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Molecular and MALDI-TOF MS characterisation of *Hyalomma aegyptium* ticks collected from turtles and their associated microorganisms in Algeria

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ABSTRACT

The identification of ticks and their associated pathogens is important for knowledge on tick-borne diseases. The objective of this study was to use morphological, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and/or molecular biology tools to identify ticks collected from turtles in north-eastern Algeria, as well as to investigate the microorganisms associated with these ticks. A total of 471 adult ticks were collected and identified morphologically as *Hyalomma aegyptium*, of which 248 (52.7%) were female and 223 (47.3%) were male. amongst them, 230 specimens were randomly selected for molecular and MALDI-TOF MS analysis. Molecular biology confirmed that our ticks were *Hy. aegyptium*. MALDI-TOF MS analysis revealed that 100% of the spectra were of excellent quality. Four spectra were selected to update our own database MALDI-TOF MS arthropod. The blind test of the 226 remaining spectra showed that all ticks were correctly identified, with scores ranging from 1.774 to 2.655 with a mean of 2.271 ± 0.16 of which, 223 (98.6%) had log score value (LSV)>1.8. Molecular biology screening showed that the ticks carried the DNA of *Borrelia turcica, Rickettsia africae, Rickettsia aeschlimannii, Rickettsia sibirica mongolitimonae* and with the Anaplasmataceae were close to a potentially new, undescribed *Ehrlichia* sp. This study confirms that MALDI-TOF MS is a reliable tool for the identification of ticks and that ticks collected from turtles in Algeria are carriers of several species of microorganisms which may be responsible for diseases in humans and animals.

1. Introduction

Ticks are obligate haematophagous arthropods that parasitize most land vertebrates and occasionally humans (Chitimia-Dobler et al., 2018; Parola et al., 2013; Parola and Raoult, 2001). While feeding, ticks may act as vectors of infectious agents such as bacterial, viral, protozoan agents and helminths to animals and humans (Hall-Mendelin et al., 2011; Parola et al., 2013).

Hyalomma aegyptium is a hard tick species with a typical three-host life cycle (Paștiu et al., 2012). In its adult stage, it mainly feeds on turtles of the genus *Testudo* (*Testudinidae*) (Tiar et al., 2016). The larvae and nymphs of *Hy. aegyptium* may also feed on various vertebrates: turtles, lizards, birds, small mammals and even humans (Apanaskevich, 2004; Bursali et al., 2010). The geographical distribution of *Hy. aegyptium* extends from north west Africa, the Mediterranean region, the Balkans, the Middle East, Turkey, Afghanistan and Pakistan, the Caucasus, and Central Asia (Tiar et al., 2016). The presence of *Hy. aegyptium* has also been confirmed in Algeria, Romania, Morocco, Syria, Lebanon, and Iran (Siroký et al., 2009). Several microorganisms have been associated with *Hy. aegyptium*, including *Rickettsia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Borrelia* spp. *Coxiella burnetii* (Kumar et al., 2020), *Theileria annulata* (Ray, 1950), *Hemolivia mauritanica* (Paștiu et al., 2012) and *Hepatozoon kisrae* (Paperna et al., 2002).

The identification of ticks and their associated pathogens is important for knowledge on tick-borne diseases. Morphological identification is mainly based on the observation of morphological characters using dichotomous keys (Parola and Raoult, 2001). It is often limited by a lack of entomological expertise, the availability of identification keys and documentation, the inability to identify species complexes, and damaged specimens (Yssouf et al., 2016). Molecular identification based

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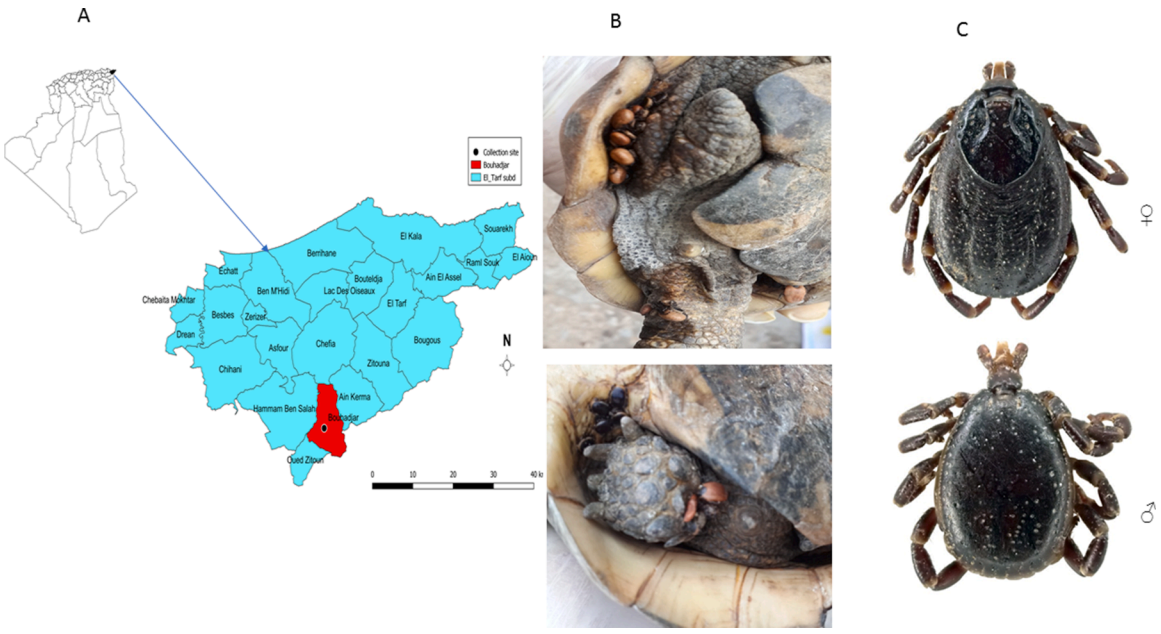

Fig. 1. Map of Algeria showing the sites where ticks used in this study were collected from turtles between June and August 2019 (A). Parts of the turtle showing attached ticks (B); Picture of a female (above) and a male (below) of *Hy. aegyptium* (C).

on amplification and sequencing targeting a portion of various genes can overcome the limitations of morphological identification (Yssouf et al., 2016). However, this method faces some problems such as high reagent costs, lack of sequences of all species described on public databases, or universal primers allowing a gene in all tick species to be identified (Yssouf et al., 2016). The use of half or part of the tick body, especially for immature stages (larvae and nymphs), which results in the destruction of the tick body, can also be a drawback of molecular biology. This destruction of the tick body can be avoided in larger adults where the legs are sufficient for molecular identification. Recently, matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used, in clinical microbiology and mycology but also in entomology (Sevestre et al., 2021). The objective of this study is, firstly, to identify, using MALDI-TOF MS, ticks collected from turtles in the eastern region of Algeria and preserved in 70% alcohol and, secondly, to search for the micro-organisms associated with these ticks using molecular biology tools.

2. Materials and methods

2.1. Study areas and period of collection

The ticks were collected from turtles belonging to the same species, *Testudo graeca nabeulensis*, between June and August 2019. This collection was carried out in Bouhadjar, a town south of El Tarf (36° 42 '2 "N, 8° 18′50″ E) (Fig. 1A). This region is located in the extreme north-east of Algeria, characterised by Mediterranean climatic conditions and an altitude of 400 m. The hot season extends from May to October, with average temperatures of above 20 °C, and the cold season extends from November to April, with average temperatures lower than 16 °C. The average annual precipitation is 556. mm, but rains are irregular. Rainfall ranges from 23 mm in summer to 221 mm in winter.

2.2. Ethics statement

Permission for sampling on wild animals was granted by the El Tarf University Animal Ethics Committee, according to Algerian legislation (Ordinance No. 06–05 of 19 Joumada Ethania 1427 corresponding to 15 July 2006 on the Protection and Preservation of Certain Animal Species Threatened with Extinction).

2.3. Tick collection and morphological identification

Ticks were collected from live turtles that were then released back into the field. One handler held the turtle in a position that allowed access to the different parts of body, namely the neck, the joints of the front and rear legs, the tail, and the extremities of the ventral face of the carapace (Fig. 1B). Another handler removed the ticks either manually or with a forceps. Ticks from the same animal were counted, stored in the same tube containing 70% ethanol, and kept at room temperature before being sent to VITROME, IHU Mediterranée Infection (Marseille, France) for analysis. In Marseille, ticks were identified morphologically at the species level using the appropriate taxonomic keys (Hillyard, 1996) under microscope at a magnification of × 56 (Zeiss Axio Zoom. V16, Zeiss, Marly le Roi, France). The sex (male and female) of each specimen was determined and pictures were taken (Fig. 1C).

2.4. Tick dissection

Each tick was rinsed with distilled water and dried with sterile filter paper. A single sterile surgical blade was used to remove all four legs on one side and the tick was cut longitudinally into two equal parts, as previously described (Boyer et al., 2019a; Kernif et al., 2012). The four legs and the half without legs of each tick were used for MALDI-TOF MS and molecular analyses, respectively. The legged half of each specimen was frozen at −20 °C.

2.5. DNA extraction and molecular identification of ticks

Half of each legless tick was incubated at 56 °C overnight with 180 µL of G2 lysis buffer (Qiagen, Hilden, Germany) and 20 µL of proteinase K (Qiagen, Hilden, Germany). DNA tracking was performed with an EZ1 DNA tissue kit (Qiagen, Hilden, Germany) using a EZ1 Robot according to the manufacturer's instructions. Total genomic DNA was eluted in 100 µL of Tris-EDTA (TE) buffer (Qiagen) and was immediately used or stored at −20 °C until use. To confirm the morphological identification of the ticks the spectra of which were used to update our own database MALDI-TOF MS arthropod spectra (see below), a standard PCR and

Table 1.
Primers and probes used for real-time quantitative and standard PCR in this study.

PRIMERS F, R (5′−3′) AND PROBES P (6FAM-TAMRA) USING FOR QPCR			
Microorganisms	Targeted sequence	Primers f, r (5′−3′) and probes p (6FAM-TAMRA)	References
Rickettsia spp.	*gltA (RKND03)*	f_GTGAATGAAAGATTACACTATTTAT r_GTATCTTAGCAATCATTCTAATAGC p_CTATTATGCTTGCGGCTGTCGGTTC	(Rolain et al., 2002)
Borrelia spp.	*(Bor ITS4)*	f_GGCTTCGGGTCTACCACATCTA r_CCGGGAGGGGAGTGAAATAG p_TGCAAAAGGCACGCCATCACC	(Mediannikov et al., 2010b)
Bartonella spp.	*(Barto ITS2)*	f_GATGCCGGGGAAGGTTTTC r_GCCTGGGAGGACTTGAACCT p_GCGCGCGCTTGATAAGCGTG	(Rolain et al., 2003)
Anaplasma spp.	*23SrRNA (TtAna)*	f_TGACAGCGTACCTTTTGCAT r_TGGAGGACCGAACCTGTTAC p_GGATTAGACCCGAAACCAAG	(Djiba et al., 2013)
Coxiella burnetii	*(IS1111)*	f_CAAGAAACGTATCGCTGTGGC r_CACAGAGCCACCGTATGAATC p_CCGAGTTCGAAACAATGAGGGCTG	(Ehounoud et al., 2016)
Piroplasmorida	*5.8S*	f_AYYKTYAGCGRTGGATGTC r_TCGCAGRAGTCTKCAAGTC p_FAM-TTYGCTGCGTCCTTCATCGTTGT-MGB	(Dahmana et al., 2020)
Primers f, r (5′−3′) using for standard PCR			
Rickettsia spp.	*ompA*	f_ATGGCGAATATTTCTCCAAAA r_AGTGCAGCATTCGCTCCCCCT	(Parola et al., 2001)
Borrelia spp.	*flaB*	f_TGGTATGGGAGTTTCTGG r_TAAGCTGACTAATACTAATTACCC	(Vial et al., 2006)
Anaplasma spp.	*23SrRNA*	f_ATAAGCTGCGGGGAATTGT r_TGCAAAAGGTACGCTGTCAC	(Djiba et al., 2013)
	groEL	f_GTTGAAAARACTGATGGTATGCA r_ACACGRTCTTTACGYTCYTTAAC	(Ouarti et al., 2021)

sequencing by amplifying a *16S rRNA* fragment of a predicted size of 400 bps using *16S* tick primers (F: 5′-CCGGTCTGAACTCAGATCAAGT-3′ and R: 5′-GCTCAATGATTTTTTAAATTGCTGT-3′) was performed, as previously described (Boyer et al., 2019a). DNA from *Rhipicephalus sanguineus* s.l. bred in our laboratory and extracted at the same time as our samples were used as a control. The sequences were assembled and analysed using the ChromasPro software (version 1.34) (Technelysium Pty. Ltd., Tewantin, Australia), and were then blasted against GenBank (http://blast.ncbi.nlm.nih.gov) (Diarra et al., 2017).

2.6. Sample preparation for MALDI-TOF MS analysis

The four legs of each tick were dried at 37 °C in an oven overnight to evaporate the organic solvents (Diarra et al., 2017), followed by incubation at 37 °C in 40 µL of high performance liquid chromatography (HPLC) water overnight. After drying on filter paper, the legs were homogenised in 30 µL of homogenisation buffer composed of a 50/50 (v/v) mixture of formic acid (70% v/v) (Sigma) plus acetonitrile (50% v/v) (Fluka, Buchs, Switzerland), using the TissueLyser (Qiagen) with bis glass (Sigma, Lyon, France), as previously described. After homogenising the sample, centrifugation was performed for one minute to granulate the debris and 1 µl of supernatant from each sample was loaded onto the MALDI-TOF MS target plate in quadruple (Bruker Daltonics, Wissembourg, France) and covered with 1 µl of CHCA matrix solution, composed of saturated alpha-cyano-4-hydroxycynnamic acid (Sigma, Lyon, France), 50% acetonitrile (v / v), 2.5% trifluoroacetic acid (v/v) (Aldrich, Dorset, UK), and HPLC grade water (Yssouf et al., 2016). After drying for several minutes at room temperature, the target was placed in the Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics) for analysis. To control matrix quality, sample loading, and MALDI-TOF MS performance, the matrix solution was duplicated on each MALDI-TOF MS plate with and without protein extracts from *Aedes aegypti* legs of our laboratory's breeding stock.

2.7. MALDI-TOF MS parameters

Protein mass profiles of tick legs were generated using a Microflex LT MALDI-TOF Mass Spectrometer (Bruker Daltonics, Germany), with detection in the linear positive-ion mode at a laser frequency of 50 Hz within a mass range of 2–20 kDa. The acceleration voltage was 20 kV, and the extraction delay time was 200 ns. Each spectrum corresponded to ions obtained from 240 laser shots performed in six regions of the same spot and automatically acquired using the AutoXecute method with the default parameters of the FlexControl v3.4 software (Bruker Daltonics).

2.8. Spectral processing and database creation

Spectral profiles were visualised with flex Analysis v3.3 software to check the quality of each spectrum (smoothing, baseline subtraction, and peak intensity) and to verify the reproducibility of the spectra (Benyahia et al., 2020). To upgrade our own database MALDI-TOF MS arthropod spectra, the MSP reference spectra of four *Hy. aegyptium* specimens were included in our reference MS spectra databases. Average spectra (MSP, Main Spectrum Profile) were created by combining the four spectra of one tested sample, using the automated function of the MALDI-Biotyper software (Bruker Daltonics). MSPs were created on the basis of an unbiased algorithm using peak position, intensity and frequency data using the default parameter set of the "Bio Typer MSP Creation Standard Method".

2.9. Validation of MALDI TOF MS identification of ticks by blind tests

A blind test was performed with the remaining spectra of tick legs not included in the reference MS spectra databases. The level of significance of the identification was determined using the log score values (LSVs) provided by the MALDI-Biotyper V.3.3 software. These LSVs correspond to the degree of similarity between the database of reference spectra MS and those submitted by blind testing. Scores ranging from 0 to 3 were obtained for each spectrum of the samples tested, based on previous studies (Sevestre et al., 2021; Yssouf et al., 2013). An LSV greater than 1.8 was considered as a cut-off for species identification (Yssouf et al., 2013).

2.10. Detection of microorganisms and phylogenetic analysis

DNA samples from each tick were tested by quantitative PCR according to the manufacturer's protocol using a PCR detection system; a CFX Connect™ Real-Time (Bio-Rad) with specific primers and probes targeting the following microorganisms: *Rickettsia* spp., Anaplasmataceae, *Borrelia* spp., *Bartonella* spp., *Coxiella burnetii, Theileria* spp. and *Babesia* spp. The composition of the reaction mixture and the parameters of the amplification reactions are the same as those described previously (Diarra et al., 2017). DNA from *Rickettsia montanensis, Bartonella elizabethae, Anaplasma phagocytophilum, Coxiella burnetii, Borrelia crocidurae* and *Babesia vogeli* were used as positive controls and *Rh. sanguineus* from our laboratory, free of bacteria, were used as negative controls. A sample was considered positive with a cycle cut-off value (Ct) of less than 36. Samples that were positive for qPCR *Borrelia* spp. were subjected to amplifying and sequencing a 300-bp fragment of the *flagB* gene (Vial et al., 2006). The samples positive for qPCR *Rickettsia* spp. were then subjected to amplifying and sequencing of a 650-bp fragment of the *ompA* gene (Parola et al., 2001). For positive samples at qPCR, Anaplasmataceae were then subjected to amplifying and sequencing of a 434-bp fragment of the *23S* gene and a 509-bp fragment of the *groEL* genes (Djiba et al., 2013). Sequences were assembled, analysed, and compared to GenBank (http://blast.ncbi.nlm.nih.gov) as above (Diarra et al., 2017). The primers and probes used in this study are listed in

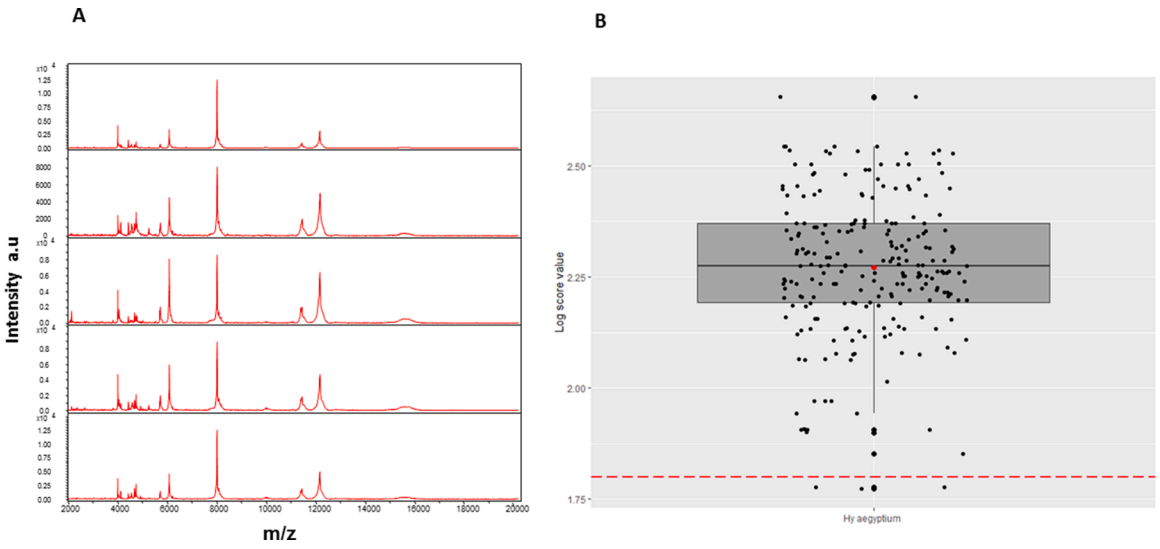

Fig. 2. Representative MALDI-TOF MS spectra from legs of five specimens of *Hy. aegyptium* (A); Bostplot showing the log score value (LSV) distribution. The red line represents the threshold of LSV above which the identification is correct, and the red dot represents the mean of the LSVs values (B).

Table 1 The different sequences were aligned using BioEdit software and phylogenetic trees were built using TOPALi 2.5 software (Biomathematics and Statistics Scotland, Edinburgh, United Kingdom. Model selection was performed in a menu containing several available models (MrBayes or PhyML). The optimal model is automatically selected and passed to the menus for launching the phylogenetic analysis and the phylogenetic trees are estimated by running for each model. The model Maximum Likelihood (ML) phylogenetic tree proposed by default in TOPALi 2.5 software was selected and used for the construction of phylogenetic trees. Node numbers are percentages of the bootstrap values obtained by repeating the analysis of 100 iterations to generate a majority consensus tree (only those with a value equal to or greater than 70 were retained.

2.11. Statistics analysis

The various statistical analyses were performed using R software version 3.4 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) and ggplot packages were used to perform the graphics.

3. Results

3.1. Tick collection and molecular identification

A total of 471 adult ticks were collected from 39 turtles in Bouhadjar between June and August 2019. Morphologically, all ticks were identified as *Hy. aegyptium*, including 223 (47.3%) males and 248 (52.7%) females. To confirm our morphological identification, four ticks were randomly selected to upgrade our MALDI-TOF spectra arthropod database and were subjected to molecular analysis. Querying the 400 base pair sequences obtained against GenBank showed that they were 99–100% identical to that of *Hy. aegyptium* (MG418680), confirming the morphological identification.

3.2. MALDI-TOF MS identification

A total of 230 ticks were selected according to host for MALDI-TOF MS analysis. The spectral analysis using Flex Analysis v3.3 software showed that 230 (100%) of the spectra had excellent quality i.e., were reproducible, had a peak intensity > 3000 au and no background noise

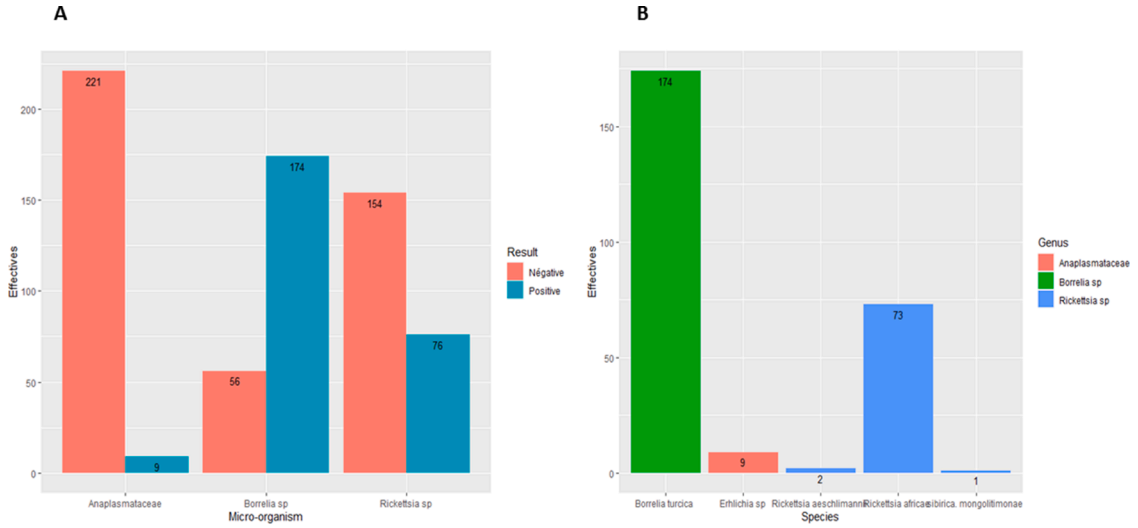

Fig. 3. Results for microorganisms screening in *Hy. aegyptium* collected from Algeria. Blue bars are the number of positive samples and red bars the number of negative samples (A). Numbers of different species of microorganisms detected in *Hy. aegyptium* collected from Algeria in 2019 (B).

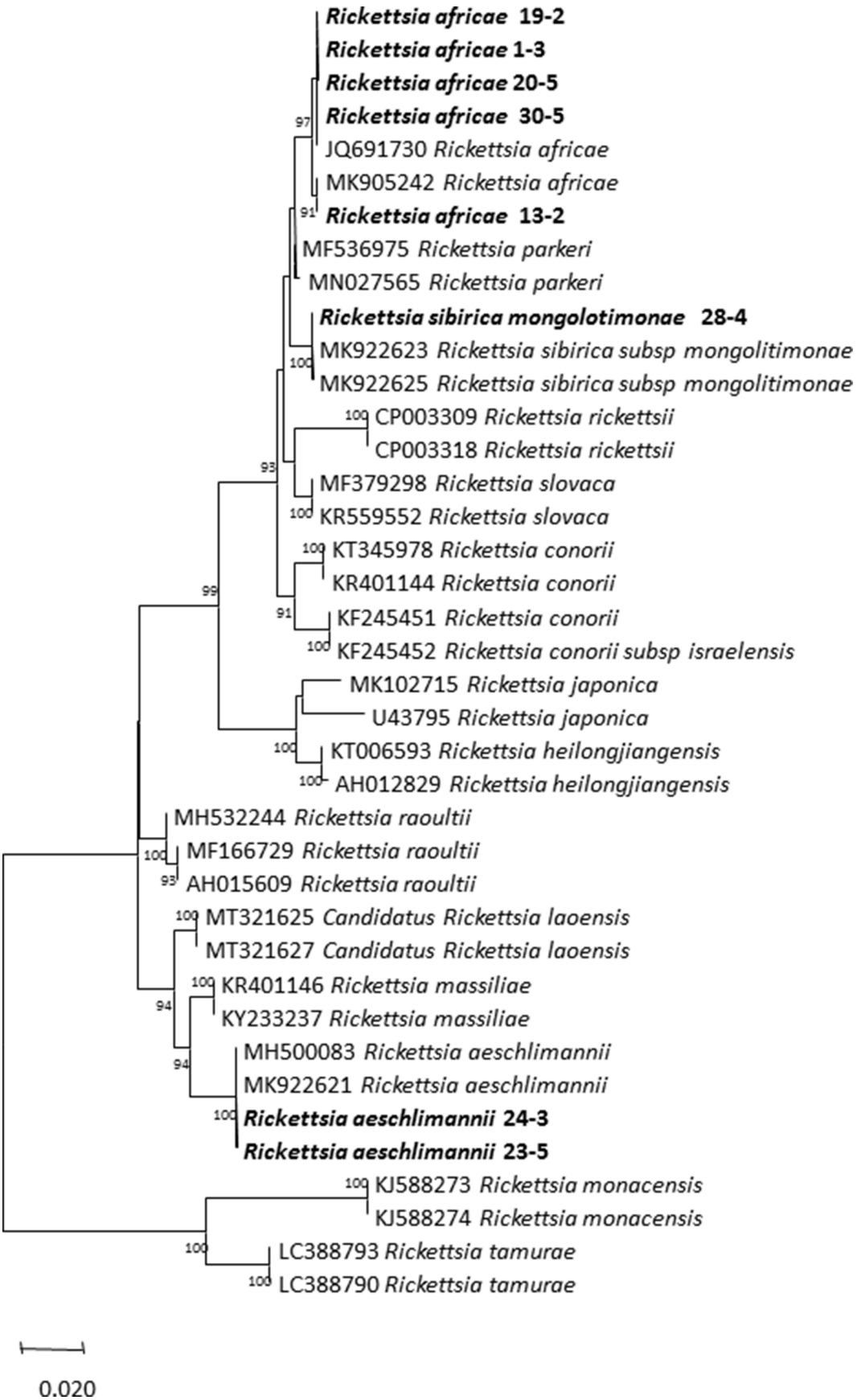

Fig. 4. Phylogenetic tree showing the relationships between *Rickettsia* spp. identified in *Hy. aegyptium* collected from turtles in Algeria in 2019 relative to other species based on a comparison of a 650 bp fragment of the *ompA* gene. The tree was generated by the maximum likelihood algorithm (PhyML) with the general time-reversible (GTR) proposed by the software. The specimens in our study are shown in bold, with the GenBank accession numbers of each sequence at the beginning.

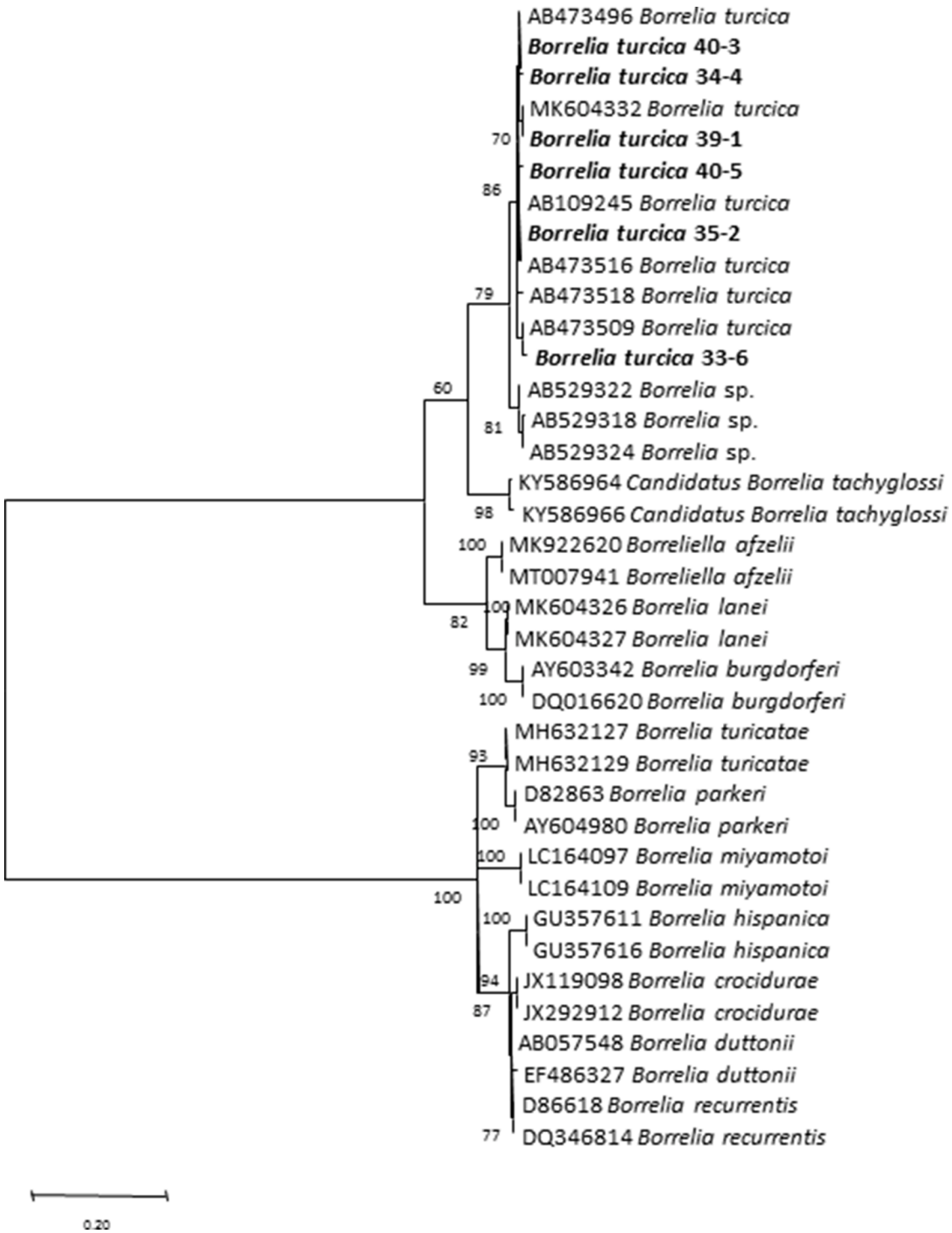

Fig. 5. Phylogenetic tree showing the relationships between *Borrelia turcica* identified in *Hy. aegyptium* collected from turtles in Algeria in 2019 relative to other species based on a comparison of a 300 bp fragment of the *Flagellin B* gene. The tree was generated by the maximum likelihood algorithm (PhyML) with the symmetrical model (SYM) proposed by the software. The specimens in our study are shown in bold, with the GenBank accession numbers of each sequence at the beginning.

(Fig. 2A). Four spectra were added to our own database MALDI-TOF MS and the blind testing of the 226 remaining spectra showed that all specimens were correctly identified, with log score values (LSVs) ranging from 1.774 to 2.655 with mean of 2.271 ± 0.16. Of these, 223 (98.6%) had LSVs >1.8 (Fig. 2B).

3.3. Detection of microorganisms and phylogenetic analysis

Of the ticks tested, 76/230 (33%) were positive for *Rickettsia* spp., 174/230 (75.6%) for *Borrelia* spp., and 9/230 (3.9%) for Anaplasmataceae (Fig. 3A). No samples were positive for *Bartonella* spp., *C. burnetii, Theileria* spp. or *Babesia* spp. Sequencing the samples which were positive for *Rickettsia* spp. using the *ompA* gene revealed that 73/76 (96%) of the obtained sequences were 99.65 to 100% identical to the

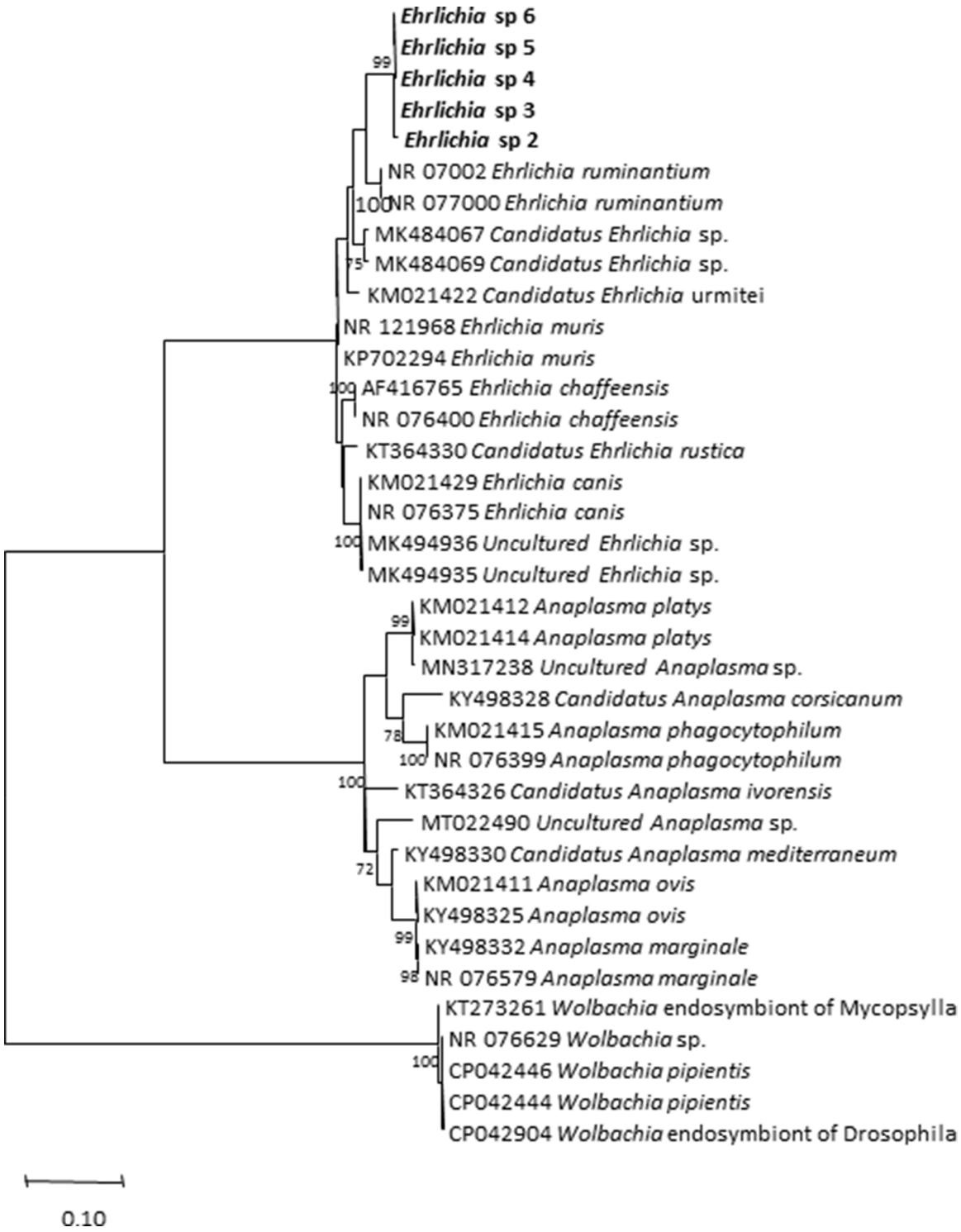

Fig. 6. Phylogenetic tree showing the relationships between *Ehrlichia* spp. identified in *Hy. aegyptium* collected from turtles in Algeria in 2019 relative to other species based on a comparison of a 434 bp fragment of the *23S* Anaplasmataceae gene. The tree was generated by the maximum likelihood algorithm (PhyML) with the general time-reversible (GTR) model proposed by the software. The specimens in our study are shown in bold, with the GenBank accession numbers of each sequence at the beginning.

corresponding sequences of *Rickettsia africae* (GenBank: JQ691730), 2/76 (2.6%) were 99.53% and 100% identical to *Rickettsia aeschlimannii* (GenBank: MK922621), and 1/76 (1.4%) was 100% identical to *Rickettsia sibirica. mongolitimonae* (GenBank: MK922625) (Fig. 3B). Sequencing amplified the *Borrelia, FlagB* gene fragment and revealed that all of the obtained sequences were 100% identical to the corresponding sequence of *Borrelia turcica* (GenBank: MK604332, AB473509 and AB109244) (Fig. 3B). For the nine Anaplasmataceae, *23S* Anaplasmataceae gene sequencing revealed that all of the obtained sequences were identical with the closest sequence in GenBank being *Ehrlichia ruminantium* (96.26% identity with GenBank: NR_077002) (Fig. 3B). Sequencing with the *groEL* gene showed that the three sequences obtained were identical to each other and were 94% identical to an incompletely described *Ehrlichia* sp. (GenBank: LC565631). In this

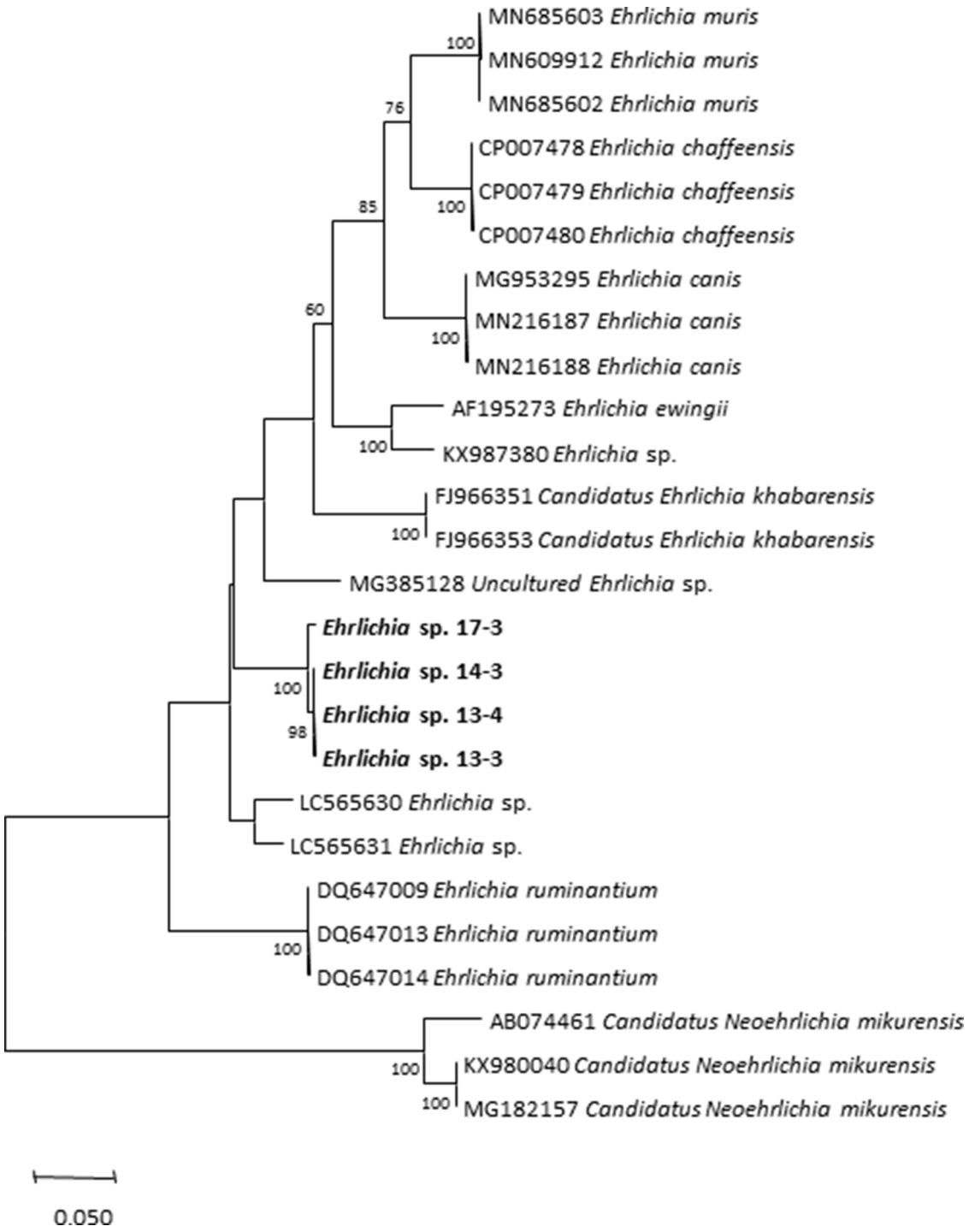

Fig. 7. Phylogenetic tree showing the relationships between *Ehrlichia* spp. identified in *Hy. aegyptium* collected from turtles in Algeria in 2019 relative to other species based on a comparison of a 509 bp fragment of the *groEL* gene. The tree was generated by the Maximum Likelihood algorithm (PhyML) with the Hasegawa, Kishino and Yana (HKY) model proposed by the software. The specimens in our study are shown in bold, with the GenBank accession numbers of each sequence at the beginning.

study, a co-infection of ticks by two or three microorganisms is possible of which we find; 20% with *R. africae* and *B. turcica*, 0.87% by *R.aeschlimannii* and *B. turcica*, 0.87% by *R. africae* and Anaplasmataceae, 0.43% by *R. sibirica mongolitimonae* and *B. turcica* and 0.43% of our samples were co-infected with *R. africae,* Anaplasmataceae and *B. turcica*. The phylogenetic position of the sequences of *Rickettsia, Borrelia* and *Ehrlichia* species detected in our study are shown in Figs. 4, 5, 6 and 7. The sequences from different microorganism species used to build phylogenetic trees were deposited in GenBank and accession numbers are listed in Supplementary Table 1.

4. Discussion

In this study, all ticks collected from *Testudo graeca nabeulensis* were morphologically identified as *Hy. aegyptium*. This was to be expected, as adult *Hy. aegyptium* are highly host specific for this tortoise, as has already been reported in Algeria (Kautman et al., 2016; Lay, 2001). However, the absence of nymphs and or larvae in our samples would

probably be due to the lack of experience of the tick collectors who did not take care of these stages which are small in size or were attached to parts of the tortures not accessible. Blasting the *16S* sequences, a reliable molecular method for tick identification (Yssouf et al., 2016), confirmed our morphological identification and allowed us to conduct an evaluation of MALDI-TOF MS for identification.

MALDI-TOF MS is a technique for identifying large biomolecules by gentle ionisation of a sample (Sevestre et al., 2021; Yssouf et al., 2016). The sample ions are separated from one another on the basis of a mass-to-charge ratio (*m/z*), and the *m/z* measurement is determined by the time it takes an ion to travel through the flight path. This process generates a spectral profile that reveals the composition of the sample being analysed, allowing both its identification and structural analysis (Sevestre et al., 2021). For several years, the MALDI-TOF MS technique has been used for the routine diagnosis of micro-organisms such as the identification of bacteria, archaea, yeasts, and filamentous fungi (Lay, 2001). This innovative proteomic tool has also been successfully applied to the detection of other parasites such as helminths and intestinal protozoa of medical and veterinary importance (Feucherolles et al., 2019). Over the past 15 years, studies have shown that MALDI-TOF MS is a reliable, rapid and powerful tool for the identification of arthropod vectors and non-vectors, but also for the determination of blood meal origins and discriminating between infected and non-infected arthropods (Sevestre et al., 2021). However, the use of MALDI-TOF MS in entomology requires the development of protocols allowing the part of the arthropod generating reproducible and species-specific spectra to be selected. The part of the arthropod used for MALDI-TOF MS analysis varies from one arthropod to another but also according to the developmental stages of the arthropod (Sevestre et al., 2021). All the ticks used in this study were correctly identified by MALDI-TOF MS as *Hy. aegyptium* and 98.6% of them had LSVs >1.8, which has been reported as an identification threshold (Diarra et al., 2017). These results confirm the recently reported ability of MALDI-TOF MS to correctly identify ticks (Boucheikhchoukh et al., 2018; Boyer et al., 2019b; Kernif et al., 2012).

In this study, the DNA of *R. africae, R. aeschlimannii, R. sibirica mongolitimonae, B. turcica*, and a potential undescribed *Ehrlichia* sp. were detected in *Hy. aegyptium* ticks. *Rickettsia africae* is the aetiological agent of African tick-bite fever in humans (ATBF), mainly vectored by ticks of the *Amblyomma* genus (Parola et al., 2013). In Algeria, *R. africae* DNA has already been detected in *Hyalomma dromedarii* (Kernif et al., 2012). Several studies have detected the DNA of *R. africae* in ticks of the *Hyalomma* genus in Mali (Diarra et al., 2017), Côte d'Ivoire (Ehounoud et al., 2016), Senegal (Mediannikov et al., 2010a), and Israel (Waner et al., 2014). Here, the detection of *R. africae* DNA in *Hy. aegyptium* does not allow us to conclude that this tick may play a role in the transmission of *R. africae*, but is may have been caused by the fact that these ticks probably feed on hosts carrying this bacterium (Orkun et al., 2019; Paștiu et al., 2012)

R. aeschlimannii is an agent of spotted fever group (SFG) in humans. Some ticks of the *Hyalomma* genus have been reported as potential vectors (Parola et al., 2013). The DNA of this bacterium was detected in several *Hyalomma* species collected from Europe, Africa and Asia (Bitam et al., 2009). Our study confirms the presence of *R. aeschlimanni* in *Hy. aegyptium* ticks collected from turtles (Bitam et al., 2009).

Rickettsia sibirica mongolitimonae is an also spotted fever group (SFG) rickettsia. It has been associated with Lymphangitis-associated rickettsiosis (LAR) and, mainly transmitted by ticks of the genus *Hyalomma* (Bitam et al., 2009). The DNA of this bacterium has been detected in *Hy. aegyptium* collected from hedgehogs in Turkey (Orkun et al., 2019), but also in other tick species in Europe, Africa and Asia (Hall-Mendelin et al., 2011). To our knowledge, this is the first time that *R. sibirica mongolitimonae* DNA has been reported in Algeria, in turtle ticks, but it should be noted that this bacterium was detected in an adult patient who returned to France after a trip to Algeria having been in contact with camels that were heavily parasitised by ticks (Hall-Mendelin et al., 2011).

Borrelia turcica is a member of the reptile-associated *Borrelia* clade, isolated for the first time in 2004 from the *Hy. aegyptium* tick in Turkey, which is considered to be its vector (Güner et al., 2004). The presence of this bacterium has been reported in a few countries of south-eastern Europe, such as Turkey, Romania, Bulgaria and Greece (Güner et al., 2004). Genetically, *B. turcica* diverges from the Borrelia species of the Lyme and relapsing fever group (Hepner et al., 2020). We report here, for the first time, the presence of *B. turcica* in *Hy. aegyptium* ticks collected from turtles in Algeria but also for the first time in Africa, because the presence of *B. turcica* has not been reported anywhere on the African continent. However, no information on the potential pathogenicity of *B. turcica* to humans has been reported (Hepner et al., 2019).

In this study, we detected DNA from bacteria of the Anaplasmataceae family using the Anaplasmataceae. Analyses of sequences of amplified fragment of the *groEL* and *23S* genes, suggested that these DNA samples were obtained from an undescribed *Ehrlichia* sp. Bacteria of the genus *Ehrlichia* are obligate Gram-negative intracellular bacteria that infect mammalian haematopoietic, endothelial or blood cells, transmitted by ticks and responsible for ehrlichiosis in animals and humans (Vieira et al., 2011). Trigger symptoms include high fever, anorexia, thrombocytopenia, haemorrhages, anaemia, and some serious problems such as splenomegaly, hepatomegaly and meningitis (Franco-Zetina et al., 2019).

In conclusion, *Hy. aegyptium* ticks from this study carried DNA of several microorganisms, including some that are capable of causing both animal and human disease. These results enrich the repertoire of microorganisms associated with ticks in Algeria. Further studies would be necessary in other localities of Algeria to see the distribution of *Hy. aegyptium* ticks and its carriage in microorganisms.

Authors' contributions

Conceived and designed the experiments: AZD, PP. Performed the experiments: HB, AZD. Analysed the data: AZD, HB. Contributed reagents/materials/analysis tools: HB, AZD, JMB, DEG. Took pictures: JMB, HB, DEG. Acquired funding: PP. Project administration: PP, AZD. Supervision: PP. Drafted the paper: HB, AZD. Critically reviewed the paper: PP, AZD. All authors reviewed and approved the final version.

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Declaration of competing interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2021.101858.

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