Midichloria mitochondrii is widespread in hard ticks (Ixodidae) and resides in the mitochondria of phylogenetically diverse species

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SUMMARY

The hard tick *Ixodes ricinus* (Ixodidae) is the sole animal thus far shown to harbour an intra-mitochondrial bacterium, which has recently been named *Midichloria mitochondrii*. The objectives of this work were (i) to screen ixodid ticks for *Midichloria*-related bacteria and (ii) to determine whether these bacteria exploit the intra-mitochondrial niche in other tick species. Our main goal was to discover further models of this peculiar form of symbiosis. We have thus performed a PCR screening for *Midichloria*-related bacteria in samples of ixodid ticks collected in Italy, North America and Iceland. A total of 7 newly examined species from 5 genera were found positive for bacteria closely related to *M. mitochondrii*. Samples of the tick species *Rhipicephalus bursa*, found positive in the PCR screening, were analysed with transmission electron microscopy, which revealed the presence of bacteria both in the cytoplasm and in the mitochondria of the oocytes. There is thus evidence that bacteria invade mitochondria in at least 2 tick species. Phylogenetic analysis on the bacterial 16S rRNA gene sequences generated from positive specimens revealed that the bacteria form a monophyletic group within the order Rickettsiales. The phylogeny of *Midichloria* symbionts and related bacteria does not appear completely congruent with the phylogeny of the hosts.

Key words: *Midichloria mitochondrii*, *Ixodes ricinus*, hard ticks, intra-mitochondrial, PCR screening, phylogeny.

INTRODUCTION

Ticks are obligate haematophagous mites parasitizing vertebrates. They act as vectors of a number of microbial agents that cause diseases in humans and animals. These pathogens include *Borrelia* spp., *Coxiella burnetii*, *Francisella tularensis*, as well as intracellular bacteria from the order Rickettsiales. Other tick-associated bacteria do not appear to be present in the salivary glands or to cause infections in humans and animals; some of these bacteria are transmitted to the progeny through the egg cytoplasm (transovarial transmission). These vertically transmitted microorganisms are usually referred to as symbionts, but their interaction with the host tick is generally not fully understood (for a general introduction to ticks, see Bowman and Nuttall, 2004).

The hard tick *Ixodes ricinus* (family Ixodidae) harbours an endosymbiont with the peculiar capacity to invade the mitochondria and multiply therein (first observation by Lewis, 1979). These mitochondrial bacteria found in *I. ricinus* have been identified based on 16S rRNA gene sequence data (Beninati *et al.* 2004), investigated at the ultrastructural level (Sacchi *et al.* 2004), and described as a new genus and species, i.e. *Midichloria mitochondrii* (Sassera *et al.* 2006). The biological role of *M. mitochondrii* in *I. ricinus* is still unknown. During

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oogenesis, the proportion of mitochondria invaded by *M. mitochondrii* is not negligible (e.g. see photos in Sacchi *et al.* 2004), and this suggests that the bacterium could have a detrimental effect on the host tick. However, the fact that *M. mitochondrii* is apparently at fixation in females of *I. ricinus* (100% prevalence according to Lo *et al.* 2006*a*) seems to indicate that its presence is somehow compatible with the survival and reproduction of the host tick (see discussion in Lo *et al.* 2006*a* and *b*).

While data have been acquired for *M. mitochondrii* in *I. ricinus*, which have permitted its description as a new genus and species, there is only circumstantial evidence for the presence of DNA sequences from related bacteria in other tick species (see Discussion section). The aim of this work was thus to obtain a more detailed picture of the distribution of bacteria related to *Midichloria* in hard ticks (family Ixodidae). The discovery of other tick species infected by bacteria related to *M. mitochondrii* could enhance investigations aimed at uncovering its biological role. For instance, a tick species could be found in which the prevalence of *Midichloria*-related symbionts is far from 100%, or in which different populations show different infection levels. This might permit both a comparison of the biology, fitness parameters and vector competence of infected versus uninfected ticks, and experiments on possible reproductive parasitism phenomena (Bandi *et al.* 2001). Furthermore, *I. ricinus* is the sole animal thus far shown to harbour an intra-mitochondrial bacterium and *M. mitochondrii* is the unique intra-mitochondrial bacterium thus far described. Determining whether intra-mitochondrial bacterial presence is an exception or a more widespread phenomenon was the main goal of this work.

The screening for *Midichloria*-related bacteria reported here focused on hard ticks. Most of the ∼900 species of ticks thus far described (suborder Ixodida in the order Acarina) are assigned to 2 main families: the Ixodidae (hard ticks) and the Argasidae (soft ticks). The Ixodidae are subdivided into 2 main lineages, the Prostriata and Metastriata (Hoogstraal and Aeschlimann, 1982). The Prostriata encompass the subfamily Ixodinae (over 200 species in the single genus *Ixodes*). The Metastriata group comprises 370 species in 4 subfamilies: the Amblyomminae, Haemaphysalinae, Hyalomminae and Rhipicephalinae (Barker and Murrel, 2004). Our screening included representatives of all of the subfamilies of the Ixodidae and led to the detection of bacteria in tick specimens belonging to 7 different species (in addition to the previously investigated *I. ricinus*). 16S rRNA sequence data generated for these bacteria revealed a very close phylogenetic relationship with *M. mitochondrii*. Nucleotide identities between the 16S rRNA from *M. mitochondrii* of *I. ricinus* and the sequences from the other tick species were above 99%. We have thus referred to all of these tick bacteria using the species name *M. mitochondrii* (Sassera *et al.* 2006).

MATERIALS AND METHODS

Tick species screened for bacteria related to Midichloria mitochondrii

Tick samples representing a total of 21 species of the Ixodidae were examined for the presence of bacteria related to *M. mitochondrii*. The Metastriata were represented by the genera *Rhipicephalus* (4 species), *Dermacentor* (4 species), *Hyalomma* (2 species), *Amblyomma* (3 species) and *Haemaphysalis* (3 species). The Prostriata were represented by 5 species of the genus *Ixodes*.

A total of 197 tick specimens were collected in Italy (Lazio, Sicily and Lombardia), Iceland (Grimsey) and USA (New Jersey, Florida, Missouri, South Carolina, Oregon, California and Georgia). Of these specimens, 125 were ethanol-preserved before DNA extraction, while the other 72 were processed immediately after collection. Ticks were collected at different developmental stages (larvae, nymphs, adults, and engorged females). Forty-two of the samples were pooled prior to DNA extraction. For the remaining 155 samples DNA was extracted from individual specimens. See Table 1 for a complete list of the specimens. All ticks were identified using standard taxonomic keys; in addition, for confirmation of tick identification, a fragment of the small subunit mitochondrial ribosomal RNA gene (12S rRNA) was sequenced for a subset of the examined specimens/pools of specimens.

DNA extraction and polymerase chain reaction (PCR) amplification of tick 12S rRNA

DNA was extracted using Qiagen tissue kit (Qiagen, Hilden, Germany). Ethanol-preserved ticks were washed and dried for 45 min before DNA extraction. Afterwards, ticks were triturated into a solution containing 180 μl of ATL lysis buffer (Qiagen) and 200 ng/μl proteinase K (Sigma Aldrich, St Louis, USA). The lysis was carried out overnight with shaking at 56 °C. The elution step was performed by 2 additions of 25 μl of hot (72 °C) deionized water in the columns; the remaining extraction steps were performed according to the manufacturer's instructions. DNA was stored at −20 °C.

PCR for amplification of 12S rRNA was performed as described by Beati and Keirans (2001). PCR products were gel-purified using the QIAquick™ Gel Extraction Kit (Qiagen) according to the manufacturer's protocols, resuspended in 30 μl of deionized water and sequenced with PCR primers using the ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit, version 3.1 (Applera Europe, Warrington, UK), and run on an automated

sequencer (ABI Prism 310 DNA sequencer, Applied Biosystems, Foster City, CA).

PCR screening for bacteria related to Midichloria mitochondrii

PCR screening for bacteria related to *M. mitochondrii* was performed using 2 sets of primers: Midi-F (5′-GTACATGGGAATCTACCTTGC-3′) and Midi-R (5′-CAGGTCGCCCTATTGCTTCTTT-3′); Midi-F2 (5′-CAACGAGCGCAACCCT TAT-3′) and MidiR2 (5′-CAGTCGTCAACCTT ACCGT-3′). All samples were tested with both primer copies, in order to double check the results obtained. These primers, targeted the 16S rRNA gene, amplify fragments of ∼1100 (Midi-F –R) and ∼350 bp (Midi-F2 –R2) and were designed to be conserved between the sequence of *M. mitochondrii* from *I. ricinus* (AJ566640) and the closely related sequences available in the data bases. In particular, an alignment was generated with the aim of representing all of the main lineages of the order Rickettsiales and the sequences already available that cluster as a monophyletic group with *M. mitochondrii* (see phylogenetic trees in Beninati *et al.* 2004; Lo *et al.* 2006 *a*; Sassera *et al.* 2006). Primers were then designed to be conserved and specific for bacteria in the *M. mitochondrii* cluster. Primer specificity was then checked by BLAST searches and by PCR on biological samples positive for bacteria of the genera *Rickettsia*, *Ehrlichia*, *Wolbachia* and *Anaplasma*.

Amplifications were performed, with both primer sets, in 20 μl of buffer (10 mM Tris-HCl (pH 8·3), 50 mM KCl, 1·5 mM MgCl$_2$, 0·001% gelatine) with 0·2 mM each deoxynucleoside triphosphate, 10 pmol of each primer, 0·5 U of *Taq* Polymerase (Eppendorf) and 2 μl of DNA sample. The thermal profile was: 2 min at 95 °C; 40 cycles of 95 °C for 30 sec, 56 °C for 30 sec and 68 °C for 45 sec; the elongation was completed at 10 min at 68 °C. PCR products obtained with primer combination MidiF and MidiR were purified and sequenced as previously described using primers Midi-F and Midi-R and the internal primers P3b (5′-CTGTTTGCT-CCCCACGCTTTC-3′) and P2b (5′-GATATT-AGGAGGAACACCGC-3′).

Phylogenetic analyses

The bacterial 16S rRNA sequences obtained from ticks were subjected to BLAST analysis. These sequences were then aligned using as a mask an alignment downloaded from the Ribosomal Database Project (Cole *et al.* 2003). The alignment was generated considering 16S rRNA secondary structures and included the sequence of *M. mitochondrii* from *I. ricinus* and the homologous *Midichloria*-related sequences available in the data bases (excluding sequences below 1000 bp). Representatives of the

main lineages of the order Rickettsiales were also included. Phylogenetic analyses were performed using the programmes Mega 3.1 (Kumar *et al.* 2004) and MrBayes (Huelsenbeck and Ronquist, 2001). Trees were generated using Neighbour Joining, Maximum Parsimony and Bayesian Inference methods. For Bayesian Inference, parameters for the model of substitution (General Time Reversible with Gamma correction for among-site heterogeneity) were estimated from the data. A total of 10 000 trees were obtained (ngen = 1 000 000, samplefreq = 100), and the first 3000 of these were considered as the 'burn in' and discarded. A 50% majority-rule consensus tree of the remaining trees, including branch lengths (sumt) was produced. For macro-taxonomic purposes, analyses were performed on alignments including representatives of the Rickettsiales (see tree in Fig. 1), while for investigating the relationships at a lower taxonomic level, sequences from Rickettsiales which are not closely related with *M. mitochondrii* were excluded (see tree in Fig. 2). The sequences obtained have been deposited in the EMBL data library (see Accession numbers in Table 1).

The 12S mitochondrial rRNA sequences obtained were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/blast) and compared to the sequences available in the data bases in order to confirm morphology-based species identification. The sequences were aligned with ClustalX (Thompson *et al.* 1997) and manually adjusted taking into account secondary structures (Hickson *et al.* 1996). Phylogenetic analyses were then performed as described for 16S rRNA sequences. These sequences have been deposited in the EMBL data library (see Accession numbers in Table 1).

Electron microscopy

Specimens of the tick species *Rhipicephalus bursa* were found positive for *M. mitochondrii* during the PCR screening; this tick species was thus chosen for transmission electron microscopy (TEM) examination. Eleven adult engorged females were collected from the field (Lazio, Italy), brought to the lab within 48 h and immediately dissected. Half of the ovary from each female specimen was fixed for TEM examination, while the remaining half was used for DNA extraction and PCR. The 4 ovarian specimens that were found to be PCR positive for *M. mitochondrii* as well as a negative ovarian specimen were then subjected to TEM examination. For TEM examination, ovaries were fixed in 0·1 M cacodylate buffer (pH 7·2) containing 2·5% glutaraldehyde for 3 h at 4 °C. The samples were then washed in the same buffer and post-fixed in 1% OsO$_4$ in the same buffer for 1·5 h at 4 °C. Successively all samples were dehydrated in ethanol and embedded in Epon 812. The semithin sections (1 μm) for light microscopy

Table 1. List of taxa, geographical origin, Accession numbers for bacterial 16S rRNA and tick 12S rRNA sequences, and screening results

Species	Subfamily	Geographical origin	Sample Accession no.	16S rRNA Accession no.	12S rRNA Accession no.	Adult ♀ +/Tot	Adult ♂ +/Tot	Juveniles[3] +/Tot	Pools +/Tot
Dermacentor marginatus[1]	Rhipicephalinae	Lazio (Italy)	MSNPVCAR321	N.A.	AM410570	0/5	0/2	0/0	0/1
Dermacentor variabilis[2]	Rhipicephalinae	Florida (USA)	RML121948 RML121533	N.A.	AF150037	0/9	0/3	0/0	0/0
Dermacentor albipictus[2]	Rhipicephalinae	Missouri/ Georgia (USA)	RML122883 RML123324	N.A.	AF150041	0/3	0/3	0/1	0/1
Dermacentor reticulatus[1]	Rhipicephalinae	Lombardia (Italy)	MSNPVCAR322	N.A.	AF150038	0/2	0/1	0/0	0/0
Rhipicephalus turanicus[2]	Rhipicephalinae	Sicilia (Italy)	MSNPVCAR323	AM411587	AM410571	2/4	1/6	0/0	0/4
Rhipicephalus sanguineus[1]	Rhipicephalinae	Lombardia (Italy)	MSNPVCAR324	N.A.	AF150020	0/11	0/0	0/0	0/0
Rhipicephalus bursa[1]	Rhipicephalinae	Lazio (Italy)	MSNPVCAR325	AM411595 AM411596 AM411597	AM410572	7/21	2/15	0/0	0/0
Rhipicephalus bursa[2]	Rhipicephalinae	Sicilia (Italy)	MSNPVCAR326	N.A.	AF150053	0/0	0/1	0/2	0/20
Rhipicephalus annulatus[1]	Rhipicephalinae	Lazio (Italy)	MSNPVCAR327	N.A.	AM410573	0/2	0/0	0/0	0/0
Haemaphysalis l. palustris[1]	Haemaphysalinae	Georgia (USA)	RML121605	N.A.	AM410574	0/0	0/0	0/0	0/2
Haemaphysalis inermis[1]	Haemaphysalinae	Lazio (Italy)	MSNPVCAR328	N.A.	AM410576	0/2	0/0	0/0	0/0
Haemaphysalis punctata[2]	Haemaphysalinae	Sicilia (Italy)	MSNPVCAR329	AM411598 AM411599	AM410575	0/1	1/6	0/0	1/2
Hyalomma marginatum[2]	Hyalomminae	Sicilia (Italy)	MSNPVCAR330	AM411590 AM411591 AM411592	AM410577	0/0	0/0	0/0	4/5
Hyalomma truncatum[2]	Hyalomminae	Sicilia (Italy)	N.A.	AM411588 AM411589	AM410578	0/0	2/2	0/0	0/0
Amblyomma americanum[2]	Amblyomminae	Georgia (USA)	RML120727 RML120732	N.A.	AF150050	0/10	0/0	0/0	0/5
Amblyomma tuberculatum[2]	Amblyomminae	Georgia (USA)	RML120880	AM411593 AM411594	AM410579	0/0	0/0	0/0	2/2
Amblyomma maculatum[2]	Amblyomminae	Florida/ S. Carolina (USA)	RML119740 RML57678	N.A.	AY342285	0/6	0/0	0/0	0/0
Ixodes ricinus[2]	Ixodinae	Sicilia (Italy)	MSNPVCAR331	AJ566640	AF150029	2/2	0/0	0/0	0/0

Ixodes scapularis[2]	Ixodinae	New Jersey (USA)	RML124024	AF150030	N.A.	0/3	0/3	0/6	0/0
Ixodes pacificus[2]	Ixodinae	Oregon/California (USA)	RML123356 RML122710 RML53718	AM410580	N.A.	0/4	0/0	0/2	0/0
Ixodes hexagonus[1]	Ixodinae	Lombardia (Italy)	MSNPVCAR333	U95886	N.A.	0/4	0/0	0/6	0/0
Ixodes uriae[2]	Ixodinae	Grimsey (Iceland)	MSNPVCAR334	AM410581	AM411600	1/15	0/0	0/0	0/0

[1] DNA extracted from living specimens.
[2] DNA extracted from samples preserved in ethanol.
[3] Nymphs or larvae.
MSNPV, Museo di Storia Naturale, Università di Pavia; RML, U.S. National Tick Collection.
N.A., Sample Accession no. not available, or sequencing not performed because the samples were negative for midichlorians.

were stained with 0·5% toluidine blue; thin sections (80 nm) were stained with uranyl acetate and lead citrate and examined under an EM900 transmission electron microscope (Zeiss).

RESULTS

All of the ticks examined have been identified using morphological keys. In addition, all specimens were examined by PCR amplification of mitochondrial 12S rRNA to check for the quality of the DNA. A fragment of about 350 bp of the 12S rRNA was sequenced for a subset of samples, in order to confirm morphological identification. The fragment was also sequenced for all samples positive for *M. mitochondrii*. In total, forty 12S rRNA sequences were generated. BLAST search of 12S rRNA sequences confirmed morphological identifications in all cases. The 12S rRNA sequences obtained have been deposited in the EMBL data library (see Accession numbers in Table 1).

The 2 PCR protocols designed to be specific for the 16S rRNA of *M. mitochondrii* and related bacteria gave amplifications of the expected size from tick specimens belonging to 8 species out of the 21 species included in this study. In total, 15 tick specimens were found positive out of the 155 individual ticks examined. In addition, out of 42 pooled tick samples, 7 were found to be positive for *M. mitochondrii*. Table 1 summarizes the results obtained.

The ~1100 bp PCR-amplified 16S rRNA gene fragments from positive specimens were then sequenced, and the sequences were, in all cases, unambiguous. The sequences obtained were run against the databases and gave the highest similarity scores with the 16S rRNA sequences of *M. mitochondrii* from *I. ricinus* and with those sequences which have already been shown to form a monophyletic group with this bacterium (see Fig. 1 and Sassera *et al*. 2006).

Phylogenetic analyses were performed on 3 different data sets. The first included 16S rRNA sequences from bacteria of the main branches of the Rickettsiales and from *M. mitochondrii* and related bacteria (Fig. 1). The second alignment involved bacterial 16S rRNA from *M. mitochondrii* of *I. ricinus* and all closely related tick endosymbionts (Fig. 2A). The last alignment focused on mitochondrial 12S rRNA from ixodid ticks (Fig. 2B). The results of the analyses performed for each data set were similar irrespective of the phylogenetic method employed.

Figure 1 shows an example of 16S rRNA-based tree (Neighbour Joining) including representatives of the genera and families of the Rickettsiales. All of the sequences generated in this study form a monophyletic branch, which also includes the sequence of *M. mitochondrii* from *I. ricinus*, and the bacterial sequences that were already available in the data bases from the ticks *Ixodes persulcatus* (Mediannikov
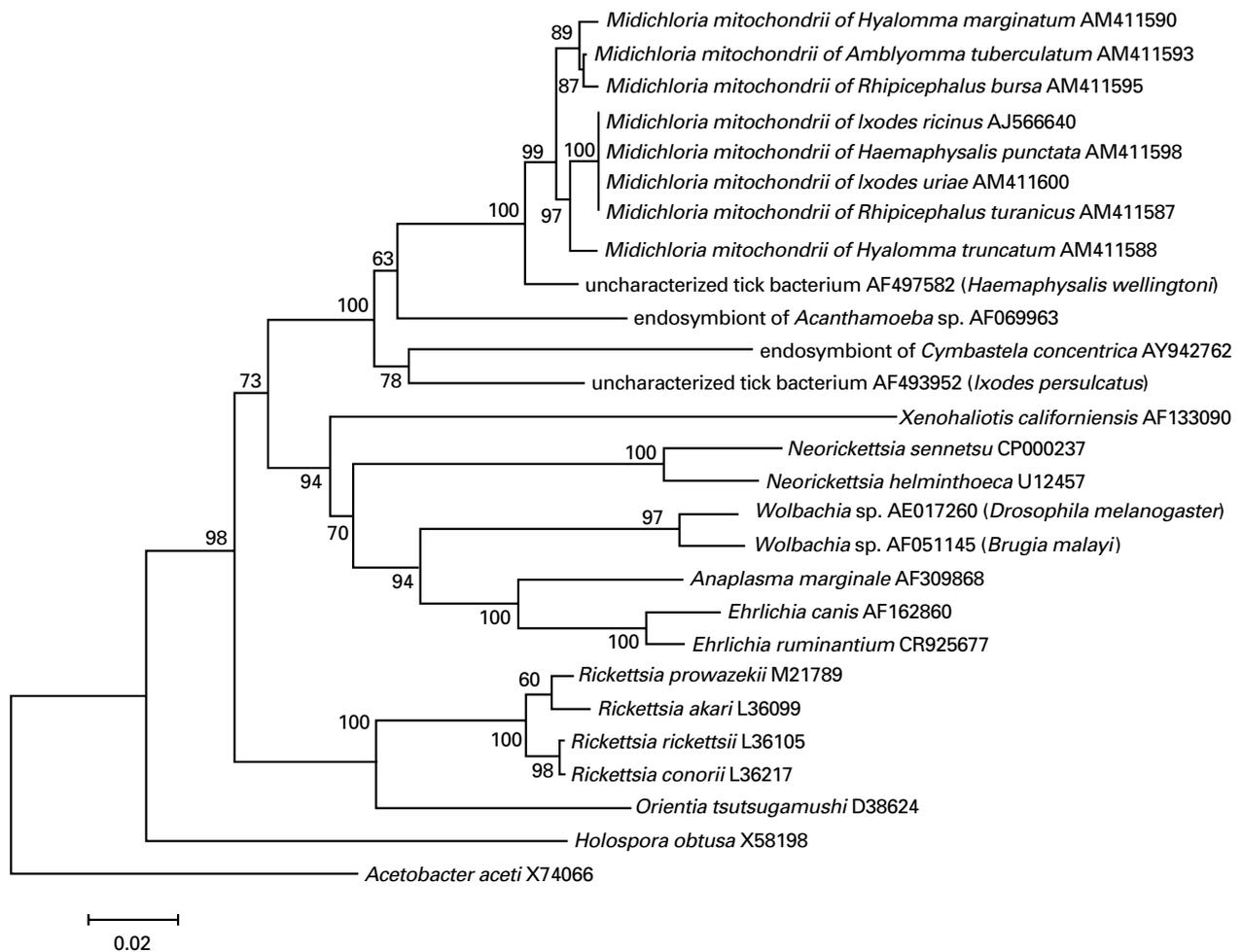
Fig. 1. Example of tree based on 16S rRNA gene sequences showing the position of *Midichloria mitochondrii* from different ticks relative to representatives of the order Rickettsiales. The tree was generated using MEGA (Neighbour Joining; Kimura correction). The Accession number for each sequence is indicated. Numbers adjacent to each node represent the bootstrap percentages (5000 repetitions). *Acetobacter aceti* (Rhodospirillales) was included as an outgroup. Additional analyses in which other Alphaproteobacteria were included as outgroups, or using other tree-building approaches, generated trees showing the same overall topology. Bar: 0·02 inferred substitutions per site.

et al. 2004) and *Haemaphysalis wellingtoni* (Parola *et al.* 2003), from the marine sponge *Cymbastela concentrica* (Taylor *et al.* 2005) and from the amoeba *Achantamoeba* sp. (Fritsche *et al.* 1999). Support for this branch was very high in all of the analyses performed (Neighbour Joining; Maximum Parsimony; Bayesian Inference), with bootstrap or posterior probability values of 97–100%. Within this branch, molecular diversity at the nucleotide level is limited: with the exclusion of the sequences from *Acantha- moeba* sp., *C. concentrica* and from *I. persulcatus*, pairwise nucleotide differences ranged from 3 to 22 nt out of 1000.

Figure 2A is a 16S rRNA-based tree (Neighbour Joining) including the *M. mitochondrii* sequences generated in this study and the sequence from the endosymbiont of *Acanthamoeba* sp. (included as an outgroup). Sequences from *M. mitochondrii* are as- signed to 3 main branches, supported by high boot- strap values. Figure 2B shows a 12S rRNA-based

tree (Neighbour Joining) including the tick species examined in this study. The tree is not fully resolved, but the main branches correspond to the traditional systematic arrangement of hard tick genera into subfamilies and of the subfamilies into the groups Prostriata and Metastriata (Hoogstraal and Aes- chlimann, 1982), with the exception of *Ixodes uriae* whose positioning relative to the two groups is not resolved. The uncertain positioning of *I. uriae* is not surprising if we consider the results of total evidence phylogenetics of hard ticks, placing this and other species of the genus *Ixodes* in different positions depending on the methods of analysis (Klompen *et al.* 2000). Indeed, phylogenetic analyses of our tick data set performed using other algorithms (Maximum Parsimony and Bayesian Inference; see Materials and Methods section) did not lead to a better resol- ution. A detailed comparison of the host and sym- biont phylogenies was therefore outside the scope of this work, considering that (i) tick phylogeny is still
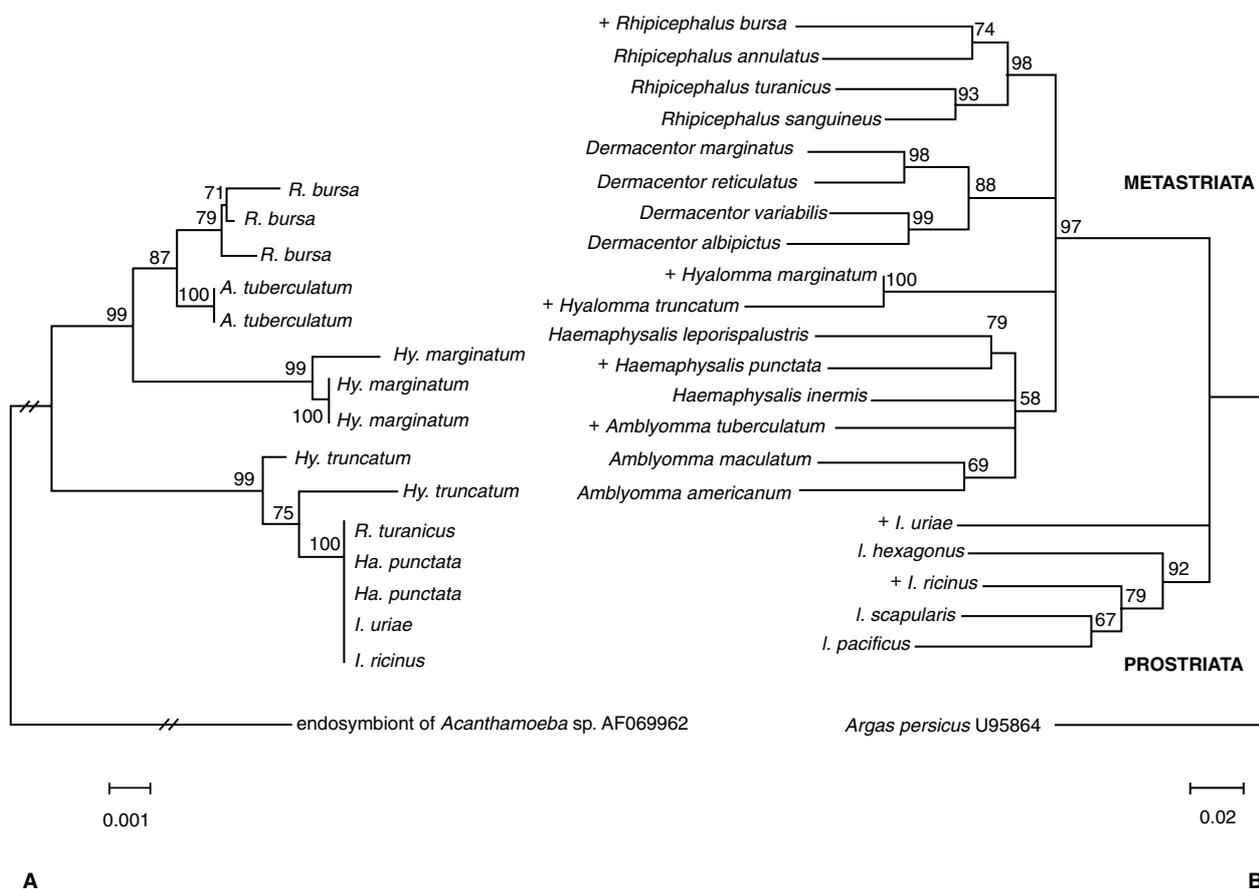
Fig. 2. Comparison of the phylogenies of *Midichloria mitochondrii* and ixodid ticks. Nucleotide sequence Accession numbers for ixodid ticks and for the different 'strains' of *M. mitochondrii* are reported in Table 1. Accession numbers for outgroup sequences are indicated. (A) Example of tree based on 16S rRNA gene sequences showing the inferred phylogeny of *M. mitochondrii*. Names at the terminal nodes are those of the host ticks (for full-length genus names, see (B). The tree was generated using MEGA (Neighbour Joining; Kimura correction). Numbers adjacent to each node represent the bootstrap values (5000 repetitions). An endosymbiont of *Acanthamoeba* sp. was used as outgroup. Additional analyses in which other Alphaproteobacteria were included as outgroups, or using other tree-building approaches, generated trees showing the same overall topology. Bar: 0·001 inferred substitutions per site. (B) Example of phylogenetic tree based on partial 12S rRNA gene sequences showing the relationships of the examined ticks. The + symbol indicates tick species that were found to be positive for *M. mitochondrii*. The tree was generated using MEGA (Neighbour Joining; Kimura correction). Numbers adjacent to each node represent the bootstrap percentages (5000 repetitions). Nodes with bootstrap values below 50% are shown as unresolved. Analyses using other tree-building approaches did not lead to a better resolution of these nodes. The argasid tick *Argas persicus* was included as outgroup. Bar: 0·02 inferred substitutions per site.

not fully resolved and (ii) the level of divergence here reported in the 16S rRNA of *M. mitochondrii* is not sufficient for the reconstruction of a robust phylogeny. Examining the symbiont (Fig. 2A) and host trees (e.g. the tree in Fig. 2B or the trees in Fig. 4 in Klompen *et al.* 2000) it is clear that the positioning of *M. mitochondrii* endosymbionts is not always congruent with the taxonomic/phylogenetic placements of the hosts. For example, endosymbionts from ticks of the *Ixodes* genus (the Prostriata of traditional taxonomic arrangements) cluster with endosymbionts from ticks of the Metastriata group. In some cases, tick species harbouring closely related *M. mitochondrii* are highly divergent at the level of their 12S rRNA gene. For example, *M. mitochondrii* from distantly related ticks of the

genus *Ixodes* (*I. ricinus* and *I. uriae*) are identical to each other in their 16S rRNA, and are also identical to *M. mitochondrii* of the Metastriata ticks *R. turanicus* and *Ha. punctata*. There are also single-tick species in which different individuals harbour *M. mitochondrii* with different 16S rRNA sequences (*Hy. truncatum*, *Hy. marginatum* and *R. bursa*).

Out of the 7 new tick species that were found positive for *M. mitochondrii*, we had the possibility to collect samples on 2 different occasions only for the species *R. bursa*. The number of specimens collected allowed us to obtain a first picture of the prevalence of *M. mitochondrii* in this tick species, with 7 positive females out of the 21 examined and 2 males out of 16. We emphasize that all positive specimens were from a single population, while the specimens from the
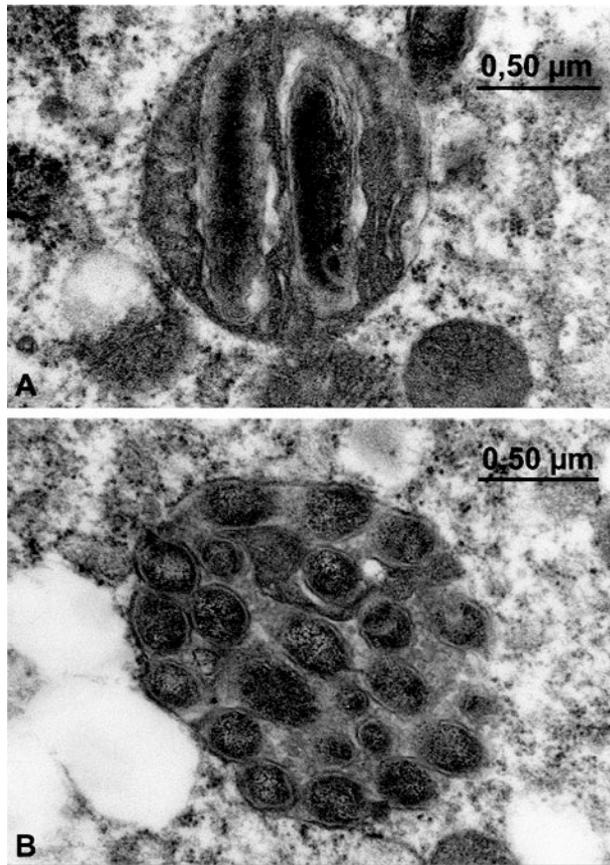
Fig. 3. Electron micrographs of ovarian cells of *Rhipicephalus bursa* showing mitochondria harbouring bacteria (A). Mitochondrion containing several bacteria does not present clear signs of reduction or degeneration of the matrix (B).

other population examined were all negative. In general, for all of the species that were found positive for *M. mitochondrii* in this screening, not all of the specimens examined were positive (see Table 1), though only for *R. bursa* were we able to collect a number of specimens sufficient for concluding that prevalence is far from 100%.

Female specimens of *R. bursa* were prepared for both PCR and TEM examinations. TEM was performed on ovaries from 4 female specimens that were found positive for *M. mitochondrii*, and from 1 specimen that was found negative. TEM examinations of the ovaries from positive females revealed the presence of bacteria in the mitochondria of the oocytes (Fig. 3A). The few bacteria that were observed outside the mitochondria were free in the cytoplasm (i.e. without surrounding vacuoles). The size of these bacteria is $\sim 1\,\mu$m in length and $\sim 0.25\,\mu$m in diameter. The number of bacteria in the mitochondria ranged from a single bacterium to over 20 observed in a single section. Infected mitochondria in *R. bursa* do not present clear signs of reduction/degeneration of the matrix, even in cases where numerous bacteria infect the mitochondria (Fig. 3B).

DISCUSSION

The screening reported here shows that *M. mitochondrii* is commonly found in ticks. Eight species out of 21 were positive, and only in 1 of the 7 genera examined (*Dermacentor*) were positive individuals not detected. The prevalence of *M. mitochondrii* in the various tick species examined here differs markedly from that observed in *I. ricinus*, where 100% of females and 44% of males have been found positive (Lo *et al.* 2006*a*). In general, the results here reported on the distribution of *M. mitochondrii* in ticks likely represent an underestimation. Firstly, for most of the species a limited number of specimens was examined, which implies that future screenings could lead to the detection of positive tick individuals from further tick species. Secondly, our screening included ethanol-preserved specimens. Partial degradation of the DNA cannot thus be excluded, even though control PCR for mitochondrial tick genes provided evidence for a good DNA quality.

Figure 2A shows a phylogenetic tree of *M. mitochondrii*; a tree representing a phylogeny of the ticks included in this study is also shown for comparison (Fig. 2B). The overall phylogeny of ticks is still unresolved (Barker and Murrel, 2004), and it was not the goal of this work to address this issue. However, an incomplete congruence in the phylogenies of ticks and their endosymbionts can be inferred, both comparing the *M. mitochondrii* tree with the host tree in Fig. 2B (or with trees in other studies; e.g. Black *et al.* 1997; Klompen *et al.* 2000), as well as considering that ticks belonging to different genera (*Ixodes* spp. and *R. turanicus*) harbour *M. mitochondrii* with identical 16S rRNA sequences. Considering the *Midichloria*-like 16S rRNA sequences already available in the data bases, we note that the endosymbiont of *I. persulcatus* belongs to the sister group of the main cluster that includes the sequences generated in this study plus those from *Ha. wellingtoni* and *Acanthamoeba* sp. In the tree in Fig. 1, the endosymbiont of *I. persulcatus* clusters with a bacterial sequence obtained from a sponge. The distance between this *Ixodes* bacterium and those found in other representatives of the genus *Ixodes* is relatively large.

Previous studies have provided microscopical and molecular evidence for vertical transmission of *M. mitochondrii* in *I. ricinus* (Sacchi *et al.* 2004; Lo *et al.* 2006*a*). The results reported here reveal that in some tick species different individuals harbour *M. mitochondrii* with identical gene sequences (i.e. in *I. ricinus*, *A. tuberculatum*, *Ha. punctata*). There is thus an overall consistency of information which indicates an effective role of vertical transmission in the diffusion of these bacteria. On the other hand, the results reported above also indicate a partial lack of congruence in the phylogenies of *M. mitochondrii* endosymbionts and their hosts, and suggest that

horizontal transmission of the symbionts might have occurred. How horizontal transmission could take place is unknown. However, based on the presence of DNA from *M. mitochondrii* in the blood of roe deer (Skarphédinsson *et al.* 2005), and the possible detection of a related bacterium in samples from human patients (Mediannikov *et al.* 2004), we might suggest that transmission to tick hosts could occur, and that blood could represent a potential source of infection for ticks. In any case, based on the above studies, the possibility that *M. mitochondrii* could infect humans and animals is worthy of further investigation.

In *R. bursa*, the species for which we could obtain the most samples, the prevalence of *M. mitochondrii* was 33% in females and 13% in males. The discovery of a tick species in which the prevalence of *M. mitochondrii* is far from fixation in both males and females will facilitate experiments aimed at uncovering the biological role of these endosymbionts. For example, fitness parameters could be compared in positive and negative individuals, as could the relative prevalence of tick-borne pathogens in positive and negative individuals. Experiments examining the possible role of *M. mitochondrii* as a reproductive parasite (e.g. cytoplasmic incompatibility, as in the case of *Wolbachia*; see Werren, 1997) could also be envisioned.

TEM observations on the ovaries from *R. bursa* gave results comparable to those obtained in *I. ricinus*, in terms of the size, shape and overall appearance of the bacterial symbionts (Sacchi *et al.* 2004). Evidence was also obtained that the bacteria reside in mitochondria. *I. ricinus* mitochondria harbouring numerous bacteria (i.e. over 10/20 per section) always present clear signs of degeneration of the matrix, in some cases resembling empty sacs; this has been interpreted as a consumption of the matrix by the bacteria (Sacchi *et al.* 2004). Such degeneration was less evident in *R. bursa*, even in mitochondria containing up to 20 bacteria per section. These TEM observations, associated with the lower prevalence of *M. mitochondrii* in *R. bursa*, may indicate that the kind of interaction between the host tick and symbionts is different in *R. bursa* and in *I. ricinus*. We note that within the monophyletic branch of *M. mitochondrii*, the bacteria of these 2 tick species are assigned to 2 different branches.

The capacity to invade the host mitochondria is thus present in different lineages of *M. mitochondrii*, infecting different tick species belonging to different genera and subfamilies. We could therefore hypothesize that the tropism for the mitochondria could represent an ancestral trait of *M. mitochondrii*, and it is reasonable to expect that other tick species in addition to the two that we investigated at the microscopic level harbour bacteria with the capacity to invade the mitochondria. In summary, intra-mitochondrial symbiosis is probably not just a

curiosity limited to *I. ricinus*, but could represent a more widespread biological phenomenon, at least in ticks of the family Ixodidae, a group of arthropods encompassing over 600 species, many of significant medical and veterinary importance. The fact that 16S rRNA gene fragments almost identical to those from *M. mitochondrii* have been found in samples from humans and deer (Mediannikov *et al.* 2004; Skarphédinsson *et al.* 2005) opens the intriguing question of whether the invasion of mitochondria by these bacteria also occurs in mammals.

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