Molecular screening for *Midichloria* in hard and soft ticks reveals variable prevalence levels and bacterial loads in different tick species

Alessandra Cafiso[a], Chiara Bazzocchi[a,b], Leone De Marco[c,d], Maxwell N. Opara[e], Davide Sassera[c,]*, Olivier Plantard[f,]

[a] Department of Veterinary Medicine, University of Milan, Via Celoria 10, 20133 Milan, Italy

[b] Joint Research Center for Epidemiology and Molecular Surveillance of Infections, University of Milan, 20133 Milan, Italy

[c] Department of Biology and Biotechnology, University of Pavia, Via Ferrata 9, 27100 Pavia, Italy

[d] School of Bioscience and Veterinary Medicine, University of Camerino, Via Gentile III Da Varano, 62032 Camerino, Italy

[e] Department of Parasitology and Entomology, Faculty of Veterinary Medicine, University of Abuja, PMB 117, FCT Nigeria

[f] BIOEPAR, INRA, Oniris, Route de Gachet, 44307 Nantes, France

* Corresponding author. Tel.: +39 0382 986028. *E-mail address*: davide.sassera@unipv.it (D. Sassera)

Abstract

Candidatus Midichloria mitochondrii, symbiont of the sheep tick *Ixodes ricinus*, was the first described member of the family *Candidatus* Midichloriaceae, order Rickettsiales. Recent reports are expanding our view of this family, now including bacteria of great biological and medical interest, indicating a widespread distribution with an increasing range of hosts, with ticks being strongly represented.

Here we present a molecular screening of 17 tick species, detecting and quantifying bacteria of the family *Midichloriaceae* in seven of them, including the first report of a representative of this family in a soft tick species (Argasidae), *Ornithodoros maritimus*. Based on sequence identity and phylogenetic analysis we propose that all these bacterial symbionts of ticks could be members of the genus *Midichloria*. The performed screening highlights different prevalence levels and variable bacterial loads in different tick species including one, *Ixodes aulacodi*, where the bacterium is present in all examined individuals, like in *I. ricinus*. This result prompts us to hypothesize different roles of *Midichloria* bacteria in different tick species.

Keywords: Midichloria spp., *Ixodes, Amblyomma, Hyalomma, Ornithodoros*.

Introduction

Interactions between arthropods and bacteria can play multiple and important roles in the biology of both. Such relationships range from obligatory mutualistic endosymbiosis, where the removal of the symbiont population results in the death of the host, to full parasitism, with the bacteria having a clear pathogenic effect on the host (Duron et al., 2008; Goebel and Gross, 2001; Moran et al., 2008; Zug and Hammerstein, 2015). In between these two extremes lay a wide range of intracellular and extracellular bacteria that establish more complex relationships with theirhosts, that are either not fully understood, or not easily defined in the mutualism/parasitism dichotomy (Hunter et al., 2015). The study of these relationships is of particular importance in hematophagous arthropods, as these bacteria can not only influence the biology of the arthropod, but can also be transmitted to the vertebrate hosts, with important pathogenic effects (Parola and Raoult, 2011). They can also be targeted in vector-control methods, using "symbiotic control approaches" (Sassera et al., 2013).

Ticks have been reported to harbor complex and highly variable microbial communities that play important roles in the biology of these arthropods (Rynkiewicz et al., 2015). Among the members of these communities are important pathogens of humans and animals that can be transmitted through the blood meal, including protozoans such as *Babesia* spp. (Stańczak et al., 2004) and a wide range of viruses (Lani et al., 2014). However, the diversity of bacterial tick-borne pathogens is even greater, including, for example, *Borrelia burgdorferi* and related species, causative agent of Lyme disease (Chomel et al., 2015). Less is known about the other members of the bacterial community associated with Ixodida, those that do not cause overt diseases. However, studies focused on reporting and comparing the presence of such bacteria are increasing, with the goal of understanding their role on host physiology. For example, in *Dermacentor andersoni*, the bacterial symbiont *Rickettsia peacockii* is known to prevent the transovarial transmission of the Rocky Mountain spotted fever agent *Rickettsia rickettsii* (Felsheim et al., 2009).

One of the most investigated endosymbionts of ticks is *Candidatus* Midichloria mitochondrii (hereafter *M. mitochondrii*) of the family *Candidatus* Midichloriaceae (hereafter *Midichloriaceae*) as this species is found in 100% of the females of *Ixodes ricinus*, the most common and investigated European tick species. Following its initial observation by electronic microscopy in the gonad of *I. ricinus* (Zhu et al., 1992) and subsequent investigations based on molecular (Beninati et al., 2004) and ultrastructural studies (Sacchi et al., 2004), *M. mitochondrii* was formally described in 2006 (Sassera et al., 2006). These investigations detected the bacterium within the oocytes of females of the tick *I. ricinus*, and also highlighted a unique feature of this intracellular microorganism, *i. e.* the capacity to colonize the intermembrane space of mitochondria. Indeed, while some *M. mitochondrii* were detected in the host cell cytoplasm (Beninati et al., 2009), others were localized in the space between the inner and the outer mitochondrial membranes. The bacterium shows 100% prevalence in females of *I. ricinus*, is vertically transmitted to the progeny and is less prevalent and abundant in males (Lo et al., 2006; Sassera et al., 2008). In addition to the demonstrated transovarial transmission, multiple lines of evidence suggest the possibility of horizontal transmission, following the detection of *M. mitochondrii* in the *I. ricinus* salivary glands. Indeed, serological and molecular screenings showed positivity of mammalian blood and sera to *M. mitochondrii* (Mariconti et al., 2012; Bazzocchi et al., 2013).

Following the discovery of *M. mitochondrii* in *I. ricinus*, multiple studies detected closely related bacteria, both in ticks and in various other hosts and biological matrices, ranging from other arthropods species (Matsuura et al., 2012) to fish (Cafiso et al., 2015), ciliates (Vannini et al., 2010; Senra et al., 2016; Szokoli et al., 2016) to amoebae (Fritsche et al., 1999). These reports led to the description of *Midichloriaceae*, a novel family within the order *Rickettsiales* (Driscoll et al., 2013; Montagna et al., 2013). Focusing on ticks, screenings of multiple Ixodida species were performed, either searching directly for bacteria closely related to *M. mitochondrii* (Epis et al., 2008; Beninati et al., 2009) or in the

context of studies assessing the microbial diversity using universal primers (Loftis et al., 2006; Dergousoff et al., 2011). The overall result was that positive specimens were detected in species belonging to each of the six most important genera of hard ticks (Ixodidae, *i.e.* the genera *Ixodes*, *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus*), while to our knowledge there are no reports indicating the presence of *Midichloria* bacteria in soft ticks (Argasidae). In Table 1 a summary of the reports of *Midichloriaceae* in ticks of previous studies is presented. While the limited number of individuals screened for most species does not allow for a precise prevalence estimate, among species analyzed to date only the Australian species *I. holocyclus* presents a 100% prevalence as in *I. ricinus* (Beninati et al., 2009). An additional study on the microbial community of *I. holocyclus* indicates that the bacterium is not only prevalent, but also extremely abundant in this tick species (Gofton et al., 2015), similarly to what was observed in *I. ricinus*. Nevertheless, the presence of *Midichloria* bacteria was not observed inside mitochondria inthis species (Beninati et al., 2009). Interestingly, a phylogenetic analysis of the *Midichloria* bacteria of multiple tick species did not show signs of co-cladogenesis between the ticks and the symbionts (Epis et al., 2008), suggesting that horizontal transfer could be the main source of diffusion of *Midichloria* among ticks.

In order to expand our knowledge of the distribution of *Midichloria* in ticks, we collected samples from multiple species and performed qualitative and quantitative molecular screenings followed by phylogenetic analysis.

Materials and methods

Tick sampling

Ninety-two tick specimens belonging to 16 species of the Ixodidae family and to one species of the Argasidae family were collected in three continents, sampled free in the environment or directly from the hosts, then conserved in ethanol at 4°C or frozen alive at -80 °C. All specimens were identified using standard taxonomic keys (including Hillyard, 1997 and Pereiz-Eid, 2007 for European species; Arthur, 1965 for the African species). Ticks genus, species, number of collected individuals, life stage, geographical origin, vertebrate host and conservation protocol are summarized in Table 2.

DNA extraction

Before proceeding with DNA extraction, ethanol preserved specimens were rehydrated and washed twice in PBS 1X for 20 minutes and then left to dry for additional 20 minutes, in order to remove all the ethanol residues. Frozen ticks were boiled for 5 minutes before processing them. After these steps, DNA was extracted from all ticks using DNeasy Blood & Tissue Kit (Qiagen) with the following changes to the manufacturer instructions: proteinase K incubation was carried on overnight at 56°C and DNA was eluted in two steps with 25 µl each of sterile water pre-heated at 72 °C (as explained in Epis et al., 2008), quantified and stored at -80 °C until use.

Qualitative PCRs

In order to evaluate the quality of the extracted DNA, a fragment of the mitochondrial *12S rRNA* of the tick was amplified using a previously published protocol (Epis et al., 2008). Qualitative PCR to detect *Midichloria* bacteria was performed using a modified version of the protocol described by Epis et al. (2008) with two sets of primers targeting the *16S rRNA* bacterial gene. The first set of primers (Midi-F: GTACATGGGAATCTACCTTGC; Midi-R: CAGGTCGCCCTATTGCTTCTTT; primers final concentration: 1 µM; amplification size: 1100 bp) was used for a first round of amplification. The second set of primers (Midi-F2: CAAAAGTGAAAGCCTTGGGC; Midi-R2: TGAGACTTAAAYCCCAACATC) was used to perform two semi-nested PCRs (Midi-F/Midi-R2,

primers final concentration: 1 μM, amplification size: 691 bp; Midi-F2/Midi-R, primers final concentration: 1 μM, amplification size: 675 bp). PCRs were carried out using the same thermal profile (30 s at 95 °C, 30 s at 58 °C and 45 s at 72 °C for 40 times).

Real Time PCR

DNA samples were subsequently subjected to a Real Time PCR (qPCR) based on a previously published protocol designed for amplification of a fragment of the *gyrB* gene from *M. mitochondrii* (Sassera et al., 2008). For bacteria quantification, a purified plasmid containing the *gyrB* fragment was serially diluted starting from 10^9 copies/μl to 1 copy/μl to evaluate the efficiency and detection limit of the PCR protocol. The amplification of *gyrB* gene (a single copy gene per genome; Sassera et al., 2008) was obtained starting from 25 ng of DNA for each tick sample and the quantification was performed comparing the qPCR results with those of serial dilutions of cloned fragments (containing known copy numbers).

DNA sequencing and phylogenetic analyses

16S rRNA and *gyrB* genes PCR products were loaded on agarose gel, excised and purified with Wizard® SV Gel and PCR Clean-Up System (Promega) and then subjected to Sanger sequencing. After manual correction of the electropherograms, the obtained *16S rRNA* sequences were added to a dataset of published *16S rRNA* sequences of *Midichloriaceae* bacteria. Sequences were aligned using the software Muscle (Edgar, 2004) and the alignment was used for phylogeny reconstruction using the software RaxML (Stamatakis et al., 2008) with the GTRCAT model and 1000 bootstrap replicates. A heatmap representing the variable levels of identity percentages was obtained from the *16S rRNA* alignment using an in-house python script. *gyrB* gene amplicons were subjected to BLAST analyses in order to verify the specificity of the amplification.

Results

A total of 92 tick individuals, belonging to 16 Ixodidae and one Argasidae species, were collected and DNA was successfully extracted, as confirmed by the *12S rRNA* gene amplification (data not shown). A qualitative semi-nested PCR to amplify a fragment of the *16S rRNA* of *Midichloria* bacteria, showed a total of 38 out of 92 specimens positive. These individuals belong to six out of 17 analyzed species (five Ixodidae and one Argasidae species), with different prevalence levels. All DNAs were subjected to a qPCR approach based on the amplification of the *gyrB* gene of *Midichloria*. This quantitative method allows the detection of as low as 10 copies of *gyrB* gene per PCR reaction. The qPCR showed a total of 48 out of 92 specimens positive, belonging to a total of nine out of 17 species (eight Ixodidae and one Argasidae species). In several samples that resulted positive in qPCR, the estimated quantification was below the sensitivity threshold of 10 copies/µl. In all cases the obtained amplicons were sequenced, confirming the specific amplification of a fragment of *gyrB*. We defined as "$< 10^1$ copies/µl" all the values from tick samples for which the quantification was lower than the sensitivity threshold (see Table S1).

Positive specimens are indicated in Table 2 and bacterial loads are shown in Table S1. DNA sequences were obtained for all *16S rRNA* and *gyrB* genes amplicons and submitted to the EMBL database (*16S rRNA* accession numbers LT575860-LT575865, *gyrB* accession numbers LT575850-LT575859).

Twenty-six individuals of the species *I. aulacodi* were examined (24 females and 2 males), and all were positive for the presence of *Midichloria* bacteria using the qualitative PCR approach, while qPCR showed positivity in 24 out of 26 ticks (92%). The two *I. aulacodi* negative in qPCR were the two females sampled from the Gambian Pouched Rat *Cricetomys gambianus* (*I. aulacodi* 23 and 24) while

187 the 24 others were sampled from Grasscutter *Tryonomys* spp. All the *16S rRNA* amplicons showed a
188 99% similarity with *M. mitochondrii* IricVA strain *16S rRNA*. *gyrB* sequences showed instead 97%
189 similarity with *M. mitochondrii* partial *gyrB* gene. Bacterial load varied substantially between ticks,
190 ranging from about 1.18 x 10^5 bacteria per tick to 2.49 x 10^8 bacteria per tick, with no evident
191 difference among female and male samples.
192
193 One *I. colasbelcouri* was positive for the presence of *Midichloria* bacteria in qualitative PCR and the
194 obtained *16S rRNA* fragment sequence showed 99% similarity with the *16S rRNA* gene of *M.*
195 *mitochondrii*. Moreover, both the *I. colasbelcouri* individuals were positive in qPCR and the *gyrB* gene
196 sequence of both specimens showed a 97% similarity with the *gyrB* gene fragment of *M. mitochondrii*
197 in *I. ricinus*. The two individuals of *I. colasbelcouri* harbored different amounts of *Midichloria*
198 bacteria: more than 3.71 x 10^6 bacteria were found in the individual that was also positive in qualitative
199 PCR and around 4.53 x 10^5 in the other specimen.
200
201 Six specimens of *I. frontalis* were positive (60%) for *Midichloria* using the qualitative approach. Five
202 out of six of these samples were also positive with the qPCR investigation. The *16S rRNA* sequences
203 were identical and related to *M. mitochondrii* (99% identity). Moreover, all the *gyrB* amplicons from
204 positive samples were identical and related to *M. mitochondrii* (97% identity). Copies of *gyrB* varied
205 from a maximum of 8.23 x 10^8 per tick sample to a minimum lower than 5 x 10^2 copies.
206
207 In *I. ventalloi*, four samples (one adult female and three nymphs) were positive only using a qPCR
208 approach, with bacterial load between a minimum lower than 5 x 10^2 and a maximum of 1.08 x 10^3
209 copies per tick. *gyrB* gene sequence was identical among the four samples and showed 99% similarity
210 with *M. mitochondrii gyrB* gene. In *I. vespertilionis* the one female sample examined was found to be
211 positive for *Midichloria* bacteria, with a *gyrB* gene sequence identical to the one of *M. mitochondrii*.

The only available *A. variegatum* sample was positive both in qualitative (99% identity with *M. mitochondrii*) and in quantitative PCR (97% similarity with *M. mitochondrii*) with *gyrB* gene copies < 5×10^2 per total extracted DNA.

All the three individuals belonging to *H. anatolicum* species harbored *Midichloria*, which was detectable only with qPCR approach (bacterial loads were < 5×10^2 *gyrB* copies for total extracted DNA) showing a 97% similarity with *gyrB* of *M. mitochondrii*.

Of the four analyzed specimens of *H. excavatum*, two showed positivity in qualitative PCR (99% identity with *M. mitochondrii* and 99% with a *Rickettsiales* bacterium found in *H. excavatum* by Loftis et al. in 2006) while qPCR detected the presence of *Midichloria* in all the four specimens (*gyrB* similarity with *M. mitochondrii* was 95%). Quantification of *gyrB* ranged from < 5×10^2 to 7.98×10^4 copies.

Two specimens out of five of the soft tick *O. maritimus* were positive with the qualitative PCR (99% identity with *M. mitochondrii*). The qPCR amplification showed positivity for *Midichloria* in four out of five samples. In this case three out of four qPCR positive samples showed a similarity of 97% with *gyrB* of *M. mitochondrii*, while one specimen showed 100% sequence identity with *M. mitochondrii*). The sample presenting the same *gyrB* sequence of *M. mitochondrii* (*O. maritimus* 3) was also the one showing the highest amount of bacteria compared to the other individuals of the same species (over 9.91×10^5 bacteria compared to an average < 5×10^2 for the other three individuals). No samples of the other 10 species were positive to either of the PCR protocols applied.

All the obtained *16S rRNA* sequences were added to an alignment of sequences of *Midichloriaceae* from ticks and other organisms retrieved from the databases and three *Rickettsiales* sequences used as

outgroups. The manually curated alignment was subjected to Maximum Likelihood phylogenetic reconstruction. The resulting tree showed that all the novel sequences belong to a highly supported monophyletic clade (in grey in Fig. 1) including sequences of *Midichloria* previously obtained from tick species, as well as sequences detected in the blood of mammals parasitized by ticks. As previously reported (Boscaro et al., 2013), *16S rRNA* single gene alignments cannot solve all nodes of *Midichloriaceae* phylogeny, and indeed our tree presents multiple polytomies. However the support for the monophyly of the clade encompassing sequences of ticks symbionts is strong, validating our claim of clustering them within the *Midichloria* genus.

Discussion

This work presents a molecular screening for the presence of bacteria related to *M. mitochondrii* in various species of ticks. Two PCR protocols were used to achieve this aim, a dual semi-nested qualitative PCR for the *16S rRNA* gene and a qPCR for the *gyrB* gene. The qPCR sensitivity was assessed to 10 copies per µl using a plasmid standard. It is interesting to note that the used methods did not give fully congruent results (Table 2), with 38/92 samples positive to one or both the *16S rRNA* semi-nested reamplifications and 48/92 samples positive to the qPCR (35 out of 92 are positive using both qPCR and at least one semi-nested). It appears that the three protocols show different sensitivities in different tick species (Table S1). These results could be due to the presence of mismatches in the primers regions among different species, resulting in cases of suboptimal primer annealing. Indeed, there was a clear pattern of specific protocols being more sensitive in single species. For example, *I. aulacodi*, showing high bacterial load, was positive with all protocols (including the *16S rRNA gene* PCR performed before the two semi-nested). *I. frontalis* samples were positive to *gyrB* qPCR and to

one of the semi-nested protocols (Midi-F/Midi-R2), while *I. colasbelcouri* was positive to the qPCR and to the second semi-nested protocol (Midi-F2/Midi-R) (Table 2).

All the PCR amplicons were sequenced, in order to verify the specificity of amplification and to determine the genetic variation within the *Midichloria* bacteria detected in all tick species. The novel *16S rRNA* sequences were used for a phylogenetic approach by adding them to the tree of *Midichloriaceae* (Fig. 1). All the novel sequences belong to the monophyletic group containing exclusively sequences of *Midichloriaceae* associated to ticks (see the group shaded in grey in Fig. 1). Within this group, *16S rRNA* identity is always above 93% (Fig. 2) and the same group formed by all the *Midichloriaceae* associated to ticks is observed in the clustering analysis based on identity percentages among sequences. In this analysis however, the group also includes the sequence associated to the rainbow trout *Oncorhynchus mykiss*. This sequence is the sister group of all "*Midichloriaceae* associated to ticks" in the phylogenetic analysis (Fig. 1), thus illustrating the proximity of this sequence to the ones associated to ticks. The unexpected location of this sequence in the clustering analysis may be due to known skews/bias of clustering – i. *e.* phenetic – methods relatively to phylogenetic reconstruction). Based on the strong phylogenetic support and a previously performed generalized mixed Yule coalescent analysis (Montagna et al., 2013), we propose that all the bacteria of this clade, the MALO cluster 2 (Montagna et al., 2013), should be classified within the genus *Midichloria*.

Even if the *16S rRNA* marker, due to limited genetic variability, is not suitable for fine phylogenetic discrimination (the *gyrB* marker being even worse due to its reduced length), the tree did not show evidence of co-cladogenesis between the bacteria and their tick hosts. For example, within the group of *Midichloria* associated to ticks, *Midichloria* associated to different species of the *Ixodes* genus do not form a monophyletic group, but are scattered in various clusters of the phylogenetic tree, sometimes

clustering with sequences of *Midichloria* associated to other tick genera or even other tick families (*id est I. aulacodi* or *I. colasbelcouri* clustering with *A. variegatum* or *O. maritimus* that belong to the Amblyommidae and Argasidae families respectively; or *I. uriae* clustering with *Haemaphysalis punctata* and *Rhipicephalus turanicus* that belong to the Amblyommidae family). This result, together with the presence of sequences obtained from mammalian blood samples (Bazzocchi et al., 2013), provides additional support to the hypothesis that bacteria of the genus *Midichloria* can transfer horizontally between ticks through vertebrate hosts, probably through the blood meal.

We believe that a multigene phylogenetic approach could be very useful in order to better define the boundaries of the genus *Midichloria*, and to provide a definite answer to the question of the co-cladogenesis. A Multi Locus Sequence Typing (MLST) effort, restricted to the symbionts of ticks, or even wider, including other members of the family, could elucidate this issue.

We examined individuals from 17 species of hard and soft ticks, detecting positive ticks in nine of them. For most species the limited number of available samples did not allow for a precise evaluation of the prevalence levels. The only species which exhibited 100% prevalence with a significant number of specimens (n=26) was *I. aulacodi*. In this tick species, both males (n=2) and females (n=24) appear to host *Midichloria* bacteria. The presence of *Midichloria* in 100% females could indicate a vertical transmission of the symbiont to the offspring, as demonstrated for *M. mitochondrii* of *I. ricinus*. The qPCR negativity of two samples could be due to a lower sensitivity of the method in this species. Unfortunately it was not possible to fully evaluate the prevalence of *Midichloria* bacteria in the males of this species in order to determine whether or not they were all infected with the bacterium, as only two males were collected (in *I. ricinus* only 44% males host the *M. mitochondrii* symbiont).

The qPCR was efficient in evaluating the *Midichloria* load in all tick samples. The numbers were very variable, ranging from values below 5 x10^2 copies to 8.23 x 10^8 gene copies per tick. While the limited number of samples did not allow for statistical analysis of these data, we observed a high variability of bacterial load in the species *I. frontalis* and *O. maritimus* (Table S1). The presence of a bacterium of the genus *Midichloria* in *I. frontalis* confirms the recent discover by Palomar and colleagues (Palomar et al., 2015). The species with the highest number of examined samples, *I. aulacodi*, has a median *Midichloria* load of 1.08 x 10^6 bacteria. Such high numbers, coupled with 100% prevalence, strongly suggest an important role for this bacterium in the physiology of the host. Indeed these data could indicate a mutualistic relationship between *I. aulacodi* and *Midichloria*, as previously suggested for the *I. ricinus – M. mitochondrii* symbiosis. It must be noted that a previous report indicates that the number of *M. mitochondrii* symbionts varies greatly through the *I. ricinus* life cycle (Sassera et al., 2008). Additional *I. aulacodi* individuals, belonging to different life stages, should be examined in order to investigate this issue.

The presence of a *Midichloria* bacterium in *H. excavatum* is in accordance with the detection of a *16S rRNA* gene sequence belonging to a Rickettsiales bacterium found by Loftis et al. (2006) in one *H. excavatum* specimen and in one pool of *Hyalomma* spp. nymphs. Three of the four *O. maritimus* individuals that were positive for *Midichloria* had low bacterial load and identical gene sequences, while the remaining positive sample presented very high load and a different *gyrB* sequence. This result could simply indicate a gene polymorphism in *Midichloria* bacteria in *O. maritimus.* However another possible explanation could be that *O. maritimus* can host multiple *M. mitochondrii* strains. Indeed it is interesting to observe that the higher bacterial load was found in the sample with a *gyrB* sequence that is identical to the one belonging to *M. mitochondrii* of *I. ricinus.* Following this reasoning, we could hypothesize that the ancestral *Midichloria* behavior is that of tick-borne bacteria that travel horizontally through ticks and mammalian hosts at low prevalence and low bacterial load, as is often the case for

tick-borne rickettsiae (which are close relatives of *Midichloriaceae*). Such behavior could have evolved, once or multiple times, in a stronger relationship, in which the bacterium assumed a mutualistic role within the host, with a strong increase in load per individual, higher prevalence and possibly vertical transmission. More studies are necessary to test whether this hypothesis is correct in *I. ricinus, I. holocyclus* and/or *I. aulacodi*.

Future studies should thus be focused on developing and applying genetic markers on faster evolving characters, in order to investigate the genetic variability of *Midichloria* in ticks using a multi-gene phylogenetic approach and test the above hypotheses. Such investigations, including the selection of a gene with a mutation rate providing suitable polymorphism, are facilitated by the knowledge of the whole genome of *M. mitochondrii* (Sassera et al., 2011). It would also be interesting to analyze tick ovaries by means of transmission electron microscopy, in order to assess if the bacteria are present inside the mitochondria of infected cells (as does *M. mitochondrii* of *I. ricinus*) or just in the cytoplasm (as observed in *I. holocyclus*). This would answer the question of whether the intramitochondrial tropism is a unique character of *M. mitochondrii* or if it is widespread in the genus *Midichloria*.

Acknowledgements

This work was partially financed by FIR-2013 fund to D.S. and PRIN-MIUR-2012 to C.B. For their help to provide samples or during field sampling, we would like to thank Francis Abioye, Albert Agoulon, Suzanne Bastian, François Berger, Mirko Berggoetz, Patrick Berthelot, Agnes Bouju, Jean-Claude Beaucournu, Alain Butet, Thierry Delhorme, Axelle Durand, Agustin Estrada-Peña, Lise Gern, Steve Goodman, Dieter Heylen, Caroline Hervet, Karine Huber, Maggy Jouglin, Olivier Lambert, Jérome Letty, Joy Amara Maxwell, Grégoire Perez, Laurent Précigout, Yann Rantier, Jean-Marc Reynes, Frédéric Stachurski, Frédéric Touzalin, Zati Vatansever and Gwenaël Vourc'h.

References

Arthur, D.R., 1965. Ticks of the genus *Ixodes* in Africa. The Athlone press, London.

Bazzocchi, C., Mariconti, M., Sassera, D., Rinaldi, L., Martin, E., Cringoli, G., Urbanelli, S., Genchi, C., Bandi, C., Epis, S., 2013. Molecular and serological evidence for the circulation of the tick symbiont *Midichloria* (Rickettsiales: *Midichloriaceae*) in different mammalian species. Parasit Vectors 6, 350–56.

Beninati, T., Lo, N., Sacchi, L., Genchi, C., Noda, H., Bandi, C., 2004. A novel alpha-Proteobacterium resides in the mitochondria of ovarian cells of the tick *Ixodes ricinus*. Appl Environ Microbiol 70, 2596–602.

Beninati, T., Riegler, M., Vilcins, I.M., Sacchi, L., McFadyen, R., Krockenberger, M., Bandi, C., O'Neill, S.L., Lo, N., 2009. Absence of the symbiont *Candidatus* Midichloria mitochondrii in the mitochondria of the tick *Ixodes holocyclus*. FEMS Microbiol Lett 299, 241–7.

Boscaro, V., Petroni, G., Ristori, A., Verni, F., Vannini, C., 2013. "*Candidatus* Defluviella procrastinata" and "*Candidatus* Cyrtobacter zanobii", two novel ciliate endosymbionts belonging to the "*Midichloria* clade". Microb Ecol 65, 302–10.

Cafiso, A., Sassera, D., Serra, V., Bandi, C., McCarthy, U., Bazzocchi, C., 2015. Molecular evidence for a bacterium of the family *Midichloriaceae* (order Rickettsiales) in skin and organs of the rainbow trout *Oncorhynchus mykiss* (Walbaum) affected by red mark syndrome. J Fish Dis 39, 497–501.

Chomel, B., 2015. Lyme disease. Rev Sci Tech 34, 569–76.

Dergousoff, S.J., Chilton, N.B., 2011. Novel genotypes of *Anaplasma bovis*, "*Candidatus* Midichloria" sp. and *Ignatzschineria* sp. in the Rocky Mountain wood tick, *Dermacentor andersoni*. Vet Microbiol 150, 100–6.

Driscoll, T., Gillespie, J.J., Nordberg, E.K., Azad, A.F., Sobral, B.W., 2013. Bacterial DNA sifted from the *Trichoplax adhaerens* (Animalia: Placozoa) genome project reveals a putative rickettsial endosymbiont. Genome Biol Evol 5, 621–45.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstadter, J., Hurst, G.D., 2013. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biol 6, 1-12.

Edgar, R.C., 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5, 113.

Epis, S., Sassera, D., Beninati, T., Lo, N., Beati, L., Piesman, J., Rinaldi, L., McCoy, K.D., Torina, A., Sacchi, L., Clementi, E., Genchi, M., Magnino, S., Bandi, C., 2008. *Midichloria mitochondrii* is widespread in hard ticks (Ixodidae) and resides in the mitochondria of phylogenetically diverse species. Parasitology 135, 485–94.

Felsheim, R.F., Kurtti, T.J., Munderloh, U.G., 2009. Genome sequence of the endosymbiont *Rickettsia peacockii* and comparison with virulent *Rickettsia rickettsii*: identification of virulence factors. PLoS One 4, e8361.

Fritsche, T.R., Horn, M., Seyedirashti, S., Gautom, R.K., Schleifer, K.H., Wagner, M., 1999. In situ detection of novel bacterial endosymbionts of *Acanthamoeba* spp. phylogenetically related to members of the order Rickettsiales. Appl Environ Microbiol 65, 206–12.

Fujita, H., Takada, N., Kawabata, H., Ishiguro, F., Yamamoto, S. et al., 2007. Some suggestive records of rickettsiae isolated from ticks in Korea and central China. Annual Report of Ohara General Hospital 47, 21–4.

Goddard, J., Sumner, J.W., Nicholson, W.L., Paddock, C.D., Shen, J., Piesman, J., 2003. Survey of ticks collected in Mississippi for *Rickettsia*, *Ehrlichia*, and *Borrelia* species. J Vector Ecol 28, 184–9.

Goebel, W., Gross, R., 2001. Intracellular survival strategies of mutualistic and parasitic prokaryotes. Trends in Microbiology 9, 267-273.

Gofton, A.W., Oskam, C.L., Lo, N., Beninati, T., Wei, H., McCarl, V., Murray, D.C., Paparini, A., Greay, T.L., Holmes, A.J., Bunce, M., Ryan, U., Irwin, P., 2015. Inhibition of the endosymbiont "*Candidatus* Midichloria mitochondrii" during 16S rRNA gene profiling reveals potential pathogens in *Ixodes* ticks from Australia. Parasit Vectors 8, 345.

Hillyard, P.D, 1997. Ticks of North-West Europe. The Dorset Press, Dorchester.

Hunter, D.J., Torkelson, J.L., Bodnar, J., Mortazavi, B., Laurent, T., Deason, J., Thephavongsa, K., Zhong, J., 2015. The *Rickettsia* Endosymbiont of *Ixodes pacificus* Contains All the Genes of De Novo Folate Biosynthesis. PLoS One 10, e0144552.

Lani, R., Moghaddam, E,, Haghani, A., Chang, L.Y., AbuBakar, S., Zandi, K., 2014. Tick-borne viruses: a review from the perspective of therapeutic approaches. Ticks Tick Borne Dis 5, 457-65.

Loftis, A.D., Reeves, W.K., Szumlas, D.E., Abbassy, M.M., Helmy, I.M., Moriarity, J.R., Dasch, G.A., 2006. Population survey of Egyptian arthropods for rickettsial agents. Ann N Y Acad Sci 1078, 364–7.

Lo, N., Beninati, T., Sassera, D., Bouman, E.A., Santagati, S., Gern, L., Sambri, V., Masuzawa, T., Gray, J.S., Jaenson, T.G., Bouattour, A., Kenny, M.J., Guner, E.S., Kharitonenkov, I.G., Bitam, I.,

Bandi, C., 2006. Widespread distribution and high prevalence of an alpha-proteobacterial symbiont in the tick *Ixodes ricinus*. Environ Microbiol 8, 1280–7.

Mariconti, M., Epis, S., Gaibani, P., Valle, C. dalla, Sassera, D., Tomao, P., Fabbi, M., Castelli, F., Marone, P., Sambri, V., Bazzocchi, C., Bandi, C., 2012. Humans parasitized by the hard tick *Ixodes ricinus* are seropositive to *Midichloria mitochondrii*: is *Midichloria* a novel pathogen, or just a marker of tick bite? Pathogens and Global Health 106, 391–396.

Matsuura, Y., Kikuchi, Y., Meng, X.Y., Koga, R., Fukatsu, T., 2012. Novel clade of alphaproteobacterial endosymbionts associated with stinkbugs and other arthropods. Appl Environ Microbiol 78, 4149–56.

Mediannikov, O., Ivanov, L.I., Nishikawa, M., Saito, R., Sidel'nikov, I.N., Zdanovskaia, N.I., Mokretsova, E.V., Tarasevich, I.V., Suzuki, H, 2004. Microorganism "Montezuma" of the order Rickettsiales: the potential causative agent of tick-borne disease in the Far East of Russia. Zh. Mikrobiol. Epidemiol. Immunobiol. l, 7–13. (In Russian)

Montagna, M., Sassera, D., Epis, S., Bazzocchi, C., Vannini, C., Lo, N., Sacchi, L., Fukatsu, T., Petroni, G., Bandi, C., 2013. "*Candidatus* Midichloriaceae" fam. nov. (Rickettsiales), an ecologically widespread clade of intracellular alphaproteobacteria. Applied and Environmental Microbiology 79, 3241–3248.

Moran, N.A., McCutecheon, J.P., Nakabachi, A., 2008. Genomics and evolution of Heritable Bacterial Symbionts. Ann Rev Genetics 42, 165-190.

Najm, N.A., Silaghi, C., Bell-Sakyi, L., Pfister, K., Passos, L.M., 2012. Detection of bacteria related to *Candidatus* Midichloria mitochondrii in tick cell lines. Parasitol Res 110, 437–42.

441 Palomar, A.M., Portillo, A., Santibáñez, P., Mazuelas, D., Roncero, L., García-Álvarez, L., Santibáñez,
442 S., Gutiérrez, Ó., Oteo, J.A., 2015. Detection of tick-borne *Anaplasma bovis*, *Anaplasma*
443 *phagocytophilum* and *Anaplasma centrale* in Spain. Med Vet Entomol 29, 349–53.

444 Parola, P., Raoult. D., 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious
445 threat. Clin Infect Dis 32, 897-928.

446 Parola, P., Cornet, J.P., Sanogo, Y.O., Miller, R.S., Thien, H.V., Gonzalez, J.P., Raoult, D., Telford III,
447 S.R., Wongsrichanalai, C., 2003. Detection of *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and
448 other eubacteria in ticks from the Thai-Myanmar border and Vietnam. J Clin Microbiol 41, 1600–8.

449 Pérez-Eid, C., 2007. Les tiques - Identification, biologie, importance médicale et vétérinaire. Tec and
450 Doc Lavoisier, Paris.

451 Qiu, Y., Nakao, R., Ohnuma, A., Kawamori, F., Sugimoto, C., 2014. Microbial population analysis of
452 the salivary glands of ticks; a possible strategy for the surveillance of bacterial pathogens. PLoS One 9,
453 e103961.

454 Rynkiewicz, E.C., Hemmerich, C., Rusch, D.B., Fuqua, C., Clay, K., 2015. Concordance of bacterial
455 communities of two tick species and blood of their shared rodent host. Mol Ecol 24, 2566–79.

456 Sacchi, L., Bigliardi, E., Corona, S., Beninati, T., Lo, N., Franceschi, A., 2004. A symbiont of the tick
457 *Ixodes ricinus* invades and consumes mitochondria in a mode similar to that of the parasitic bacterium
458 *Bdellovibrio bacteriovorus*. Tissue Cell 36, 43–53.

459 Sassera, D., Beninati, T., Bandi, C., Bouman, E.A., Sacchi, L., Fabbi, M., Lo, N., 2006. "*Candidatus*
460 Midichloria mitochondrii", an endosymbiont of the tick *Ixodes ricinus* with a unique intramitochondrial
461 lifestyle. Int J Syst Evol Microbiol 56, 2535–40.

462 Sassera, D., Lo, N., Bouman, E.A., Epis, S., Mortarino, M., Bandi, C., 2008. "*Candidatus* Midichloria"
463 endosymbionts bloom after the blood meal of the host, the hard tick *Ixodes ricinus*. Appl Environ
464 Microbiol 74, 6138–40.

465 Sassera, D., Lo, N., Epis, S., D'Auria, G., Montagna, M., Comandatore, F., Horner, D., Peretó, J,,
466 Luciano, A.M., Franciosi, F., Ferri, E., Crotti, E., Bazzocchi, C., Daffonchio, D., Sacchi, L., Moya, A.,
467 Latorre, A., Bandi, C, 2011. Phylogenomic evidence for the presence of a flagellum and cbb(3) oxidase
468 in the free-living mitochondrial ancestor. Mol Biol Evol 28, 3285–96

469 Sassera, D., Epis, S., Pajoro, M., Bandi, C., 2013. Microbial symbiosis and the control of vector-borne
470 pathogens in tsetse flies, human lice, and triatomine bugs. Pathog Glob Health 107, 285–92.

471 Senra, M.V., Dias, R.J., Castelli, M., Silva-Neto, I.D., Verni, F., Soares, C.A., Petroni, G., 2016. A
472 House for Two-Double Bacterial Infection in *Euplotes woodruffi* Sq1 (Ciliophora, Euplotia) Sampled
473 in Southeastern Brazil. Microb Ecol 71, 505–17.

474 Stamatakis, A., Hoover, P., Rougemont, J., 2008. A rapid bootstrap algorithm for the RAxML Web
475 servers. Syst Biol 57, 758–71.

476 Stańczak, J., Gabre, R.M., Kruminis-Łozowska, W., Racewicz, M., Kubica-Biernat, B., 2004. *Ixodes*
477 *ricinus* as a vector of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Babesia*
478 *microti* in urban and suburban forests. Ann Agric Environ Med 11, 109–14.

479 Szokoli, F., Sabaneyeva, E., Castelli, M., Krenek, S., Schrallhammer, M., Soares, C.A., da Silva-Neto,
480 I.D., Berendonk, T.U., Petroni, G., 2016. "*Candidatus* Fokinia solitaria", a Novel "Stand-Alone"
481 Symbiotic Lineage of *Midichloriaceae* (Rickettsiales). PLoS One 11, e0145743.

Vannini, C., Ferrantini, F., Schleifer, K.H., Ludwig, W., Verni, F., Petroni, G., 2010. "*Candidatus* Anadelfobacter veles" and "*Candidatus* Cyrtobacter comes," two new rickettsiales species hosted by the protist ciliate *Euplotes harpa* (Ciliophora, Spirotrichea). Appl Environ Microbiol 76, 4047–54.

Williams-Newkirk, A.J., Rowe, L.A., Mixson-Hayden, T.R., Dasch, G.A., 2012. Presence, genetic variability, and potential significance of "*Candidatus* Midichloria mitochondrii" in the lone star tick *Amblyomma americanum*. Exp Appl Acarol 58, 291–300.

Zhu, Z., Aeschlimann, A., Gern, L., 1992. *Rickettsia*-like microorganisms in the ovarian primordia of molting *Ixodes ricinus* (acari: Ixodidae) larvae and nymphs. Ann Parasitol Hum Comp 67, 99–110.

Zug, R., Hammerstein, P., 2015. Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. Biol Rev Camb Philos Soc 90, 89–111.

Figures and tables

Fig. 1 Phylogenetic tree of the family *Midichloriaceae*, obtained after Maximum likelyhood analysis of a *16S rRNA* gene alignment. For each analyzed sequence, the name of the bacterium, the accession number, and the host/collection site are indicated. Sequences obtained in this study are shown in bold, the clade proposed to represent the genus *Midichloria* is shaded in grey, bootstrap values are shown above each node. Branches with support values below 50 were collapsed.

Fig. 2 Heatmap representing the identity percentages in the *16S rRNA* gene between members of the *Midichloriaceae*. Bacteria belonging to the *Midichloria* genus are indicated in green in the clustering analysis, other *Midichloriaceae* are indicated in red, while *Ehrlichia canis* AF162860.1 and *Neorickettsia sennetsu* NR 074386.1 are in blue.

Table 1 Summary of the *Midichloria* sequences obtained during previous screenings, indicating the tick species analyzed.

Table 2 List of the analyzed samples, indicating tick species, life stage, sex, geographical origin, host (when present), method of preservation

and overall positive samples obtained from both qualitative PCR (*16S rRNA* gene) and qPCR (*gyrB* gene).

Table S1 Quantifications of the *gyrB* gene through qPCR in tick samples positive for *Midichloria*.

516

517 **Table 1**

Host		Author
Genus	**Species**	
Amblyomma	*americanum*	Williams-Newkirk et al., 2012
Amblyomma	*tuberculatum*	Epis et al., 2008
Dermacentor	*andersonii*	Dergousoff et al., 2011
Haemaphysalis	*punctata*	Epis et al., 2008
Haemaphysalis	*wellingtoni*	Parola et al., 2003
Hyalomma	*excavatum*	Loftis et al., 2006
Hyalomma	spp. (nymphs)	Loftis et al., 2006
Hyalomma	*marginatum*	Epis et al., 2008
Hyalomma	*truncatum*	Epis et al., 2008
Ixodes	*brunneus*	Goddard et al., 2003
Ixodes	*frontalis*	Palomar et al., 2015
Ixodes	*holocyclus*	Beninati et al., 2009
Ixodes	*ovatus*	Fujita et al., 2007
Ixodes	*persulcatus*	Qiu et al., 2014 Mediannikov et al., 2002
Ixodes	*ricinus*	Beninati et al., 2004
Ixodes	*uriae*	Epis et al., 2008
Rhipicephalus	*bursa*	Epis et al., 2008
Rhipicephalus	*decoloratus*	Najm et al., 2012
Rhipicephalus	*turanicus*	Epis et al., 2008

518

519 **Table 2**

Genus	Species	No. of specimens	Biological stage/Sex	Geographical origin	Host (if present)	Positive samples for *16S rRNA* PCR	Positive samples for *gyrB* PCR
Ixodes	*arboricola*	1*	♀	Belgium	Laboratory strain	ND	ND
Ixodes	*aulacodi*	14*	♀	Akure (Nigeria)	*Thryonomis* spp.	14	14
		2*	♂	Akure (Nigeria)	*Thryonomis* spp.	2	2
		1*	♀	Owerri (Nigeria)	*Thryonomis* spp.	1	1
		2*	♀	Owerri (Nigeria)	*Cricetomys gambianus*	2	0
		7*	♀	Ondo (Nigeria	*Thryonomis* spp.	7	7
Ixodes	*acuminatus*	2	♀	Pleine-Fougere (France)	Laboratory strain		
		4	♀	Pleine-Fougere (France)	Micromammal	ND	ND
		4	♀	Pleine-Fougere (France)	*Mustela nivalis*		
Ixodes	*canisuga*	2*	♀	Bernay (France)	close to *Vulpes vulpes*	ND	ND
		2*	nymph	Bernay (France)	close to *Vulpes vulpes*		
Ixodes	*colasbelcouri*	2*	♀	Anjozorobe (Madagascar)	*Rattus rattus*	1	2
Ixodes	*frontalis*	8*	♀	Sautron (France)	*Streptopelia decaocto*	4	3
		1*	♀	Nantes (France)	*Gallinula chloroplus*	1	1
		1*	♀	Nantes (France)	*Melopsittacus undulatus*	1	1
Ixodes	*lividus*	3	nymph	Anetz (France)	*Riparia riparia*	ND	ND
		3*	nymph	Anetz (France)	*Riparia riparia*		
Ixodes	*loricatus*	1*	♀	Morteros (Argentina)	*Didelphis albiventris*	ND	ND
Ixodes	*rubicundus*	1*	♀	Tüssen-Die-Riviere (South	*Tragelaphus oryx*	ND	ND

				Africa)			
Ixodes	*simplex*	1□	♀	Rancognes (France)	close to *Miniopterus schreibersi*	ND	ND
		1□	♂	Rancognes (France)	close to *Miniopterus schreibersi*		
Ixodes	*trianguliceps*	4*	nymph	Combraille (France)	Micromammal	ND	ND
Ixodes	*ventalloi*	4*	♀	Saint Pierre de Quiberon (France)	*Oryctolagus cuniculus*	0	1
		6*	nymph	Portugal	-	0	3
Ixodes	*vespertilionis*	1□	♀	Bernay (France)	close to *Rhinolophus ferrumequium*	0	1
		1□	♂	Bernay (France)	close to *Rhinolophus ferrumequium*	0	0
Amblyomma	*variegatum*	1*	♀	Guadeloupe (France)	Laboratory strain	1	1
Hyalomma	*anatolicum*	3*	♀	Turkey	-	0	3
Hyalomma	*excavatum*	4*	♀	Turkey	-	2	4
Ornithodoros	*maritimus*	5*	♀	Valuec Island (France)	*Phalacrocorax aristotelis*	2	4

520

521

522 **Table S1**

523

Genus	Species	Sample code	Sex/Stage	Positivity for *16S rDNA* gene	*gyrB* copies in 25 ng DNA	*gyrB* copies in total DNA eluted in 50 µl
Ixodes	*aulacodi*	1	♀	+	1.52×10^3	7.61×10^5
		2	♀	+	1.31×10^3	2.62×10^5
		3	♀	+	6.29×10^3	1.26×10^6
		4	♀	+	1.47×10^5	2.20×10^7
		5	♀	+	1.58×10^4	7.89×10^5
		6	♀	+	9.93×10^3	2.98×10^6
		7	♀	+	4.04×10^4	1.29×10^8
		8	♀	+	3.48×10^4	1.25×10^8
		9	♀	+	6.57×10^3	2.13×10^7
		10	♀	+	2.22×10^4	1.67×10^7
		11	♀	+	1.91×10^3	4.78×10^5
		12	♀	+	7.20×10^3	7.20×10^5
		13	♀	+	6.34×10^4	6.34×10^6
		14	♀	+	1.15×10^4	2.31×10^6
		15	♀	+	2.77×10^5	2.49×10^8
		16	♀	+	6.03×10^3	6.03×10^5
		17	♀	+	9.05×10^3	9.05×10^5
		18	♀	+	2.35×10^3	1.18×10^5
		19	♀	+	3.41×10^3	5.11×10^5
		20	♀	+	5.69×10^4	8.54×10^6
		21	♀	+	5.30×10^3	5.30×10^5
		22	♀	+	5.49×10^3	5.49×10^5
		23	♀	+	0	0
		24	♀	+	0	0
		25	♂	+	3.74×10^4	1.87×10^6
		26	♂	+	2.56×10^3	1.28×10^5
Ixodes	*colasbelcouri*	1	♀	+	3.71×10^4	3.71×10^6
		2	♀	-	9.05×10^3	4.53×10^5

Ixodes	*frontalis*	1	♀	+	$< 10^1$	$< 5 \times 10^2$
		2	♀	+	1.98×10^5	8.23×10^8
		3	♀	+	1.01×10^5	3.37×10^8
		4	♀	+	0	0
		5	♀	+	$< 10^1$	7.81×10^3
		6	♀	+	$< 10^1$	$< 5 \times 10^2$
Ixodes	*ventalloi*	1	♀	-	$< 10^1$	$< 2.8 \times 10^3$
		5	nymph	-	1.50×10^1	9.01×10^2
		7	nymph	-	1.80×10^1	1.08×10^3
		10	nymph	-	$< 10^1$	1.50×10^1
Ixodes	*vespertilionis*	1	♀	-	$< 10^1$	$< 5 \times 10^2$
Amblyomma	*variegatum*	1	♀	+	$< 10^1$	$< 5 \times 10^2$
Hyalomma	*anatolicum*	1	♀	-	$< 10^1$	$< 5 \times 10^2$
		2	♀	-	$< 10^1$	$< 5 \times 10^2$
		3	♀	-	$< 10^1$	$< 5 \times 10^2$
Hyalomma	*excavatum*	1	♀	+	1.33×10^2	7.98×10^4
		2	♀	-	$< 10^1$	$< 5 \times 10^2$
		3	♀	-	$< 10^1$	$< 5 \times 10^2$
		4	♀	+	1.10×10^1	1.17×10^3
Ornithodoros	*maritimus*	1	♀	-	$< 10^1$	$< 5 \times 10^2$
		2	♀	-	$< 10^1$	$< 5 \times 10^2$
		3	♀	+	1.98×10^4	9.91×10^5
		4	♀	+	$< 10^1$	$< 5 \times 10^2$

Figure1

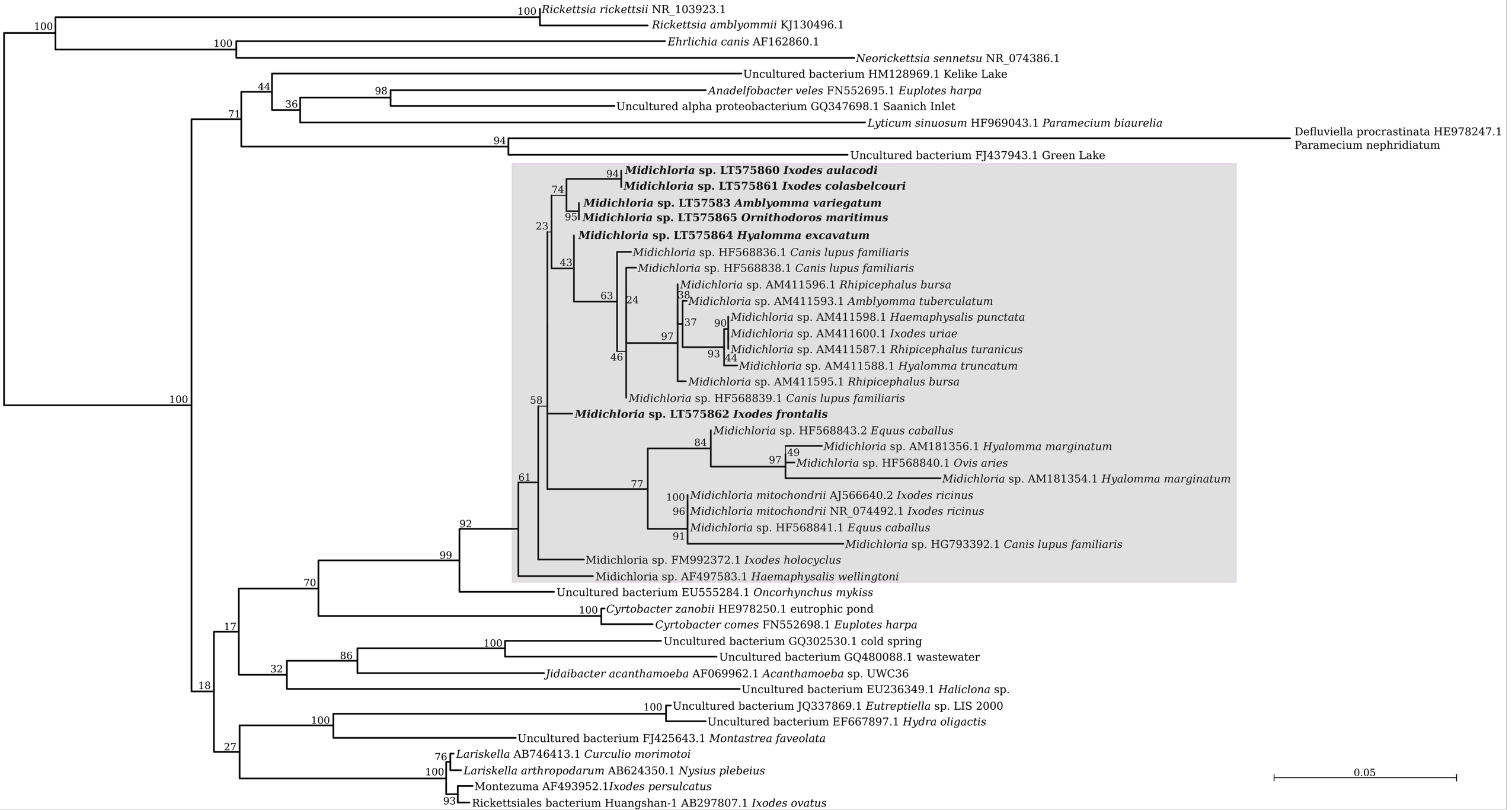
Rickettsia rickettsii NR_103923.1
Rickettsia amblyommii KJ130496.1
Ehrlichia canis AF162860.1
Neorickettsia sennetsu NR_074386.1
Uncultured bacterium HM128969.1 Kelike Lake
Anadelfobacter veles FN552695.1 Euplotes harpa
Uncultured alpha proteobacterium GQ347698.1 Saanich Inlet
Lyticum sinuosum HF969043.1 Paramecium biaurelia
Defluviella procrastinata HE978247.1
Paramecium nephridiatum
Uncultured bacterium FJ437943.1 Green Lake
Midichloria sp. LT575860 Ixodes aulacodi
Midichloria sp. LT575861 Ixodes colasbelcouri
Midichloria sp. LT57583 Amblyomma variegatum
Midichloria sp. LT575865 Ornithodoros maritimus
Midichloria sp. LT575864 Hyalomma excavatum
Midichloria sp. HF568836.1 Canis lupus familiaris
Midichloria sp. HF568838.1 Canis lupus familiaris
Midichloria sp. AM411596.1 Rhipicephalus bursa
Midichloria sp. AM411593.1 Amblyomma tuberculatum
Midichloria sp. AM411598.1 Haemaphysalis punctata
Midichloria sp. AM411600.1 Ixodes uriae
Midichloria sp. AM411587.1 Rhipicephalus turanicus
Midichloria sp. AM411588.1 Hyalomma truncatum
Midichloria sp. AM411595.1 Rhipicephalus bursa
Midichloria sp. HF568839.1 Canis lupus familiaris
Midichloria sp. LT575862 Ixodes frontalis
Midichloria sp. HF568843.2 Equus caballus
Midichloria sp. AM181356.1 Hyalomma marginatum
Midichloria sp. HF568840.1 Ovis aries
Midichloria sp. AM181354.1 Hyalomma marginatum
Midichloria mitochondrii AJ566640.2 Ixodes ricinus
Midichloria mitochondrii NR_074492.1 Ixodes ricinus
Midichloria sp. HF568841.1 Equus caballus
Midichloria sp. HG793392.1 Canis lupus familiaris
Midichloria sp. FM992372.1 Ixodes holocyclus
Midichloria sp. AF497583.1 Haemaphysalis wellingtoni
Uncultured bacterium EU555284.1 Oncorhynchus mykiss
Cyrtobacter zanobii HE978250.1 eutrophic pond
Cyrtobacter comes FN552698.1 Euplotes harpa
Uncultured bacterium GQ302530.1 cold spring
Uncultured bacterium GQ480088.1 wastewater
Jidaibacter acanthamoeba AF069962.1 Acanthamoeba sp. UWC36
Uncultured bacterium EU236349.1 Haliclona sp.
Uncultured bacterium JQ337869.1 Eutreptiella sp. LIS 2000
Uncultured bacterium EF667897.1 Hydra oligactis
Uncultured bacterium FJ425643.1 Montastrea faveolata
Lariskella AB746413.1 Curculio morimotoi
Lariskella arthropodarum AB624350.1 Nysius plebeius
Montezuma AF493952.1Ixodes persulcatus
Rickettsiales bacterium Huangshan-1 AB297807.1 Ixodes ovatus
0.05

Figure2

Click here to download high resolution image

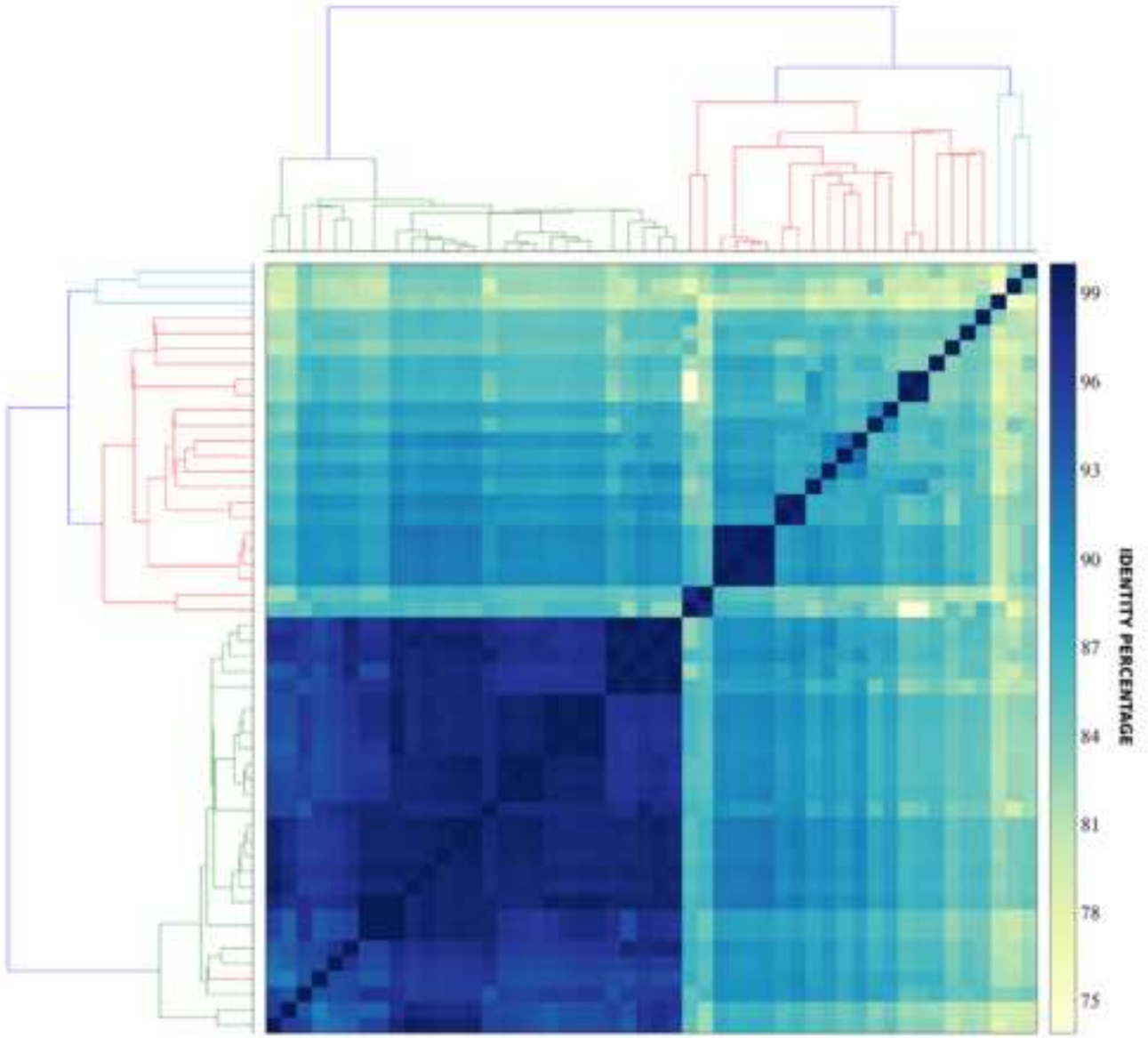

Table S1

Genus	Species	Sample code	Sex/Stage	Positivity for *16S rDNA* gene	*gyrB* copies in 25 ng DNA	*gyrB* copies in total DNA eluted in 50 µl
Ixodes	*aulacodi*	1	♀	+	1.52×10^3	7.61×10^5
		2	♀	+	1.31×10^3	2.62×10^5
		3	♀	+	6.29×10^3	1.26×10^6
		4	♀	+	1.47×10^5	2.20×10^7
		5	♀	+	1.58×10^4	7.89×10^5
		6	♀	+	9.93×10^3	2.98×10^6
		7	♀	+	4.04×10^4	1.29×10^8
		8	♀	+	3.48×10^4	1.25×10^8
		9	♀	+	6.57×10^3	2.13×10^7
		10	♀	+	2.22×10^4	1.67×10^7
		11	♀	+	1.91×10^3	4.78×10^5
		12	♀	+	7.20×10^3	7.20×10^5
		13	♀	+	6.34×10^4	6.34×10^6
		14	♀	+	1.15×10^4	2.31×10^6
		15	♀	+	2.77×10^5	2.49×10^8
		16	♀	+	6.03×10^3	6.03×10^5
		17	♀	+	9.05×10^3	9.05×10^5
		18	♀	+	2.35×10^3	1.18×10^5
		19	♀	+	3.41×10^3	5.11×10^5
		20	♀	+	5.69×10^4	8.54×10^6
		21	♀	+	5.30×10^3	5.30×10^5
		22	♀	+	5.49×10^3	5.49×10^5
		23	♀	+	0	0
		24	♀	+	0	0
		25	♂	+	3.74×10^4	1.87×10^6
		26	♂	+	2.56×10^3	1.28×10^5
Ixodes	*colasbelcouri*	1	♀	+	3.71×10^4	3.71×10^6
		2	♀	-	9.05×10^3	4.53×10^5
Ixodes	*frontalis*	1	♀	+	$< 10^1$	$< 5 \times 10^2$
		2	♀	+	1.98×10^5	8.23×10^8
		3	♀	+	1.01×10^5	3.37×10^8
		4	♀	+	0	0
		5	♀	+	$< 10^1$	7.81×10^3
		6	♀	+	$< 10^1$	$< 5 \times 10^2$

Ixodes	*ventalloi*	1	♀	-	$< 10^1$	$< 2.8 \times 10^3$
		5	nymph	-	1.50×10^1	9.01×10^2
		7	nymph	-	1.80×10^1	1.08×10^3
		10	nymph	-	$< 10^1$	1.50×10^1
Ixodes	*vespertilionis*	1	♀	-	$< 10^1$	$< 5 \times 10^2$
Amblyomma	*variegatum*	1	♀	+	$< 10^1$	$< 5 \times 10^2$
Hyalomma	*anatolicum*	1	♀	-	$< 10^1$	$< 5 \times 10^2$
		2	♀	-	$< 10^1$	$< 5 \times 10^2$
		3	♀	-	$< 10^1$	$< 5 \times 10^2$
Hyalomma	*excavatum*	1	♀	+	1.33×10^2	7.98×10^4
		2	♀	-	$< 10^1$	$< 5 \times 10^2$
		3	♀	-	$< 10^1$	$< 5 \times 10^2$
		4	♀	+	1.10×10^1	1.17×10^3
Ornithodoros	*maritimus*	1	♀	-	$< 10^1$	$< 5 \times 10^2$
		2	♀	-	$< 10^1$	$< 5 \times 10^2$
		3	♀	+	1.98×10^4	9.91×10^5
		4	♀	+	$< 10^1$	$< 5 \times 10^2$