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Abstract

Mutualistic interactions with microbes have facilitated the adaptation of major eukaryotic lineages to restricted diet niches. Hence, ticks with their strictly blood-feeding lifestyle are associated with intracellular bacterial symbionts through an essential B vitamin supplementation. In this study, examination of the whole bacterial diversity in 25 tick species of the *Amblyomma* genus showed that three intracellular bacteria, *Coxiella*-like endosymbionts (LE), *Francisella*-LE and *Rickettsia*, are remarkably common. No other bacterium is so uniformly present in *Amblyomma* ticks. Almost all *Amblyomma* species were found to harbour a nutritive obligate symbiont, *Coxiella*-LE or *Francisella*-LE, able to synthesize B vitamins. However, despite the co-evolved and obligate nature of these mutualistic interactions, the structure of microbiomes does not mirror the *Amblyomma* phylogeny with a clear exclusion pattern between *Coxiella*-LE and *Francisella*-LE across tick species. *Coxiella*-LE, but not *Francisella*-LE, form evolutionarily stable associations with ticks commonly leading to co-cladogenesis. We further evidenced symbiont replacements during radiation of *Amblyomma*, with recent, and likely ongoing, invasions by *Francisella*-LE and subsequent replacements of ancestral *Coxiella*-LE through transient co-infections. Nutritional symbiosis in *Amblyomma* ticks is thus not a stable evolutionary state, but instead arises from conflicting origins between unrelated but competing symbionts with similar metabolic capabilities.

Introduction

Macro-organisms harbour complex microbial communities living inside and on their body (Margulis, 1993; Theis et al., 2016). These microbial communities, known as the microbiomes, can determine pivotal phenotypic traits of their hosts, driving a variety of ecological and evolutionary processes including major nutritive, reproductive and immune functions (Gould et al., 2018; Groussin et al., 2017; Hanning & Diaz-Sanchez, 2015; Ley et al., 2008; Turner, James, & Poole, 2013). As hosts vary in the microbiomes they harbour, an associated functionally important phenotypic variation exists within host populations (Falony et al., 2016; Ferrari & Vavre, 2011; Jaenike, 2012; Oliver, Russell, Moran, & Hunter, 2003; Scarborough, Ferrari, & Godfray, 2005). In arthropods, these microbiomes notably include highly specialized intracellular bacteria depending almost exclusively on maternal (transovarial) transmission to ensure their persistence in host populations (Moran, McCutcheon, & Nakabachi, 2008; Wernegreen, 2012). Some of these maternally inherited symbionts are essential for the life cycle of their arthropod hosts: They are obligate mutualists able to synthesize biochemical products favouring the specialization of arthropods to novel habitats or to particular feeding niches such as strict haematophagy or phloemophagy (Moran et al., 2008; Wernegreen, 2012). Overall, these mutualistic interactions have facilitated the radiation of major arthropod lineages, leading to remarkable host–symbiont phylogenetic congruence with a strict co-cladogenesis pattern in many cases (Chen, Li, & Aksoy, 1999; Duron et al., 2017; Jousselin, Desdevises, & Coeur d'acier, 2009; Moran, Tran, & Gerardo, 2005; Takiya, Tran, Dietrich, & Moran, 2006).

Arthropods and beneficial maternally inherited symbionts can form evolutionary stable associations lasting for millions of years, but that are not necessarily permanent (Bennett & Moran, 2015; McCutcheon, Boyd, & Dale, 2019; Moran et al., 2008; Wernegreen, 2012).

Recent phylogenetic reconstructions suggest that beneficial symbiotic relationships can break down: Recently acquired symbionts can replace ancestral beneficial symbionts and provide similar benefits to the host (McCutcheon et al., 2019; Sudakaran, Kost, & Kaltenpoth, 2017). An alternative scenario is that recently acquired symbionts may cooperate with ancestral beneficial symbionts (Moran et al. 2008; Vautrin & Vavre, 2009). Vertical transmission actually locks the different symbionts together as coinfection and creates then priviliged situations for symbiont–symbiont interactions, especially cooperation and dependence between symbionts. Functions of ancestral beneficial symbionts may be complemented by recently acquired cosymbionts and their coexistence can be ultimately stable over millions of years (Meseguer et al 2017). New beneficial symbionts often originate from microbes abundant in the host environment, potentially including entomopathogens, parasites vectored by arthropods or other maternally inherited symbionts, primarily facultative (i.e. not essential) for host survival (Koga & Moran, 2014; Matsuura et al., 2018; McCutcheon et al., 2019; Sachs, Skophammer, & Regus, 2011). These facultative symbionts, however, determine important traits in arthropods: protection against natural enemies, adaptation to changing environments or reproductive traits (Engelstädter & Hurst, 2009; Ferrari & Vavre, 2011; Moran et al., 2008; Oliver et al., 2003). Contrary to beneficial obligate symbionts, facultative symbionts undergo occasional horizontal transfers (HT) across arthropod species, resulting in limited phylogenetic congruence between hosts and symbionts (Duron, Wilkes, & Hurst, 2010; Jousselin, Cœur d'Acier, Vanlerberghe-Masutti, & Duron, 2013; Russell et al., 2009). Overall, the diverse range of microbial lifestyle strategies creates a complex web of interactions mediating the dynamics of beneficial symbioses in arthropods (McCutcheon et al., 2019).

Co-existence of symbionts within microbial communities is expected to involve interactions ranging from cooperation to competition and that can, in turn, determine aggregation and exclusion patterns (Ferrari & Vavre, 2011; Moran et al., 2008; Vautrin & Vavre, 2009). Exclusion patterns have been recently detected between maternally inherited symbionts of ticks, suggesting that replacements of beneficial symbionts occur in this system (Duron et al., 2017). Among arthropods, ticks (Arachnida: Ixodidae) are well known to engage in symbiotic associations with at least 10 different genera of maternally inherited bacteria (Ahantarig, Trinachartvanit, Baimai, & Grubhoffer, 2013; Duron et al., 2017). Ticks are specialized for an exclusive diet of vertebrate blood, and have evolved intimate interactions with beneficial symbionts that provide essential B vitamins and co-factors deficient in the blood diet (Bonnet, Binetruy, Hernández-Jarguín, & Duron, 2017; Duron et al., 2017, 2018; Gerhart, Moses, & Raghavan, 2016; Gottlieb, Lalzar, & Klasson, 2015; Guizzo et al., 2017; Hunter et al., 2015; Olivieri et al., 2019; Smith, Driscoll, Gillespie, & Raghavan, 2015). Approximately two thirds of tick species harbour *Coxiella*-like endosymbionts (*Coxiella*-LE hereafter), which are required for tick survival and reproduction (Gottlieb et al., 2015; Guizzo et al., 2017; Smith et al., 2015; Zhong, Jasinskas, & Barbour, 2007). *Coxiella*-LE genomes encode pathways for the biosynthesis of major B vitamins and co-factors that fit closely with the expected nutritional complements required for strict haematophagy (Gottlieb et al., 2015; Guizzo et al., 2017; Smith et al., 2015). *Coxiella*-LE are abundant in two organs of ticks: ovaries, that is consistent with vertical transmission into developing oocytes, and Malpighian tubules, where B vitamins are possibly synthesized (Buysse, Plantard, McCoy, Duron, & Menard, 2019; Wang et al., 2018)(Lalzar et al 2012). Owing to their maternal inheritance and beneficial nature, *Coxiella*-LE are present in most individuals within infected host species [16, 41]. In the *Rhipicephalus* tick genus, the acquisition of *Coxiella*-LE was followed by co-

diversification resulting in deeply congruent *Rhipicephalus–Coxiella*-LE phylogenies (Duron et al., 2017).

In a few tick species, however, *Coxiella*-LE are present at much lower frequencies than expected for obligate nutritional symbionts, suggesting that they are instead facultative symbionts in these hosts (Duron et al., 2015). Phylogenetic evidence corroborates this hypothesis, since closely related *Coxiella*-LE may infect distantly related tick species suggesting recurrent HT of some *Coxiella*-LE (Duron et al., 2015). In other tick species, no *Coxiella*-LE were detected at all, but alternative obligate beneficial symbionts have been identified or hypothesized (Duron et al., 2017, 2018; Gerhart et al., 2016; Kurtti et al., 2015; Olivieri et al., 2019). Indeed, *Francisella*-like endosymbionts (*Francisella*-LE) are commonly found in tick species lacking *Coxiella*-LE. A recent analysis of endosymbiotic communities in 81 tick species also showed that there is a significant exclusion pattern between *Francisella*-LE and *Coxiella*-LE (Duron et al., 2017). Like *Coxiella*-LE, *Francisella*-LE are essential for tick nutrition: Ticks deprived of their *Francisella*-LE completely cease development but resume normal growth upon supplementation with B vitamins (Duron et al., 2018). Genomes of *Francisella*-LE contain roughly the same biosynthesis pathways of B vitamins and co-factors as observed in *Coxiella*-LE genomes (Duron et al., 2018; Gerhart et al., 2016). *Francisella*-LE also presents the same tropism than *Coxiella*-LE: *Francisella*-LE are abundant in ovaries and Malpighian tubules of the ticks they infect (Duron et al 2018, current biology). Although *Francisella*-LE and *Coxiella*-LE are distantly related, they have converged towards an analogous nutritional mutualism with ticks [32, 33].

While *Coxiella*-LE symbioses are likely ancestral in ticks, replacements by *Francisella*-LE having recently transitioned to an endosymbiotic lifestyle (Duron et al., 2018; Gerhart et al.,

2016) appear across the tick phylogeny (Duron et al., 2017). Yet, the factors favouring *Francisella*-LE over *Coxiella*-LE in this evolutionary dynamic are not well understood. In this study, we examined the evolutionary dynamic of tick microbiomes, with a focus on beneficial nutritional symbioses, in an ecologically diverse genus of hard ticks, *Amblyomma*. This genus is the third largest in the family Ixodidae, with its species primarily occupying the tropical zones. The centre of species diversity is on the American continent, where half of all the 130 *Amblyomma* species are found (Guglielmone, Estrada-Peña, Keirans, & Robbins, 2003). The *Amblyomma* genus includes major vectors of tick-borne disease agents, including the lone star tick, *A. americanum*, which is the primary vector of *Ehrlichia* spp. (Childs & Paddock, 2003). Only a few studies have examined the microbial diversity in *Amblyomma* species, showing that they are infected either by *Coxiella*-LE or by *Francisella*-LE in addition to other maternally inherited bacteria (Binetruy, Dupraz, Buysse, & Duron, 2019; Budachetri et al., 2014; Clay et al., 2008; Duron et al., 2017; Gerhart et al., 2016). Here, we investigated the variation in tick microbial communities at different geographic and phylogenetic scales using a representative collection of specimens covering ca. 20% of *Amblyomma* species diversity. First, we reconstructed the *Amblyomma* phylogeny through the sequencing of large nuclear rDNA sequences (18S, ITS1, 5.8S, ITS2, and 28S rDNA). Second, we extensively characterized bacterial communities in *Amblyomma* species through a DNA barcoding approach targeting the 16S rDNA. Third, we traced the evolutionary histories of *Coxiella*-LE and *Francisella*-LE using multilocus sequence typing (MLST) systems. While the *Coxiella*-LE MLST already exists (Duron et al., 2015), in this study we developed a specific *Francisella*-LE MLST. Finally, we compared *Amblyomma* phylogeny with microbiome structure and further used co-phylogenetics, by comparing *Amblyomma*, *Coxiella*-LE and *Francisella*-LE phylogenies, to reveal the global dynamics of symbiotic interactions.

Materials and methods

Tick collection and processing

We examined a total of 144 tick specimens belonging to 25 *Amblyomma* species (1–11 specimens per species) collected from field sites in America and Africa or from laboratory colonies (Supplementary Table S1). Samples were preserved in 70% ethanol until use. To eliminate external (i.e. cuticular) microbes, tick specimens were surface cleaned with bleach prior to DNA extraction (Binetruy, Dupraz, et al., 2019). A few specimens (*A. loculosum*, *n*=4; *A. sculptum*, *n*=1; *Amblyomma* sp., *n*=1, obtained from a previous study (Duron et al., 2017)) were, however, not bleach-treated prior to DNA extraction (Supplementary Table S1). All tick DNA was individually extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany).

Molecular typing of ticks

To reconstruct *Amblyomma* phylogeny, we sequenced almost complete rDNA sequences, including the 5′ end of 18S, the entire ITS1, 5.8S and ITS2 sequences, and the 3′ end of 28S rDNA (Supplementary Table S2) from 44 specimens (indicated in yellow in supplementary Table S1). Depending on the tick species, the size of the rDNA amplicon obtained varies from 4270 bp to 5054 bp. PCR products were purified using the kit Cleanpcr (CleanNA, Waddinxveen, The Netherlands), fragmented to a size of 300 bp and further used to construct libraries with the kit Nextera XT (Illumina, San Diego, California, USA). These libraries were then pooled together, indexed and pair-end sequenced on a Miseq (Illumina) sequencer using a flow cell equipped with a V3, 600-cycle reagent cartridge.

We obtained >24 million reads after quality filtering and removing adaptors using the Cutadapt tool on a Galaxy workbench (Goecks, Nekrutenko, Taylor, & The Galaxy Team,

178 2010; M. Martin, 2011). Paired-end reads were *de novo* assembled using metaSPAdes v3.11
179 with k-mer sizes of 21, 33, 55, and 73 bp (Nurk, Meleshko, Korobeynikov, & Pevzner, 2017).
180 The resulting metaSPAdes contigs were binned into tick nuclear DNA and non-tick nuclear
181 DNA groups through megablast in the GenBank nucleotide collection (Boratyn et al., 2013).
182 These contigs were scaffolded, when applicable, by aligning to the complete sequences of the
183 rRNA genes operon of *A. americanum* (GenBank AF291874), *A. hebraeum* (GenBank
184 KY457489) and the partial sequence of *A. marmoreum* (GenBank KY457492) using MEGA
185 (Kumar, Stecher, & Tamura, 2016).
186
187 *Bacterial metabarcoding*
188 A 251-bp portion of the V4 variable region of the bacterial 16S rRNA gene was amplified
189 individually for each DNA sample using a Multiplex PCR Kit (Qiagen) and universal primers
190 (16SV4F: 5′-GTGCCAGCMGCCGCGGTAA-3′ and 16SV4R: 5′-
191 GGACTACHVGGGTWTCTAATCC-3′) (Galan et al., 2016). Amplified bacterial 16S rDNA
192 products were purified and sequenced using an Illumina MiSeq platform (GenSeq,
193 Montpellier University) and 251-bp end sequence reads were obtained,. All bioinformatic
194 analyses were conducted using the pipeline Frogs
195 (https://github.com/geraldinepascal/FROGS) (Escudié et al., 2018) as previously described
196 (Binetruy et al., 2019). One step of post-process operational taxonomic unit (OTU) affiliation
197 was additionally performed through the Frogs pipeline on a Galaxy workbench (Escudié et
198 al., 2018; Goecks et al., 2010). This step consists in aggregating OTUs that share 97% of
199 identity within 99% of the amplicon length, and it reduced the probability of keeping
200 artefactual OTUs by resolving multi-hit ambiguities. To control the contamination during
201 these procedure negative controls were performed (three negative extraction controls were
202 included in all extraction series, two negative PCR controls were included in all PCR series).

Moreover. OTUs having a maximal abundance in negative controls were discarded and false-positive OTUs were removed by filtering OTU representing less than 0.005% of the OTU total abundance (Bokulich et al., 2013). Following this procedure, the microbiome was determined individually for almost all *Amblyomma* specimens (*n*=142/143); however, the amount of DNA for the single specimen of *A. sculptum* was not sufficient.

A phylogenetic tree using OTU sequences and beta-diversity matrices based on this tree was assessed with FastTree and GUnifrac packages in R (J. Chen et al., 2012; Price, Dehal, & Arkin, 2009), using the computational procedure described in (Binetruy, Dupraz, et al., 2019). Multidimensional scaling (MDS) plots were then generated using the package ggplot2 in R (Wickham, 2016). Permutational multivariate analysis of variance (PERMANOVA) implemented in the vegan package in R or pairwise PERMANOVA (Arbizu, 2017/2018) was further performed on the generalized UniFrac ($\alpha = 0.5$) dissimilarity matrix to evaluate the potential impact of the presence of symbionts and co-infection on bacterial diversity. The *P*-values of the pairwise PERMANOVA were corrected for multiple comparisons using Holm's method (Holm, 1979). A Mantel test was used to examine the association between microbial diversity (GUnifrac distance) and *Amblyomma* phylogeny using the ecodist package in R (*n*=9999 permutations) (Goslee & Urban, 2007).

Multilocus typing of Coxiella-LE and Francisella-LE

Coxiella-LE were genotyped through nested or semi-nested PCR amplification and sequencing of three housekeeping genes (16S rRNA, *rpoB* and *groEL*) previously developed for the *Coxiella* MLST methodology (Duron et al., 2015) (Supplementary Table S2). No *Francisella*-LE MLST was previously developed and we thus used the published genome of *Francisella*-LE F-Om strain (isolated from the soft tick *Ornithodoros moubata* [32]; GenBank accession number: QAPC00000000) as reference to design specific PCR primers

for five genes (16S rRNA, *rpoB*, *groEL*, *ftsZ* and *gyrB*; Supplementary Table S2). Positive PCR products were purified and sequenced in both directions by Eurofins (Ebersberg, Germany). Sequence chromatograms were manually cleaned with Chromas Lite (http://www.technelysium.com.au/chromas_lite.html) and aligned with CLUSTALW implemented in MEGA 7 (Kumar et al., 2016; Thompson, Gibson, & Higgins, 2002).

Phylogenetic analyses

Phylogenetic relationships were assessed using *Amblyomma*, *Coxiella*-LE and *Francisella*-LE sequences produced in this study and additional sequences available in GenBank (including *Amblyomma*, *Coxiella*-LE and *Francisella*-LE relatives and outgroups). GBLOCKS (Castresana, 2000) was used to remove poorly aligned positions and to obtain unambiguous sequence alignments. All sequence alignments were also checked for putative recombinant regions using the RDP3 analysis package (D. P. Martin et al., 2010). The best fitting evolutionary models were determined using the Akaike information criterion and Bayesian information criterion with MEGA 7 (Kumar et al., 2016). Phylogenetic analyses were based on Bayesian inferences (BI) with MrBayes v3.2.7 (Ronquist et al., 2012). Two replicate analyses were run for 1 million generations. For each replicate, we ran one cold chain and three hot chains of the Markov chain Monte Carlo method, using a random starting tree and sampling trees every 100 generations and discarding 25% as burn-in. The remaining trees were used to calculate 50% majority-rule consensus trees.

The BI phylogenies of *Amblyomma*, *Coxiella*-LE and *Francisella*-LE were then used to conduct co-phylogenetic analyses using the Procrustean Approach to Cophylogeny (PACo) package in R (Balbuena, Míguez-Lozano, & Blasco-Costa, 2013; Hutchinson, Cagua, Balbuena, Stouffer, & Poisot, 2017). The significance of the co-phylogenetic tests was

established by 100,000 random permutations of the two-association matrix. To test an effect of *Rickettsia* co-infection on the evolution of *Francisella*-LE and *Coxiella*-LE, linear regressions were computed in R using the matrix of genetic distances of these symbionts and the presence/absence of *Rickettsia* as an explicative variable.

Ethics statement

The use of the genetic resources was declared to the French Ministry of the Environment (reference TREL19028117S/156) and to Gabon government (entry authorization #AE16008/PR/ANPN/SE/CS/AEPN and #research authorization #AR0013/16/MESRS/CENAREST/CG/CST/CSAR).

Results

Phylogeny of Amblyomma ticks

We first reconstructed the phylogenetic relationships among the 25 *Amblyomma* species using BI analyses based on large fragments of nuclear rDNA sequences obtained from one-to-two specimens per species (Figure 1). The findings support a monophyletic origin of the five African *Amblyomma* species (AF group): They cluster together in a robust clade nested among the 20 New World *Amblyomma* species (NW group), suggesting an American origin of these African species (Figure 1). Moreover, the phylogeny of *Amblyomma* also parallels the tick host-range at least in the NW group: Closely related *Amblyomma* species often share the same host species, such as *A. dissimile* and *A. rotundatum* feeding on poikilotherms (reptiles and amphibians) or *A. latepunctatum*, *A. scalpturatum* and *A. naponense* feeding mainly on tapirs and Suidae (Figure 1). However, in other cases, closely related *Amblyomma* species use different host species, such as *A. ovale*, a generalist species feeding on a diversity

277 of domestic and wild animals, and *A. varium*, a specialized species feeding on arboreal
278 vertebrates.
279
280 *Microbial diversity and symbiont prevalence*
281 We further examined the whole bacterial diversity of 24 out of the 25 *Amblyomma* species
282 (*n*=142 specimens, one-to-ten specimens were examined per species) via high-throughput 16S
283 rDNA sequencing. The amount of DNA for the 25th *Amblyomma* species, *A. sculptum* (*n*=1),
284 was not sufficient to perform bacterial barcoding (this specimen was already PCR-typed for
285 *Coxiella*-LE and *Francisella*-LE, as further detailed). After filtration of false-positive OTUs
286 and contaminants (Binetruy, Dupraz, et al., 2019; Birer, Tysklind, Zinger, & Duplais, 2017),
287 4,364,360 reads distributed in 195 OTUs were obtained (Table S3). *Coxiella*-LE and
288 *Francisella*-LE were the most abundant bacterial genera representing 33.6% and 33.1% of the
289 total number of reads, respectively (Figure 2 and Supplementary Table S1). Other bacterial
290 genera were found: In most cases, each represented a negligible part of the 16S rDNA reads
291 when present. A remarkable exception to this pattern was the presence of abundant
292 intracellular bacteria belonging to the *Rickettsia* genus in 16 *Amblyomma* species (Figure 2
293 and Supplementary Table S1). Other exceptions included the *A. romitii* samples for which no
294 *Coxiella*-LE and *Francisella*-LE reads were detected: Most reads were assigned to a tick-
295 borne pathogen, *Ehrlichia* sp. In *A. loculosum* and *Amblyomma* sp. samples, *Coxiella*-LE
296 reads were detected but reads of other bacteria were more abundant. Since the *A. loculosum*
297 and *Amblyomma* sp. samples were not bleach-treated prior to DNA extraction (as done for the
298 other samples), the abundant presence of these bacteria may be due to the cuticular
299 bacteriome, as recently observed in another *Amblyomma* species (Binetruy, Dupraz, et al.,
300 2019).
301

The structure of microbial diversity was not globally impacted by the phylogenetic proximity among *Amblyomma* species: The dendrogram of microbial diversity did not parallel the *Amblyomma* phylogeny (Mantel two-tailed test, R=0.16, P=0.19) (Supplementary Figure S1). However, the MDS plot suggested an effect of tick species on bacterial diversity (Supplementary Figure S2), as confirmed by the PERMANOVA analysis (R^2= 0.51, P=0.001). Further testing showed a clear separation between *Coxiella*-LE-infected tick species/specimens and *Francisella*-LE-infected ones (pairwise PERMANOVA analysis, R^2=0.31, adjusted P for multiple comparisons=0.0003; Figure 3A). *Rickettsia* also structures the bacterial diversity but to a lesser extent than *Coxiella*-LE and *Francisella*-LE (PERMANOVA, R^2= 0.11, P=0.001; Figure 3B). The *Coxiella*-LE and *Francisella*-LE clusters are actually structured into two sub-clusters each, fitting with the presence of *Rickettsia* (Figure 3c) as corroborated by PERMANOVA analyses: (1) *Coxiella*-LE without *Rickettsia* vs. *Coxiella*-LE with *Rickettsia* (R^2=0.2, adjusted P for multiple comparisons=0.0015), and (2) *Francisella*-LE without *Rickettsia* vs. *Francisella*-LE with *Rickettsia* (R^2=0.22, adjusted P for multiple comparisons=0.0015).

Of the 25 *Amblyomma* species (including *A. sculptum*) examined for the presence of *Coxiella*-LE and *Francisella*-LE, 24 were infected by one or both of these symbionts: 11 *Amblyomma* species harbour only *Coxiella*-LE, 13 species only *Francisella*-LE and three species both (Figure 2, Supplementary Table S1). Only *A. romitii* was not infected by *Coxiella*-LE and *Francisella*-LE but this may be explained by the presence of the tick-borne pathogen *Ehrlichia* sp., which may mask the presence of other bacteria. In the 24 infected *Amblyomma* species, *Coxiella*-LE and *Francisella*-LE were not randomly associated (Fisher's exact test, P=0.001): These two symbionts co-occurred in the same tick species less frequently than expected by chance (exclusion pattern), meaning that their distribution across tick species was

strongly dependent on each other. Conversely, neither *Coxiella*-LE nor *Francisella*-LE showed a non-random association with *Rickettsia* (Fisher's exact tests, P=0.99 and 0.68, respectively). However, *Coxiella*-LE and *Francisella*-LE had a patchy and quite uniform distribution along the *Amblyomma* phylogeny: While some closely related *Amblyomma* species were infected by the same symbiont genus (e.g. *A. dissimile* and *A. rotundatum* by *Francisella*-LE, or *A. latepunctatum*, *A. scalpturatum* and *A. naponense* by *Coxiella*-LE), others were not (e.g. *A. americanum*, infected by *Coxiella*-LE, and *A. oblongoguttatum* by *Francisella*-LE) (Figure 2). This distribution pattern paralleled partly the tick host-range: Indeed, *A. dissimile* and *A. rotundatum* that are related and specialized for poikilotherms were both infected by *Francisella*-LE (Figure 1, 2).

In the tick species they infect, *Coxiella*-LE and *Francisella*-LE were present in most specimens examined (Figure 2, Supplementary Table S1). However, a more contrasted pattern was apparent in the three *Amblyomma* species that were co-infected by *Coxiella*-LE and *Francisella*-LE: (i) in *A. geayi*, of the 10 examined specimens, seven were infected by *Coxiella*-LE in one locality, but in other localities, two specimens were infected by *Francisella*-LE and one was co-infected by *Coxiella*-LE and *Francisella*-LE; (ii) in *A. latepunctatum*, of the four examined specimens, three specimens of the same locality were co-infected by *Coxiella*-LE and *Francisella*-LE but one specimen from another locality was only infected by *Coxiella*-LE; (iii) in *A. sculptum*, the single examined specimen was co-infected by *Coxiella*-LE and *Francisella*-LE (Figure 2, Supplementary Table S1). In contrast to *Coxiella*-LE and *Francisella*-LE, the prevalence of *Rickettsia* was heterogeneous, with infection frequencies ranging from 14% to 100% depending on tick species (Figure 2, Supplementary Table S1). In all cases, *Rickettsia* was found with either *Coxiella*-LE (seven

Amblyomma species) or *Francisella*-LE (seven species) or both (two species). Neither *Rickettsia* nor *Coxiella*-LE nor *Francisella*-LE shows infection-biased sex ratio: within each *Amblyomma* species, the prevalence of infection did not differ between males and females (Fisher's exact test, all $P>0.1$).

Evolutionary history of Coxiella-LE and Francisella-LE symbioses

Sequencing of three *Coxiella* MLST genes (16S rRNA, *rpoB* and *groEL*) led to the identification of 14 genetically different *Coxiella*-LE in a subset of 32 specimens representing the 14 infected *Amblyomma* species (one to four specimens per species were examined). Each *Amblyomma* species was infected by a genetically distinct *Coxiella*-LE and no variation of *Coxiella*-LE was observed among specimens belonging to the same *Amblyomma* species. We observed no sign of recombination in the *Coxiella*-LE data set (all $P>0.05$ for the GENECONV and RDP recombination-detection tests) and we thus used the 16S rRNA, *rpoB* and *groEL* concatenated sequences for BI analyses. Comparisons with other sequences available on GenBank showed that the *Coxiella*-LE of *Amblyomma* are polyphyletic: They were scattered into different well-supported clusters among *Coxiella*-LE of other tick species (Supplementary Figure S3). Indeed, the *Coxiella*-LE of *A. variegatum*, *A. tholloni* and *A. splendidum* form a monophyletic clade that is more closely related to the *Coxiella*-LE of *Ixodes* tick species than to the *Coxiella*-LE of other *Amblyomma* species. Similarly, the *Coxiella*-LE clade of *A. cajennense*, *A. sculptum* and *A. americanum* is more closely related to the *Coxiella*-LE of *Dermacentor* tick species. This pattern is suggestive of recurrent HT events of *Coxiella*-LE among tick species. However, the *Coxiella*-LE clusters of *Amblyomma* species can be gathered into two main groups, one with all the *Coxiella*-LE of NW *Amblyomma* species and the other with all the *Coxiella*-LE of AF *Amblyomma* species (Supplementary Figure S3). This pattern suggests an effect of phylogeographic drivers in

structuring the evolution of *Coxiella*-LE. This is strongly supported by the co-phylogeny analysis between *Coxiella*-LE and *Amblyomma* phylogenies: There is a significant topological congruence between their phylogeny (PACo analysis, P=0.0001; Figure 4A). This shows that co-cladogenesis with *Coxiella*-LE occurred during the radiation of *Amblyomma*.

Sequencing of five *Francisella* MLST genes (16S rRNA, *rpoB*, *groEL*, *ftsZ* and *gyrB*) led to the identification of 15 genetically different *Francisella*-LE in a subset of 28 tick specimens representing the 13 infected *Amblyomma* species (one to four specimens per species were used). There was only one *Francisella*-LE in each *Amblyomma* species, except *A. pacae* and *A. rotundatum* in which two and three genetically distinct *Francisella*-LE, respectively, were present in specimens from different localities. Each *Amblyomma* species harbours genetically distinct *Francisella*-LE, except for *A. geayi* and *A. latepunctatum* that harbour identical *Francisella*-LE on the basis of their MSLT sequences (Supplementary Figure S4). Owing to the lack of *Francisella*-LE *rpoB*, *groEL*, *ftsZ* and *gyrB* gene sequences available in GenBank before this study (with the exception of two published *Francisella*-LE genomes), the BI phylogenetic analysis between the *Francisella*-LE of *Amblyomma* and those of other tick species (with sequences available in GenBank) was made using only their 16S rRNA nucleotidic sequences (Supplementary Figure S5). No *Francisella*-LE subclade specific to *Amblyomma* exists along the 16S rRNA phylogenetic tree: The *Francisella*-LE of *Amblyomma* are instead scattered among *Francisella*-LE of other tick genera suggesting recurrent HT events among unrelated tick species (Supplementary Figure S5). However, the inner topology of the *Francisella*-LE clade based on 16S rRNA gene sequences remained too poorly resolved in many cases (as shown by low support values of inner branches) to infer the exact relatedness among all *Francisella*-LE. We thus further reconstructed the phylogenetic relationships between *Francisella*-LE using BI analyses based on their 16S rRNA, *rpoB*,

401 *groEL*, *ftsZ* and *gyrB* nucleotidic sequences. We observed no sign of recombination in the
402 *Francisella*-LE data set (all $P>0.05$ for the GENECONV and RDP recombination-detection
403 tests) and we thus used the 16S rRNA, *rpoB*, *groEL*, *ftsZ* and *gyrB* concatenated sequences
404 for analyses. Conversely to *Coxiella*-LE, there was no apparent co-cladogenesis or
405 phylogeographic pattern along the *Francisella*-LE phylogeny (Supplementary Figure S4).
406 Indeed, the *Francisella*-LE of *A. sculptum* (NW group) is closely related to the *Francisella*-
407 LE of an unrelated *Amblyomma* species, *A. paulopunctatum* (AF group). In addition, the
408 *Francisella*-LE of *A. sculptum* and *A. paulopunctatum* are more closely related to the
409 *Francisella*-LE of the soft tick *O. moubata* than to the *Francisella*-LE of other *Amblyomma*
410 species. The co-phylogeny analysis also showed no significant signal of congruence between
411 the *Francisella*-LE and *Amblyomma* phylogenies (PACo analysis, $P = 0.06$): Only HT events
412 seem to have impacted the distribution *Francisella*-LE across *Amblyomma* phylogeny (Figure
413 4B). Interestingly, while co-infection with *Rickettsia* along the *Coxiella*-LE phylogeny is
414 random (linear model, adjusted $R^2= -0.01$, F-stat=0.60, P=0.56; Supplementary Figure S6A),
415 it is not so along the *Francisella*-LE phylogeny (adjusted R^2=0.15, F-stat=6.90, P=0.002;
416 Figure S6B): Co-infections with *Rickettsia* are more common with certain *Francisella*-LE
417 subclades than with others.
418
419 **Discussion**
420 Three intracellular bacterial genera, *Coxiella*-LE, *Francisella*-LE and *Rickettsia*, are
421 widespread across the 25 species of *Amblyomma* ticks we examined in this study. Only a few
422 other bacteria have been detected and none is so uniformly present in *Amblyomma*. However,
423 the structure of the microbiomes does not mirror the *Amblyomma* phylogeny and closely
424 related *Amblyomma* commonly harbour divergent microbiomes. As expected, almost all
425 *Amblyomma* species were found to harbour a nutritive obligate symbiont, *Coxiella*-LE or

Francisella-LE, both able to synthesize B vitamins but with a clear exclusion pattern between them: *Coxiella*-LE was found as a single infection in 11 *Amblyomma* species, *Francisella*-LE in 13 species and co-infection was seen in only three species. Despite the co-evolved and obligate interactions of ticks with their mutualistic partners, we detected evidence of symbiont replacements during radiation of *Amblyomma*, raising questions regarding the ecological and evolutionary factors underlying replacements.

The comparison of symbiont and tick phylogenies revealed that the *Coxiella*-LE symbiosis is ancient and arose in the early evolution of the *Amblyomma* genus. Hence, some *Coxiella*-LE are specialized for their *Amblyomma* hosts, with an ancient acquisition followed by co-diversification, meaning that the persistence of *Coxiella*-LE through vertical transmission is stable over the duration of *Amblyomma* species diversification. A very similar co-diversification pattern has also been reported for *Coxiella*-LE symbiosis in the *Rhipicephalus* tick genus (Duron et al., 2017). However, the spread of *Coxiella*-LE was more complex in *Amblyomma*: The infections found in some *Amblyomma* species are distantly related and do not form an *Amblyomma*-specific clade. Rather, phylogenetics shows that *Coxiella*-LE of *Amblyomma* are actually scattered among *Coxiella*-LE of other tick genera such as *Ixodes* and *Dermacentor*. Only extensive HT of *Coxiella*-LE among tick genera may explain these phylogenetic incongruences. Since facultative, but not obligate, symbionts can undergo HT between host species (Bennett & Moran, 2015; McCutcheon et al., 2019; Nancy A. Moran et al., 2008; Wernegreen, 2012), this suggests that some *Coxiella*-LE are facultative symbionts of ticks. Interestingly, *Coxiella*-LE is a facultative symbiont in some *Ixodes* species, such as *I. ricinus* and *I. uriae* (Duron et al., 2017; Duron, Jourdain, & McCoy, 2014; Duron et al., 2015). The phylogenetic proximity of *Coxiella*-LE of *Ixodes* spp. with the *Coxiella*-LE of *A. variegatum*, *A. splendidum* and *A. tholloni* thus suggests that a facultative *Coxiella*-LE of

Ixodes spp. had an early jump to the *Amblyomma* ancestor of these species before replacing the ancestral obligate symbiont and evolving obligate nutritional symbioses with current species.

The infection dynamics of *Francisella*-LE is different to that of *Coxiella*-LE. While the *Francisella*-LE of ticks form a monophyletic clade within the *Francisella* genus (Duron et al., 2017, 2018), we observed frequent HT events between unrelated tick species. No co-cladogenesis signal with *Amblyomma* is apparent along the phylogenies, meaning that current *Francisella*-LE arose only recently in this genus. The presence of unrelated *Francisella*-LE in *Amblyomma* further indicates that several independent acquisitions of *Francisella*-LE have occurred during the radiation of this tick genus. These acquisitions have likely come at the expense of the *Coxiella*-LE with their ultimate replacement by *Francisella*-LE. The AF *Amblyomma* group is illustrative of this process: Most species (*A. splendidum*, *A. variegatum*, *A. tholloni* and *A. loculosum*) are infected by *Coxiella*-LE with a strong co-cladogenesis pattern, but one species (*A. paulopunctatum*) is infected by *Francisella*-LE. This pattern suggests that *Francisella*-LE has replaced the *Coxiella*-LE primarily present in the *A. paulopunctatum* ancestor. Other examples include the monophyletic group formed by *A. americanum*, *A. oblongoguttatum*, *A. cajennense* and *A. sculptum*: *Coxiella*-LE has co-diverged with all species but one, since here *Francisella*-LE eliminated the *Coxiella*-LE primarily present in the *A. oblongoguttatum* ancestor. In addition, *A. sculptum* is co-infected at the individual level by an ancestral *Coxiella*-LE (i.e. showing a co-cladogenesis pattern with *Coxiella*-LE of the *A. sculptum* relatives) and a recently acquired *Francisella*-LE. This pattern suggests that HT of *Francisella*-LE within *Amblyomma* communities is recent, likely ongoing, and that co-infections with ancestral *Coxiella*-LE are only transitory. The preferential association of some *Francisella*-LE with *Rickettsia* further implies that these co-

476 infections may be important drivers since some *Rickettsia* are also able to synthesize folate
477 (B9 vitamin) (Hunter et al., 2015) and thus to participate in nutritional symbiosis along with
478 *Francisella*-LE. Under this hypothesis, *Francisella*-LE and *Rickettsia* are cooperating
479 together, and each may fulfil essential metabolic functions not ensured by the others. They
480 may also act together to replace ancestral *Coxiella*-LE.
481
482 Some biological traits of these symbioses are indicative of how and why *Francisella*-LE and
483 some *Coxiella*-LE are both eliminating ancestral *Coxiella*-LE in *Amblyomma* ticks.
484 *Francisella*-LE or *Coxiella*-LE were occasionally detected in the salivary glands of several
485 tick species (Budachetri et al., 2014; Buysse et al., 2019; Klyachko, Stein, Grindle, Clay, &
486 Fuqua, 2007) suggesting that ticks may inject part of their symbionts during feeding. Ticks,
487 unlike other arthropod vectors, often attach and aggregate on the host for several days to
488 obtain a meal, a process termed 'co-feeding'. The spatiotemporal proximity of ticks during
489 co-feeding may favour the HT of *Francisella*-LE and *Coxiella*-LE between conspecifics but
490 also between different tick species, as commonly observed for tick-borne pathogens
491 (Voordouw, 2015; Wright, Sonenshine, Gaff, & Hynes, 2015). This process may lead to local
492 or systemic infections in vertebrates since a few cases of opportunistic *Coxiella*-LE infections
493 have been reported after tick feeding (Shivaprasad et al., 2008; Vapniarsky, Barr, & Murphy,
494 2012; Woc-Colburn et al., 2008, p.). This mode of transmission may be particularly
495 significant for *Francisella*-LE and *Coxiella*-LE by leading to co-infections with ancestral
496 *Coxiella*-LE in ticks. Interestingly, we found two *Amblyomma* species, *A. geayi* and *A.*
497 *latepunctatum*, that are co-infected by their respective ancestral *Coxiella*-LE but that also
498 share the same *Francisella*-LE, which is also closely related to the *Francisella*-LE of *A.*
499 *dissimile*. The genetic proximity of *Francisella*-LE in these three unrelated *Amblyomma*
500 species is suggestive of recent HT events through co-feeding: While these three *Amblyomma*

501 species feed on very different vertebrate hosts, hampering the possibility of co-feeding, the
502 immature stages of *A. dissimile* are commonly found in diverse mammals or birds (Binetruy,
503 Chevillon, de Thoisy, Garnier, & Duron, 2019; Guglielmone & Nava, 2010; Scott & Durden,
504 2015), suggesting that they may be ecological bridges driving HT of *Francisella*-LE across
505 tick species.
506
507 By replacing the ancestral *Coxiella*-LE, the novel symbiont colonizes a pre-adapted tick
508 physiological environment that requires the provision of B vitamin: Thus, it must be able to
509 synthesize these compounds as the ancestral *Coxiella*-LE did. The presence of several B
510 vitamin biosynthesis pathways is ancestral in the *Coxiella* and *Francisella* genera, and all
511 members of these genera, including pathogenic species that are also all intracellular, have
512 conserved these abilities through their radiation (Duron et al., 2018; Gerhart et al., 2016;
513 Meibom & Charbit, 2010; Rowe & Huntley, 2015; Smith et al., 2015; van Schaik, Chen,
514 Mertens, Weber, & Samuel, 2013): All *Francisella*-LE and *Coxiella*-LE are already pre-
515 adapted to nutritional symbioses with ticks. Beyond B vitamins, the replacement of ancestral
516 *Coxiella*-LE suggests that the new symbiont could supply an additional benefit that the
517 ancestral *Coxiella*-LE was unable to supply to ticks, thereby out-competing them. The
518 genomes of ancient beneficial endosymbionts have lost most of their gene contents from their
519 ancestor, being usually small in size and dense in gene content but also suffering Muller's
520 ratchet, with fixation of deleterious mutations through genetic drift (McCutcheon et al., 2019;
521 McCutcheon & Moran, 2012; N. A. Moran, 1996; Rispe & Moran, 2000). This evolution
522 towards massive genomic reduction is obvious for the *Coxiella*-LE of *A. americanum*, which
523 have a genome of only 0.66 Mb (Smith et al., 2015), but not for the *Francisella*-LE of *A.*
524 *maculatum*: Its genome is 1.56 Mb, and although half is pseudogenized, it may have a higher
525 biosynthetic capability (Gerhart et al., 2016). Similar variation is also reported between

Coxiella-LE with some genomes reaching ca. 1.5 Mb (Gottlieb et al., 2015; Ramaiah & Dasch, 2018), suggesting that some *Coxiella*-LE have greater biosynthetic capabilities than others. However, other processes may act on the *Coxiella*-LE replacement. Indeed, ancestral *Coxiella*-LE may have evolved too reduced (degraded) genomes and become maladapted, opening the road to replacement by a new symbiont. This degeneration–replacement model has been proposed for other arthropods such as cicadas (Campbell et al., 2015; Łukasik et al., 2018; Matsuura et al., 2018), but replacements are expected to be transient making them difficult to observe (McCutcheon et al., 2019). In *Amblyomma*, the observations of three species with co-infections by ancestral *Coxiella*-LE and recently acquired *Francisella*-LE may correspond to this transient state before extinction of *Coxiella*-LE.

That ecological specialization to strict haematophagy is driven by nutritional symbiotic interactions is beyond doubt for ticks. The present study, nevertheless, shows that nutritional symbiosis in the *Amblyomma* genus is not stable state, being impacted by competition between *Coxiella*-LE and *Francisella*-LE or between *Coxiella*-LE themselves. We potentialy underestimate the amplitude of this dynamics: our intraspecific sampling was low for some tick species and this may have led to an underestimation of the *Francisella*-LE frequency. Indeed, in the cases of low prevalence, most samples will be found not infected by *Francisella*-LE while other members of the species, not sampled and tested, are in fact infected. Precisely, this pattern is found in this study: we found variation *Francisella*-LE infection pattern between sampling localities in three tick species in which just few few specimens were infected, which clearly indicates this potential for false negatives arising from insufficient sampling. Anyway, ticks now march on with their recently acquired *Francisella*-LE, at the expense of *Coxiella*-LE, but the precise mechanisms providing the advantage to *Francisella*-LE remain to be determined, including endosymbiosis intrinsic factors, such as

competition between symbionts with similar metabolic capabilities and the differential degree of genome reduction.

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Conflict of interest

The authors declare that they have no conflict of interest.

851 **Figure legends**

852 **Figure 1** Phylogenetic relationship of the 25 *Amblyomma* species examined in this study. The
853 phylogenetic tree was inferred using Bayesian inferences (BI) from a large fragment of rDNA
854 sequences (18S, ITS1, 5.8S, ITS2, and 28S rDNA; 3312 bp unambiguously aligned; best-fit
855 approximation for the evolutionary model: GTR+G+I), the numbers on nodes indicate the BI
856 probability. One to two specimens per *Amblyomma* species (45 specimens in total) were
857 analysed. The vertebrate species on which tick species mainly feed (host-range) is shown.
858 White circles, African group (AF); black circles, New Word group (NW).
859
860 **Figure 2** Diversity of the microbiome and bacterial prevalence in *Amblyomma* species. The
861 left part of the Figure shows the *Amblyomma* phylogenetic tree adapted from Figure 1. One to
862 two specimens per *Amblyomma* species (45 specimens in total) were included in the
863 phylogenetic analysis. A the middle part, the heatmap shows the abundance and diversity of
864 the nine most abundant OTUs characterized in the 16S rRNA bacterial data set for each tick
865 species. The number on the right of the heatmap indicates the number of tick specimens per
866 species used to construct the heatmap (one to ten specimens per *Amblyomma* species (142
867 specimens in total) were included in the microbiote analysis). On the right of the figure, the
868 prevalence of the three most common symbionts is illustrated by the coloured squares
869 (*Coxiella*-LE*,* blue square; *Francisella*-LE, green square and *Rickettsia,* red square): full
870 square indicate a prevalence of 100%, while empty or partly colored squared indicate
871 prevalences from 0 to <100% (the numbers besides these squares indicate the prevalences).
872 The amount of DNA for the single specimen of *A. sculptum* was not sufficient to perform the
873 16S rRNA barcoding, therefore the heatmap for this species is not applicable (N/A). In this
874 latter species, the presence of *Coxiella*-LE and *Francisella*-LE was asserted through specific

875 PCR assays as detailed in the text. White circles, *Amblyomma* African group (AF); black
876 circles, *Amblyomma* New Word group (NW).
877
878 **Figure 3** Structuration of the *Amblyomma* microbiome. **A** Multidimensional scaling plot
879 (MDS) of the microbial diversity of each *Amblyomma* species depending on the presence of
880 both *Coxiella*-LE (red dots) and *Francisella*-LE (green dots) or the absence of obligate
881 symbiont (blue dots). **B** MDS plot of the microbial diversity depending on the presence (red
882 dots) / absence (blue dots) of *Rickettsia*. **C** MDS plot of the microbial diversity depending on
883 the association / no association between the three symbionts (*Coxiella*-LE*, Francisella*-LE
884 and *Rickettsia*): C_R (red dots) correspond to *Amblyomma* specimens co-infected by
885 *Coxiella*-LE and *Rickettsia*; C (brownish dots) to specimens only infected by *Coxiella*-LE; F
886 (green dots) to specimens only infected by *Francisella*-LE; F_R to specimens co-infected by
887 *Francisella*-LE and *Rickettsia*; C_F_R (light blue dots) to specimens multi-infected by
888 *Coxiella*-LE, *Francisella*-LE and *Rickettsia*; dark blue dots to specimens not infected by these
889 symbionts. Each MDS plot is based on the generalized Unifrac ($\alpha = 0.5$) distance matrix and
890 all confidence ellipses are been drawn with the confidence level of 0.95.
891
892 **Figure 4** Evolutionary histories between *Amblyomma* and their nutritional symbionts. **A** Co-
893 phylogeny between the phylogenies of *Amblyomma* (on the left) and *Coxiella*-LE (on the
894 right): The tick phylogeny is based on 3312-bp nuclear rDNA sequences (18S, ITS1, 5.8S,
895 ITS2, and 28S rDNA; best-fit approximation for the evolutionary model: GTR+G+I); the
896 *Coxiella*-LE phylogeny was reconstructed using a concatenated gene sequence (16S rRNA,
897 *rpoB* and *groEL* concatenated sequences, 2045 unambiguously aligned base pairs; best-fit
898 approximation for the evolutionary model: GTR+G+I). **B** Cophylogeny between the
899 phylogenies of *Amblyomma* (on the left) and *Francisella*-LE (on the right): The tick

900 phylogeny is based on 3312-bp nuclear rDNA sequences (18S, ITS1, 5.8S, ITS2, and 28S
901 rDNA; best-fit approximation for the evolutionary model: GTR+G+I); the *Francisella*-LE
902 phylogeny was reconstructed using a concatenated gene sequence (16S rRNA, *rpoB*, *groEL*,
903 *ftsZ* and *gyrB* concatenated sequences, 3506 unambiguously aligned base pairs; best-fit
904 approximation for the evolutionary model: GTR+G+I). Each phylogeny was reconstructed
905 using BI and node numbers are the posterior probabilities (only values >70 are shown). White
906 circles, *Amblyomma* African group (AF); black circles, New Word group (NW).