Argas sp. EEZA-CRETAV3 larvae, collected in Almería in 2020, showed presence of a coccidian parasite Adelina bamberooniae (AF494059) (MIR = 0.7%) (Table 3).

**Sequences Submission to a Public Database**

The nucleotide sequences of ticks and microorganisms detected in this study (n = 42) were deposited in the GenBank database under accession numbers showed in Table 6.

**DISCUSSION**

Soft ticks are important vectors of microbial agents of animal and human diseases. Despite this, the knowledge of argasid tick species and their associated microorganisms is generally scarce. Herein, the occurrence of five novel Argas spp. genotypes, in addition to A. reflexus, collected in the Iberian Peninsula (Spain) in nests occupied by little owls and European rollers is reported. Moreover, we detected the presence of tick-borne microorganisms belonging to genera Rickettsia, Coxiiella, Francisella, Rickettsiella, and Babesia in Argas ticks. In contrast,
### TABLE 3 | Microorganisms detected in this study.

| Tick species | Developmental stage/Gender | No. of specimens (No. of pools) | Host | Origin | Date of collection | Rickettsia | Coxiella | Rickettsiella | Francisella | Babesia | Adelina |
|--------------|----------------------------|----------------------------------|------|--------|-------------------|-----------|----------|--------------|-------------|---------|---------|
| Argas reflexus | Nymphs | 7 (2) | Athene noctua | Tabernas (Almería) | 01/06/2018 | 0 (0) | 0 (0) | 2 (28.6) | 0 (0) | 0 (0) | 0 (0) |
| Argas reflexus | Nymph | 1 (1) | Coracias garrulus | Tabernas (Almería) | 28/06/2020 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Argas reflexus | Nu | 1 (1) | C. garrulus | Tabernas (Almería) | 31/05/2012 | 1 (100) | NP | 0 (0) | 0 (0) | 1 (100) | 0 (0) | 0 (0) |
| Males | 2 (2) | C. garrulus | Uleila del Campo (Almería) | 14/06/2015 | 2 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 1 (50) | 0 (0) |
| Adults or nymphs (last stage) | 3 (1) | A. noctua | Tabernas (Almería) | 09/05/2018 | 3 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Nymphs | 6 (2) | A. noctua | Tabernas (Almería) | 09/05/2018 | 6 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Adult | 1 (1) | A. noctua | Tabernas (Almería) | 08/06/2018 | 1 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Nymphs | 3 (1) | A. noctua | Tabernas (Almería) | 08/06/2018 | 3 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Nymphs | 12 (2) | A. noctua | Tabernas (Almería) | 23/05/2019 | 12 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Nymph | 1 (1) | A. noctua | Tabernas (Almería) | 30/05/2019 | 1 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Male | 1 (1) | A. noctua | Tabernas (Almería) | 10/06/2019 | 1 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Larva | 1 (1) | A. noctua | Tabernas (Almería) | 17/06/2019 | 1 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Nymphs | 2 (1) | A. noctua | Tabernas (Almería) | 17/06/2019 | 1 (50) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Nymphs | 5 (1) | A. noctua | Tabernas (Almería) | 13/05/2020 | 5 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Nymphs | 7 (1) | A. noctua | Tabernas (Almería) | 27/05/2020 | 7 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Adult | 1 (1) | A. noctua | Tabernas (Almería) | 11/06/2020 | 1 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Nymphs | 12 (2) | A. noctua | Tabernas (Almería) | 11/06/2020 | 11 (91.6) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Nymphs | 25 (5) | A. noctua | Tabernas (Almería) | 15/06/2020 | 25 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

(Continued)
| Tick species | Developmental stage/Gender | No. of specimens (No. of pools) | Host | Origin | Date of collection | Rickettsia | Coxiella | Rickettsiella | Francisella | Babesia | Adelina |
|--------------|----------------------------|---------------------------------|------|--------|--------------------|-----------|---------|--------------|-------------|---------|---------|
| Argas sp. EEZA-CRETAV2 | Male | 1 (1) | C. garrulus | Tabernas (Almería) | 2012 | 1 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Nymph | 1 (1) | C. garrulus | Uleila del Campo (Almería) | 14/06/2015 | 1 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Female | 1 (1) | C. garrulus | Uleila del Campo (Almería) | 14/06/2015 | 1 (100) | NP | 1 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Nymphs | 3 (1) | A. noctua | Tabernas (Almería) | 09/05/2018 | 3 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Nymphs | 1 (1) | C. garrulus | Tahal (Almería) | 18/06/2018 | 1 (100) | NP | 0 (0) | 1 (100) | 0 (0) | 0 (0) | 0 (0) |
| | Nymphs | 4 (1) | A. noctua | Tabernas (Almería) | 23/05/2019 | 4 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Nymphs | 2 (1)<sup>b</sup> | A. noctua | Tabernas (Almería) | 30/05/2019 | 2 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Nymph | 1 (1)<sup>b</sup> | A. noctua | Tabernas (Almería) | 10/06/2019 | 1 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Nymph | 1 (1)<sup>c</sup> | A. noctua | Tabernas (Almería) | 27/05/2020 | 1 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Adult | 1 (1)<sup>c</sup> | A. noctua | Tabernas (Almería) | 11/06/2020 | 1 (100) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Nymphs | 5 (1)<sup>c</sup> | A. noctua | Tabernas (Almería) | 11/06/2020 | 4 (80) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Nymphs | 12 (2) | A. noctua | Tabernas (Almería) | 15/06/2020 | 11 (91.6) | NP | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Argas sp. EEZA-CRETAV3 | Nymphs | 2 (1) | C. garrulus | Tabernas (Almería) | 17/06/2009 | 0 (0) | 0 (0) | 1 (50) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Nymph | 1 (1) | C. garrulus | Tabernas (Almería) | 08/06/2015 | 0 (0) | 1 (100) | 1 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Larvae | 2 (1) | C. garrulus | Tabernas (Almería) | 17/06/2018 | 0 (0) | 0 (0) | 1 (50) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Larvae | 7 (1) | C. garrulus | Tabernas (Almería) | 27/06/2020 | 0 (0) | 0 (0) | 1 (14.3) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Larvae | 10 (2) | C. garrulus | Tabernas (Almería) | 28/06/2020 | 0 (0) | 1 (10) | 2 (20) | 0 (0) | 0 (0) | 0 (0) | 1 (10) |
| Tick species        | Developmental stage/Gender | No. of specimens (No. of pools) | Host   | Origin              | Date of collection | Rickettsia (No. of pools (MIR %)) | Coxiella (No. of pools (MIR %)) | Rickettsiella (No. of pools (MIR %)) | Francisella (No. of pools (MIR %)) | Babesia (No. of pools (MIR %)) | Adelina (No. of pools (MIR %)) |
|---------------------|----------------------------|--------------------------------|--------|---------------------|--------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|---------------------------------|----------------------------------|
| Argas sp.           | larvae                     | 2 [2]                           | C. garrulus | Pinarejos (Segovia) | 15/07/2004         | 0 (0)                            | 2 (100)                           | 1 (50)                            | 0 (0)                            | 0 (0)                           | 0 (0)                            |
| Argas sp.           | Males                      | 2 [2]                           | C. garrulus | Illana (Guadalajara)| 05/07/2004         | 0 (0)                            | 2 (100)                           | 0 (0)                            | 0 (0)                            | 0 (0)                           | 0 (0)                            |
| Total               |                            |                                 |        |                     |                    | 112 (74.7)                       | 10 (6.7)                          | 1 (0.7)                           | 1 (0.7)                           | 1 (0.7)                         | 1 (0.7)                          |
| A. reflexus         |                            | 8 (3)                           |        |                     |                    | 0 (0)                            | 0 (0)                             | 2 (25)                            | 0 (0)                            | 0 (0)                           | 0 (0)                            |
| Argas sp.           |                            | 83 (20)                         |        |                     |                    | 81 (97.6)                        | NP                                | 0 (0)                             | 1 (1.2)                          | 1 (1.2)                         | 0 (0)                            |
| Argas sp.           |                            | 33 (10)                         |        |                     |                    | 31 (93.9)                        | NP                                | 1 (3)                             | 1 (3)                            | 0 (0)                           | 0 (0)                            |
| Argas sp.           |                            | 22 (6)                          |        |                     |                    | 0 (0)                            | 2 (9.1)                           | 6 (27.3)                          | 0 (0)                            | 0 (0)                           | 0 (0)                            |
| Argas sp.           |                            | 2 (2)                           |        |                     |                    | 0 (0)                            | 2 (100)                           | 1 (50)                            | 0 (0)                            | 0 (0)                           | 0 (0)                            |
| Argas sp.           |                            | 2 (2)                           |        |                     |                    | 0 (0)                            | 2 (100)                           | 0 (0)                             | 0 (0)                            | 0 (0)                           | 0 (0)                            |

PI, Prevalence of infection = (No. of positive ticks/total No. of individual ticks); MIR, Minimum infectious rate = (No. of positive pools/total No. of individual ticks) × 100; NP, not processed; a,b,c,d: same pool. Positive results are shown in bold.
Anaplasmataceae, Bartonella, and Borrelia bacterium species and viruses belonging to the families Flaviviridae, Orthonairoviridae, and Phenuiviridae have not been detected.

**Tick Identification**

Of the six *Argas* genotypes detected in this study, only one could be identified to the species level, namely, *A. reflexus*. The morphological identification of ticks is challenging, even for experts (27), while the molecular approach appears to be an accurate tool for tick identification (25, 27). Nevertheless, increased effort for molecular characterization of more *Argas* species is needed for a reliable taxonomic inference based on molecular tools. In order to confirm if the genotypes identified in this study represent validated or potentially novel tick species, a further morphological analysis including unfed larva specimens, should be performed along with rigorous molecular analyses of *Argas* ticks from diverse geographical locations.

*Argas reflexus* specimens, known as the pigeon tick, have been collected in two bird nests in Almería (Southern Spain), one occupied by little owl (*n* = 7) and one by roller (*n* = 1). This species occurs in Spain, and it is a well-known ectoparasite of little owls, whereas information regarding roller infestations is scarce (6, 20, 28). *Argas* sp. EEZA-CRETAV4 and *Argas* sp. EEZA-CRETAV5, genotypes amplified in Central Spain, clustered molecularly with *A. persicus* from China (29), but in a different branch than other *A. persicus* specimens (Figure 1). Nevertheless, the broad genetic divergence of this group (29, 30), also revealed in the phylogeny inferred herein (Figure 1), suggests that cryptic species could occur in this taxon known as fowl tick. This worldwide distributed tick has been previously reported in Spain and is known to infest wild birds also in other countries (6, 28). *Argas* sp. EEZA-CRETAV1–2 genotypes clustered together and appear to be closely related with *A. vulgaris* and *A. polonicus*, respectively (Figure 1). Neither of these two tick species have been previously reported from western Europe and their occurrence is only documented in a few eastern European countries (20, 21, 31). These genotypes have been found in the nest material of roller and little owls, occurring in the same nests in several cases (Table 1). In the study area, there is a high competition among cavity-nesting birds for suitable cavities and the same cavity can be successively used by little owls, pigeons and rollers (32). This fact could explain the infestation of both bird species by the same nidicolous tick taxa, i.e., *A. reflexus* and *Argas* sp. EEZA-CRETAV1–2. In contrast, *Argas* sp. EEZA-CRETAV3, closely related to *A. africolumbae*, has been found only in nests occupied by rollers. The roller is a long-distance migrant species (trans-Saharan migrant) and the Spanish populations overwinter in different African regions (33). It is worth mentioning that *A. africolumbae* is an ornithophilic tick that occurs in Africa: South Africa, Kenya, Tanzania and Burkina Faso (34, 35). It is well-known that birds can serve as dispersers of ticks and tick-borne microorganisms, even though this information pertains mainly to hard ticks (36, 37). Some studies suggest that the role of birds as dispersers of soft ticks is less important, due to the biology of these ticks (nidicolous behavior and shorter blood-feeding time), but the role of migratory birds as reservoirs or amplifiers of tick-borne microorganisms associated with soft ticks remains to be better investigated (38, 39).

**Tick-Borne Microorganisms**

The microbiological screening of ticks is important to identify the local risks of emergence of tick-borne diseases. *Argas* species, including *A. reflexus* and *A. persicus*, have been described as important pests and vectors of diseases in poultry and wild birds, being responsible for high economic losses (7, 11, 40). These tick species have also been recorded biting humans and causing anaphylaxis episodes (8, 10). Although humans are accidental hosts of *Argas* ticks and the ticks carry numerous microorganisms, human pathogens among them, the role of these ticks as vectors of human infectious agents has not been proven.

The most prevalent microorganisms amplified in this study belong to the *Rickettsia* genus (α-Proteobacteria; Rickettiaceae). The high prevalence of *Rickettsia* spp. found in ticks of *Argas* sp. EEZA-CRETAV1–2 specimens suggests that the bacterium is a common endosymbiont of ticks of the two genotypes. The detection of the newly described *Rickettsia* strain in tick-body samples, but not in tick-leg samples of ticks of other genotypes (*Argas* sp. EEZA-CRETAV3–5), suggests that this *Rickettsia* species may not be a true intracellular endosymbiont and its presence in the former genotypes could be acquired through feeding on infected hosts or by co-feeding. The phylogenetic analysis of *Rickettsia* sp. EEZA-CRETAV reveals its close relation with *R. fournieri* and *Ca. R. vini*, both *Rickettsia* species associated with ornithophilic nidicolous ticks (41, 42). While *R. fournieri*...
FIGURE 2 | Phylogenetic tree showing the relationships between Rickettsia sp. EEZA-CRETA and published Rickettsia spp. taxa. The evolutionary analysis was inferred using the maximum likelihood method and general time reversible + G model with Mega X, by concatenating fragments of six genes (sca4, 16s rRNA, ompB, ompA, 17-kDa, and gltA). The analysis involved 34 nucleotide sequences and a total of 4,120 positions in the final dataset. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers (>65%) shown at the nodes correspond to bootstrapped percentages (for 500 repetitions). The GenBank accession number of the sequences used in the analysis is shown in brackets after Rickettsia taxon name and the corresponding strain. Sequences obtained in this study are marked with diamond.
Phomrmar et al.

Argas and Tick-Borne Microorganisms, Spain

TABLE 5 | Highest identities reached between fragment genes of Francisella, Coxiella, and Rickettsiella spp. detected in the present study and published sequences.

| Bacteria | Gene (GenBank accession No.) | Identity (%) | Species (GenBank accession No.) |
|----------|-------------------------------|--------------|----------------------------------|
| Francisella sp. EEZA-CRETAV | rpoB MW287617 | 97.4 | Francisella persica ATCC (CP013202, CP012505) |
| Coxiella sp. of Argas reflexus | rpoB MW287616 | 100 | Coxiella-like endosymbiont of Argas reflexus (KY677983, KY677982) |
| Coxiella sp. EEZA-CRETAV1 | rpoB MW287614 | 96.7 | Coxiella-like endosymbiont of Ornithodoros rostratus (KP985288-91) |
| Coxiella sp. EEZA-CRETAV2 | rpoB MW287615 | 96.5 | Coxiella-like endosymbiont of Ornithodoros rostratus (KP985288-91) |
| | groEL MW287611 | 97.0 | Uncultured Coxiella sp. (KJ459055-6; detected in Ornithodoros capensis) |
| | groEL MW287612 | 97.4 | Coxiella-like endosymbiont of Ornithodoros peruvianus (KP985476-7) |
| Rickettsiella sp. EEZA-CRETAV | groEL MW287613 | 99.7 | Rickettsiella endosymbiont of Ornithodoros normandi (KP985530, KP985531) |

has been described only once from A. lagenoplastis in Australia (41), Ca. R. vini has been detected in several European countries in Ixodes spp. (36, 42–45). The single study performed suggests that Ca. R. vini is not pathogenic (43). Nevertheless, the two Rickettsia taxa are closely related to other Rickettsia species that are recognized as human pathogens, specifically Rickettsia japonica and Rickettsia heilongjiangensis (46, 47). Thus, the epidemiology and pathological potential of Rickettsia strains such as Rickettsia sp. EEZA-CRETAV, in addition to R. fournieri and Ca. R. vini, should be further investigated. Likewise, the isolation of Rickettsia sp. EEZA-CRETAV is necessary to gain an insight into the epidemiological importance of this strain.

In addition to the Rickettsia taxon, this study has revealed for the first time different proteobacterial tick endosymbionts in Argas spp. from Spain, namely, Coxiella, Rickettsiella (Gamma-proteobacterium; Coxiellaceae) and Francisella (Gamma-proteobacterium; Francisellaceae) species. The detected species, commonly known as Rickettsiella-like, Coxiella-like, and Francisella-like, are intracellular obligatory endosymbionts important for tick survival. They play some role in B vitamins biosynthesis and their presence may interfere with the transmission (positively or negatively) of other microorganisms, including tick-borne pathogens (48). They are related to species responsible for important human diseases. For instance, Coxiella burnetii and Francisella tularensis cause Q fever and tularemia, respectively (49, 50). Coxiella-like species have been implicated in human and avian diseases (51, 52). Three different Coxiella strains have been successfully detected in this study. Of them, the Coxiella sp. from A. reflexus was homologous to the species previously detected in the same tick species, but the remaining two strains, designated as Coxiella sp. EEZA-CRETAV1–2, differ from the scarcely-known Coxiella-like strains of Argas species (14, 53–55). To date, Rickettsiella has been mainly reported in hard ticks (Ixodes spp. and Haemaphysalis spp.) and soft ticks belonging to the genus Ornithodoros (14). The presence of Rickettsiella in Argas ticks has been suggested for the bat tick A. transgariepinus from Namibia, but the available 16S rRNA sequences shared low identities with the published Rickettsiella sequences (17). In the present study, a Rickettsiella sp. similar to that of O. normandi from Tunisia (55) was detected in an Argas sp. EEZA-CRETAV2 specimen. Lastly, a strain closely related to F. persicus was found in an Argas sp. EEZA-CRETAV1 specimen. Francisella persicus, formerly Wolbachia persica, is an endosymbiont of Arbusarbus (previously referred to as A. persicus) (56). This bacterium has not been identified as an animal or human pathogen, but the analysis of its genome shows that this species conserves an important number of potentially functional virulence-associated genes, suggesting that it could be pathogenic to mammals (57).

Bacteria of anaplasmatraceae family, Bartonella, and Borrelia spp. have not been detected in the ticks analyzed. Argas spp. are recognized vectors of B. anserine, the agent of the avian
FIGURE 3 | Maximum likelihood trees of Babesia species based on 18S rRNA analysis. The evolutionary analysis was inferred using Tamura-Nei model + G model with Mega X. The analysis involved 39 nucleotide sequences and a total of 483 positions in the final dataset. The tree is drawn to scale, with branch lengths (Continued)
spirochetosis, a worldwide distributed disease of veterinary importance that has not been reported from Spain (11, 40). The lack of virus detection in this study was unexpected, because diverse viruses are readily detected in *Argas* ticks (7). Scarce tick-borne viruses have been described from Spain and all but Meaban-like virus, a flavivirus found in *Ornithodoros maritimus* (38), are associated to ixodid ticks. Some of these viruses have a great relevance for human health, e.g., Crimean-Congo hemorrhagic fever virus, whose arrival in infected ticks has been suggested to take place through migratory birds (37,58). This fact highlights the importance of studying viruses in soft ticks associated to birds in Spain, an important area in the migratory routes of many avian species, because what it is not sought, it is not found (59).

Two apicomplexan parasites have been found in this work, *Babesia* sp. and *A. bambarooniae*, though the latter species is not known as a tick-borne microorganism. In turn, the apicomplexan piroplasms *Babesia* (Aconoidasida; Babesiidae) are mainly vectored by ixodid ticks, though argasid ticks also were suggested as potential vectors (60). *Babesia* sp. EEZA-CRETAV has been amplified from an *Argas* sp. EEZA-CRETAV3 tick associated with rollers and the presence of the protozoan in the blood of rollers cannot be rejected. Sixteen *Babesia* species responsible for avian piroplasmosis, in addition to several strains that are not fully identified, are known (61). Of these, *Babesia fragilica*, *Babesia shortti* and *Babesia benneti* have been reported from Spain (62–64). *Babesia* sp. EEZA-CRETAV is closely related to some of the scarcely genetically characterized *Babesia* species, mainly to *B. ardeae* (Figure 3). This species has been detected in Asia and its pathogenicity is unknown (61). This strain is also close to the human-pathogenic *B. ducanii* that has been identified in North America, the United Kingdom and Australia (65). To our knowledge, *Babesia* spp. have not been identified in owls.

CONCLUSIONS

The sedentary lifestyle of soft ticks could imply a limited role of these ticks in the circulation of infectious agents (66). However, as indicated by this study, the high re-use of cavities within and between years by different bird species could importantly enhance the spread of microorganisms associated with soft nidicolous ticks, such as *Argas* ticks.

This study highlights the richness of nidicolous *Argas* ticks associated with cavity-nesting birds in a semi-desert area in Western Europe, and suggests that the diversity of this genus in Spain might be underestimated. Moreover, this work provides the first report of *Rickettsia* sp., *Coxiella* spp., *Rickettsiella* sp., *Francisella* sp. and *Babesia* sp., from soft ticks in Spain, and *A. bambarooniae* from Ixodida.

Further research should be carried out to confirm if the new genotypes of ticks and their microorganisms represent novel taxa and, additionally, to understand their role in the epidemiology of zoonoses using the One Health approach.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.
AUTHOR CONTRIBUTIONS

APa, RV, JO, and FV designed the initial study. JV and FV carried out the field work. APa performed the tick identification and tick processing. APa, APO, SS, and PS implemented the analysis of microorganisms. APa and FV wrote the first draft of the manuscript. All authors contributed to data interpretation and revisions.

FUNDING

RV was funded by the Research Grant Agency (VEGA) of the Ministry of Education, Science, Research and Sport of the Slovak Republic and Slovak Academy of Sciences (2/0023/20). FV received financial support from the projects CGL2014-55969 and PGC2018-097426-B-C22 (Spanish Ministry of Universities, Spanish State Research Agency, FEDER Program, European Union). JV was funded by a predoctoral grant (BES-2015-075951) of the Spanish Ministry of Science and Innovation.

ACKNOWLEDGMENTS

We would like to thank Agustín Estrada-Peña (University of Zaragoza) for helping with the taxonomy of Argas spp. We thank L. Bolonio and I. E. Cardiel (CEBIME, Conservación y Estudio de la Biodiversidad en Medios Esteparios) for providing tick samples. Teresa Martínez, Maite Amat, Miguel Ángel Calero, and Stanislav Kolenick helped with fieldwork. Junta de Andalucía kindly provided permits to sample birds and their nests. We thank Dr. Didier Raoult (Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, France), Dr. J. Stephen Dumler (The Johns Hopkins Hospital, USA), Dr. Houssam Attoui [UMR Virologie, National Institute for Agriculture, Food, and Environment (INRAE), France], Dr. Luis M. Hernández-Triana [Wildlife Zoonooses and Vector-borne Diseases Research Group, Animal and Plant Health Agency, United Kingdom], Dr. Joaquim Ruiz (Universidad Científica del Sur, Peru), Dr. Volker Fingerle (German National Reference Centre for Borrelia), and Dr. Matthias Niedrig (Centre for Biosafety, Robert Koch Institute, Germany) for providing positive controls.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.637837/full#supplementary-material

REFERENCES

1. Guglielmone AA, Robbins RG, Apanaskevich DA, Petney TN, Estrada-Peña A, Horak IG, et al. The argasidae, ixodidae and nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. Zootaxa. (2010) 2528:1–28. doi: 10.11646/zootaxa.2528.1.1
2. Dushabek F. Argas (Argas) vulgaris Filippova, 1961, a new member of Czechoslovak tick fauna. Folia Parasitol. (1976) 23:281–8.
3. Dikaei B. Argasid tick fauna (Argasidae) of the Chechen-Ingush ASSR. Parasitologia. (1981) 15:76–8.
4. Estrada-Peña A, Lucientes J, Sánchez C. Argas (Persicargas) gilcolla n. sp. (Acariinae: Argasidae): a parasite of the griffon vulture, Gyps fulvus, in Spain. J Parasitol. (1987) 73:824–8.
5. Estrada-Peña A, Pfaffle M, Baneth G, Kleinerman G, Petney TN. Ixooideae of the western Palearctic: a review of available literature for identification of species. Ticks Tick Borne Dis. (2017) 8:512–25. doi: 10.1016/j.ttbdis.2017.02.013
6. Cordero del Campillo M, Castaño Ordóñez L, Reguera Feo A. [Índice Catálogo de Zooparásitos Ibéricos]. Spain: Ediciones Universidad de León (1994).
7. Hoogstraal H. Argasid and nuttalliellid ticks as parasites and vectors. Adv Parasitol. (1985) 24:135–238. doi: 10.1016/S0065-308X(08)60563-1
8. Estrada-Peña A, Jongejans F. Ticks feeding on humans: a review of records on human-biting ixoidea with special reference to pathogen transmission. Exp Appl Acarol. (1999) 23:685–715. doi: 10.1023/a:100621108739
9. Rosenstein M. Paralysis in chickens caused by larvae of the poultry tick, Argas persicus. Avian Dis. (1976) 20:407–9. doi: 10.2307/1589261
10. Boni E, Inconvaia C. Near-fatal anaphylaxis with Kounis syndrome caused by Argas reflexus bite: a case report. Clin Mol Allergy. (2020) 18:4. doi: 10.1186/s12948-020-01121-w
11. Gothe R, Buchheim C, Schrecke W. Argas (Persicargas) persicus and Argas (Argas) afriicolus as natural vectors of Borrelia anserina and Aegyptienna pullorum in upper Volta. Berliner Munchener Tierarztlche Wochenschrift. (1981) 94:280–5.
12. Vargina SG, Kuchuk LA, Gershtein VI, Karas FR. [Transmission of Isykk Kul virus by Argas persiculans ticks in experiment]. Sborn nauch Tr Inst Virus Im Ivanov Akad Med Nauk SSSR (1982). p. 123–7. doi: 10.1128/genomeA.00662-15
13. Karadjian G, Chavatte J, Landau I. Systematic revision of the adelie haemogregarines, with creation of Bartazoan n. g., reassignment of Hepatozoon argantis Garnham, 1954 to Helomelia, and molecular data on Helomelia stellata. Parasite. (2015) 22:31. doi: 10.1051/parasite/2015031
14. Duron O, Binetruy F, Noël V, Cremaschi J, McCoy KD, Arnathau A, et al. Evolutionary changes in symbiont community structure in ticks. Mol Ecol. (2017) 26:2905–21. doi: 10.1111/mec.14094. doi: 10.1111/mec.14094
15. Lafi I, El Hamzaoui B, Bitam I, Leulmi H, Lahou R, Mediannikov O, et al. Detection of relapsing fever Borrelia spp., Bartonella spp. and Anaplasmataceae bacteria in argasid ticks in Algeria. PLoS Negl Trop Dis. (2017) 11:e0006064. doi: 10.1101/journal.pntd.0006064
16. Lv J, Fernández de Marco MM, Goharriz H, Phipps LP, McElhinney LM, Hernández-Triana LM, et al. Detection of tick-borne bacteria and babesia with zoonotic potential in Argas (Carvis) vespertilionis (Latreille, 1802) ticks from British bats. Sci Rep. (2018) 8:1865. doi: 10.1038/s41598-018-20138-1
17. Reeves WK, Mans BJ, Durden LA, Miller MM, Gratton EM, Laverty TM, Rickettsia hongkongensis and a Rickettsia from the Bat Tick Argas sp. N. Curr Genet. (2020) 66:663–720. doi: 10.1007/s00294-020-02105-z
18. Lázaro R, Rodrigo FS, Gutiérrez L, Domingo F, Puigdefábregas J. Analysis of a thirty-year rainfall record (1967–1997) from semi-arid SE Spain: a plant ecological perspective. J Arid Environ. (2001) 48:373–95. doi: 10.1016/j.jaridenv.2000.0735
19. Cramp S. The Complete Birds of the Western Palearctic on CDROM. Oxford: Oxford University Press (1998).
20. Filippova NA. [Argasid Ticks (Argasidae)]. Fauna of USSR, Arachnoidae 4(3]. Moskw: ‘Nauka’ Publishing House (1966).
21. Siuda K, Hoogstraal H, Clifford CM, Wassef HY. Observations on the subgenus Argas (Ixoidea: argasidae: Argas). 17. Argas (A.) polonicus sp. N. parasitizing domestic pigeons in Krakow, Poland. J Parasitol. (1979) 65:170–81.
22. Morin G. Fauna D’Italia Ixodida. Bologna: Calderini (1998).
23. Estrada-Peña A, Mihalca AD, Petney T. Ticks of Europe North Africa. A Guide to Species Identification. Switzerland: Springer International Publishing AG (2017). doi: 10.1007/978-3-319-63576-0

24. Portillo A, Santos AS, Santibáñez S, Pérez-Martínez L, Blanco JR, Ibarra V, et al. Detection of a non-pathogenic variant of Anaplasma phagocytophilum in Ixodes ricinus from La Rioja, Spain. Ann NY Acad Sci. (2005) 1063:333–6. doi: 10.1196/annals.1355.053

25. Black WC, Piesman J. Phylogeny of hard and soft tick taxa (Acari: Ixodida) and their hosts. In: Palomar AM, Portillo A, Santibáñez S, García-Álvarez L, Oteo JA. Prevalence of ‘pathogenic’ rickettsiae in Ixodes arboricola ticks in the north of Spain, 2011–2013. Parasit Vectors. (2015) 8:110. doi: 10.1186/s13071-015-0724-6

26. Palomar AM, Portillo A, Crespo A, Santibáñez S, Mazuelas D, Oteo JA. Prevalence of ‘pathogenic’ rickettsiae in Ixodes arboricola ticks in the north of Spain, 2011–2013. Parasit Vectors. (2015) 8:110. doi: 10.1186/s13071-015-0724-6

27. Estrada-Peña A, D’Amico G, Palomar AM, Dupraz M, Fonville M, Heylen D, et al. A comparative test of ixodid tick identification by a network of European researchers. Ticks Tick Borne Dis. (2017) 8:540–6. doi: 10.1111/tid.12700

28. Doss MA, Farr MM, Roach KE, Anastas G. Special Publication No. 3, Ticks Tick Borne Diseases, II. Hosts, Part 1. A-F. United States Government Printing Office. (1974). Available in: http://hdl.handle.net/1969.1/239068 (accessed January 24, 2021).

29. Feng J, Wu M, Wulan, Huang T, Zhang J, Renbati N, et al. Identification of two genotypes of argas persicus and associated rickettsia-specific genes from different regions of inner mongolia. J Parazitol. (2019) 95:92–101. doi: 10.1645/18-15

30. Muñoz-Leal S, Venzal JM, Nava S, Reyes M, Martins TF, Leite RC, et al. The geographic distribution of Argas (Persicargas) miniatus and Argas (Persicargas) persicus (Acari: Argasidae) in America, with morphological and molecular diagnoses from Brazil, Chile and Cuba. Ticks Tick Borne Dis. (2018) 9:44–56. doi: 10.1111/tid.12700

31. Dusbábek F. Identity of Argas (Argas) polonicus populations in Czechoslovakia and Poland. Folia Parasitol. (1985) 32:163–71.

32. Valera F, Václav R, Calero-Torralbo MA, Martínez T, Veiga J. Natural cavity network of European researchers. Ticks Tick Borne Dis. (2017) 8:1001. doi: 10.1111/tid.12700

33. Rodríguez-Ruiz J, de la Puente J, Parejo D, Valera F, Calero-Torralbo MA, Martínez T, Veiga J. Natural cavity network of European researchers. Ticks Tick Borne Dis. (2017) 8:1001. doi: 10.1111/tid.12700

34. Hoogstraal H, Kaiser MN, Walker JB, Ledger JA, Converse JD, Rice RGA. Rickettsia fournieri sp. nov, a novel spotted fever group rickettsia from Argas persicus and associated rickettsia-specific genes. Ann NY Acad Sci. (2012) 1239:103902. doi: 10.1111/j.1748-0469.2012.103902.x

35. Bonnet SF, Binetruy F, Hernández-Larguin AM, Durón O. The Tick Microbiome: why non-pathogenic Microorganisms Matter in Tick Biology and Pathogen Transmission. Front Cell Infect Microbiol. (2017) 7:236. doi: 10.3389/fcimb.2017.00236

36. Ellis J, Oyston PC, Green M, Tibball RW. Tularemia. Clin Microbiol Rev. (2002) 15:631–46. doi: 10.1128/CMR.15.4.631-646.2002

37. Oteo JA, Pérez-Cortés S, Santibáñez P, Gutiérrez E, Portillo A, Blanco JR, et al. Q fever endocarditis associated with a cardiovascular implantable electronic device. Clin Microb Infect. (2012) 18:E482–4. doi: 10.1111/j.1469-0691.2012.03992.x

38. Reaves WK. Molecular evidence for a novel Crimean-Congo hemorrhagic fever virus in Ixodes arboricola ticks and propagation in tick cell lines. Ticks Tick Borne Dis. (2020) 11:101511. doi: 10.1111/tid.12700

39. Ando S, Kurosawa M, Sakata A, Fujita H, Sakai K, Sekine M, et al. Human Rickettsia helongiangensis infection, Japan. Emerg Infect Dis. (2010) 16:1306–8. doi: 10.3202/jid.2015.100049

40. Uchida T. Rickettsia japonica, the etiologic agent of Oriental spotted fever. Microbiol. Immunol. (1993) 37:91–102. doi: 10.1111/j.1440-1625.1993.tb03185.x

41. Duron O, Calero-Torralbo MA, Portillo A, Blanco JC, et al. Q fever endocarditis associated with a cardiovascular implantable electronic device. Clin Microb Infect. (2012) 18:E482–4. doi: 10.1111/j.1469-0691.2012.03992.x

42. Palomar AM, Portillo A, Santibáñez S, Santibáñez P, García-Álvarez L, Oteo JA. Prevalence of ‘pathogenic’ rickettsiae in Ixodes arboricola ticks in the north of Spain, 2011–2013. Parasit Vectors. (2015) 8:110. doi: 10.1186/s13071-015-0724-6

43. Nováková M, Costa FB, Krause F, Literak I, Labruna MB. Multiple Acquisitions of Pathogen-Derived piroplasm from Phalacrocoracidae in South Africa. Ticks. Genomes. (2020) 12:0187. doi: 10.1002/tgs.20699

44. Beringer JM, Parola P, RaoULT D. Isolation of Wolbachia persica from La Rioja, Spain. Ann NY Acad Sci. (2018) 139:103902. doi: 10.1111/micp.13902

45. Reeves WK. Molecular evidence for a novel Coxella from Argas monolakensis (Acari: Argasidae) from Mono Lake, California, USA. Exp Appl Acarol. (2008) 44:57–60. doi: 10.1007/s10393-008-9128-x

46. Angelakis E, Mediannikov O, Jos SL, Berenger JM, Parola P, Raoult D. Crimean-Congo tick-borne flavivirus infection, Japan. Emerg Infect Dis. (2016) 22:285–8. doi: 10.3202/eid2201.150106

47. Hosseini-Chegeni A, Kayedi MH. Molecular detection of Coxella (Gammaproteobacteria: Coxillaceae) in Argas persicus and Alveonasus canestrini (Acari: Argasidae) from Iran. Microb Pathog. (2020) 139:103902. doi: 10.1016/j.micpath.2019.103902

48. Reaves WK. Molecular evidence for a novel Coxella from Argas monolakensis (Acari: Argasidae) in the United States. Emerg Infect Dis. (2012) 18:2057–60. doi: 10.3202/jemdoc.2012.08025

49. Díaz-Arós A, Buerki S, Sleigh AM, Harder P, Capron A. Cross-species transmission of Rickettsia microti to Chasmatodon septemlineatus (Aves: Phaethontidae) by Dermacentor variabilis (Acari: Ixodidae). Emerging Microb Infect. (2016) 5:297–301. doi: 10.1186/s13241-016-0297-9

50. Peirce MA, Parson NJ. Babesia ugwidiensis, a new species of avian Babesia from Phalacrocoracidae in South Africa. Parasite. (2012) 19:375–9. doi: 10.1051/parasite/2012194375
61. Chavatte J, Okumura C, Landau I. Redescription of Babesia ardeae Toumanoff, 1940, a parasite of Ardeidae, including molecular characterization. Parasitol Res. (2017) 116:1089–97. doi: 10.1007/s00436-017-5394-1
62. Blanco G, Merino S, Tella JL, Fargallo JA, Gajón A. Hematozoa in two populations of the threatened red-billed chough in Spain. J Wildl Dis. (1997) 33:642–5. doi: 10.7589/0090-3558-33.3.642
63. Merino S. Babesia bennetti n. sp. from yellow-legged gull (Larus cachinnans, Aves, Laridae) on Benidorm Island, Mediterranean Sea. J Parasitol. (1998) 84:422–4. doi: 10.2307/3284504
64. Muñoz E, Molina R, Ferrer D. Babesia shortti infection in a common kestrel (Falco tinnunculus) in Catalonia (northeastern Spain). Avian Pathol. (1999) 28:207–9. doi: 10.1080/03079459994957
65. Young KM, Corrin T, Wilhelm B, Uhland C, Greig J, Mascarenhas M, et al. Zoonotic Babesia: a scoping review of the global evidence. PLoS ONE. (2019) 14:e0226781. doi: 10.1371/journal.pone.0226781
66. Rataud A, Dupraz M, Toty C, Blanchon T, Vittecoq M, Choquet R, et al. Evaluating Functional Dispersal in a Nest Ectoparasite and Its Eco-Epidemiological Implications. Front Vet Sci. (2020) 7:570137. doi: 10.3389/fvets.2020.570137

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Palomar, Veiga, Portillo, Santibáñez, Václav, Santibáñez, Oteo and Valera. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.