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

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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 numerous 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.

1. 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., 2013; 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 their hosts, 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, 2001). These microorganisms 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, 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 hinder the transovarial transmission of the Rocky

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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 bacterium is found in 100% of the females of *Ixodes ricinus*, the most common 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, 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 and Chilton, 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 in this species (Beninati et al., 2009). Interestingly, a phylogenetic analysis of *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.

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

Host		Author
Genus	Species	
Amblyomma	*americanum*	Williams-Newkirk et al., 2012
Amblyomma	*tuberculatum*	Epis et al., 2008
Dermacentor	*andersonii*	Dergousoff and Chilton, 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., 2004
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

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.

2. Materials and methods

2.1. 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 Pérez-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.

2.2. DNA extraction

Before proceeding with DNA extraction, ethanol preserved specimens were rehydrated and washed twice in PBS 1X for 20 min and then left to dry for additional 20 min, in order to remove all the ethanol residues. Frozen ticks were boiled for 5 min 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.

2.3. 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