Comparative genomics of *Rickettsiella* bacteria reveal variable metabolic traits involved in symbiotic interactions with arthropods

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Abstract

Members of the *Rickettsiella* genus (Legionellales) are emerging as widespread bacteria associated with insects, arachnids, and crustaceans. While some *Rickettsiella* strains are highly virulent entomopathogens, others are maternally inherited endosymbionts that manipulate arthropod phenotypes, including the induction of defensive symbiosis and cytoplasmic incompatibility. However, the genomic diversity of *Rickettsiella* remains largely unexplored, and their genetic potential to induce complex phenotypes in arthropods is only partially understood. In this study, we sequenced five novel *Rickettsiella* genomes isolated from three tick species. Through comparative genomics, we observed that *Rickettsiella* members share similar metabolic capabilities, and collectively lack virulence genes from pathogenic Legionellales. Additional analysis of *Rickettsiella* genomes revealed significant variability in metabolic properties related to endosymbiosis. Specifically, their capacity to biosynthesize certain B vitamins and heme varies, suggesting a functional role of some *Rickettsiella* strains in the nutrition of their arthropod hosts. Some *Rickettsiella* genomes harbour homologs of *Wolbachia cif* genes, encoding for the effectors of cytoplasmic incompatibility, suggesting that *Rickettsiella* may use a similar molecular mechanism similar to manipulate the reproduction of their arthropod hosts. Phylogenomics further revealed that tick-borne *Rickettsiella* exhibit distinct evolutionary origins within the genus, indicating that *Rickettsiella* have undergone repeated horizontal transfers between ticks and other arthropods.

Keywords: symbiosis, *cif* genes, cytoplasmic incompatibility, ticks, entomopathogenicity

Introduction

Bacteria of the genus *Rickettsiella* (Legionellales: Coxiellaceae) are emerging as both widespread and genetically diverse in arthropods [1–7]. Historically, *Rickettsiella* have been identified as contagious entomopathogens infecting a broad range of insects, but various pathotypes have also been reported in other arthropods, including arachnids and crustaceans [1–3, 8–11]. In infected arthropods, *Rickettsiella*-induced diseases often replace body cavity tissue with a white iridescent liquid, leading to the death of hosts [9–11], which has spurred research into using entomopathogenic *Rickettsiella* as a biocidal agent [12],[13]. However, further surveys have revealed that non-pathogenic *Rickettsiella* are common in field populations of arthropods [14–18]. Most of these non-pathogenic *Rickettsiella* are predominantly maternally transmitted, and have a variety of repercussions on the arthropod's phenotypes, ranging from providing fitness advantages to manipulating reproduction [16, 19-21]. In aphids, *Rickettsiella viridis* can alter body colour, reducing attractiveness to predators and parasitoids [16, 21], or directly protect from entomopathogenic fungi, significantly lowering aphid mortality [16, 19]. In the poultry red mite *Dermanyssus gallinae*, *Rickettsiella* may be an essential nutritional endosymbiont providing B vitamins that are lacking in the blood diet of its host [22]. In the agricultural spider *Mermessus fradeorum*, a *Rickettsiella* endosymbiont induces cytoplasmic incompatibility (CI), that results in embryonic mortality in the offspring of matings between infected males and uninfected females [20]. This reproductive manipulation reduces the production of uninfected progeny and increases the proportion of infected females (*i.e.*, the transmitting sex), thereby promoting the spread of CI-inducing bacteria within the host population [23]. Overall, members of the genus *Rickettsiella* have evolved complex lifestyles that can significantly impact the ecology and evolution of their arthropod hosts [24].

Rickettsiella is remarkably common in the two main tick families, Ixodidae (hard ticks) and Argasidae (soft ticks), with at least 15% of species infected and prevalence ranging from 5% to 100% at the population level [4, 5, 25–31]. *Rickettsiella* also exhibit significant genetic diversity both within and between tick species [4, 5, 25–31]. Indeed, genetically distinct strains of *Rickettsiella* were detected in populations of the castor bean tick *Ixodes ricinus* and the polar seabird tick *Ixodes uriae* [25, 26]. The *Rickettsiella*-induced phenotypes in ticks have not been clearly identified yet, but a *Rickettsiella* endosymbiont was observed in a parthenogenetic laboratory colony of the wood tick *Ixodes woodi* [30, 31]. Both males and females are normally present in this tick species, hence *Rickettsiella* might manipulate the tick reproduction to induce asexuality [30]. A bacterium was identified in *I. ricinus*, and further in the blacklegged tick *Ixodes scapularis* [32] as a new genus and species, *Diplorickettsia massiliensis* [33], but it was further synonymised as *Rickettsiella massiliensis* [34] since the bacterium is nested within the current *Rickettsiella* genus [31]. *Rickettsiella massiliensis* has been proposed as a potential agent of opportunistic infections in humans [35], but no clear clinical cases have been reported to date.

Rickettsiella is currently among the lesser-known tick-borne bacteria [24], whereas other Legionellales, particularly those that infect humans, are better understood. The order *Legionellales* includes two families, *Legionellaceae* and *Coxiellaceae*, with several clinically significant microorganisms, such as *Legionella pneumophila*, the causative agent of Legionnaires' disease, and *Coxiella burnetii*, responsible for Q fever, both of which have been the focus of extensive genomic studies [24, 36]. These pathogenic *Legionellales* have evolved specific mechanisms to infect mammalian alveolar macrophages, such as a *Dot*/*Icm* type IV secretion system to translocate effectors and inhibit host cell apoptosis [37, 38]. However, not all Legionellales are pathogens: Some are B vitamin provisioning endosymbionts essential for the nutrition of arthropods with an obligate blood-feeding habit [39, 40], including *Coxiella*-like endosymbionts in ticks [41–43] and *Legionella polyplacis* in rat lice [22, 24, 36, 44]. Blood is nutritionally unbalanced, and these obligate blood feeders have evolved narrow associations with B vitamin provisioning symbionts, as they themselves cannot synthesise the essential cofactors and vitamins that are lacking in their diet [39, 40]. Additionally, *Coxiella*-like endosymbionts produce chorismate, a tryptophan precursor that regulates serotonin biosynthesis, actively promoting tick feeding activity and blood intake, and modifying behaviour [45].

Compared to other Legionellales, few *Rickettsiella* genomes have been sequenced. They have circular 1.3-1.9 Mb genomes, smaller than the genomes of other *Legionellales* such as the pathogens *L. pneumophila* (3.4 Mb) and *C. burnetii* (2.0 Mb), and the members of the genera *Aquicella* (2.5 Mb) and *Berkiella* (3 - 3.6 Mb), associated with amoebae living in aquatic environments [24, 34, 46]. While *Rickettsiella* genomes are known to encode 1,400-2,200 protein-coding genes [2, 22, 47, 48], no comparative studies have been conducted. So far, the genomes have been used in phylogenomic studies [34, 42], and a few investigations have been conducted focusing on single strains. Indeed, the *Rickettsiella* genome of the poultry red mite *D. gallinae* contains more than 300 pseudogenized protein-coding sequences, but has retained several B vitamin biosynthesis pathways, suggesting the importance of these pathways in evolution of a nutritional endosymbiosis [22]. Other examples include the genomes of *R. grylli*, infecting isopods, and *R. viridis*, infecting aphids, which both contain a complete *Dot*/*Icm* type IV secretion system [47, 48]. However, many phenotypically characterised *Rickettsiella* strains, as the one inducing CI in spiders [20], have never been sequenced. Among tick-borne *Rickettsiella*, only *R. massiliensis* has had its genome sequenced, though it was published as a brief genome announcement that included only a basic genome analysis [2].

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In this study, we present five novel genomes of *Rickettsiella* naturally infecting ticks, retrace their evolutionary history, characterise their genomic content, and infer their metabolic capacities. To this aim, we sequenced five microbial metagenomes, including three from *I. ricinus*, one from *Ornithodoros erraticus* and one from *Ornithodoros phacochoerus*. We then compared these genomes with published genomes of *Rickettsiella* and representative *Legionellales*, both pathogenic (*L. pneumophila, C. burnetii*) and symbiotic (*Coxiella*-like endosymbionts of ticks), to assess gene content and metabolic similarities. We further used these genomic analyses to predict the putative phenotypes of these *Rickettsiella* on their arthropod hosts.

Methods

Sample collection, DNA extraction and sequencing

Specimens of three tick species, *I. ricinus*, *O. erraticus* and *O. phacochoerus*, were collected in this study. Different methods for DNA extraction and sequencing were applied depending on tick species. For *I. ricinus*, questing ticks were collected in September 2020 by dragging in "De Dorschkamp", a deciduous forest east of Wageningen, the Netherlands (51°58’39.5"N, 5°41’59.3"E). Species and stage of collected ticks were morphologically identified using an identification key [49]. A first metagenome was sequenced from a pool of twelve males, leading to the recovery of a genome of *Rickettsiella* later designated as Iric. Another batch of questing *I. ricinus* was collected in September 2021 by dragging in "De Amsterdamse Waterleiding Duinen", a coastal sand dune area in the Netherlands (52°32’79.9"N, 4°54’38.6"E). From this batch, DNA from two females was separately extracted and sequenced, and two *Rickettsiella* genomes, designated as Ird1 and Ird6, were recovered among genomes of other tick-borne bacteria [50]. *Ornithodoros phacochoerus* specimens were sampled from warthog (*Phacochoerus africanus*) burrows in the Coutada 9 Game Reserve in Mozambique (17°42'33.1"S 33°50'29.5"E) in 2021. *Ornithodoros erraticus* specimens were obtained from a laboratory colony derived from field species collected in a pigsty in the Alentejo region of Portugal in 2016. For *O. phacochoerus* (Opha) and *O. erraticus* (Oerr), genomic DNA extractions enriched in endosymbiont DNA were obtained as previously described [41, 51]. Briefly, Malpighian tubules and ovaries were dissected from four ticks of *O. phacochoerus* and three ticks of *O. erraticus*. The organs were pooled and homogenised in 100µl sterile double-distilled water. The obtained homogenate was diluted and incubated for 1h at 20°C in 10ml sterile double-distilled water. To remove host nuclei and other cell debris, the homogenate was filtered using a 5µm Minisartfilter (Sartorius). The remaining cells in the homogenate were pelleted by centrifugation (15min at 20,000 x g at 4ºC). Total genomic DNA (gDNA) was extracted from the obtained pellet using the DNeasy Blood and Tissue Kit (Qiagen). The obtained gDNA was quantified on Qubit using the dsDNA high-sensitivity kit (Invitrogen).
In all cases, a shotgun metagenomics approach was applied, obtaining reads belonging to the ticks, the *Rickettsiella* strains, and any other microbial organisms infecting the same ticks. For the metagenomes Iric, Ird1, and Ird6, DNA extracted from whole tick bodies was first sequenced using PromethION flow cells (FLO-PRO002) with the Ligation Sequencing Kit (SQK-LSK110) from Oxford Nanopore Technologies, and simultaneously sequenced on the NovaSeq 6000 platform with the Nextera DNA Flex Library Prep Kit (Illumina). For the metagenomes Oerr and Opha, endosymbionts DNA enriched samples were sequenced using HiSeq2000 technology using the TruSeq Nano DNA library construction and HiSeq SBS v3 kits (Illumina).

Genome assembly

For the Oerr and Opha metagenomes, the reads were subjected to a modified version of the Blobology pipeline [52], available on github. The modified version allows an initial filtering of the host reads using the genome of *I. scapularis* (GCA_016920785.2) as reference, to ease the toll on computing resources, and includes the use of Python3, SPAdes v.3.15.4, bowtie2 v.2.1.0, blast v.2.12.0. Finally, the obtained genome assemblies were refined with Bandage v.0.8.1 [53], by manual removal of contigs unrelated to the assembly graph with no BLAST hits against the *R. viridis* genome as reference (GCF_003966755.1).

For the metagenomes Iric, Ird1 and Ird6, Minimap2 vs. 2.24 was used to map all nanopore reads against five known *Rickettsiella* genome sequences (GCF_000168295.1_ASM16829v1, GCF_000257395.1_ASM25739v1, GCF_001881495.1_ASM188149v1, NZ_AP018005.1 and NZ_CP079094.1). Reads that mapped against the *Rickettsiella* reference genomes were collected using Samtools vs. 1.15.1 and then *de novo* assembled using Flye vs. 2.9.2_b1786 (error rate 0.03), which resulted in 43 contigs with sizes ranging from 0.5 kb to 1.81 Mb. Medaka vs. 1.7.2 was used to polish the contigs based on the filtered *Rickettsiella* nanopore reads. Blastn against the *Rickettsiella* reference genomes confirmed that the 1.81 Mb contig corresponded to a near-complete genome assembly.

The completeness of retrieved Metagenome-Assembled Genomes (MAGs hereafter) was estimated using miComplete v.1.1.1 [54] (--hmms Bact105). The completeness estimation was performed on a dataset including 12 representative species of *Legionellales* available on NCBI and using the larger genome of the aquatic *Legionellales* "*Candidatus* Berkiella aquae" (GCF_001431295.2) as reference (Supplementary Table 1). This dataset of organisms was used in several analyses in our study and we will therefore refer to it as "Dataset 1". The creation of this dataset was completed on February 1st, 2024. Three *R. isopodorum* and one *R. grylli* genome sequences were excluded from the dataset as they do not show significant differences in terms of sequence similarity (ANI higher than 98, calculated with the ANI calculator implemented in EZBioCloud [55]) and gene content (no strain specific ortholog clusters) when compared to their reference genomes. A summary of the organisms included in Dataset 1 and their hosts is presented in Table 1, while more information about the dataset, including the average nucleotide identity among genomes, can be found in Supplementary Table 1.

Organism	Host	Host generic
Aquicella lusitana	Aquatic environment	Aquatic
"Candidatus Berkiella aquae"	*Acanthamoeba polyphaga*	Amoeba
"Candidatus Berkiella cookevillensis"	*Acanthamoeba polyphaga*	Amoeba
Coxiella burnetii RSA 493	*Dermacentor andersoni*	Tick
Coxiella endosymbiont of Amblyomma sculptum strain CeAS-UFV	*Amblyomma sculptum*	Tick
Coxiella endosymbiont of Ornithodoros maritimus CLEOmar	*Ornithodoros maritimus*	Tick
Legionella pneumophila	*Homo sapiens*	Human
Rickettsiella endosymbiont of Dermanyssus gallinae	*Dermanyssus gallinae*	Mite
Rickettsiella grylli	Armadillium sp.	Isopod
Rickettsiella isolated from Ixodes ricinus, Ird1 strain	*Ixodes ricinus*	Tick
Rickettsiella isolated from Ixodes ricinus, Ir_d6 strain	*Ixodes ricinus*	Tick
Rickettsiella isolated from Ixodes ricinus, Iric strain	*Ixodes ricinus*	Tick

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Rickettsiella isolated from Ornithodoros erraticus, Oerr strain	*Ornithodoros erraticus*	Tick
Rickettsiella isolated from Ornithodoros phacochoerus, Opha strain	*Ornithodoros phacochoerus*	Tick
"Candidatus Rickettsiella isopodorum"	*Trachelipus rathkii*	Isopod
Rickettsiella massiliensis 20B	*Ixodes ricinus*	Tick
"Candidatus Rickettsiella viridis"	*Acyrthosiphon pisum*	Hemipteran

Table 1: Summary of Dataset 1.

Genome annotation

Genomes were annotated with Prokka v.1.14.6 [56] using the “compliant” mode and default parameters. The resulting annotations in GenBank format, together with the other 12 annotated *Legionellales* of Dataset 1 downloaded from NCBI, were subjected to pseudogene analysis with PseudoFinder v.1.1.0 [57] with default parameters. The counts of intact and pseudo- genes have been graphically represented with an in-house R script available on github. KEGG orthology assignments of intact genes and pseudogenes of each genome were predicted using BlastKoala v.3.0 [58]. The outputs were then merged using in-house scripts available on github and github. Finally, we screened for mobile elements with MobileElementFinder v.1.1.2 [59] using the database MGEdb v.1.1.1 and default parameters.

Phylogeny of *Rickettsiella* organisms

The phylogenetic relationships within Dataset 1 were inferred through a phylogenomic approach on single copy panorthologous nucleotide sequences using *Buchnera aphidicola* (GCF_003099975.1) as an outgroup. The pipeline is available on github and includes the use of the tools OrthoFinder v.2.5. [60], SeaView v.5.0.5 [61], ModelTest-NG v0.2.0 [62], RAxML (raxmlHPC-PTHREADS v.8.2.12, [63], 100 bootstraps), and in-house scripts in Python3, bash, and R. All of the softwares have been run in default settings, except for RAxML, where the model was set to GTR+G4. Additionally, in order to catch a glimpse on *Rickettsiella* evolution on a larger scale, a second dataset (Dataset 2) was created by adding 16S rRNA sequences (n = 446) of members of the *Rickettsiella*, *Aquicella*, and *Berkiella* genera. These sequences were downloaded from SILVA RefNR SSU r138.1 to the 16S rRNA sequences of the organisms of Dataset 1, the 16S rRNA sequence from the CI-inducing *Rickettsiella* strain from spiders (MK627517.1), and the 16S rRNA sequence of *Buchnera aphidicola* str. Sg (NR_145527.1) predicted with barrnap v.0.9 [64]. The sequences were aligned with MUSCLE v3.8.1551 [65] and the phylogeny was inferred with RAxML (100 bootstraps, rapid bootstrap random number seed: 1234, random number seed for the parsimony inferences: 123). The obtained 16S rRNA-based tree was then pruned using iTOL v.6.9 [66] to ease tree visualisation.

Comparative genomics

In order to obtain a description of common and strain-specific genomic features and metabolic capabilities of *Rickettsiella*, we analysed the clusters of orthologous intact genes detected for Dataset 1, regardless of phylogenetic relationships. The OrthoFinder output table from which the single copy panorthologs used in the phylogenomics-based approach for the phylogeny inference (Orthogroups.GeneCount.tsv, Supplementary Table 2) was analysed for intersections using the R package UpSetR [67], and the results were graphically represented using the same tool. All the commands are available on github.

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Particular attention was given to clusters uniquely shared by (i) all *Rickettsiella* species and strains, (ii) *Rickettsiella* strains isolated from hematophagous arthropods, for traces of adaptation to host blood feeding (iii) *Rickettsiella* strains isolated from phytophagous/detritivorous hosts, for traces of adaptation to other diets (iv) *Rickettsiella* isolated from the same host, for adaptation to hosts/environments and (v) *Rickettsiella* and *Coxiella* isolated from ticks, for traces of adaptation to tick hosts. We also investigated gene orthology in respect to the phylogenetic clusters that appeared well-defined in the phylogenetic trees. Orthology was performed using OrthoFinder on all translated proteins encoded in genomes, as well as on translated sequences of intact genes and pseudogenes as previously predicted with PseudoFinder, in order to obtain more information about the evolution of the strains. The results were graphically represented as Venn diagrams using the R package VennDiagram [68], and the KEGG and Prokka annotations of the clusters were obtained (commands available on GitHub).

We also analysed specific pathways that could be involved in nutritional symbioses with blood-feeding arthropods (B vitamins, chorismate and heme metabolism [45, 51, 69, 70]), using subsets of Supplementary Table 3 to obtain graphical representation of the gene presence/absence/pseudogenization along the phylogenomics-obtained tree with an in-house R script available on github. The analysis showed differences in some of the pathways, we thus aligned the amino acid sequences of the said genes, with MUSCLE and inferred their respective phylogeny with RAxML (100 bootstraps, rapid bootstrap random number seed: 1234, random number seed for the parsimony inferences: 123). We further checked for the presence of virulence factors known in *Legionellales* [37, 38]. Protein sequences of virulence factors annotated in *Legionella longbeachae* NSW150 (NC_013861), *L. pneumophila* str. Corby (NC_009494) and *L. pneumophila* subsp. *pneumophila* str. Philadelphia 1 (NC_002942) were downloaded from VFDB [71] and used as queries in a blast search against the translated sequences of intact genes and pseudogenes of Dataset 1. The phylogeny of the identified virulence factors was inferred using the same pipeline applied for the phylogeny of the pathways potentially involved in the nutritional symbiosis (see above).

Finally, we also looked for the presence of CI genes. The CI phenotype is encoded by a pair of syntenic genes, *cifA* and *cifB*, whose direct genotype-phenotype association has been demonstrated in *Wolbachia* [72, 73]. *cifA* and *cifB* possess homologs that have been categorised into five phylogenetic clusters [74–77], although type V has recently been split into type V-to-X [77]. To determine the presence of *cif* gene homologs on the *Rickettsiella* genomes, we used Orthofinder to search for specific orthologs of the *cif* gene groups identified in a previous study [74]. These searches were conducted on a database of CDS from Dataset 1, as well as from published *Wolbachia*, known to induce CI, and *Rickettsia* genomes (Supplementary Table 4). Protein domains were predicted for the *cifA* and *cifB* homologs detected using the HHpred webserver (https://toolkit.tuebingen.mpg.de/#/tools/hhpred/, [78]) with default parameters [79], and using the following databases: SCOPe70 v.2.08, Pfam v.36.0, SMART v.6.0, and COG/KOG v.1.0. The sequences of each detected domain were retrieved and manually aligned using Clustal Omega [80], independently for each *cifA* and *cifB* homologs, to complete the domain detection of some false negatives generated by HHpred. Visualisation of protein domains within the *cif* genes was performed using RStudio v4.3.1 with the ggplot2 v.3.4.4 [81], cowplot v1.1.1 [82], and gridExtra v.2.3 [83] packages. A maximum likelihood phylogeny of *cifA* and *cifB* pair genes has been inferred on the basis of three conserved domains (RNA-binding-like, AAA-ATPases-like and PD-(D/E)XK nucleases) independently aligned and concatenated.

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Results

Microbial metagenomes

We sequenced and assembled three microbial metagenomes from *I. ricinus* (Ird1, Ird6 and Iric), one from O. *phacochoerus* (Opha) and one from *O. erraticus* (Oerr). We obtained an average of 71.86 Gb of paired-end reads (from 2.2 gb to 192 bp) of 150 nt in length for each sample (see Supplementary Table 1 for details). For each metagenome, we extracted the reads belonging to the target organisms and obtained the complete sequences of five novel *Rickettsiella* MAGs (see Supplementary Table 1 for details). The *Rickettsiella* MAGs are all of similar size (~1.7 Mb), with the exception of *Rickettsiella* Oerr MAG (~1.3 Mb), and they are all estimated to be complete. However, the estimation attributes to *Rickettsiella* strain Oerr MAG lower completeness scores (0.9048; weighted completeness 0.8109) among the MAGs sequenced in this study. Lower scores are expected for endosymbiotic, host-restricted bacteria, whose genomes undergo erosion [84–87], and observed in *Rickettsiella* relatives, as the *Coxiella*-like-endosymbionts of ticks. The completeness score of *Rickettsiella* strain Oerr MAG thus reflects more the genomic erosion in *Rickettsiella* than a misassembled genome. The GC content varies between 36% and 39%, values also consistent with those from endosymbiotic bacteria. Metagenomes also contain reads of other bacterial endosymbionts as *Midichloria* and *Francisella*-like endosymbiont for *I. ricinus* and *O. phacochoerus*, respectively (data not shown). These endosymbionts were not further considered in this study. No other endosymbiont than *Rickettsiella* was detected for *O. erraticus*.
The annotation of the five *Rickettsiella* MAGs revealed a somewhat similar number of functional genes, ranging from 1,268 to 1,473 (Supplementary Table 1). Including all *Rickettsiella* genomes shows that the number of functional genes is between 1,189 for *R. isopodorum* and 1,707 for the *Rickettsiella* endosymbiont of *D. gallinae* (Supplementary Table 1, Supplementary Figure 1). *Rickettsiella* genomes display similar levels of pseudogenization to other *Legionellales*, though some variation is observed. For instance, higher numbers of pseudogenes were predicted for the *Rickettsiella* MAGs Iric and Oerr (340 and 237 respectively), *R. massiliensis* 20B (508) and a few other *Legionellales*, including *Coxiella*-like endosymbiont of *Ornithodoros maritimus* strain CLEOmar (778). In contrast, lower numbers of pseudogenes were predicted in other *Legionellales*, such as the *Coxiella*-like endosymbiont of *Amblyomma sculptum* strain CeAS-UFV (31) (Supplementary Table 1, Supplementary Figure 1). Concerning mobile elements, only one was detected in the analysed dataset, and only in *R. grylli* (Supplementary Table 1). Both recently host-restricted bacteria and long term symbionts generally possess a low number of mobile elements in their genomes, although recent symbionts are usually characterised by a greater number. However, long term symbionts are additionally characterised by fewer pseudogenes and smaller genome sizes [85] than the *Rickettsiella* strains here investigated. These features taken together suggest a relatively recent infection of the hosts by *Rickettsiella*.
Most Average Nucleotide Identities (ANIs) between the five *Rickettsiella* MAGs range in similar values, varying from 69.39 to 72.61%, suggesting that they may be distinct species (Supplementary Table 1). A notable exception is the 99.77% ANI between Iric and Ird6, showing that these two *Rickettsiella* MAGs are very similar. The five *Rickettsiella* MAGs have ANIs ranging from 69.36-to-98.46% with other *Rickettsiella* genomes. Indeed, ANI is 98.46% between Iric/Ird6 and *R. massiliensis*, and 98.26% between Ird1 and *R. isopodorum* (Supplementary Table 1).

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Phylogeny of *Rickettsiella*

Phylogenomic analysis based on 92 single-copy panorthologs showed that *Rickettsiella*, *Coxiella*, *Legionella*, *Aquicella*, and *Berkiella* genera cluster together in a robust monophyletic clade forming the Legionellales order (Figure 1). The inner tree topology is also congruent with the known genera of Legionellales [24]. Indeed, genomes of *Rickettsiella*, *Coxiella*, and *Berkiella* cluster together according to their assigned genus and form robust monophyletic subclades, with *Aquicella* and *Coxiella* forming sister genera of *Rickettsiella*. Two *Rickettsiella* MAGs obtained in this study from *I. ricinus*, Iric and Ird6, cluster with *R. massiliensis* sharing a high ANI from 98.5 to 99.8% (node 15, Figure 1B). The three other *Rickettsiella* MAGs obtained in this study cluster with diverse *Rickettsiella* species: Opha with the defensive symbiont of aphid, *R. viridis*, and the putative nutritional *Rickettsiella* symbiont of poultry red mite (node 13), Ird1 with *R. isopodorum* (node 12) and Ierr with a group formed by *R. grylli*, *R. isopodorum* and Irid6 (node 10, Figure 1B). The *Rickettsiella* found in different tick species are thus distantly related and do not form a tick-specific clade (Figure 1).

The 16S rRNA-based phylogeny shows a well-supported monophyletic *Rickettsiella* clade, confirming the robustness of the genus (Supplementary Figure 2). No *Rickettsiella* subclade specific to ticks are evident along the tree: the *Rickettsiella* found in ticks are rather scattered among *Rickettsiella* of woodlice, spiders and insects and associated with diverse phenotypic effect as nutritional symbiosis, defensive symbiosis and entomopathogenicity (Supplementary Figure 2). Particularly striking is the separation of *Rickettsiella* strain Ird1, isolated from an *I. ricinus* individual, and the other strains isolated from *I. ricinus* individuals (Iric, Ird6, and *R. massiliensis* 20B [2]). The Ird1 *Rickettsiella* strain appears to be phylogenetically closer to a *Rickettsiella* strain isolated from *Proasellus aquaticus*, an aquatic crustacean with a detritivorous diet. However, the inner topology of the genus *Rickettsiella* based on 16S rRNA gene sequences remains too poorly resolved in many other cases (as shown by low support values) for inferring the exact relatedness between all *Rickettsiella* from different arthropod species.

Genome comparisons

Rickettsiella genome stability

No mobile elements were detected by MobileElementFinder in the *Rickettsiella* genomes apart from one insertion sequence in *R. grylli*.

Orthology analysis

The analysis of the intact orthologous genes detected for Dataset 1 (Figure 2, Supplementary Figure 3, Supplementary Table 2) indicates huge variation among Legionellales, with just 135 clusters shared among all *Legionellales*, most of which involved in basic bacterial processes. Only 25 clusters of intact genes are shared exclusively by members of the genus *Rickettsiella*, most of them associated with no function (Supplementary Table 2). No clusters of intact genes specific to *Rickettsiella* and *Coxiella* strains isolated from hematophagous arthropod hosts were detected. We further performed a phylogeny-driven analysis of Dataset 1, focusing on the phylogenetic clusters that could be identified on the phylogenomics-based tree shown in Figure 1. The core *Rickettsiella* genome appears to contain 606 clusters of orthologs, that drop to 362 when pseudogenes are removed from the analysis; core pseudogenes in the *Rickettsiella* genus (29 clusters of pseudogenized sequences) appear to be mainly associated with transcription and regulation functions (Supplementary Table 5).

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In Figure 1, node 10 supports the cluster encompassing two previously described and potentially pathogenic *Rickettsiella* species isolated from isopods (*R. grylli* [3] and *R. isopodorum* [88]), and two of the newly *Rickettsiella* strains identified in ticks. Notably, these two new strains were isolated from very distant ticks, both in terms of host species and in geographic location: *Rickettsiella* Ird1 from the hard tick *I. ricinus* collected in the Netherlands and *Rickettsiella* Oerr from the soft tick *O. erraticus* collected in Mozambique (Supplementary Table 1). The core genome of these organisms contains 823 clusters of orthologs, which drop to 704 when pseudogenes are not considered in the analysis (Figure 2). Furthermore, when only intact genes are retained in the orthology inference, *R. isopodorum* does not present any specific genes, the number of specific clusters of orthologs slightly decreases in the *Rickettsiella* strain Oerr (16 to 13), whereas the number of clusters of orthologs specific to *Rickettsiella* strain Ird1 increases, and remains unvaried for *R. grylli*. Of the 704 clusters of intact genes shared by the members of this node, 198 are uncharacterised proteins, and 27 appear to be involved in secretion of effectors and pumps. Furthermore, five were annotated by Prokka as multidrug resistance proteins. However, the putative pathogen *R. grylli* possesses 22 additional transport proteins compared to the rest of this dataset. In addition, *Rickettsiella* Oerr uniquely possesses 35 uncharacterised proteins (Supplementary Table 5). Interestingly, *Rickettsiella* Oerr and Ird1 exclusively share 15 clusters of intact orthologs, and 9 clusters of intact orthologs. The intact shared orthologs include 13 uncharacterised protein sequences, a transposase, a methylase, and the T4SS components VirB4 and VirB9; whereas the shared clusters of pseudogenes include one uncharacterised protein and the Cell division coordinator CpoB (Supplementary Table 5).

Node 13 supports the cluster encompassing the host colour-altering and defensive symbiont *R. viridis* (aphid host, [14]), the *Rickettsiella* endosymbiont of *D. gallinae* (poultry red mite host, [89]), and the newly sequenced *Rickettsiella* str. Oerr isolated from the soft tick *O. phacochoerus*. The core genome of this node contains 935 clusters of orthologous intact genes (1061 clusters when pseudogenes are maintained in the analysis; Figure 1B). All the *Rickettsiella* strains encompassed in this node appear to maintain a discrete, albeit low, number of unique clusters. Among others, the core intact genes include T4SS components, ATPase subunits, biotin genes, 79 hypothetical proteins, four ankyrin repeat domain-containing proteins, and 14 DUF domain-containing proteins. Notably, *Rickettsiella* strain Opha appears to share more clusters of orthologous genes with the endosymbiont of *D. gallinae* (65, encoding for hypothetical proteins, several ATPases, DUF containing-proteins, and transcription regulators) than with *R.viridis* (37, most of them being potentially involved in adhesion). However, the *Rickettsiella* endosymbiont of *D. gallinae* and *R. viridis* share a large number of clusters as well (90, including 69 with unknown function). Interestingly, the core pseudogenes include many response regulators and translocators, and the *Rickettsiella* endosymbiont of *D. gallinae* and the *Rickettsiella* strain Opha share no additional pseudogenes, whereas the *Rickettsiella* endosymbiont of *D. gallinae* and *R. viridis* share 9 more clusters of pseudogenes. Finally, the aerobic respiration control sensor protein ArcB is pseudogenized in the strain Opha only, and the endosymbiont of *D. gallinae* contains 10 more unique pseudogenes, that include regulators and transposases (Supplementary Table 5).

Finally, Node 15 encompasses three *Rickettsiella* strains isolated from different individuals of the hard tick *I. ricinus*: *R. massiliensis*, *Rickettsiella* Iric and Ird6. The core of 929 clusters of intact genes (1384 clusters when pseudogenes are included in the analysis) contains mainly hypothetical proteins and house-keeping genes, but also genes involved in energy production. Interestingly, *Rickettsiella* strain Iric exhibits distinctive clusters of orthologous sequences exclusively when pseudogenes are analysed, revealing just a single cluster associated with amino sugar and nucleotide sugar metabolism (Supplementary Table 5). However, the other strains possess only five unique clusters of orthologous intact sequences as well. A relatively high number of intact sequences is shared by any two organisms in these subset of

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genomes: 74 clusters between *R. massiliensis* and *Rickettsiella* str. Ird6, 82 between *R. massiliensis* and *Rickettsiella* str. Iric, and 314 between *Rickettsiella* str. Iric and *Rickettsiella* str. Ird6. Most sequences shared by *R. massiliensis* and the strain Iric are annotated as hypothetical proteins, whereas most sequences shared by *R. massiliensis* and the strain Ird6 have an annotated function, most of them being housekeeping genes and genes encoding for chaperons. The *Rickettsiella* Iric and Ird6 share mostly uncharacterised proteins, but also several dehydrogenases, ATPase synthase subunits A and B, and a queuosine precursor transporter (Supplementary Table 5).

Metabolic capabilities

The annotation of metabolic pathways (Supplementary Table 3) shows overall similar metabolic capabilities among the members of the *Rickettsiella* genus. Major differences can be identified, however, in specific pathways: the respiration and ATP production appears to be most degraded in *Rickettsiella* strain Iric and R. *massiliensis* 20B, *i.e.*, the two *Rickettsiella* strains with the higher number of predicted pseudogenes that form a monophyletic cluster together with the Rickettsiella Ird6. Furthermore, B vitamin and heme pathways show significant differences, appearing most degraded in R. *massiliensis* 20B, but still relatively conserved in *Rickettsiella* strain Iric (*e.g.*, biotin, folate). Strikingly, the heme biosynthesis pathway is absent in *Rickettsiella* strain Oerr, which has also lost most of the thiamine biosynthesis pathway, yet retains a unique segment of this pathway not found in other *Rickettsiella* strains sequenced in this study. However, although it is not possible to fully reconstruct the evolution of these sequences, an online blast search and inferred phylogenies for each gene shows that they are of Gammaproteobacteria origin (Supplementary Table 3, five best hits shown, and Supplementary Figure 4), often found in *Legionella* spp. and in two additional *Rickettsiella* organisms. This suggests either multiple horizontal transfers from other Gammaproteobacteria or Legionellales to these *Rickettsiella* strains, or a loss of the genes in the other *Rickettsiella* strains here analysed.
Finally, while *Coxiella*-like endosymbionts can produce a tryptophan precursor, chorismate, which regulates serotonin biosynthesis in ticks [44], *Rickettsiella* genomes do not harbour chorismate biosynthesis genes, suggesting that *Rickettsiella* infection do not impact tick behaviour through this mechanism.

Identification of virulence factors

Further genomic comparisons showed that abundance of virulence genes varies between Legionellales members (Table 2, Supplementary Table 6). While large repertories of virulence genes associated with adherence, secretion systems, effectors and exotoxin RtxA are common in *L. pneumophila* genomes, these genes are rare or absent in most other Legionellales as *Coxiella*-like endosymbionts associated with ticks. Indeed, some virulence genes are absent in *Rickettsiella* genomes including the exotoxin rtxA, which is involved in adherence, cytotoxicity, and pore formation in vertebrate cells by *Legionella* species [90]. *Aquicella lusitana* do not possess the exotoxin as well, suggesting that the loss of this gene might be ancestral to *Rickettsiella* and *Aquicella*. Of the other factors associated with virulence in *Legionella*, *Rickettsiella* genomes conserve the ones involved in adherence, in regulation, and in nutritional metabolism (particularly, heme metabolism cofactors [91]). Furthermore, the *Rickettsiella* strains maintain the effector delivery systems, but do not retain the secreted effectors associated with virulence identified in *Legionella*, suggesting that this machinery could be exploited by *Rickettsiella* to perform different functions. The same occurs in *Aquicella*, *Berkiella* and *C. burnetii*, whereas the two *Coxiella*-like endosymbionts present neither the delivery system nor the genes encoding for its virulence secreted effectors. Furthermore, all the investigated *Legionellales*, with the exception of

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Legionella and several *Rickettsiella* strains, do not possess the Type IV A secretion system (TIVASS). The strains that maintain most of this system are *R. viridis* and the tick strains Ird1, Oerr and Opha, whereas the *Rickettsiella* endosymbiont of *D. gallinae*, *R. grylli* and *R. isopodorum* maintain only a few genes involved in this machinery. No evidence of horizontal gene transfer could be obtained. Finally, the enhancer-binding protein (Enh) loci are not found in *Rickettsiella*, whereas they can be found in *Aquicella*, *Berkiella*, *C. burnetii*, and *Legionella*.

Category	*Aquicella*	*Berkiella*	*Coxiella burnetii*	*Coxiella* endosymbionts	*Rickettsiella* strains	*Legionella*
Adherence						
Dot/Icm						
Dot/Icm secretedEffectors						
lsp_TIISS						
LvhTIVASS						
Exotoxin RtxA						
Nutritional Metabolic Function						
PostTranslational Modification						
Regulation						
Enh_loci						

Table 2: Simplified scheme of presence (light blue) and absence (white) of virulence factors categories in the organisms of Dataset 1. The organisms were grouped by genus, separating only *Coxiella* endosymbionts from the proved pathogenic relative *C. burnetii*. The virulence factor category was considered present if at least three of the representatives of the *Rickettsiella* strains and one of the *Berkiella* and *Coxiella* strains contained at least half of the virulence factors of that category. The search was performed using reference *Legionella* sequences from VFDB as queries in a local blast search (default settings, results were manually curated). Details on the annotation can be found in Supplementary table 6.

***Rickettsiella* and cytoplasmic incompatibility**

We detected homologs of the CI genes *cifA* and *cifB* in the genomes of (i) *Rickettsiella* strain Opha, and (ii) *R. isopodorum* where a CifB-like protein has been previously identified (Figure 3, [92]). Both *Rickettsiella* strains harbour the RNA-binding-like domain in *cifA*, and the AAA-ATPase-like and PD-(D/E)XK nuclease domains in *cifB*, both conserved in all *cif* genes [74, 93]. Additionally, the *cifB* gene in *Rickettsiella* strain Opha has a weakly aligned latrotoxin-like fragment, while *R. isopodorum* has a large TcdA/TcdB pore-forming toxin domain, also found in other *cifB* homologs of *Wolbachia* and *Rickettsia*. Furthermore, we also identified a unique *cifB* homolog (without *cifA*) in a *Rickettsiella* sp. genome reconstructed from eDNA isolated from hot springs and volcanic lakes (GCA_037439325.1) and in the distant relative "*Ca.* Aquirickettsiella gammari" (GCA_002290645.2).

Both the detected *cifA* and *cifB* homologs of *Rickettsiella* cluster within the Type V *cif* paraphyletic group, which is the most diverse in terms of size and domain composition [74, 93]. Both the *cif* genes from *Rickettsiella* strain Opha and *R. isopodorum* group together in a highly supported clade comprising *cif* genes from *Rickettsia* and *Wolbachia*,

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originating from at least two supergroups (*w*Meg and *w*Con from supergroup A; *w*Oegib and *w*Bor from supergroup B), supporting the occurrence of gene transfers between these phylogenetically distinct endosymbionts. However, the long branch lengths and low intra-group bootstrap values create uncertainty regarding the exact position of the *Rickettsiella cif* genes, challenging the reconstruction of their evolutionary history and origin.

Discussion

We show here that members of the genus *Rickettsiella* share a set of similar metabolic capabilities, as pathways involved in energy production, including glycolysis and a normal TCA cycle. Most notably, the *Rickettsiella* species and strains lack most key virulence genes responsible for persistence and replication of pathogenic *Legionella* and *Coxiella* in vertebrate cells. Indeed, while the *Rickettsiella* genomes harbour the virulence genes encoding the *Dot*/*Icm* secretion system, the genes encoding the Legionellales *Dot*/*Icm* secreted effectors and the exotoxin RtxA have been pseudogenized or are completely absent. However, examination of *Rickettsiella* genomes further reveals a greater variability in metabolic properties associated with endosymbiosis: They have variable capacity to biosynthesize *de novo* certain B vitamins (biotin, riboflavin, pyridoxal phosphate, pantothenate) and heme, and some harbour CI effectors, suggesting that they are engaged in complex interactions with their respective arthropod hosts. Phylogenomics further confirmed that members of the genus *Rickettsiella* form a robust monophyletic clade of Legionellales associated with arthropods but that tick-borne *Rickettsiella* do not cluster within a tick-specific subclade. Tick-borne *Rickettsiella* rather exhibit distinct evolutionary origins in the genus, revealing that *Rickettsiella* have undergone repeated horizontal transfers between ticks and other arthropods, including aphids and woodlice.

The virulence factors presence/absence pattern across Legionellales reveals key past events in the evolutionary history of *Rickettsiella*. Indeed, the absence of major Legionellales virulence factors in all *Rickettsiella* strains, including the putative human pathogen *R. massiliensis* 20B, suggests that these microorganisms are not strongly pathogenic to mammals as *L. pneumophila* and *C. burnetii*. Concerning the exotoxin RtxA, the closest relative of *Rickettsiella*, *Aquicella lusitana*, does not possess the exotoxin as well, suggesting that the loss of this gene might be ancestral to *Rickettsiella* and *Aquicella* and extend deep in the evolutionary past of Coxiellaceae. Several *Legionella* virulence factors are conserved in *Rickettsiella* and to some extent in *Aquicella*, though they may have alternative functions. Notably, traits such as adherence, stress response regulation, nutritional metabolism (particularly involving heme cofactors), and the type IV secretion systems are common to both pathogenic and mutualistic bacteria [87,90-97] the *Dot*/*Icm* effector delivery system has been exclusively associated only with pathogenesis thus far [42]. Indeed, genes encoding the *Dot*/*Icm* system are either absent or pseudogenized in *Coxiella*-like endosymbionts associated with nutritional symbiosis in ticks [42,98]. The conservation of the system *Dot*/*Icm* in *Rickettsiella* suggests either that this machinery is being exploited by *Rickettsiella* to perform functions other than virulence by secreting different molecular effectors, or that *Rickettsiella* maintain a degree of virulence but using different virulence effectors than *Legionella*. Alternatively, since the *Rickettsiella Dot*/*Icm* system lacks a few genes compared to *L. pneumophila* and *C. burnetii*, this machinery may undergone a gradual degradation and they mirrored a global genome decay, as commonly observed in endosymbionts of arthropods [84, 85, 87, 99, 100]. Whatever the explanation, virulence against mammals is clearly being gradually lost in *Rickettsiella*, leaving opportunities for the evolution of other interactions with their arthropod hosts.

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The genomes of *Rickettsiella* consistently harbour genes involved in narrow interactions with arthropods, although these genes vary between *Rickettsiella* strains and species. Indeed, the biosynthesis genes of several B vitamins (biotin, riboflavin, and folate) have been found all intact in most *Rickettsiella* genomes, suggesting that they could provide these nutrients to their arthropod hosts. In this context, *Rickettsiella* may be essential mutualistic partners for hematophagous arthropods, such as ticks and certain mites, which need these essential B vitamins for their growth and to achieve their life cycle [39]. Most tick species harbour *Coxiella*-like endosymbionts that ensure obligate B vitamins provisioning, while certain tick species lacking *Coxiella*-LE instead host *Francisella*-like endosymbionts [39, 101] or *Midichloria* [102, 103] as we observed in this study in *O. phacocherus* and *I. ricinus*, respectively. However, co-infections (i.e., the simultaneous infection of *Rickettsiella* with one or more different microorganisms) have been previously documented in ticks [4, 5, 101], hence, the identification of *Rickettsiella* tick species previously associated with other symbionts is not surprising. However, in *O. erraticus*, we observed no other endosymbionts than *Rickettsiella* suggesting that this bacterium is a obligate nutritional partner of this tick species. In addition, certain *Rickettsiella* genomes possess biosynthesis genes for heme, a nutrient which is essential for regulating protein synthesis and cell differentiation in animals. Unlike other eukaryotes, ticks lack most of the genes encoding proteins necessary for heme production and degradation [104–106]. Some *Rickettsiella* endosymbionts could thus potentially influence tick metabolism through the production of this essential cofactor.

Further examinations of *Rickettsiella* genomes also suggest that at least two *Rickettsiella* strains may be reproductive parasites of the soft tick *O. phacochoerus* and the woodlouse *Trachelipus rathkei*. Although no phenotypic data are currently available for these arthropod species, their *Rickettsiella* possess CI-inducing genes with key functional domains, including a RNA-binding-like domain in *cifA*, a the AAA-ATPase-like and PD-(D/E)XK nuclease domains in *cifB*, as observed in all *cif* genes of CI-inducing *Wolbachia* [74, 93]. The observed pattern suggests acquisitions of *cif* genes in *Rickettsiella* through lateral gene transfers from phylogenetically distant endosymbionts also inhabiting arthropods, *Rickettsia* and *Wolbachia*. Both are common in arthropods [107, 108], especially *Rickettsia* in ticks [4, 109] in which co-infections with *Rickettsiella* have been reported [4]. These co-infections create physical proximity between these endosymbionts and may have facilitated lateral DNA transfers between them [110, 111], which could explain the intertwined evolutionary history of *cifA* and *cifB* genes among endosymbionts of arthropods.

The phylogenetic diversity pattern strongly suggests that horizontal transfers between arthropod species are key drivers of *Rickettsiella* spread, dictating global incidence of infections across arthropod communities. Repeated horizontal transfers results to a widespread and global distribution in diverse arthropod hosts, but also to multiple acquisitions of unrelated *Rickettsiella* strains in the same host species as in *I. ricinus* (this study) and the polar seabird tick *Ixodes uriae* [25]. The capacity of *Rickettsiella* to move between host species can thus explain why no clusters specific to tick-borne *Rickettsiella* were identified. However, exceptions exist: one uncharacterised protein is shared by all *Rickettsiella* isolated from *I. ricinus*, the fatty acid oxidation complex subunit alpha is shared by all strains isolated from *I. ricinus* with the exception of *R. massiliensis* 20 B, five clusters of hypothetical proteins specific to the Oerr strain, and nine clusters of uncharacterised proteins specific to the Opha strain. While most of these proteins have not been associated with any function yet, the fatty acid oxidation complex is quite interesting. Indeed, bacteria may exploit fatty acids from the host and utilise them for their own sustainment [112]. On the other hand, the oxidation of fatty acids might allow *Rickettsiella* to produce energy-rich compounds that could be used by *Rickettsiella* itself but also its tick host, aiding the

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host's metabolism, as previously suggested for the tick microbiome [113, 114] and *Coxiella*-like endosymbionts [41, 115].

Conclusions

Rickettsiella is a diverse group of endosymbiotic bacteria that exhibit a range of adaptations, from pathogenic to mutualistic, enabling them to thrive within arthropod cells and manipulate their hosts' biology. Over the past decade, significant progress has been made in understanding their impact on arthropods and introducing these bacteria into the field of genomics. However, *Rickettsiella* remains an understudied aspect of tick biology, and a comprehensive approach is needed to fully understand its interactions with various arthropod hosts. Key questions persist, such as: How are the global abundance, diversity, and distribution of *Rickettsiella* maintained? Can *Rickettsiella* be effectively used in pest control? How do they manipulate host reproduction? What roles do they play in the evolution of their arthropod hosts? Importantly, future research is needed to more thoroughly investigate how *Rickettsiella* affects the fitness of arthropods, including ticks, and to what extent they can cause diseases in humans.

List of abbreviations

Ca.	*Candidatus*
CI	Cytoplasmic incompatibility
R.	*Rickettsiella*
TCA	Tricarboxylic acid (cycle)
TIVASS	Type IV A secretion system
min	minutes
nt	Nucleotides
ANI	Average Nucleotide Identity

Declarations

The authors have complied with all ethical standards required for conducting this research. Consents and approvals are not applicable to this research. The genomes are available on ENA under project number PRJEB70514 (Ird1 and Ird6), PRJEB82506 (Oerr and Opha), and PRJNA816462 (Iric). All the bioinformatics pipelines are available on GitHub (https://github.com/annamariafloriano/RickettsiellaComparative). This work was funded by French Agence Nationale de la Recherche (ANR, France, ref. ANR-21-CE02-0002, Laboratoire d'Excellence CEBA, ref. ANR-10-LABX-25-01 and LabEx CeMEB, ref. ANR-10-LABX-04-01), and by the United States Department of Agriculture (USDA, USA, grant no. 2019-67015-28981; https://www.asf-nifnaf.org/).

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Keywords

Symbiosis; comparative genomics; evolutionary biology; phylogeny; ticks; bacterial communities

Figure captions

Figure 1: Phylogeny of Legionellales (Dataset 1) focusing on the Rickettsiella genus inferred with RAxML on 92 single copy panorthologous intact genes (100 bootstraps, evolutionary model: GTR+G4, rapid bootstrap random number seed: 1234, the random number seed for the parsimony inferences: 123), using B. aphidicola as outgroup to root the tree. Only bootstrap supports lower than 98 are shown. The Rickettsiella organisms whose genomes were sequenced in this study are indicated in bold. On the right, the presence (green), absence (grey) and pseudogenization (yellow) of genes of specific metabolic pathways annotated with KEGG is represented as a heatmap. B. aphidicola sequences were not annotated. On the bottom, Venn diagrams of clusters of orthologs of all encoded genes, intact genes, and pseudogenes is provided for the specified nodes of the tree (Node 10, Node 13, Node 15).

Figure 2: Intersection plot displaying shared and specific clusters of orthologous proteins between Rickettsiella and other Legionellales. Intersection plot is based on Dataset 1 and was produced with the R package UpSetR. In the left side, the horizontal bar plot represents the number of orthogroups present in each organism of the dataset. In the top, the vertical bar plot represents the number of orthogroups that belong to specific intersections of organisms in the dataset. The height of each bar indicates how many orthogroups are in that particular intersection, i.e., shared by the combination of organisms indicated by the intersection matrix below. In the intersection matrix, on the bottom, each row represents an organism and each column represents a possible intersection of these organisms. Filled circles indicate that the organisms in that row are part of the intersection shown in the corresponding column. The lines connecting the filled circles indicate which organisms are included in each specific intersection. This plot is limited to six orthogroups in the intersections, for the full plot of all intersections until one shared orthogroup please see Supplementary Figure 2.

Figure 3: Phylogeny and domain architecture composition of cifA and cifB homologs in Rickettsiella and other endosymbionts. The maximum-likelihood tree was reconstructed from an alignment concatenated with RNA-binding-like, AAA-ATPase-like, and PD-(D/E)XK nuclease domain sequences (191 amino acid positions, JTT+G substitution model). The tree is midpoint rooted. The type I-V cif representation, as well as the sizes and domain compositions of the cifA and cifB genes, were drawn relative to those described in Martinez et al. (2021, 2022). The scale bar represents the inferred number of substitutions per site. Bootstrap values were estimated from 1000 replicates. Accession numbers of the Rickettsiella, Wolbachia, and Rickettsia genomes used in this analysis are listed in Supplementary Table S4.

Table 1: Simplified scheme of presence (✓) and absence (X) of virulence factors categories in the organisms of Dataset 1. The organisms were grouped by genus, separating only Coxiella endosymbionts from the proved pathogenic relative C. burnetii. The virulence factor category was considered present if at least three of the representatives of the Rickettsiella strains and one of the Berkiella and Coxiella strains contained at least half of the virulence factors of that category. The search was performed using reference Legionella sequences from VFDB as queries in a local blast search (default settings, results were manually curated). Details on the annotation can be found in Supplementary table 6.

Supplementary Figure 1: barplot of intact genes and pseudogenes detected by PseudoFinder in each organism of Dataset 1. The height of each bar represents the number of intact genes (blue) and pseudogenes (magenta).

Supplementary Figure 2: 16S rRNA based phylogeny of Rickettsiella and other Legionellales. The dataset includes the sequences of Dataset 1 (indicated in bold), all the available Diplorickettsiales 16S rRNA sequences on SILVA RefNR SSU r138.1 (446 at 10th April 2024), and the 16S rRNA of the outgroup B. aphidicola. The phylogenetic inference was

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performed using RAxML (100 bootstraps, rapid bootstrap random number seed: 1234, random number seed for the parsimony inferences: 123, default setting for model of nucleotide substitution). The tree was pruned with iTOL collapsing nodes distantly related to the target strains from Dataset 1 (bootstrap supports of each node, including the collapsed ones, were maintained). The Rickettsiella strains sequenced in this study are indicated in bold red. The arrow indicates the phylogenetic cluster supported by Node 15 in the phylogenomics-based tree (Figure 1) and the arrowhead indicates the phylogenetic cluster supported by Node 10 in the phylogenomics-based tree (Figure 1).

Supplementary Figure 3: Intersection plot of Dataset 1 produced with the R package UpSetR, where all intersections are presented. In the left side, the horizontal bar plot represents the number of orthogroups present in each organism of the dataset. In the top, the vertical bar plot represents the number of orthogroups that belong to specific intersections of organisms in the dataset. The height of each bar indicates how many orthogroups are in that particular intersection, i.e., shared by the combination of organisms indicated by the intersection matrix below. In the intersection matrix, on the bottom, each row represents an organism and each column represents a possible intersection of these organisms. Filled circles indicate that the organisms in that row are part of the intersection shown in the corresponding column. The lines connecting the filled circles indicate which organisms are included in each specific intersection.

Supplementary Figure 4: Phylogenies of the thiamine genes present in Oerr and not in the other Rickettsiella strains of Dataset 1, in order: thiD, thiDE, thiO, thiG, thiS. Although it is not possible to clearly establish the evolutionary origin of these sequences, it is clear that they are of Gammaproteobacteria/Pseudomonadota origin, often found in Legionella and in two other Rickettsiella organisms (See also Supplementary Table 3).

Supplementary Table 1: Metadata, genome statistics and number of intact and pseudo- genes of the organisms included in Dataset 1. The ANI values for Dataset 1 and for the redundant genomes that were excluded from the analyses are also presented, as well as the mobile elements for each genome in Dataset 1.

Supplementary Table 2: Orthogroups.GeneCount.tsv contains the orthologous clusters matrix produced with OrthoFinder on the translated intact genes of Dataset 1 and on the translated gene sequences of the outgroup B. aphidicola. This matrix was used to select the clusters for the phylogenomics-based tree and for the intersection plots. In the following sheets, the intersections of clusters of specific organisms of the datasets are presented, together with their KEGG and Prokka annotations for one of the organisms in the intersection (sequence ID reported). SharedByAll: clusters of intact genes shared by all organisms in the dataset, i.e. Dataset 1 and B. aphidicola; AllNoBuchnera: shared by all Legionellales of Dataset 1 and not present in B. aphidicola; SharedByAllRickettsiella: shared by all Rickettsiella strains of Dataset 1; SharedAllIricinus: shared by all Rickettsiella strains isolated from I. ricinus included in Dataset 1; SharedAllIricinus_NoRmassiliensis: as SharedAllIricinus, but not present in the putative pathogen R. massiliensis 20B; Shared_MostPSstrains_IricRmassiliensis: shared exclusively by the Rickettsiella strains with the highest number of pseudogenes, i.e. R. massiliensis 20B and Rickettsiella strain Iric; SpSpecific_RDG: clusters exclusively present in the genome of the Rickettsiella endosymbiont of D. gallinae; SpSpecific_Rgrylli: clusters exclusively present in the genome of R. grylli; SpSpecific_Ird1: exclusively present in the genome of Rickettsiella strain Ird1; SpSpecific_Oerr: exclusively present in the genome of Rickettsiella strain Oerr; SpSpecific_Opha: exclusively present in the genome of Rickettsiella strain Opha; SpSpecific_Rmassiliensis: exclusively present in the genome of R. massiliensis 20B; SpSpecific_Rviridis: exclusively present in the genome of R. viridis.

Supplementary Table 3: KEGG annotation of the organisms of Dataset 1 obtained through BlastKoala (default settings). The column Knumbers indicates the KEGG ID. The values indicate presence (2), pseudogenization (1), and absence (0) in each organism. The sheets Summary NutrMutPaths, NutrMutPaths and EnergyPaths are focused on specific metabolic pathways potentially involved in nutritional mutualisms (B vitamins and heme metabolism, energy production); in these tables, “B”s mean null values in correspondence of the pathway names and were inserted as information for the script to obtain the heatmap shown in Figure 1. Thiamine_Oerr contains the amino acid sequences of the thiamine pathway components identified in Rickettsiella strain Oerr and not in the other Rickettsiella strains included in Dataset 1, together with the five best-hits of blast against the non-redundant NCBI database, ordered by identity percentage (ID of the hit sequences, identity percentage, query cover, E value and available taxonomic annotation of the hit organism reported). The organisms and their sequence accession number used to infer the

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phylogenies of the Oerr thiamine genes (Supplementary Figure 4) are reported in the sheet Thiamine Phylogenies datasets.

Supplementary Table 4: Accession number of the Rickettsiella, Wolbachia, and Rickettsia genomes from public databases used to screen the cifA and cifB genes pair homologs.

Supplementary Table 5: Intersections of clusters of all encoded sequences, all intact sequences, and all pseudogenes of specific phylogenetic clades represented in Figure 1. A reference sequence ID is provided for each cluster, together with the KEGG (obtained with BlastKoala, default settings) and Prokka annotation.

Supplementary Table 6: Annotation of putative virulence factors in the organisms of Dataset 1, annotated using amino acid Legionella queries downloaded from VFDB as a reference in a local blast search (default parameters) against translated all genes of the organisms of Dataset 1. Sequence IDs are reported for each organism, cells are highlighted in yellow when the sequence was identified as a pseudogene by PseudoFinder. The hyphen ("-") indicates complete absence of the sequence.

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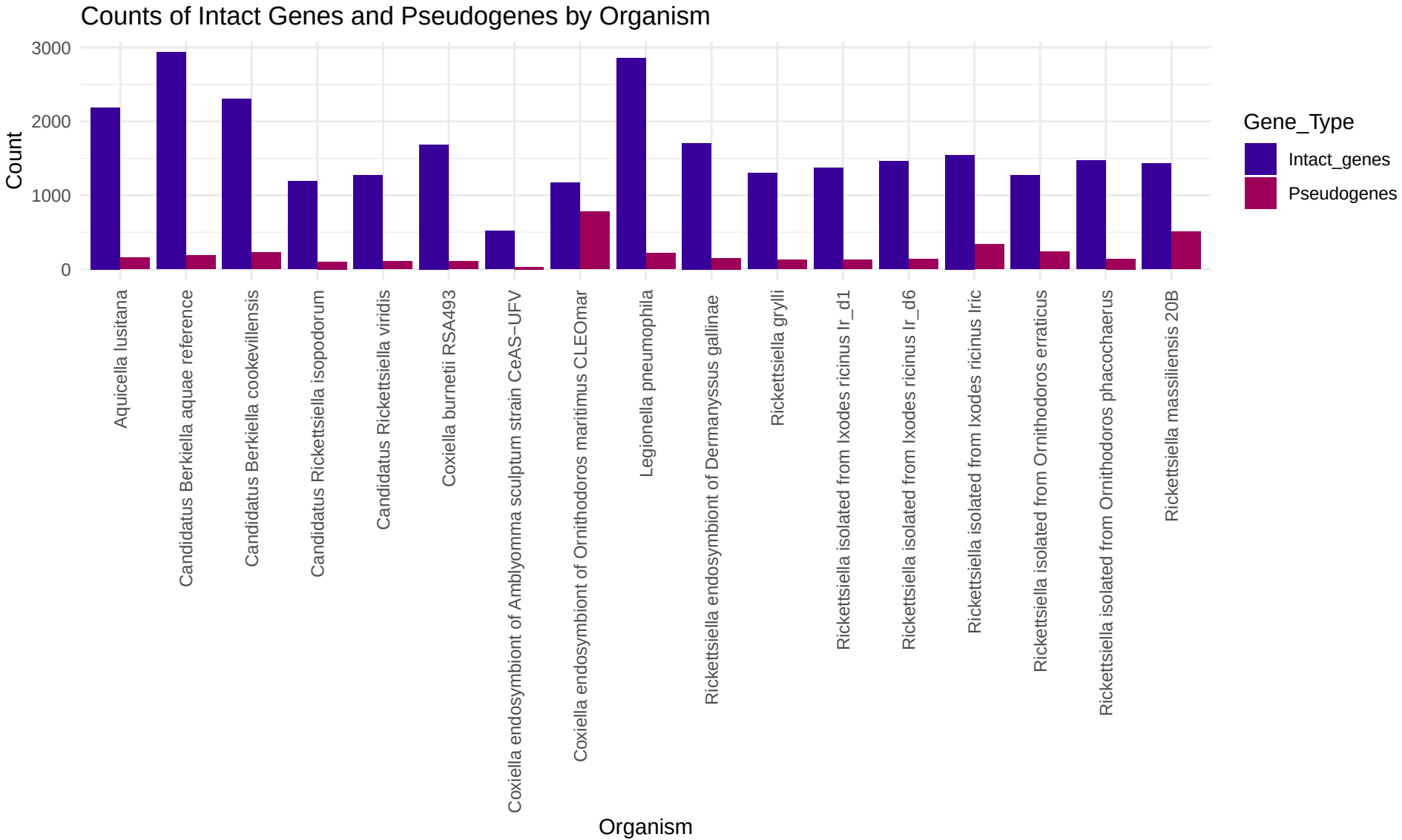
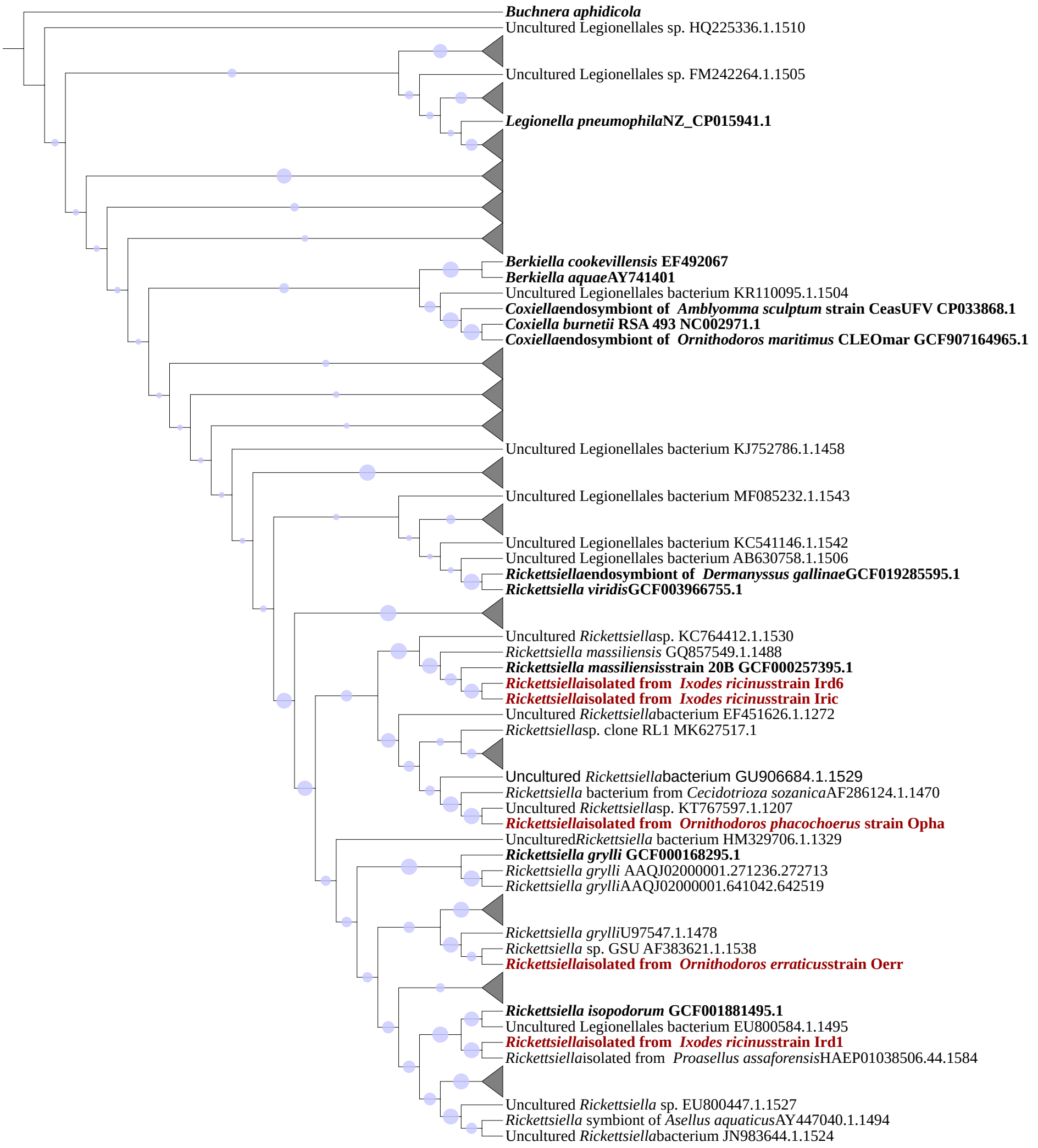
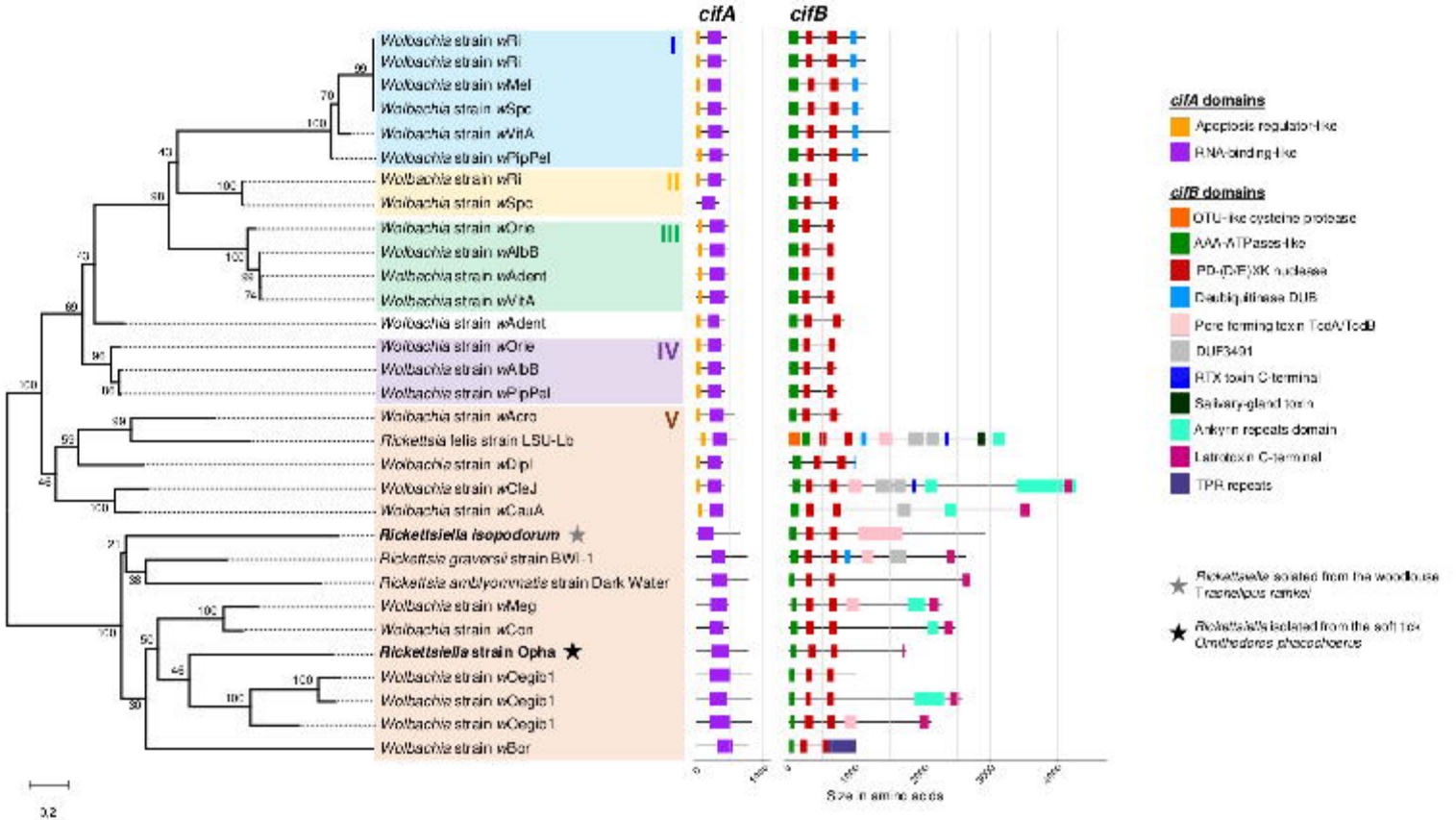

Counts of Intact Genes and Pseudogenes by Organism
Count
3000
2000
1000
0
Aquicella lusitana
Candidatus Berkiella aquae reference
Candidatus Berkiella cookevillensis
Candidatus Rickettsiella isopodorum
Candidatus Rickettsiella viridis
Coxiella burnetii RSA493
Coxiella endosymbiont of Amblyomma sculptum strain CeAS−UFV
Coxiella endosymbiont of Ornithodoros maritimus CLEOmar
Legionella pneumophila
Rickettsiella endosymbiont of Dermanyssus gallinae
Rickettsiella grylli
Rickettsiella isolated from Ixodes ricinus Ir_d1
Rickettsiella isolated from Ixodes ricinus Ir_d6
Rickettsiella isolated from Ixodes ricinus Iric
Rickettsiella isolated from Ornithodoros erraticus
Rickettsiella isolated from Ornithodoros phacochaerus
Rickettsiella massiliensis 20B
Organism
Gene_Type
Intact_genes
Pseudogenes


Buchnera aphidicola
Uncultured Legionellales sp. HQ225336.1.1510
Uncultured Legionellales sp. FM242264.1.1505
Legionella pneumophilaNZ_CP015941.1
Berkiella cookevillensis EF492067
Berkiella aquaeAY741401
Uncultured Legionellales bacterium KR110095.1.1504
Coxiellaendosymbiont of Amblyomma sculptum strain CeasUFV CP033868.1
Coxiella burnetii RSA 493 NC002971.1
Coxiellaendosymbiont of Ornithodoros maritimus CLEOmar GCF907164965.1
Uncultured Legionellales bacterium KJ752786.1.1458
Uncultured Legionellales bacterium MF085232.1.1543
Uncultured Legionellales bacterium KC541146.1.1542
Uncultured Legionellales bacterium AB630758.1.1506
Rickettsiellaendosymbiont of Dermanyssus gallinaeGCF019285595.1
Rickettsiella viridisGCF003966755.1
Uncultured Rickettsiellasp. KC764412.1.1530
Rickettsiella massiliensis GQ857549.1.1488
Rickettsiella massiliensisstrain 20B GCF000257395.1
Rickettsiellaisolated from Ixodes ricinusstrain Ird6
Rickettsiellaisolated from Ixodes ricinusstrain Iric
Uncultured Rickettsiellabacterium EF451626.1.1272
Rickettsiellasp. clone RL1 MK627517.1
Uncultured Rickettsiellabacterium GU906684.1.1529
Rickettsiella bacterium from Cecidotrioza sozanicaAF286124.1.1470
Uncultured Rickettsiellasp. KT767597.1.1207
Rickettsiellaisolated from Ornithodoros phacochoerus strain Opha
UnculturedRickettsiella bacterium HM329706.1.1329
Rickettsiella grylli GCF000168295.1
Rickettsiella grylli AAQJ02000001.271236.272713
Rickettsiella grylliAAQJ02000001.641042.642519
Rickettsiella grylliU97547.1.1478
Rickettsiella sp. GSU AF383621.1.1538
Rickettsiellaisolated from Ornithodoros erraticusstrain Oerr
Rickettsiella isopodorum GCF001881495.1
Uncultured Legionellales bacterium EU800584.1.1495
Rickettsiellaisolated from Ixodes ricinusstrain Ird1
Rickettsiellaisolated from Proasellus assaforensisHAEP01038506.44.1584
Uncultured Rickettsiella sp. EU800447.1.1527
Rickettsiella symbiont of Asellus aquaticusAY447040.1.1494
Uncultured Rickettsiellabacterium JN983644.1.1524


cifA
cifB
Wolbachia strain wRi
Wolbachia strain wRi
Wolbachia strain wMel
Wolbachia strain wSpc
Wolbachia strain wVitA
Wolbachia strain wPipPel
Wolbachia strain wRi
Wolbachia strain wSpc
Wolbachia strain wOri
Wolbachia strain wAlbB
Wolbachia strain wAdent
Wolbachia strain wVitA
Wolbachia strain wAdent
Wolbachia strain wOri
Wolbachia strain wAlbB
Wolbachia strain wPipPel
Wolbachia strain wAcro
Rickettsia felis strain LSU-Lb
Wolbachia strain wDpl
Wolbachia strain wCleJ
Wolbachia strain wCauA
Rickettsiella isopodorum
Rickettsia gravesii strain BWI-1
Rickettsiella endosymbiont strain Dark Water
Wolbachia strain wMeg
Wolbachia strain wCon
Rickettsiella strain Opha
Wolbachia strain wOegic1
Wolbachia strain wOegic1
Wolbachia strain wOegic1
Wolbachia strain wBor
I
II
III
IV
V
0.2
Size in amino acids
cifA domains
Apoptosis regulator-like
RNA-binding-like
cifB domains
OTU-like cysteine protease
AAA-ATPases-like
PD-(D/E)XK nuclease
Deubiquitinase DUB
Pore forming toxin TcdA/TcdB
DUF3401
RTX toxin C-terminal
Salivary-gland toxin
Ankyrin repeats domain
Latrotoxin C-terminal
TPR repeats
Rickettsiella isolated from the woodlouse Trachelipus rathke
Rickettsiella isolated from the soft tick Ornithodoros phacochoerus