Ecological contacts and host specificity promote replacement of nutritional endosymbionts in ticks

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Abstract

Symbiosis with vitamin-provisioning microbes is essential for the nutrition of animals with specialized feeding habits. While coevolution stabilizes the interactions between symbiotic partners, their associations are not necessarily permanent: Recently acquired symbionts can replace ancestral symbionts. In this study, we demonstrate successful replacement dynamics of *Francisella*-Like Endosymbionts (-LE), a group of invasive B-vitamin-provisioning endosymbionts, across tick communities driven by a complex web of horizontal transfers. Using a broad collection of *Francisella*-LE-infected tick species, we determined the diversity of *Francisella*-LE haplotypes through a multi-locus strain typing approach, and further characterized their phylogenetic relationships and their association with biological traits of their tick hosts. The patterns observed showed that *Francisella*-LE commonly transfer through similar ecological networks and geographic distributions shared among different tick species, and, in certain cases, through preferential shuffling across congeneric tick species. Altogether, these findings reveal the importance of both routes in shaping the invasive pattern in which new nutritional symbioses are initiated.

Introduction

Mutualisms with microbes are at the origin of animal lineages feeding on nutritionally incomplete food resources [1–3]. Microbes can synthesize essential amino acids and vitamins that animals cannot, and thus have vital roles in compensating for nutritional deficiencies. Through this mechanism, symbiont acquisition enabled the emergence and expansion of many animal lineages, such as aphids, bed bugs, and leeches, which would otherwise not exist [1, 3, 4]. Over evolutionary times, hosts and nutritional symbionts coevolve traits that stabilize their interactions, leading often to strict host–symbiont co-cladogenesis [2, 4]. However, nutritional symbioses can be much more dynamic: Ancestral nutritional symbionts can be replaced by recently acquired symbionts able to provide similar or additional benefits to the hosts [1, 4–6]. Indeed, sap-feeding insects display a complex mosaic of nutritional symbiont combinations, reflecting repeated symbiont acquisitions, replacements, and losses [4]. These novel symbioses typically originate either following horizontal transfer from one host species to another or from uptake of novel symbionts from the environment, and thus primarily depend on the symbiont's ability to successfully shift hosts across species boundaries [4, 5]. Yet, it remains unclear how invasive symbionts are primarily acquired by novel host species.

Nutritional symbiotic systems in ticks (Arachnida: Ixodidea) have been impacted by repeated symbiont acquisitions, replacements, and losses [7–9]. Ticks are strict blood-feeders: They do not use any other food sources and, as such, they ingest high levels of protein, iron, and salt, but few carbohydrates, lipids, or vitamins [1]. The genomes of ticks have evolved large repertoires of genes related to this nutritional challenge, but they themselves cannot synthesize the essential vitamins that are lacking in blood meal [10, 11]. To overcome these nutritional deficiencies, ticks have evolved obligate interactions with nutritional endosymbionts [1, 9, 12–18]. The most common obligate endosymbionts, *Coxiella*-like endosymbionts (*Coxiella*-LE hereafter; Legionellales: Coxiellaceae) and *Francisella*-like

endosymbionts (*Francisella*-LE; Thiotrichales: Francisellaceae), are maternally inherited intracellular bacteria specifically associated with ticks [8, 19]. Although *Coxiella*-LE and *Francisella*-LE are distantly related, they have converged towards an analogous B-vitamin-based nutritional mutualism with ticks [1, 9, 12–16]. Their experimental elimination typically results in decreased tick survival, molting, fecundity, and egg viability, as well as in physical abnormalities [12, 15, 18, 20–22], which all are fully restored with an oral supplement of B vitamins [12]. The sequencing of *Coxiella*-LE and *Francisella*-LE genomes confirmed that they consistently produce three B vitamin types, biotin (vitamin B_7), riboflavin (B_2), and folate (B_9), which are required for the tick life cycle [9, 12–16]. As they are required for the tick life cycle, these obligate endosymbionts are present in all individuals of their tick host species, at least at early stages of development [8, 12, 19, 23, 24]. However, in few tick species, *Francisella*-LE are facultative endosymbionts (i.e., not required for the tick life cycle, and have low prevalence in the tick population) and coexist with obligate endosymbionts (as *Coxiella*-LE) that ensure the tick's requirements for B vitamins [7, 8].

Recent surveys suggest that *Francisella*-LE are invasive endosymbionts, spreading through tick communities at the expense of *Coxiella*-LE [7, 8]. These endosymbionts rarely coexist together and a tick species hosting one usually does not harbor the other one. Phylogenetic reconstructions revealed that *Coxiella*-LE, but not *Francisella*-LE, are ancestral endosymbionts in most tick lineages: *Coxiella*-LE commonly form evolutionarily stable associations lasting for millions years and leading to strict co-cladogenesis [7, 8, 17]. However, repeated replacements of *Coxiella*-LE by *Francisella*-LE are apparent across the tick phylogeny, with recent acquisitions of *Francisella*-LE through horizontal transfers and extinctions of ancestral *Coxiella*-LE in several tick lineages [7, 8]. Thanks to these dynamics, at least 20% of tick species may be infected either by obligate or by facultative *Francisella*-LE [8].

In this study, we explored the routes of *Francisella*-LE horizontal transmission to determine their impact on the stability of tick nutritional symbioses. We tested for the existence of two distinct mechanisms: (1) Direct or indirect ecological contacts that may create primary opportunities for horizontal transfers, and ultimately result in the distribution of related *Francisella*-LE in unrelated tick species, and (2) specificity towards current tick hosts that may impose on *Francisella*-LE a distribution restricted to certain tick species and their close relatives. Each of these mechanisms have been reported in common facultative endosymbionts of arthropods (e.g. 25–28), but none has been examined in the context of nutritional symbioses in ticks. To investigate these routes of transmission, we first characterized *Francisella*-LE diversity using a multi-locus typing system, which was recently developed to study *Francisella*-LE evolution in the *Amblyomma* tick genus [7]. Here, we extended this multi-locus analysis to a more cosmopolitan tick collection and used phylogenetic reconstructions to estimate the proximity of *Francisella*-LE haplotypes, including obligate and facultative endosymbiotic forms, and retraced their evolutionary histories across tick species. We also compared this diversity with other members of the *Francisella* genus, including virulent intracellular pathogens of vertebrates such as the agent of tularemia, *F. tularensis*. We next traced the network of movements of *Francisella*-LE among tick species by

examining the association of *Francisella*-LE haplotypes with tick phylogeny and their geographical and ecological traits.

Methods

Tick collection

A collection of 51 individual DNA templates obtained from 51 specimens of 14 tick species was used (**Table S1**). For each DNA template, tick identification and infection by *Francisella*-LE have been formally characterized in previous studies by molecular and/or morphological characteristics (for ticks) and single-locus DNA sequencing (for *Francisella*-LE) (**Table S1**). Of the 14 tick species examined, one belongs to the Argasidae family (soft ticks), and to the *Ornithodoros* genus. The 13 other species belong to the Ixodidae family (hard ticks), and to the *Amblyomma* (one species), *Dermacentor* (three species), *Hyalomma* (seven species), *Ixodes* (two species), and *Rhipicephalus* (one species) genera. Most specimens were collected on vegetation or on taxonomically diverse bird and mammal hosts while a few others were from a laboratory colony (**Table S1**). The 14 tick species were infected either by putative obligate *Francisella*-LE (11 species) or by facultative *Francisella*-LE (three species) (**Table S1**). The use of the genetic resources was declared to the French Ministry of the Environment (reference TREL19028117S/156).

Multi-locus typing of Francisella-*LE*

Francisella-LE were genotyped through nested or semi-nested PCR assays and sequencing of five housekeeping genes (16S rRNA [695 bp], *rpoB* [379 bp], *groEL* [981 bp], *ftsZ* [713 bp], and *gyrB* [1,035 bp]) recently developed for *Francisella*-LE multi-locus typing [7]. Primers and PCR conditions are detailed in **Table S2**. Following visualization via electrophoresis in 1.5% agarose gel, positive PCR products were sequenced by Eurofins. Sequence chromatograms were cleaned with Chromas Lite (http://www.technelysium.com.au/chromas_lite.html), and alignments were performed using ClustalW, implemented in the MEGA software [29].

Alleles of *Francisella*-LE were determined on the basis of sequence identity in nucleotide alignments. Analyses of allelic profiles included multi-locus sequences of (1) the *Francisella*-LE of the 51 specimens belonging to the 14 tick species characterized in this study, (2) the *Francisella*-LE of 25 additional specimens belonging to 12 *Amblyomma* species and available on GenBank from Binetruy et al. (2020) [7] (16S rRNA: MN998628-MN998651; *rpoB*: MT000818-MT000841; *ftsZ*: MT000842-MT000865; *groEL*: MT000866-MT000889; *gyrB*: MT000890-MT000913), and (3) the four published *Francisella*-LE genomes from three other tick species, including the only formally described *Francisella*-LE species, *F. persica* (formerly known as *Wolbachia persica*) (F-Om: QAPC00000000; FLE-Om: LVCE00000000; FLE-Am: LNCT00000000; *F. persica*: CP012505) [9, 12, 13, 30] (**Table 1**). Overall, this dataset included *Francisella*-LE multi-locus sequences from 76 tick specimens and four *Francisella*-LE genomes. It included data from 29 tick species (three belonging to the Argasidae family, and 26 to the Ixodidae family), including 22 species for which *Francisella*-LE was previously identified as putative obligate endosymbionts, six

species with *Francisella*-LE as facultative endosymbionts, and one species with a *Francisella*-LE of undetermined status (**Table 1**). Key traits of the 29 tick species (family, geographic distribution, and vertebrate types on which they usually feed) are detailed in **Table S3**.

Molecular phylogenetic analyses

Phylogenetic analyses were based on sequence alignments of single or concatenated *Francisella*-LE gene sequences obtained for analyses of allelic profiles. Sequences of other *Francisella* species (*F. opportunistica*: CP022377; *F. tularensis*: AJ749949; *F. novicida*: CP009633; *F. hispaniensis*: CP018093) obtained from GenBank were also included in the analyses. The Gblocks program with default parameters was used to obtain non-ambiguous sequence alignments [31]. All sequence alignments were also checked for putative recombinant regions using the RDP3 analysis package [32]. Tree-based phylogenetic analyses were performed using maximum-likelihood (ML) analyses using MEGA. The evolutionary models that best fit the sequence data were determined using the Akaike information criterion. Clade robustness was assessed by bootstrap analysis using 1 000 replicates.

Tick phylogeny was constructed as a simplified cladogram of tick genera adapted from Burger et *al*. [33]. This tree was used as for some tick species used in the *Francisella*-LE phylogeny there was no available tick sequence in GenBank and lack of DNA for certain ticks prevent novel sequencing. To test for associations between *Francisella*-LE and tick phylogenies, we used the Procrustean Approach to Cophylogeny (PACo) method [34] implemented in R (http://www.r-project.org) using the APE [35] and VEGAN [36] packages. PACo is a global-fit method that assesses phylogenetic congruence with the explicit aim to test the dependency of one phylogeny on another. For *Francisella*-LE, the phylogeny was obtained with the 16S rRNA, *rpoB*, *groEL*, *ftsZ*, and *gyrB* concatenated dataset as described above. To avoid spurious clustering because of multiple *Francisella*-LE haplotypes per host species, we conducted cophylogenetic analyses by using only one representative *Francisella*-LE haplotype per host species. Because all *Francisella*-LE haplotypes found in a same tick species are always closely related (see Results), we randomly sampled one *Francisella*-LE haplotype per host species and used it in further cophylogenetic analyses. The significance of cophylogenetic tests was established by 10 000 random permutations of the association matrix.

Statistical analyses

We explored the association of the phylogenetic distribution of *Francisella*-LE haplotypes with key traits, including endosymbiosis type (obligate vs. facultative endosymbiosis), geographic distribution (Old World vs. New World), tick family (Argasidae vs. Ixodidae), and feeding preferences (i.e., vertebrate classes on which the tick host species usually feed: mammals, birds, and reptiles) (**Table S3**). There is a great variation of feeding preference in ticks: Some tick species (*A. latum* and *Hy. aegyptium*) feed exclusively on reptiles while other species (*O. moubata* and *D. nitens*) only feed on mammals. Some species (*A. maculatum* and *I. scapularis*) are more generalist species feeding on birds and mammals, but never on reptiles. A few tick species (*I. ricinus*) are also generalist, feeding on birds, mammals and reptiles (**Table S3**).

We used the D metric [37] to estimate the overall degree of clustering of these traits on *Francisella*-LE phylogeny. If a trait shows a phylogenetic signal, it may be assumed that *Francisella*-LE haplotypes sharing this trait are not randomly distributed over the phylogeny: They are more phylogenetically clustered than expected by chance. The D metric provides an estimate of phylogenetic conservatism for binary traits that can be compared either with a random shuffle of trait values at the tips of a phylogeny, or with a Brownian motion model of evolution that allows to depict evolutionary diversification processes along a topology [37]. A D value of 1 indicates a phylogenetically random distribution, whereas a D value inferior to 1 indicates phylogenetic clustering. A D value of 0 indicates that the trait is clustered as if it had evolved under Brownian motion of evolution (i.e., in a random dispersal with constant trait variance over time [38]), whereas a D value inferior to 0 indicates an extremely clustered trait [37]. To assess the significance of the D metric estimates, two *p*-values were furthermore calculated using permutation tests: $p(D<1)$ indicates whether the D metric is significantly smaller than 1, meaning that the trait is not randomly distributed over the phylogeny, and $p(D>0)$ indicates whether the D metric is significantly greater than 0, meaning that the trait has a significantly different distribution on the phylogeny from the Brownian threshold model of evolution. We calculated the D metric implemented by the function "phylo.d" in the R package *caper* [39] with the default parameter of 1 000 permutations. Multiple testing was taken into account using the sequential Bonferroni procedure, according to Hochberg (1988) [40]. As done for cophylogenetic analyses to avoid spurious clustering, we calculated the D metric using only one representative *Francisella*-LE haplotype per host species.

We applied a second approach to examine the phylogenetic clustering of *Francisella*-LE haplotypes from the same tick genus, and thus to test for the level of specificity of *Francisella*-LE. To this aim, we computed the pairwise distances between all *Francisella*-LE haplotypes from the concatenated ML phylogenetic tree using its branch lengths. We further partitioned this dataset into (1) pairwise phylogenetic distances between *Francisella*-LE haplotypes from a same tick genus (intrageneric pairwise phylogenetic distances), and (2) pairwise phylogenetic distances between *Francisella*-LE haplotypes from this tick genus vs. *Francisella*-LE haplotypes from all other tick genera (intergeneric pairwise phylogenetic distances). Intrageneric pairwise phylogenetic distances for a given tick genus that are lower than intergeneric pairwise phylogenetic distances indicate specificity of these *Francisella*-LE haplotypes. We compared intrageneric vs. intergeneric pairwise phylogenetic distances using Wilcoxon signed-rank tests.

Data availability

Nucleotide sequences of *Francisella*-LE were deposited in the GenBank nucleotide database (16S rRNA: MW287912-MW287986; *rpoB*: MW286019-MW286093, *groEL*: MW285869-MW285943; *ftsZ*: MW285794-MW285868; *gyrB*: MW285944-MW286018).

Results

Characterization of Francisella-*LE haplotypes*

We amplified and sequenced the *Francisella*-LE 16S rRNA, *rpoB*, *groEL*, *ftsZ*, and *gyrB* gene sequences from the 51 DNA templates belonging to 14 tick species (**Table 1**). We completed these data with additional sequences from 15 other tick species that include genomic and multi-locus typing of *Francisella*-LE datasets available on GenBank (listed in **Table 1**). Overall, the complete multi-locus dataset included *Francisella*-LE sequences from 29 tick species.

On the basis of 16S rRNA, *rpoB*, *groEL*, *ftsZ*, and *gyrB* gene sequences, we characterized 24 to 32 distinct alleles depending on the gene, leading to the identification of 38 genetically different *Francisella*-LE haplotypes in the 29 tick species (**Table 1**). For 22 tick species, we only observed one *Francisella*-LE haplotype per tick species. For the seven other tick species, we detected sequence variation at one to five *Francisella*-LE genes between conspecific specimens, and up to four distinct *Francisella*-LE haplotypes could be present in the same tick species as observed in *Hyalomma excavatum* (**Table 1**). Of the 38 *Francisella*-LE haplotypes, 37 are specific to their respective tick species and are not shared by two or more tick species. Only the *Francisella*-LE haplotype #6 was detected in three tick species (*A. dissimile*, *A. geayi*, and *A. latepunctatum*) (**Table 1**).

Phylogenetic and statistical analyses

ML analyses based on 16S rRNA, *rpoB*, *groEL*, *ftsZ*, and *gyrB* gene sequences were conducted to examine the *Francisella*-LE phylogeny (**Fig. S1−S5**). We observed no sign of recombination in the dataset (all *p*> 0.17) and we thus further conducted a new ML analysis based on the 16S rRNA, *rpoB*, *groEL*, *ftsZ*, and *gyrB* concatenated dataset (**Fig. 1**). All but one phylogenetic reconstructions showed that the *Francisella*-LE, including *F. persica*, delineate a robust monophyletic clade within the *Francisella* genus (**Fig. 1**, **Fig. S2−S5**). Only the topology of the16S rRNA gene tree is poorly resolved due to insufficient sequence polymorphism (**Fig. S1**). The closest relative of *Francisella*-LE is an opportunistic *Francisella* pathogen (*F. opportunistica* [41]), as well as other *Francisella* pathogens, including the agent of tularemia, *F. tularensis* (**Fig. 1**, **Fig. S1−S5**).

Phylogenetic reconstructions showed that the different *Francisella*-LE haplotypes found in the same tick species always cluster together (**Fig. 1**). Indeed, the four *Francisella*-LE haplotypes of *Hy. excavatum* are more closely related to each other than to any other *Francisella*-LE haplotype. A similar pattern was observed for the six other tick species hosting more than one *Francisella*-LE haplotype.

The cophylogeny analysis returns a significant cophylogenetic signal between *Francisella*-LE and ticks (global sum of squared residuals: =0.002, *p*<0.001, *n*=10 000) (**Fig. 2**). This supports the hypothesis that the *Francisella*-LE phylogeny tracks the tick phylogeny, and that the two partners have undergone coupled evolutionary change. Indeed, *Francisella*-LE of some tick species belonging to the same genus can cluster together along the phylogeny, as shown with the *Francisella*-LE of *A. varium*, *A. goeldi*, and *A. humerale* (**Fig. 1**, **Fig. 2**). Further comparisons of pairwise phylogenetic distances showed evidence on *Francisella*-LE specificity in two tick genera, *Hyalomma* (Ixodidae) and *Ornithodoros* (Argasidae) (**Fig. 3**). *Francisella*-LE haplotypes of the *Hyalomma* genus are more closely related to each other (intrageneric pairwise phylogenetic distances, mean ± SE: 0.0094 ± 0.0008, *n*=36 pairwise comparisons) than to *Francisella*-LE

haplotypes of other tick genera (intergeneric pairwise phylogenetic distances, 0.0152 ± 0.0002, n=667) (Wilcoxon test, W=4710, p<10^{-9}). A similar pattern was observed in the *Ornithodoros* genus with intrageneric pairwise phylogenetic distances (0.0030 ± 0.0010, n=3) significantly lower than intergeneric pairwise phylogenetic distances (0.0150 ± 0.0002, n=700) (W=59, p= 0.0048). No evidence of *Francisella*-LE specificity was observed in other tick genera (*Amblyomma*: W=42146, p=0.97; *Dermacentor*: W=1529, p=0.26; *Ixodes*: W=330, p=0.92; *Argas* and *Rhipicephalus*: not applicable because only one haplotype was observed in each of these genera) (**Fig. 3**). However, it is noteworthy that some *Francisella*-LE of several *Amblyomma* species (e.g., *A. pacae*, *A. latum*, *A. oblongoguttatum*, *A. maculatum*, *A. longirostre*, *A. dissimile*, *A. geayi*, *A. latepunctatum*, and *A. rotundatum*) cluster together (**Fig. 1**, **Fig. 2**): It suggests that some degree of *Francisella*-LE specificity in these *Amblyomma* species may also exist.

Worthy of note is that the tick phylogeny is incompletely resolved: While relationships between tick genera is fully resolved, all congeneric tick species were arbitrarily considered here as equally distant because of the lack of data for some tick species (see Materials and Methods). It implies that the cophylogenetic signal is certainly significant between *Francisella*-LE haplotypes and tick genera, but not necessarily with tick species. Furthermore, the diagram of the interaction network shows some major phylogenetic incongruences (**Fig. 2**). As can be seen, no tick genus harbors a specific and monophyletic *Francisella*-LE subclade: The *Francisella*-LE of *Amblyomma* are scattered among *Francisella*-LE of other tick genera, as best shown with the *Francisella*-LE of *A. sculptum* and *A. paulopunctatum* that are more closely related to the *Francisella*-LE of the soft ticks *O. moubata* and *O. porcinus* than to the *Francisella*-LE of other *Amblyomma* species (**Fig. 1**). Hence, the *Francisella*-LE of *Amblyomma* belonged to a minimum of three distinct phylogenetic clusters (**Fig. 1**, **Fig. 2**). Similarly, the *Francisella*-LE of *Dermacentor* tick species, as well as for *Ixodes* species, were each scattered among two different *Francisella*-LE branches (**Fig. 1**, **Fig. 2**). In addition, we found a non-significant clustering signal for tick families (Argasidae and Ixodidae) on the phylogeny of *Francisella*-LE haplotypes (D=-0.43): Their distribution on the tree is significantly random ($p(D$<$1)$= 0.04, non-significant after sequential Bonferroni correction), and statistically distinguishable from a clustered distribution expected by Brownian motion ($p(D$>$0)$=0.67) (**Fig. 2**). Overall, these patterns are the signatures of repeated horizontal transfer events, revealing the ability of *Francisella*-LE to extensively move among tick species.

We found no signal of phylogenetic clustering for *Francisella*-LE endosymbiosis types (D=1.81): The distribution of obligate and facultative *Francisella*-LE on the tree is random ($p(D$<$1)$=0.92) and facultative *Francisella*-LE are scattered along the phylogeny among obligate *Francisella*-LE (Fig. 2). The *Francisella*-LE haplotype #6 illustrates this pattern well, as it was associated with obligate endosymbiosis in *A. dissimile* but with facultative endosymbiosis in *A. geayi* and *A. latepunctatum* (**Table S3**).

The geographic origin of *Francisella*-LE haplotypes showed a significant phylogenetic signal (D=-0.08, $p(D$<$1)$=0.008, $p(D$>$0)$=0.59) with a clear non-random distribution of *Francisella*-LE haplotypes between ticks from the Old and New World (**Fig. 2**). The best examples include *Francisella*-LE haplotypes of American *Dermacentor* and *Ixodes* species that cluster together within the same subclade, while *Francisella*-LE haplotypes of European *Dermacentor* and *Ixodes* species cluster together in another

subclade. There are only a few exceptions to this geographical pattern as shown with the *Francisella*-LE haplotype of an African *Amblyomma* species, *A. latum*, which clusters within a clade otherwise only composed of American *Amblyomma* species (**Fig. 2**).

We also found a significant signal of phylogenetic clustering for certain vertebrates on which ticks feed (**Fig. 2**) : *Francisella*-LE haplotypes cluster with tick species feeding on birds ($D=0.14$, $p(D{<}1)= 0.02$, $p(D{>}0)=0.39$), but not with tick species feeding on mammals ($D=0.78$, $p(D{<}1)=0.36$, $p(D{>}0)=0.10$) or on reptiles ($D=0.99$, $p(D{<}1)=0.51$, $p(D{>}0)=0.06$). However, although globally non-significant, some tick species that exclusively feed on mammals (*e.g.*, *O. moubata*, *O porcinus*, *A. sculptum*, and *A. paulopunctatum*) harbor closely related *Francisella*-LE haplotypes (**Fig. 2**). Similarly, *A. rotundatum* and *A. dissimile*, which both feed on reptiles, harbor closely related *Francisella*-LE haplotypes. However, exceptions are also observed on the haplotype-based tree as shown with *A. longirostre*: This species feeds on birds, but harbors a *Francisella*-LE haplotype more closely related to haplotypes of *Amblyomma* species feeding on mammals and reptiles than to haplotypes of other tick species feeding on birds (**Fig. 2**).

Discussion

We identified 38 distinct *Francisella*-LE haplotypes, including obligate and facultative forms, from a broad collection of 29 tick species. All *Francisella*-LE haplotypes were clustered in a monophyletic clade nested within the *Francisella* genus among virulent intracellular pathogens of vertebrates. This confirms early studies showing that *Francisella*-LE emerged from a pathogenic *Francisella* ancestor of vertebrates that had evolved a specialized endosymbiotic lifestyle with ticks [9, 12, 13]. The distribution of current *Francisella*-LE haplotypes reveals how these invasive endosymbionts spread in tick communities presumably at the expense of *Coxiella*-LE [7, 8].

Francisella-LE commonly move along ecological networks connecting tick species

The cophylogenetic analysis revealed that some *Francisella*-LE haplotype groups are consistently associated with certain tick genera, as *Ambylomma* and *Hyalomma*, suggesting a stable association with these ticks. However, the sitribution of *Francisella*-LE is more complex: For instance, the infections found in some *Amblyomma* species are distantly related and do not form an *Amblyomma*-specific clade. Rather, phylogenetic analyses show that *Francisella*-LE of *Amblyomma* are actually scattered among *Francisella*-LE of other tick genera such as *Ixodes* and *Dermacentor*. Only extensive horizontal transfer of *Francisella*-LE among tick genera may explain these phylogenetic incongruences. Several lines of evidence indicate that related *Francisella*-LE haplotypes infect tick species that are ecologically interconnected. For instance, *Francisella*-LE haplotypes are deeply isolated by geographic barriers: Considerations of phylogenetic distributions revealed a strong split between *Francisella*-LE haplotypes from New World and Old World ticks. Furthermore, examination of feeding preferences revealed that two tick species are more likely to harbor related *Francisella*-LE haplotypes if they feed on birds than if they feed on different vertebrate types. This observation could also be extended to tick species feeding on other vertebrate

types as mammals: The *A. paulopunctatum*, *O. moubata*, and *O. porcinus* ticks are phylogenetically distantly related but they are all African species specialized on Suidae (pigs, warthogs) and harbor very closely related *Francisella*-LE haplotypes. Other noteworthy examples include the *Dermacentor* and *Ixodes* genera, with species that all feed, at least partially, on mammals. The *Dermacentor* and *Ixodes* genera are distantly related since they belong to two divergent lineages within the Ixodidae (hard ticks) family: Metastriata and Prostriata, respectively. However, the *Francisella*-LE of American *Dermacentor* species, *D. occidentalis*, and *D. nitens*, are more closely related to the *Francisella*-LE of an American *Ixodes* species, *I. scapularis*, than to the *Francisella*-LE of a European *Dermacentor* species, *D. reticulatus*. In the same way, the *Francisella*-LE of a European *Ixodes* species, *I. ricinus*, is more closely related to the European *D. reticulatus* than to the American *I. scapularis*. Altogether, this shows that similar ecologies and similar geographical distributions facilitate the horizontal transfer of *Francisella*-LE across tick species. As a result, the structure of tick communities is a major driver of *Francisella*-LE horizontal transfers.

The importance of intimate ecological contacts for *Francisella*-LE horizontal transfers may be favored by traits inherited from their pathogenic *Francisella* ancestor. *Francisella*-LE were occasionally detected in the salivary glands of several tick species [12, 23, 42, 43], suggesting that ticks could inject these endosymbionts during feeding. Ticks, unlike other arthropod vectors, often attach and aggregate on vertebrates for several days to obtain a blood meal, a process termed "co-feeding". The spatiotemporal proximity of ticks during co-feeding may favor the horizontal transfers of *Francisella*-LE between different tick species, as commonly observed for tick-borne pathogens [44, 45]. This process could also potentially lead to opportunistic infections in vertebrates, but such infection by *Francisella*-LE was only documented once and under laboratory conditions: The *Francisella*-LE of the soft tick *A. arboreus*, *F. persica*, was primarily isolated following injection of a tick crush into chick embryo yolk sacs, suggesting that it may be an opportunistic pathogen able to grow in vertebrate cells [46]. Using artificial infection protocols, *F. persica* was also shown to be slightly moderately pathogenic for the guinea pig, mouse, and newborn chick, but not for the cotton rat, adult chicken or rabbit [46]. Interestingly, the genome of *F. persica*, but not those of other sequenced *Francisella*-LE, contains genes of the type VI secretion system (T6SS), and its associated *Francisella* pathogenicity island (FPI) [12] which are used for *Francisella* pathogenic species such as *F. tularensis* to infect macrophages of vertebrates [47, 48]. Certain *Francisella*-LE may thus induce opportunistic infections in vertebrates through their T6SS and FPI virulence genes and use vertebrates as an ecological arena for transfer across tick species.

Specificity of Francisella*-LE in some tick genera*

We observed related *Francisella*-LE haplotypes in congeneric tick species, such as in the *Hyalomma*, *Ornithodoros*, and, to a lesser extent, *Amblyomma* genera. Because of maternal inheritance, co-divergence between *Francisella*-LE and ticks may explain this pattern, as recently observed in the *Hyalomma* genus [23]. However, multiple lines of evidence indicate that in *Amblyomma*, it was the consequence of multiple horizontal transfers between congeneric species rather than of co-divergence. For instance, *A. goeldi*, and *A. humerale* harbored closely related *Francisella*-LE haplotypes although they are not closely related

species within the *Amblyomma* genus [7]. A similar pattern is also observed with the *Francisella*-LE haplotype #6 found in *A. dissimile*, *A. geayi*, and *A. latepunctatum* that are not related tick species although they belong to the same genus [7]. Co-divergence alone is thus insufficient to explain why related *Francisella*-LE haplotypes are present in certain tick genera such as *Amblyomma*. The pattern observed suggests instead that some *Francisella*-LE are specific to related tick species, preferentially moving horizontally among congeneric species. Under this scenario, *Francisella*-LE may be preadapted to infect related tick species because they share similar physiological traits with their current tick hosts, a pattern also observed in endosymbionts of other arthropod species [25, 26]. Thus, certain *Francisella*-LE may have the capacity to maintain infections in only a limited range of related tick species. This level of specificity seems variable depending on *Francisella*-LE haplotypes: High in some tick genera (*Hyalomma*, *Ornithodoros*, *Amblyomma*), but not in others (*Dermacentor*, *Ixodes*). As such, this diversity of specificity levels should impact movements of *Francisella*-LE across tick communities. However, it is also important to point out that association of some *Francisella*-LE haplotypes restrictively to a particular tick genus may not imply specificity, as suggested by the presence of a *Francisella*-LE haplotype in *R. decoloratus* closely related to haplotypes found in *Hyalomma* species. Instead, it could simply reflect a higher chance of *Francisella*-LE transfer and successful establishment of the endosymbiosis among interconnected tick species: *Hyalomma* species examined here were from the Old World, as *R. decoloratus*. Hence, the pattern of *Francisella*-LE diversity observed in *Hyalomma* could reflect geographical structuring (as discussed above) rather than tick specificity.

Underestimation of *Francisella*-LE diversity

The *Francisella*-LE diversity is potentially largely underestimated. Our sampling was highly biased toward obligate *Francisella*-LE since they are fixed in tick populations, and thus more easily sampled. Inversely, facultative *Francisella*-LE have more variable infection frequencies in tick populations [7, 8], and they are rarely detected. For instance, surveys of 91 specimens of the African blue tick *R. decoloratus* from five distinct populations detected the presence of *Francisella*-LE in only one specimen [8]. This means that we have probably typed the diversity of obligate *Francisella*-LE well, but only of a small fraction of facultative *Francisella*-LE. Accounting for these missed facultative *Francisella*-LE, a large diversity of facultative *Francisella*-LE may be widely circulating, but at low infection frequencies, within tick communities.

Facultative *Francisella*-LE are probably pivotal to establishing novel infections. In arthropods, obligate endosymbionts commonly enter into an evolutionary route that leads to irreversible codependence with hosts and the secondary loss of the capability to move horizontally between host species [4, 5]. *A contrario*, facultative endosymbionts have more labile interactions with their hosts, and undergo occasional horizontal transfers across arthropod species [4, 5], as commonly observed for diverse endosymbionts in ticks [8, 49, 50]. This suggests that novel nutritional *Francisella*-LE symbioses are initiated by facultative forms that further evolved to obligate forms. Once established as obligate endosymbionts, *Francisella*-LE may, however, enter into the same evolutionary route that limits any further horizontal transfers. The mixed facultative and obligate forms on the *Francisella*-LE tree with no

clear phylogenetic signal of clustering suggests that the transition from facultative to obligate forms is a common feature in the adaptive process of these endosymbionts.

Concluding remarks

This study confirmed that nutritional symbiosis in ticks is not a stable evolutionary state, but rather a dynamic system impacted by repeated acquisition of novel potential nutritional endosymbionts through horizontal transfers. We have determined that ecological networks within tick communities, along with variable levels of *Francisella*-LE specificity to their current tick hosts, are important drivers of this invasive dynamics. These endosymbionts notably combine maternal inheritance with infectious transmission between tick species in a number of cases, but also potentially using vertebrates as occasional hosts. The overall probability that such tick-to-vertebrate transfers of *Francisella*-LE occur may be high because ticks are found worldwide and feed on many different hosts. However, apart from cases obtained in artificial conditions with *F. persica* [46], all other *Francisella*-LE described to date seem to be confined to ticks. Nonetheless, future research will be necessary to describe the global diversity of *Francisella*-LE, to characterize the presence of virulence genes in their genomes, and then to assess the potential infection risk to vertebrates.

Declarations

Conflicts of interest

The authors declare no competing interest.

Availability of data and material

Nucleotide sequences of *Francisella*-LE were deposited in the GenBank nucleotide database (16S rRNA: MW287912-MW287986; *rpoB*: MW286019-MW286093, *groEL*: MW285869-MW285943; *ftsZ*: MW285794-MW285868; *gyrB*: MW285944-MW286018).

Code availability

Scripts used for the statistical analyses are available on GitHub (https://github.com/mariebuysse/pending).

Authors' contributions

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Table

Table 1 Allelic profile of the five polymorphic genes in *Francisella*-LE from the 14 tick species examined in this study. Previously published sequences of *Francisella*-LE from additional 15 tick species (including genomic and multi-locus typing *Francisella*-LE data sets) were also included for analysis. Identification of *Francisella*-LE haplotypes were based on 16S rRNA, *rpoB*, *groEL*, *ftsZ*, and *gyrB* allelic profiles. *, number of specimens (n) used to determine allelic profiles from multi-locus typing assays; NA, not applicable (for *Francisella*-LE genomes).

Tick species	Francisella-LE type	n*	Allelic profile					Francisella-LE haplotype	Reference for Francisella-LE typing
			16S rRNA	rpoB	groEL	ftsZ	gyrB		
Argasidae (soft ticks)									
Ornithodoros porcinus	Obligate	3	#1	#1	#1	#1	#1	#1	Multi-locus typing, this study
Ornithodoros moubata	Obligate	NA	#2	#2	#2	#2	#2	#2	F-Om genome [12]
	Obligate	NA	#3	#2	#2	#3	#2	#3	FLE-Om genome [9]
Argas arboreus	Unknown	NA	#4	#3	#3	#4	#3	#4	Francisella persica genome [30]
Ixodidae (hard ticks)									
Amblyommalatum	Obligate	2	#5	#4	#4	#5	#4	#5	Multi-locus typing, this study
Amblyomma dissimile #1	Obligate	1	#6	#5	#5	#6	#5	#6	Multi-locus typing [7]
Amblyomma dissimile #2	Obligate	1	#6	#5	#6	#6	#5	#7	Multi-locus typing [7]
Amblyomma geayi	Facultative	1	#6	#5	#5	#6	#5	#6	Multi-locus typing [7]
Amblyomma goeldii	Obligate	3	#7	#6	#7	#7	#6	#8	Multi-locus typing [7]
Amblyomma humerale #1	Obligate	3	#8	#7	#8	#8	#7	#9	Multi-locus typing [7]
Amblyomma humerale #2	Obligate	1	#8	#7	#9	#8	#7	#10	Multi-locus typing [7]
Amblyomma latepunctatum	Facultative	1	#6	#5	#5	#6	#5	#6	Multi-locus typing [7]
Amblyomma longirostre	Obligate	3	#9	#8	#10	#9	#8	#11	Multi-locus typing [7]
Amblyomma maculatum	Obligate	NA	#10	#9	#11	#10	#9	#12	FLE-Am genome [13]
Amblyomma oblongoguttatum	Obligate	3	#11	#4	#4	#11	#10	#13	Multi-locus typing [7]
Amblyomma pacae #1	Obligate	1	#12	#4	#12	#12	#11	#14	Multi-locus typing [7]
Amblyomma pacae #2	Obligate	1	#13	#4	#13	#12	#12	#15	Multi-locus typing [7]
Amblyomma paulopunctatum	Obligate	1	#14	#10	#14	#1	#13	#16	Multi-locus typing [7]
Amblyomma rotundatum #1	Obligate	1	#15	#11	#15	#13	#14	#17	Multi-locus typing [7]
Amblyomma rotundatum #2	Obligate	1	#16	#11	#15	#14	#14	#18	Multi-locus typing [7]
Amblyomma rotundatum #3	Obligate	1	#17	#12	#16	#15	#15	#19	Multi-locus typing [7]
Amblyomma sculptum	Facultative	1	#18	#13	#17	#1	#1	#20	Multi-locus typing [7]
Amblyomma varium #1	Obligate	1	#19	#14	#18	#16	#16	#21	Multi-locus typing [7]
Amblyomma varium #2	Obligate	2	#19	#14	#19	#16	#16	#22	Multi-locus typing [7]
Dermacentor nitens #1	Obligate	1	#20	#15	#20	#17	#17	#23	Multi-locus typing, this study
Dermacentor nitens #2	Obligate	1	#20	#15	#20	#17	#18	#24	Multi-locus typing, this study
Dermacentor occidentalis	Obligate	4	#21	#16	#21	#18	#19	#25	Multi-locus typing, this study
Dermacentor reticulatus	Obligate	1	#22	#17	#22	#19	#20	#26	Multi-locus typing, this study

| Species	Type	Count	L1	L2	L3	L4	L5	L6	Source
Hyalomma aegyptium	Obligate	5	#23	#18	#23	#20	#21	#27	Multi-locus typing, this study
Hyalomma excavatum #1	Obligate	2	#24	#19	#24	#1	#22	#28	Multi-locus typing, this study
Hyalomma excavatum #2	Obligate	1	#25	#19	#25	#1	#22	#29	Multi-locus typing, this study
Hyalomma excavatum #3	Obligate	1	#24	#19	#25	#1	#22	#30	Multi-locus typing, this study
Hyalomma excavatum #4	Obligate	4	#26	#19	#26	#27	#22	#31	Multi-locus typing, this study
Hyalomma impeltatum	Obligate	5	#27	#20	#27	#21	#23	#32	Multi-locus typing, this study
Hyalomma lusitanicum	Obligate	1	#28	#21	#28	#22	#24	#33	Multi-locus typing, this study
Hyalomma marginatum	Obligate	5	#29	#19	#29	#23	#25	#34	Multi-locus typing, this study
Hyalomma rufipes	Obligate	7	#29	#22	#30	#24	#26	#35	Multi-locus typing, this study
Ixodes ricinus	Facultative	3	#30	#17	#22	#19	#20	#36	Multi-locus typing, this study
Ixodes scapularis	Facultative	2	#31	#23	#31	#25	#27	#37	Multi-locus typing, this study
Rhipicephalus decoloratus	Facultative	1	#32	#24	#32	#26	#28	#38	Multi-locus typing, this study

Figures
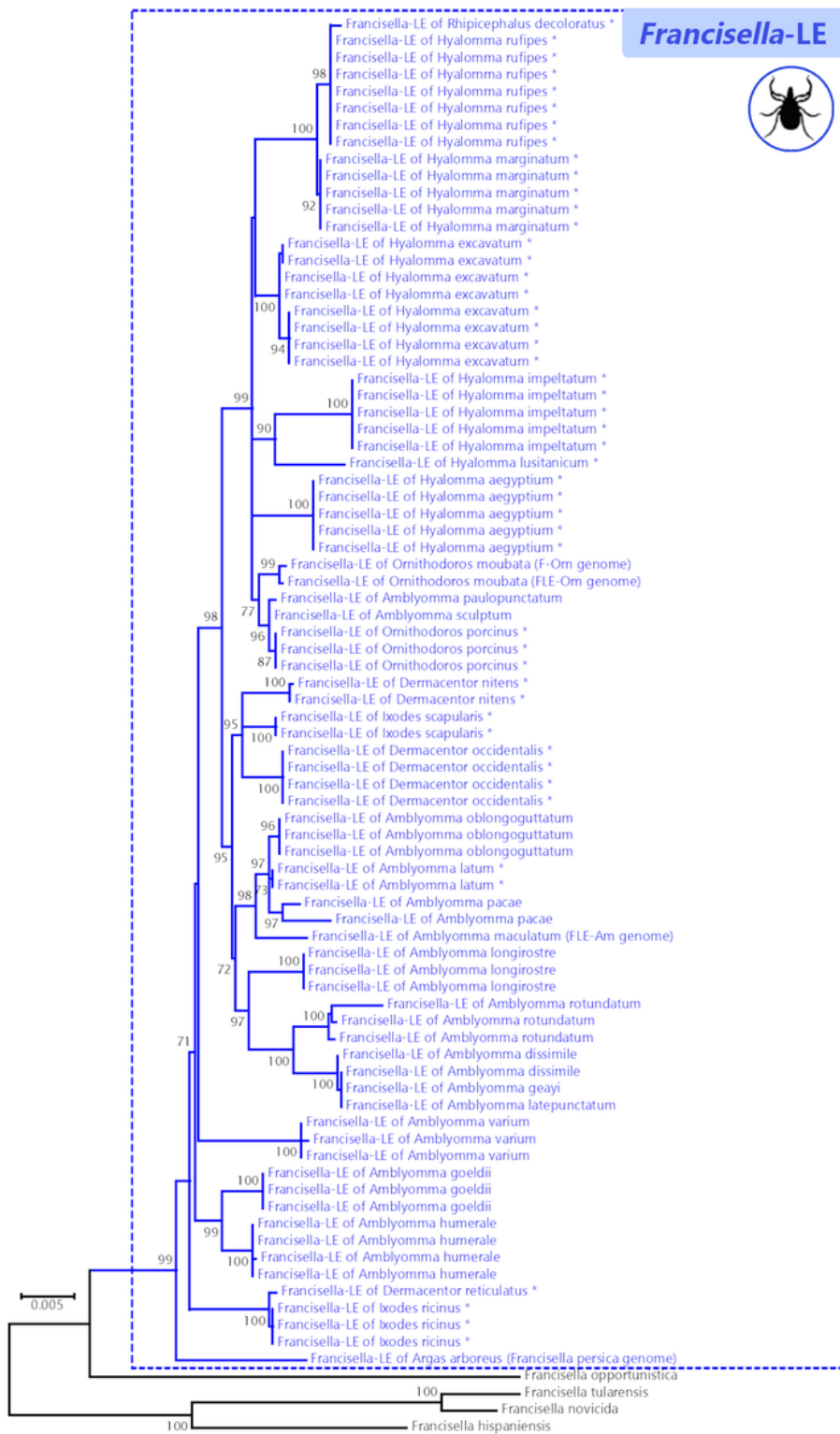
Figure 1

Francisella phylogenetic tree constructed using maximum-likelihood (ML) estimations based on concatenated 16S rRNA, rpoB, groEL, ftsZ, and gyrB nucleotide sequences (3 232 unambiguously aligned bp; best-fit approximation for the evolutionary model: GTR+G) from Francisella-LE of 29 tick species and from other Francisella species. Branch numbers indicate percentage bootstrap support (1 000 replicates).

Only bootstrap supports >70% are shown. The scale bar is in units of substitution/site. *, Francisella-LE sequences obtained in this study. Francisella-LE sequences are indicated by a blue font.
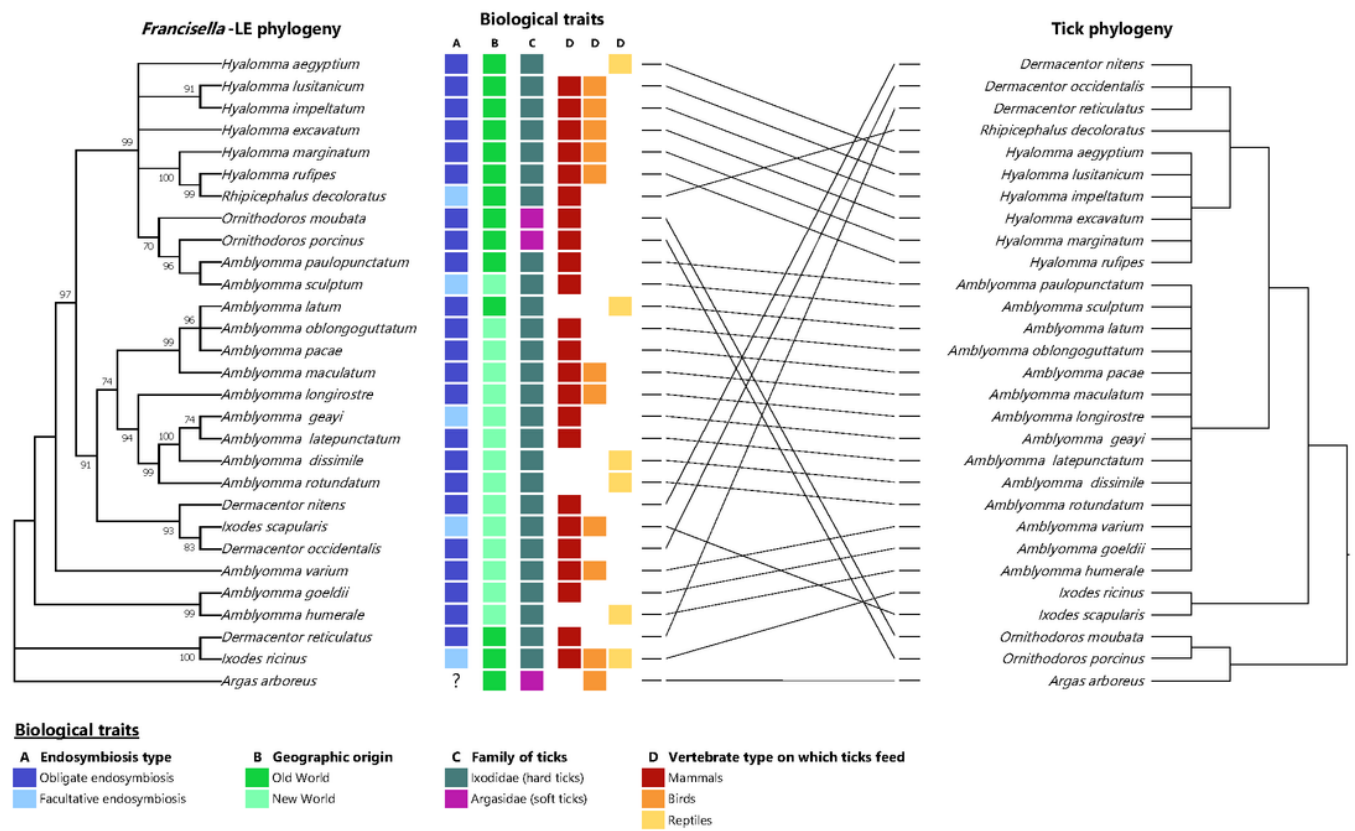

Figure 2

Cladogram depicting the 50% majority-rule consensus of Francisella-LE haplotype phylogenetic trees (left part), association with key biological traits (middle) and network association with tick phylogeny (right part). The Francisella-LE tree was constructed with the 29 Francisella-LE haplotypes characterized in the 29 tick species used in this study and using maximum-likelihood (ML) estimations based on concatenated 16S rRNA, rpoB, groEL, ftsZ, and gyrB nucleotide sequences (3 232 unambiguously aligned bp; best-fit approximation for the evolutionary model: GTR+G). Branch numbers indicate percentage bootstrap support for major branches (1 000 replicates). Only bootstrap supports >70% are shown. The tick tree is a simplified cladogram of tick genera adapted from Burger et al. [33], and all congeneric tick species were arbitrarily considered as equally distant.
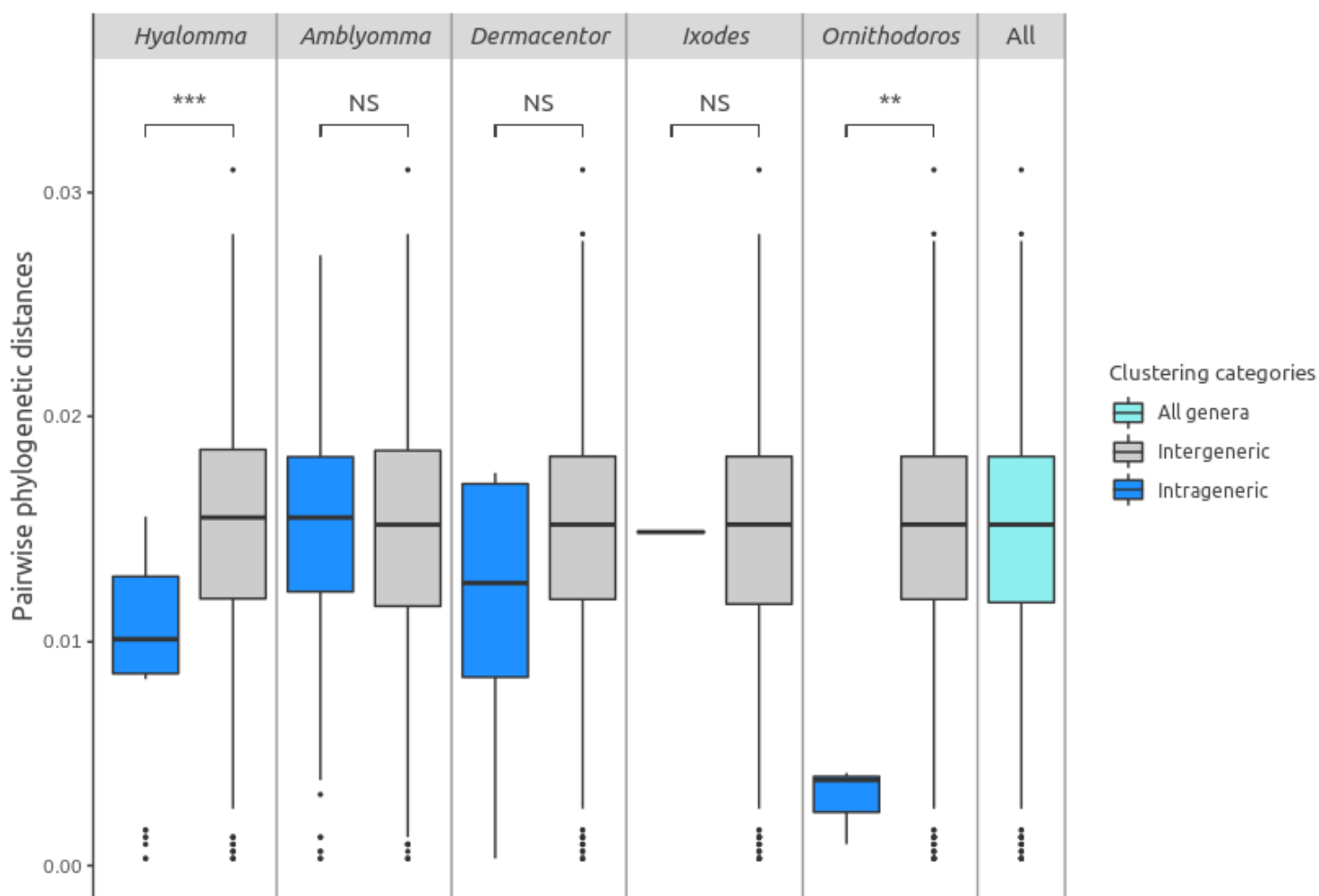
Figure 3

Intrageneric and intergeneric pairwise phylogenetic distances of Francisella-LE haplotypes. Comparisons were conducted for each tick genus harboring more than one Francisella-LE haplotype. **, p<0.005; ***, p<0.001; NS, not significant (p>0.05).

Supplementary Files

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