1 **Title: Sequence of a *Coxiella* endosymbiont of the tick**
2 ***Amblyomma nuttalli* suggests a pattern of convergent genome**
3 **reduction in the *Coxiella* genus**

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12

13 Abstract

14 Ticks require bacterial symbionts for the provision of necessary compounds that are absent in
 15 their hematophagous diet. Such symbionts are frequently vertically transmitted and, most
 16 commonly, belong to the *Coxiella* genus, which also includes the human pathogen *Coxiella*
 17 *burnetii*. This genus can be divided in four main clades, presenting partial but incomplete co-
 18 cladogenesis with the tick hosts. Here we report the genome sequence of a novel *Coxiella*,
 19 endosymbiont of the African tick *Amblyomma nuttalli*, and the ensuing comparative
 20 analyses. Its size (~1 Mb) is intermediate between symbionts of *Rhipicephalus* species and
 21 other *Amblyomma* species. Phylogenetic analyses show that the novel sequence is the first
 22 genome of the B clade, the only one for which no genomes were previously available.
 23 Accordingly, it allows to draw an enhanced scenario of the evolution of the genus, one of
 24 parallel genome reduction of different endosymbiont lineages, which are now at different
 25 stages of reduction from a more versatile ancestor. Gene content comparison allows to infer
 26 that the ancestor could be reminiscent of *Coxiella burnetii*. Interestingly, the convergent loss
 27 of mismatch repair could have been a major driver of such reductive evolution. Predicted
 28 metabolic profiles are rather homogenous among *Coxiella* endosymbionts, in particular
 29 vitamin biosynthesis, consistently with a host-supportive role. Concurrently, similarities
 30 among *Coxiella* endosymbionts according to host genus and despite phylogenetic
 31 unrelatedness hint at possible host-dependent effects.

32

34 **Keywords:** *Amblyomma nuttalli*, *Coxiella*-like endosymbiont, genome reduction,
 35 phylogeny, comparative genomics, symbiont evolution

36 **Significance statement**

37 The genus *Coxiella* includes the pathogen *Coxiella burnetii* and widespread nutritional
38 mutualists in ticks. Current knowledge on their evolution is hampered by the limited genomic
39 resources available.

40 Here we provide the first genome sequence of a *Coxiella* endosymbiont of clade B, the only
41 clade for which none was available.

42 These data allow to infer an evolutionary scenario of parallel genome reduction among
43 *Coxiella* endosymbionts, with similar constraints, leading to selective retention of
44 biosynthetic pathways beneficial for the host. The combined predicted functional capabilities
45 of the symbionts appear to be a subset of those of *C. burnetii*. Accordingly, this pathogen
46 could be closer to an ancestral state of the endosymbionts, rather than being derived from an
47 endosymbiotic ancestor, as previously hypothesized.

48 Introduction

49 Mutualistic associations with bacteria are widespread and can allow eukaryotes to colonise
50 novel ecological niches (Bennett & Moran 2015). In arthropods, intracellular and maternally
51 transmitted bacterial mutualists are particularly common (Douglas 1998). A typical role of
52 these endosymbionts is providing essential nutrients that are absent in the host's diet
53 (Sandström & Moran 1999). The classical example is that of sap-feeding insects, such as
54 aphids, acquiring essential amino acids and vitamins from intracellular bacteria (Wernegreen
55 2012).

56 Similarly, blood-feeding arthropods also rely on an incomplete source of nutrients and
57 harbour bacterial mutualists. For instance, “*Candidatus RIESIA*” bacteria supply their host, the
58 human louse, with several compounds, in particular B vitamins (Boyd et al. 2017; Sasaki-
59 Fukatsu et al. 2006). A better understanding of these symbioses and their role can foster the
60 development of control strategies for hematophagous arthropod vectors of diseases (Zindel et
61 al. 2011). Ticks in particular are extensively studied due to their prominent role of disease
62 vectors for humans and domestic animals (Dantas-Torres et al. 2012). The most important
63 pathogens vectored by ticks include *Borrelia*, the tick-borne encephalitis virus, *Coxiella*
64 *burnetii*, and multiple *Rickettsiales* bacteria (Kernif et al. 2016).

65 Most tick species present at least one bacterial endosymbiont, and many species more than
66 one (Cafiso et al. 2016; Moutailler et al. 2016; Duron et al. 2017). Transmission of symbionts
67 is mainly dependent on maternal inheritance through transovarial transmission, but horizontal
68 transfer, possibly during co-feeding, also plays a role. Indeed, closely related tick species, and
69 even different individuals of the same species, may harbour different sets of bacterial
70 symbionts (Duron et al. 2017).

71 Experimental evidence indicates a major role of such symbionts in tick physiology, as their
72 depletion resulted in impaired growth and reproduction in multiple species belonging to

different genera (Zhong et al. 2007; Guizzo et al. 2017; Ben-Yosef et al. 2020). Interestingly, in *Ornithodoros moubata* such developmental defects were rescued by supplementation with B vitamins (Duron et al. 2018). Collectively, these data suggest a common role as B vitamins providers of different, phylogenetically unrelated, symbionts. Other parallel roles have been hypothesized, including protection from pathogens, provision of energy, support for feeding, protection against oxidative and osmotic stress, and waste molecule recycling (Buysse et al. 2019; Olivieri et al. 2019).

Most of characterized tick symbionts are affiliated to *Coxiella* (*Gammaproteobacteria*) (Duron et al. 2017), and will now be abbreviated as CEs (*Coxiella* endosymbionts). Besides many CEs of ticks, this genus includes the tick-borne pathogen *Coxiella burnetii*, causative agent of the Q-fever (Angelakis & Raoult 2010). It has been hypothesized that the latter arose from a CE ancestor, but the origin of its virulence is unclear (Duron et al. 2015).

Currently, a total of seven genomes of CEs are available (Gottlieb et al. 2015; Smith et al. 2015; Guizzo et al. 2017; Ramaiah & Dasch 2018; Tsementzi et al. 2018). These present the hallmarks of genome reduction in bacterial symbionts (McCutcheon & Moran 2012), but at different stages. CEs of *Rhipicephalus* spp. have comparatively larger genomes (1.2-1.7 Mb) with a higher number of pseudogenes (Gottlieb et al. 2015; Guizzo et al. 2017; Ramaiah & Dasch 2018; Tsementzi et al. 2018), characteristics of relatively recent symbioses with ongoing genome reduction. Conversely, the CEs of *Amblyomma* ticks have more streamlined genomes (0.6-0.7 Mb), with high gene density and low number of mobile elements (Smith et al. 2015), typical of a later stage of symbiosis.

Nevertheless, all CEs possess the pathways for production of many B vitamins, consistently with the hypothesised role and the general trend observed in nutritional symbionts, which retain some host-supportive pathways even in case of severe genome reduction (Nakabachi et al. 2006; López-Madrigal et al. 2011).

Four phylogenetic clades (A-D) were identified in the genus *Coxiella*, exhibiting only partial congruence with their hosts' phylogeny (partial co-cladogenesis). Clade C displays a good

degree of co-cladogenesis with *Rhipicephalus* hosts, while CEs of two unrelated clades (B and D) were found in *Amblyomma* hosts (Duron et al. 2015). Interestingly, the known clade B *Amblyomma* hosts came from the African continent, while clade D hosts are American (Duron et al. 2015; Binetruy et al. 2020). Currently, genomes of representatives of three of the four *Coxiella* clades have been sequenced, i.e. CEs from clades C and D, and *Coxiella burnetii* from clade A.

Here, we sequenced the genome of a novel CE of *Amblyomma nuttalli* belonging to the fourth clade (B), and used it for comparative analyses, providing a basis for improving the understanding of the diversity and evolution of CEs.

Materials and methods

An adult female of *Amblyomma nuttalli* was collected from a white rhinoceros in the Masai Mara National Reserve, Kenya in February 2016. The tick was morphologically identified following standard taxonomic keys (Theiler & Salisbury 1959) and subjected to DNA extraction, using NucleoSpin® Tissue Kit (Macherey Nagel, Duren, Germany), according to the manufacturer's instructions. DNA was subjected to Illumina HiSeq X by Admera Health (South Plainfield, NJ, USA) using a Nextera XT library, obtaining 27,351,224 150-nt paired-end reads.

The reads were assembled using SPAdes (3.6.0), and subjected to a modified version of the blobology pipeline (Kumar et al. 2013), in order to select only the symbiont sequences (for a complete description of the process see (Castelli et al. 2019)). Briefly, we selected contigs with a $\log_{10}$ coverage higher than 2.5, extracted and reassembled separately the reads mapping on those contigs (Langmead & Salzberg 2012), and extensively revised manually the results (Supplementary Figure 1-2).

The completeness level of the genome was confronted with all published CE genomes using BUSCO, using the *Gammaproteobacteria* lineage dataset (Seppey et al. 2019).

Genome annotation was performed using Prokka 1.11 (Seemann 2014) and manually curated by inspecting blastp hits of predicted ORFs on NCBI nr, Uniprot, and *Legionellales* sequences.

ISEScan (Xie & Tang 2017) and ISfinder (Siguier et al. 2006) were used to identify insertion sequences and PHASTER (Arndt et al. 2016) for prophages. Pseudogene prediction on the novel genome, all published CE genomes, and representative *C. burnetii* genomes (Supplementary table 1) was performed using Pseudo-finder (Syberg-Olsen & Husnik 2018). COGs were predicted on the same dataset using the NCBI pipeline (Galperin et al. 2015) on validated genes (i.e. ORFs excluding predicted pseudogenes). COG repertoires were used for comparative analyses. Metabolic pathways were manually reconstructed employing the BioCyc database reference (Karp et al. 2019).

Two datasets were used for phylogeny. The first one involved a wide taxonomic sampling, analysed through MLST (multilocus sequence typing) as in (Duron et al. 2015), thus employing five genes and 96 organisms (published dataset plus all available CEs).

The second set was analysed by using a phylogenomic approach, and included the previous selection of *Coxiella* genomes, a representative selection of *Coxiellaceae*, including 1 MAG (metagenome assembled genome), and two other *Legionellales* as outgroup (Supplementary table 1). Using OrthoFinder (2.3.3) (Emms & Kelly 2019), 213 single copy conserved orthologs were identified.

Then, for the two sets, respectively the nucleotide and protein sequences of each single gene were aligned separately using Muscle (Edgar 2004), polished with Gblocks (Talavera & Castresana 2007), and finally concatenated (3,118 and 59,256 total positions, respectively).

For each set, we inferred the best model (GTR+I+G and LG+I+G, respectively) using modeltest-ng 0.1.3 (Darriba et al. 2019), built a maximum likelihood tree with RAxML 8.2.4 (Stamatakis 2014) with 1000 bootstrap pseudo-replicates, and a Bayesian inference tree with MrBayes (Ronquist et al. 2012) using three independent runs for 1 million and 250,000 generations, respectively, with a burn-in of 25%.

152 Results and discussion

153 The obtained genome assembly of the CE of *A. nuttalli* has a total length of 1,001,386 bp (9
154 contigs; N50: 229,733 bp; GC: 35,95%). BUSCO completeness score was 79.2%, similar to
155 other CEs (Supplementary Figure 3). A total of 45 RNA genes (including 38 tRNAs and 3
156 rRNAs) and 730 ORFs were found. Among these, we identified 696 functional CDSs and 34
157 pseudogenes, accounting for a total of 658,600 bp (65.7%) coding (including structural RNA
158 genes). Neither prophages nor ISs were found.

159 MLST phylogeny provided an overall consistent topology with most previous studies (Duron
160 et al. 2015; Gottlieb et al. 2015), in particular for the major *Coxiella* clades and their
161 relationships (clade A earliest divergent, clade B sister group of clades C+D), with moderate
162 to high support (Figure 1A, Supplementary Figure 4-5). Consistently with what could be
163 expected based on geographical origin, the CE of *A. nuttalli* lies in the clade B, composed by
164 CEs of African ticks.

165 For the available *Coxiella* genomes, phylogenomic analysis showed the same relations of the
166 MLST phylogeny (Figure 1B, Supplementary Figure 6). Interestingly, for all *Coxiella*,
167 including the novel CE of *A. nuttalli*, branch lengths are proportional to the degree of genome
168 reduction (Supplementary Table 2), consistently with previous analyses (Duron et al. 2015;
169 Gottlieb et al. 2015). This would indicate a higher evolutionary rate in smaller genomes, as
170 predicted for obligate symbionts by genome reduction models (McCutcheon & Moran 2012).

171 Specifically, *C. burnetii*, a pathogen capable of living in different environments and hosts
172 (Angelakis & Raoult 2010), presents the largest genome (2 Mb) and, with a high coding
173 density (79.9%), the highest amount of coding DNA (1.8 Mb). All the host-restricted CEs
174 have smaller genomes, with similar sizes within each clade: 1.2-1.7 Mb for CEs of
175 *Rhipicephalus* (clade C), 1.0 Mb for the novel CE of *A. nuttalli* (clade B), and 0.6-0.7 Mb for
176 CEs of other *Amblyomma* species (clade D). However, the degree of genome reduction does
177 not correlate with the phylogenetic branching pattern between clades, in particular the CEs

178 with more reduced genomes (clades B, D) do not form a single monophyletic group (Figure
179 1B). Accordingly, considering also the novel CE of *A. nuttalli*, a scenario with parallel
180 independent genome reduction in genus *Coxiella* (at least for monophyletic CEs of B-D
181 clades together) appears plausible.

182 Interestingly, most size variation resides in the non-coding genome (from 88 Kb to 816 Kb),
183 while the length of the functional coding genome is overall less variable in CEs, ranging from
184 540 Kb of CE of *A. sculptum* (Clade D), to 917 Kb of CE of *R. turanicus* (Clade C). These
185 features as well are consistent with a recent and still ongoing parallel genome reduction of
186 CEs under similar constraints, possibly due to an equivalent role for the host. Accordingly,
187 the CE of *A. nuttalli* has traits of a relatively long-term obligate symbiont, at an intermediate
188 stage among CEs for its genome size and coding density (Supplementary Table 2), and
189 having no predicted mobile elements.

190 The functional capabilities, as represented by COG repertoires, are consistent with the
191 observed pattern of genome reduction (Figure 2A). *C. burnetii* is the richest in COGs for all
192 functional categories. CEs have lower COGs numbers, roughly proportional to the respective
193 coding genomes. Many core functions are highly conserved, such as translation machinery
194 (J), coenzyme (H) and nucleotide (F) metabolism, energy production (C), protein
195 modification and chaperones (O), lipid synthesis (I), cell cycle regulation (D). This is
196 consistent with their expected major role for bacterial survival and/or host-support
197 (coenzymes). On the other side, all CEs are more pronouncedly depleted in accessory and
198 regulative functions, including poorly characterized ones (R and S), signal transduction (T),
199 secondary metabolite metabolism (Q), cell motility (N), secretion systems (U), extracellular
200 and defense structures (W and V). Such functionalities are probably less important in strictly
201 host-associated bacteria. Notable is the case of type IV secretion, probably ancestral in
202 *Legionellales* (Hugoson et al. 2019) and an important virulence factor in *C. burnetii* (Luedtke
203 et al. 2017), but absent in all CEs. Some functions display gradients of conservation along the
204 genome size, e.g. membrane structure biogenesis (M), which correlates with decrease in

205 lipopolysaccharide complexity, while peptidoglycan synthesis is conserved.

206

207 Such scenario is reflected at the level of single COGs (Figure 2B), as *C. burnetii* (clade A)
208 presents the highest number of unique COGs. CEs of all clades are a substantial subset of *C.*
209 *burnetii*, which lacks only 60 of the 1207 total COGs in the dataset. Similar observations can
210 be drawn among progressively more reduced CE clades, with highly streamlined clade D CEs
211 almost as a subset of the other CEs.

212 Few lineage-specific peculiarities were found, and the functional significance of many of
213 these is unclear, e.g. the *A. nuttalli*-specific COGs display redundant or poorly characterized
214 functions (Supplementary table 3). However, some relevant variability was also observed. All
215 CEs retain the predicted capability to perform glycolysis, Krebs cycle and oxidative
216 phosphorylation (Supplementary table 4). However, contrarily to the other CEs, the symbiont
217 of *A. nuttalli* does not possess a conventional citrate synthase, but instead presents a 2-
218 methylcitrate synthase, which may also catalyse the same reaction (Patton et al. 1993). As *C.*
219 *burnetii* has both genes, this partial redundancy could have been ancestral, and independently
220 lost in different CE lineages.

221 In general, while biosynthetic abilities for amino acids are scarce in all *Coxiella* (E), those for
222 vitamins and cofactors (H) are, as expected, abundant and highly conserved (Supplementary
223 table 4). CEs are in particular rich in genes for the synthesis for riboflavin (B2), pantothenate
224 (B5) and its derivative CoA, pyridoxine (B6), folic acid (B9), and biotin. For biotin (and
225 lipid) synthesis, missing FabI functionality is possibly replaced by FabV (Massengo-Tiassé &
226 Cronan 2008). Interestingly, despite overall smaller functional capabilities, the CEs of
227 *Amblyomma* display complete biosynthetic pathways for thiamine (B1) and NAD (B3)
228 (except the last thiamine step in clade D), while symbionts of *Rhipicephalus* retain only
229 partial pathways (including final steps from thiamine phosphate and β -nicotinate D-
230 ribonucleotide). Such differences can be explained by the presence of not yet identified
231 transporters and/or non-canonical enzymes (Gottlieb et al. 2015). They might also indicate

different, not yet clarified, metabolic requirements of the tick hosts, with *Amblyomma* species requiring a full pathway while *Rhipicephalus* being permissive for the loss of some genes.

A similar scenario may hold for nucleotide metabolism (F), which is also more reduced in the CEs of *Rhipicephalus*, lacking the initial path for the synthesis of purines (up to 5-aminoimidazole ribonucleotide) (Supplementary table 4).

Consistently with their symbiotic condition (McCutcheon & Moran 2012), CEs are depleted in DNA repair abilities (L), with lineage-specific features (Supplementary table 4). For example both CEs of *R. microplus* are devoid of the RecFOR pathway and RecA, involved in homologous recombination (Kuzminov 1999). Interestingly, the MutSL pathway is fully absent in the smaller genomes of CEs of *Amblyomma* of clades B and D, but complete in *C. burnetii* (clade A) and in most members of clade C. Among those, the exception is the CE of *R. microplus* “2”, found to have a full *mutL* gene, but a truncated *mutS* pseudogene, probably retaining only partial or no functionality. Parsimoniously, we can identify at least three multiple convergent losses of this pathway among CEs (complete in the clades B and D, and still ongoing within clade C). Considering the strong correlation with the degree of genome reduction, it is reasonable to hypothesise that this loss may have had a major evolutionary impact, possibly directly causing increased mutation rates (Schofield & Hsieh 2003), and eventually resulting in accelerated and more pronounced genome reduction. Thus, the seminal speculations by Gottlieb and co-authors (2015) on a smaller dataset are reinforced. This effect would be particularly evident from the differences in coding size and functional categories among the two closely related CEs of *R. microplus* (Figure 1B).

Conclusions

The novel sequence of CE of *Amblyomma nuttalli* expands the available diversity of

Coxiella genomes, being the first obtained from clade B. Despite reduced genome size, biosynthetic pathways for vitamins appear to be conserved, as in other CEs, supporting a role of CEs in dietary supplementation of these compounds to the hosts. At same time, some variations were found in vitamin and purine synthesis, possibly dependent on the host species.

Combining phylogenetic and genomic data, an evolutionary scenario of parallel genome reduction with analogous constraints among CEs (clades B-D) can be drawn. Consistently with previous observations (Gottlieb et al. 2015), the convergent loss of MutSL could have been a driver of such reduction, with the CE of *A. nuttalli* representing an intermediate level between clade C and D, and all CEs substantially being a subset of *C. burnetii*. Accordingly, and differently from previous views (Duron et al. 2015), CEs could have evolved from a more versatile *C. burnetii*-like ancestor, analogously to other unrelated symbionts (Taylor et al. 2005; Gerhart et al. 2016). Genomic and phylogenomic analyses of representatives of clade A other than *C. burnetii* may provide further insights.

Data Availability Statements

The data underlying this article are available in the NCBI GenBank Database at ncbi.nlm.nih.gov/, and can be accessed with JACBPR000000000 (CE genome sequence) and with SRR12168527 (total reads in SRA).

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414

Figure legends

416

417 Fig. 1: (a) Maximum likelihood MLST phylogenetic tree of *Coxiella* and other *Legionellales*
 418 (full tree in Supplementary Figure 4), and (b) maximum likelihood phylogenomic tree of
 419 *Coxiellaceae*. In (a,b), the four major *Coxiella* clades are evidenced by different colors,
 420 numbers on branches stand for bootstrap support after 1000 pseudo-replicates, CE stands for
 421 *Coxiella* endosymbiont, and respective host organisms are indicated. In (a), symbionts with
 422 available genomes are highlighted. In (b), scale bar stands for estimated proportional
 423 sequence divergence, and the bar plot on the right shows the respective coding (blue) and
 424 non-coding (red) genome sizes.

425

426 Figure 2. (a) Heatmap showing variations in number of pseudogenes (blue, indicated with the
 427 Ψ), genome size (green) and COG (Clusters of Orthologous Groups) repertoire for each
 428 functional category (orange-purple) in the *Coxiella* genus. CE stands for *Coxiella*
 429 endosymbiont. Organisms are grouped according to phylogenetic clades, in turn sorted
 430 according to coding DNA size. The color intensity is independently scaled for each column in
 431 proportion to its maximum value. (b) Venn diagram representing COG distribution on
 432 *Coxiella* clades. For each clade, COGs identified in at least one of the listed genomes are
 433 counted.

A

B

Size (Mb):

Coding DNA

Non coding DNA

0

0.5

1

1.5

2

2.5

A

B