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Presence, genetic variability, and potential significance of "*Candidatus* Midichloria mitochondrii" in the lone star tick *Amblyomma americanum*

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Abstract

We used next generation sequencing to detect the bacterium "*Candidatus* Midichloria mitochondrii" for the first time in lone star ticks (*Amblyomma americanum*) from the eastern United States. 177 individuals and 11 tick pools from seven sites in four states were tested by pyrosequencing with barcoded 16S rRNA gene eubacterial primers targeting variable regions 5–3. Average infection prevalence was 0.15 across all surveyed populations (range 0–0.29) and only the site with the smallest sample size (n = 5) was negative. Three genotypes differing by 2.6–4.1 % in a 271 bp region of 16S rRNA gene were identified. Two variants co-occurred in sites in North Carolina and New York, but were not observed in the same tick at those sites. The third genotype was found only in Georgia. Phylogenetic analysis of this fragment indicated that the three variants are more closely related to "*Candidatus* Midichloria mitochondrii" genotypes from other tick species than to each other. This variation suggests that multiple independent introductions occurred in *A. americanum* which may provide insight into bacterial spread within its ecosystem and parasitism on this tick. Whether the presence of this bacterium affects acquisition or maintenance of other pathogens and symbionts in *A. americanum* or the survival, biology and evolution of the tick itself is unknown.

Keywords

Midichloria mitochondrii; Amblyomma americanum; 16S rRNA; Metagenomics; Tick control; Pathogenic bacteria; Rickettsiales

Introduction

"*Candidatus* Midichloria mitochondrii" (hereafter *M. mitochondrii*) is a newly described Rickettsiales from ixodid ticks that has an unusual tropism for mitochondria in some hosts (Beninati et al. 2004; Epis et al. 2008). In ticks where mitochondria infection has been observed, the bacterium replicates within the organelles and may cause extensive damage to them without observable impact on host cell function (Sacchi et al. 2004). Efficient vertical transmission of and high infection rates with *M. mitochondrii* (females, nymphs, and larvae

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100 %; males 44 %) have been demonstrated in *Ixodes ricinus* (Lo et al. 2006; Sassera et al. 2008). The infection prevalence of *M. mitochondrii* remains imprecisely known in most other surveyed tick species (7–100 %) due to small sample sizes (Epis et al. 2008; Venzal et al. 2008).

Neither the significance of *M. mitochondrii* as a tick symbiont nor its potential for vertebrate pathogenicity has been established, although evidence supporting its importance in both roles exists. Declining prevalence in laboratory-reared *I. ricinus* strongly suggests the bacterium is a facultative symbiont providing benefit to ticks under some environmental conditions (Lo et al. 2006), but is too energetically costly to maintain once environmental selection pressure is removed (Pistone et al. 2012). Understanding its benefit to tick hosts may provide a novel method of control for ticks that vector disease.

Many Rickettsiales, including the *Ixodes persulcatus* tick-associated relative of *M. mitochondrii* known as the 'Montezuma agent' (Fig. 2), are pathogenic for man (Mediannikov et al. 2004). *Midichloria mitochondrii* DNA has been detected in roe deer blood (Skarphédinsson et al. 2005) and tick salivary glands (Pistone et al. 2012), indicating vertebrates are, at minimum, exposed to the organism. Consequently, the potential of *M. mitochondrii* as a vertebrate pathogen cannot be ignored, although its active infection of a vertebrate has not yet been documented. The discordant phylogenies of tick and *M. mitochondrii* strains are most parsimoniously explained by horizontal transmission through a shared vertebrate host (Epis et al. 2008), thus also suggesting some vertebrates may be susceptible to infection. Considering that numerous tick species host this bacterium and its high abundance in the average infected individual tick (Sassera et al. 2008), it is important that the host range, role, and potential for pathogenicity of *M. mitochondrii* be defined.

Epis and colleagues recently used PCR to survey 21 tick species for the presence of *M. mitochondrii* (Epis et al. 2008). The lone star tick (*Amblyomma americanum*), the most abundant and aggressive ixodid tick in the southeastern United States and a vector of several known and potential human pathogens (Childs and Paddock 2003; Goddard and Varela-Stokes 2009), was reported as negative for *M. mitochondrii* based on a screen of 10 individual adults and 5 pools from Georgia. However, while conducting a metagenomic survey of the bacterial community of *A. americanum* from four states in the United States, we detected *M. mitochondrii* DNA in multiple tick samples. Here we describe the distribution and prevalence of the bacterium in *A. americanum*, as well as its unusually high level of genetic variation compared to strains previously characterized from other ticks. We further consider its potential significance for the biology and control of this tick.

Materials and methods

Tick collection and DNA extraction

Host-seeking *A. americanum* were collected by dragging a 1 m^2 white cotton cloth through ground-level vegetation (Ginsberg and Ewing 1989). Collection sites, tick preservation, and DNA extraction are described in Mixson et al. (2006) for all samples collected in New York (1999, 2003), New Jersey (2003), and North Carolina (2002); DNAs were stored at 4 °C until tested in this study. Ticks were collected in 2010 from Panola Mountain and

Sweetwater Creek State Parks near Atlanta, Georgia. Most ticks collected in 2010 were preserved in 70 % ethanol immediately after collection and stored at 4 °C. A subset of adults were placed live into individual, sterile vials and washed with lysis buffer from the QIAamp DNA Mini Kit (Qiagen, Valencia, California) for 20 min to collect surface bacteria for metagenomic analysis. DNA was extracted from the lysis buffer rinse following the manufacturer's instructions. Following the surface wash, ticks were preserved in 70 % ethanol. DNA was extracted from all ethanol preserved nymphs and adults individually and pools of 50 larvae from single egg masses as described in Bermúdez et al. (2009).

16S rRNA gene pyrosequencing

PCR primer and barcode designs were from the Human Microbiome Project's Provisional 16S 454 Protocol (Broad primer sequences for variable regions 5–>3) (HMP website) and modified to be compatible with the Roche (Indianapolis, Indiana) 454 sequencer's Titanium chemistry. Modified primer sequences were F: 5′-CTA TGC GCC TTG CCA GCC CGC TCA GCC TAC GGG AGG CAG CAG-3′ and R: 5′-CGT ATC GCC TCC CTC GCG CCA TCA G [barcode] CCC GTC AAT TCM TTT RAG T-3′. For PCR, each 20 μL reaction mix contained 1× AccuPrime PCR Buffer II (Invitrogen, Carlsbad, California, USA), 0.5 U AccuPrime Taq High Fidelity, 13.5 μL water, 0.3 μM each forward and reverse primer, and 2 μL DNA. All reactions were performed in an Eppendorf Master Gradient thermocycler (Brinkmann Instruments, Westbury, New York, USA) under the following program: one cycle of 94 °C (2 min), 35 cycles of 94 °C (30 s), 50 °C (30 s), and 68 °C (1 min), and one cycle of 68 °C (5 min). Reaction products were analyzed by electrophoresis on 1 % agarose gels stained with ethidium bromide to ensure reaction success, then quantitated using the Quant-iT PicoGreen dsDNA kit (Invitrogen) modified from the manufacturer's protocol to 50 % of the recommended final assay volume. Amplicons were pooled in equal concentrations, purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin), then sequenced at the Centers for Disease Control and Prevention (CDC) Biotechnology Core Facility. Amplicons from 50 samples collected prior to 2010 were sequenced on one quarter of a 454 Titanium plate. Amplicons from 132 ticks collected in 2010 were sequenced on a whole plate; 49 samples were amplified and sequenced twice. Amplicons from the external rinses of 6 adults were also sequenced on this plate.

Pyrosequence analysis

Raw sequences were processed to remove low quality reads using the software mothur v. 1.22.0 (Schloss et al. 2009) following the trim.seqs alternative approach described in mothur's online documentation (Schloss 2011). The remaining trimmed, high quality reads were assigned to operational taxonomic units (OTUs) by clustering sequences at ≥97 % identity, and OTUs were identified by comparison to reference databases. Because this report is primarily concerned with the presence or absence of *M. mitochondrii* within ticks, the number of sequences analyzed across samples was not standardized.

Phylogenetic trees

We retrieved all GenBank sequences that overlapped 100 % and shared ≥94 % BLAST identity with our 16S rRNA gene fragment variants in addition to representative sequences from other clades within the Rickettsiales. All sequences were aligned and trimmed to the

same length in the software package Geneious (Drummond et al. 2010). Using the Tamura-Nei genetic distance model, a consensus neighbor-joining tree was constructed based on 1,000 bootstrap iterations with a 50 % support threshold.

Results

Midichloria mitochondrii abundance and prevalence

Not all 188 tick DNA samples generated a sufficient number of sequence reads to reliably detect low numbers of *M. mitochondrii*. Of the 50 DNA samples from New York, New Jersey, and North Carolina and the 138 samples from Georgia, only 35 and 96 DNA samples, respectively, produced ≥1,000 high quality reads each. Across all samples surveyed, 27 individuals, four pools of 5 nymphs, and the exterior wash of one tick contained at least one *M. mitochondrii* sequence (mean = 118 sequences/sample, range = 1–555). The minimum infection rate was calculated for pooled nymphs, but each pool of larvae was considered a single sample because each pool originated from a single female. Total infection prevalence was 0.15 and within site prevalence ranged from 0 to 0.29 (Table 1). The only site with no evidence of infection was also the site with the smallest sample size (Fire Island, NY, n = 5 nymphs, probability of detection 0.11).

Midichloria mitochondrii genetic variation

Three distinct sequence variants of the 16S rRNA gene fragment were identified from the pyrosequencing data (Fig. 1); these differed in pairwise comparisons by 2.58 % (variant A–B), 3.69 % (A–C), and 4.06 % (B–C). Genotypes differed in their distribution between sites and samples. Variant A (GenBank accession number JQ678693) was found exclusively in ticks from Georgia and was the sole genotype found there. Variants B (JQ678691) and C (JQ678692) were identified individually in North Carolina and New Jersey sites, respectively, and also together in North Carolina and New York sites (Table 2). The only DNA that contained multiple variants was extracted from a pool of 5 nymphs from Shelter Island, New York, where both genotypes B and C were also frequently observed in individual ticks (variant B: 1 male and 3 nymph pools; variant C: 2 females, 3 males, and 2 nymph pools).

Phylogenetic analysis

The phylogenetic tree based on the 271 bp 16S rRNA gene sequence fragments (Fig. 2) was highly similar in its organization within the *M. mitochondrii* clade to published trees based on 1,000 bp to near full length 16S rRNA gene fragments (Epis et al. 2008; Vannini et al. 2010). However, unlike other *M. mitochondrii* sequences obtained from the same tick species, the *A. americanum*-derived variants were more closely related to those from other ticks than to each other. The position of several other *M. mitochondrii*-like organisms and some related clades did not fully resolve with the amount of sequence data available.

Discussion

Midichloria mitochondrii has been previously reported in 11 ixodid tick species from Europe, Asia, North America, South America, and Iceland (Parola et al. 2003; Epis et al.

2008; Venzal et al. 2008; Beninati et al. 2009; Dergousoff and Chilton 2011; Pistone et al. 2012) and from horse flies (*Tabanus bovinus* and *Tabanus tergestinus*) and bed bugs (*Cimex lectularius*) collected in Europe (Hornok et al. 2008; Richard et al. 2009). The wide distribution of this bacterium in haematophagous arthropods and the lack of concordance between the phylogenies of *M. mitochondrii* and its tick hosts strongly suggest that the bacterium is transmitted horizontally between tick species or into tick species from some other host(s) (Epis et al. 2008). The data reported here provide further support for this hypothesis by identifying three distinct genotypes of *M. mitochondrii* in *A. americanum*. While the full 16S rRNA gene sequence is not yet available for these new variants, previous work on *M. mitochondrii* within a single tick species has shown variation in this gene to be limited or absent in all ticks (Pistone et al. 2012) except *I. holocyclus* where two strains are known that differ by 2.5 % (Lo et al. 2006; Beninati et al. 2009).

The structure of the *M. mitochondrii* clade in the phylogenetic tree shown in Fig. 2 is very similar to the trees based on the longer 16S rRNA gene sequences published previously (Epis et al. 2008; Vannini et al. 2010). The most notable characteristic of the tree was the distribution of *A. americanum*-derived genotypes throughout the *M. mitochondrii* clade, rather than clustering together as is seen in other tick species. This suggests that *M. mitochondrii* may have been introduced into *A. americanum* multiple times from different vertebrate or invertebrate hosts. However, of the three tick species hosting closely related strains, only the geographic range of *Amblyomma tuberculatum* overlaps with that of *A. americanum*, indicating that additional undiscovered hosts of *M. mitochondrii* likely exist in North America.

As yet, there is no evidence that individual ticks were co-infected by multiple variants despite indications that infection prevalence may be relatively high in populations hosting multiple strains. Several mechanisms may give rise to this pattern. One possibility is that, like in *I. ricinus* (Sassera et al. 2008), the dominant mode of transmission for *M. mitochondrii* in *A. americanum* may be vertically from female to eggs, creating very little opportunity for the acquisition of multiple infections by superinfection. Additional mechanisms to prevent or reduce the incidence of co-infections in ticks may be similar to those seen in other arthropod-bacterial symbiont systems. Examples include, but are not limited to, a bacterial population bottleneck during the colonization of host oocytes (Mira and Moran 2002), cytoplasmic incompatibility between hosts infected with different bacterial strains (Stouthamer et al. 1999), and competitive exclusion of additional strains by previous host infection (Macaluso et al. 2002). Additional work is needed to confirm the pattern of single genotype infections within individual ticks and to determine its significance either at the population level of *A. americanum* or by its mode of acquisition and maintenance.

The detection of *M. mitochondrii* DNA in the external rinse of a single male was interesting, but also puzzling because the male's corpuscular DNA sample did not test positive. Nearly three times the number of sequence reads were obtained from the male's rinse compared to its residual DNA sample. This suggests that the buffers used to rinse the tick may have extracted large amounts of DNA from the body cavity of the tick. Further work is needed to

determine if *M. mitochondrii* is a component of the external bacterial community of some *A. americanum*.

The relationship dynamics between *M. mitochondrii* and its tick hosts are still under investigation. Other bacteria have been shown to provide a competitive advantage to ticks in specific environmental conditions. For example, *Borrelia burgdorferi* increases the tolerance of *I. ricinus* for hot, dry habitats (Herrmann and Gern 2010). The fluctuating infection prevalence of *M. mitochondrii* in *I. ricinus* (Lo et al. 2006; Sassera et al. 2008) and its frequent discovery in other tick species may indicate a similarly beneficial association, possibly involving a role in energy metabolism given the preference of *M. mitochondrii* for tick cell mitochondria in some hosts (Pistone et al. 2012). This is especially interesting considering the ongoing expansion of *A. americanum* out of its historical range in the southeastern United States and into the northeastern and midwestern states (Ginsberg et al. 1991; Means and White 1997; Keirans and Lacombe 1998; Merten and Durden 2000). *Amblyomma americanum* is the most frequently reported tick attached to humans in the southeast and Atlantic states (Merten and Durden 2000), and although once considered merely a nuisance species, the importance of *A. americanum* as a disease vector in the United States is now well recognized (Childs and Paddock 2003). It is important that future research addresses the question of *M. mitochondrii*'s potential contribution to the range expansion of *A. americanum* so that manipulation of the relationship may be explored as a method of control for this important vector. Genetic modification of this agent to suppress acquisition of pathogenic agents vectored by *A. americanum* may be possible if one or more *M. mitochondrii* variants are stably inherited both transovarially and transstadially, similar to the introduction of *Wolbachia* into *Aedes aegypti* to suppress arbovirus and protozoa transmission (Moreira et al. 2009).

This is the first report of *M. mitochondrii* in *A. americanum*. Estimates of prevalence were highly variable between sites, an unsurprising finding given the small within-site sample sizes. With our limited sample sizes and number of sites tested, the true prevalence, genetic diversity and distribution of genotypes of *M. mitochondrii* in *A. americanum* is likely only roughly approximated. However, our results have demonstrated that *M. mitochondrii* is widely distributed, genetically diverse, and common in *A. americanum*.

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1   TTTTGAAACTACTAAGCTTG AGTACTGTAGGGGATAGCGG AATTCCTAGTGTAGGGGTGA AATTCGTAGATATTAGGAGG AACACCGGAGGCGAAAGCGG
A   .................... ....T............... .................... .................... ......A.............
B   .................... ............G....... .................... .................... ....................
C   ..........G...G..... .................... .................... .................... ....................

101 TTATCTGGGCAGTCACTGAC GCTGTTGCACGAAAGCGTGG GGAGCAAACAGGATTAGATA CCCTGGTAGTCCACGCAGTA AACGATGAGTGCTAGATGTT
A   ...........A........ .................... .................... .................... ....................
B   ........A........A.. .................... .................... .................... ....................
C   ............T...... .................... .................... .................... ....................

201 GGGGTTTAAGTCTCAGTGTC GCAGCTAACGCATTAAGCAC TCCGCCTGGGGAGTACGGTC GCAAGATTAAA
A   .................... .................... .................... ...........
B   ....G............... .................... .................... ...........
C   ...A..C.TT.......... .................... .................... ...........
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Fig. 1.

Three variants of the *Midichloria mitochondrii* 16S rRNA gene fragment identified in *Amblyomma americanum.* The *first row* of each section represents the consensus sequence, and the number to the left of the line the position of the first nucleotide in the line. Variants are labeled *A*, *B*, and *C* with their differences from the consensus indicated. Total fragment length is 271 bp and spans a portion of variable region 4 and all of variable region 5

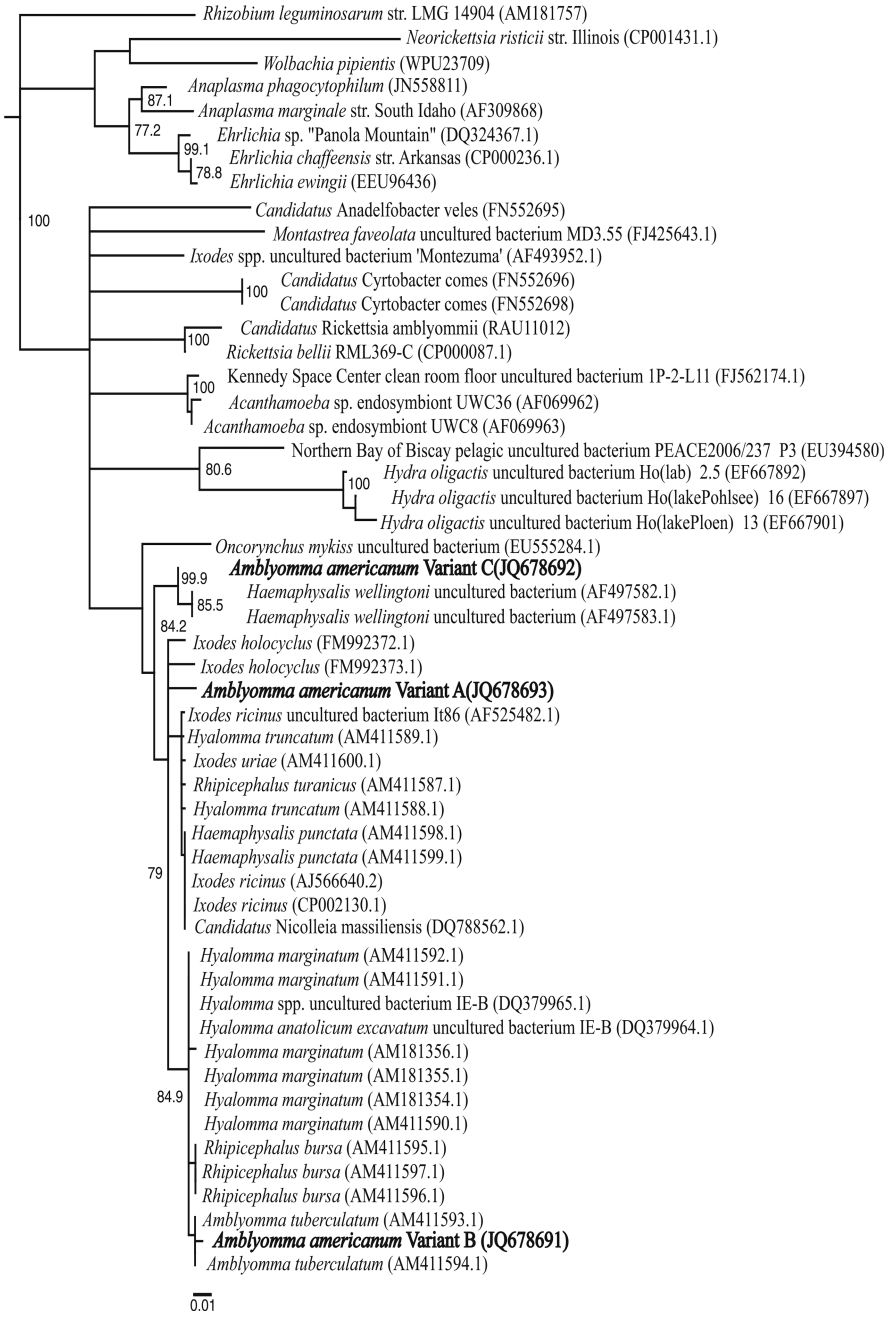

Fig. 2.

Phylogenetic tree of *Midichloria mitochondrii* 16S rRNA gene fragments obtained from *Amblyomma americanum* and related sequences from GenBank. *Bold type* is used to highlight the position of the *A. americanum* variants. The tree was constructed from a 271 bp fragment using neighbor-joining with *Rhizobium leguminosarum* as the out group. Unless noted, all *terminal node labels* indicate the tick host from which the *M. mitochondrii* sequence was obtained. GenBank accession numbers are provided in parentheses. Branch

support is given for bootstraps greater than 75 %. *Scale bar* indicates the number of substitutions per site

Table 1

Prevalence of *Midichloria mitochondrii* infection in *Amblyomma americanum* collected from the eastern United States

Site	Pooled larvae	Nymph	Pooled nymphs	Male	Female	Adult rinse	Site prevalence
Bodie Island, NC	NT	NT	NT	1/5	1/5	NT	0.20 (n = 10)
Buxton Woods, NC	NT	NT	NT	0/5	2/5	NT	0.20 (n = 10)
Earle, NJ	NT	NT	NT	2/5	0/5	NT	0.20 (n = 10)
Fire Island, NY	NT	0/5	NT	NT	NT	NT	0 (n = 5)
Shelter Island, NY	NT	NT	4/5	4/5	2/5	NT	0.29 (n = 35)
Panola Mountain, GA	0/3	6/60	NT	1/3	0/3	1/6	0.11 (n = 75)
Sweetwater Creek, GA	0/3	8/60	NT	NT	NT	NT	0.13 (n = 63)
Life stage prevalence	0 (n = 6)	0.11 (n = 125)	0.16 (n = 25)	0.35 (n = 23)	0.22 (n = 23)	0.17 (n = 6)	

Minimum infection rate is given for pooled nymphs. Fraction indicates the number positive/number sampled
Numbers in parentheses are the sample size for a given site or life stage
NT not tested

Table 2

Distribution of *Midichloria mitochondrii* 16S rRNA gene variants at *Amblyomma americanum* sample sites

Site	16S rRNA gene variant			Total no. samples
	A	B	C	
Bodie Island, NC	0	2	0	2
Buxton Woods, NC	0	1	1	2
Earle, NJ	0	0	2	2
Fire Island, NY	0	0	0	0
Shelter Island, NY	0	4	7	10
Panola MTN, GA	8	0	0	8
Sweetwater Creek, GA	8	0	0	8

Values indicate the number of samples where a variant was detected. Note that one pool of 5 nymphs from Shelter Island, NY contained large numbers of both B and C sequences, so the pool was counted in both columns