The Genome Sequence of "Candidatus Fokinia solitaria": Insights on Reductive Evolution in Rickettsiales

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Abstract
“Candidatus Fokinia solitaria” is an obligate intracellular endosymbiont of a unicellular eukaryote, a ciliate of the genus Paramecium. Here, we present the genome sequence of this bacterium and subsequent analysis. Phylogenomic analysis confirmed the previously reported positioning of the symbiont within the “Candidatus Midichloriaceae” family (order Rickettsiales), as well as its high sequence divergence from other members of the family, indicative of fast sequence evolution. Consistently with this high evolutionary rate, a comparative genomic analysis revealed that the genome of this symbiont is the smallest of the Rickettsiales to date. The reduced genome does not present flagellar genes, nor the pathway for the biosynthesis of lipopolysaccharides (present in all the other so far sequenced members of the family “Candidatus Midichloriaceae”) or genes for the Krebs cycle (present, although not always complete, in Rickettsiales). These results indicate an evolutionary trend toward a stronger dependence on the host, in comparison with other members of the family. Two alternative scenarios are compatible with our results; “Candidatus Fokinia solitaria” could be either a recently evolved, vertically transmitted mutualist, or a parasite with a high host-specificity.

Key words: genome reduction, bacterial symbiont, Paramecium, Midichloriaceae, blobology.

Introduction
Bacterial endosymbionts that live within eukaryotic cells establish highly diverse relationships with their hosts, ranging from mutualism to parasitism. Furthermore, obligate intracellular endosymbionts, which have become host-dependent, tend to experience reductive genome evolution (McCutcheon and Moran 2011). Such phenomenon is observed in bacteria with very different behaviors, from the infectious pathogen Chlamydia (Nunes and Gomes 2014) to the noninfectious, vertically transmitted mutualist Buchnera (Shigenobu et al. 2000). In the case of mutualist bacteria, especially for those that provide metabolites necessary for the hosts’ survival, genomes can undergo extreme reduction, pushing toward the limit of becoming an organelle—for example, “Candidatus Tremblaya princeps” genome is ~139 kb, and the mtDNA of Reclinomonas americana is ~69 kb (Sagan 1967; Schwartz and Dayhoff 1978; McCutcheon and Moran 2007, 2011). On the other side, genomic shrinkage can occur also in infectious parasites or pathogens, and, in this case, it can be considered a signature of host specificity (Moran 2002; Dagan et al. 2006; Bäumler and Fang 2013).

The order Rickettsiales encompasses intracellular bacteria that infect highly diverse hosts. Rickettsiales, as most intracellular bacteria, possess small genomes, however they do not show extreme reduction. Their genomes are mainly in the range of 1–1.5 Mb (Darby et al. 2007; Gillespie et al. 2012). Most Rickettsiales show the capability to transfer horizontally,
either as a part of their life cycle (Vaughan et al. 2012; Dumler and Walker 2015; Schulz et al. 2016; Senra et al. 2016), or in their recent evolutionary history (Epis et al. 2008; Gillespie et al. 2012; Castelli et al. 2016). Some Rickettsiales are pathogenic to humans or other vertebrates (Dumler and Walker 2015; Thomas et al. 2016), while others have a strong interdependence with their host (Comandatore et al. 2013; Nikoh et al. 2014). However, often the relationships between Rickettsiales and their hosts are not easily classified or are yet to be explained.

“Candidatus (Ca.) Midichloriaceae” (hereafter Midichloriaceae) is the most recently described family of the order Rickettsiales (according to the definition by Szokoli et al. 2016a). Its members are widespread and live in association with diverse hosts, including amoebae, ticks, corals, flagellates, fish, and mammals (Montagna et al. 2013; Senra et al. 2016; Szokoli et al. 2016b). However, only three genomes, derived from two species, of the family are available on databases (Sassera et al. 2011; Wang and Wu 2014a; Schulz et al. 2016). “Ca. Fokinia solitaria” (hereafter, F. solitaria), the first representative of a novel genus within the family, was recently detected in the ciliate Paramecium sp. collected from a wastewater treatment plant in Rio de Janeiro, Brazil (Szokoli et al. 2016b). Ciliates are unicellular eukaryotes that frequently harbor endosymbiotic bacteria (Fokin 2004; Schweikert et al. 2013), including several Rickettsiales (Castelli et al. 2016). Endosymbionts of ciliates may entertain a wide range of relationships with their hosts, including necessary mutualists (Vannini et al. 2012) and parasites (Kalz and Koella 2003). Nevertheless, in most cases the effect on host fitness has not been yet elucidated, although in some conditions an apparent benefit for the host was observed (Soldo and Godoy 1973; Kusch et al. 2013; Nikoh et al. 2014). The obtained results with the manually curated annotation.

Materials and Methods

Total DNA was extracted from a culture of Paramecium sp. strain Rio ETE_ALG 3VII using a modified CTAB procedure (Gustinich, et al. 1991). Prior to DNA extraction, cells were starved for 2 days and filtered through eight layers of sterile gauze before cell-harvesting via gentle centrifugation (Szokoli et al. 2016b). After DNA quality check, sequencing was performed using Illumina HiSeq 2500, to generate 14,783,394 150-nt paired-end reads. Reads were assembled using SPAdes v3.9.0 (Bankevich et al. 2012; Nurk et al. 2013), and the resulting preliminary assembly was subject to the blobology pipeline (Kumar et al. 2013), to select the putative F. solitaria reads based on contig coverage, GC%, and taxonomy. The contigs belonging to the 100–1,000× coverage, estimated using Bowtie2 (Langmead and Salzberg 2012), and >30 GC% on the blobology plot were thus selected (Supplementary fig. S1, Supplementary Material online). In parallel, contigs outside this range were scanned to detect potential signatures suggesting they could belong to the F. solitaria genome, performing a BLAST search using a database of Rickettsiales genomes, and manually inspecting contigs showing significant hits (e-value <10−5). In addition, the extracted DNA was subjected to Pacific Biosciences Sequel sequencing, obtaining 12,243,617 reads. All PacBio reads and the selected Illumina reads were used to perform a second assembly step using the software Unicycler (Wick et al. 2017). The softwares Bandage (Wick et al. 2015) and BLAST were then used to manually perform bioinformatic joins on the result of this second assembly. The obtained genome was annotated using Prokka (Seemann, 2014) followed by extensive manual curation.

A data set of 13 Rickettsiales and 4 outgroup organisms was constructed (Supplementary table S7, Supplementary Material online), selecting all other available Midichloriaceae genomes (n= 3), a similar number of Anaplasmataceae genomes (n= 5) and Rickettsiaceae (n= 4), more closely related (n= 2) and more distant (n= 2) outgroups. The data set was used to infer the phylogenetic position of F. solitaria through two different approaches. In the phylogenomic approach, we identified conserved orthologs using OrthoMCL (Li et al. 2003) (inflation value: 1.1; %identity cutoff: 30). Subsequently, the orthologous protein sequences were aligned using Muscle (Edgar 2004), polished with Gblocks (Talavera and Castresana 2007) and used to build a phylogenomic tree using the software RAxML (Stamatakis 2015), with 100 bootstraps (LG +I+G+F model inferred by using ProtTest 3.4.2; Darriba et al. 2011, sorting models according to AIC and BIC). In the conserved genes data set approach, protein sequences of 24 highly conserved genes, previously selected to give a stable phylogenetic signal were utilized (Lang et al. 2013), aligned with Muscle and used to build a phylogenomic tree using RAxML, with 100 bootstraps (LG +I+G+F model inferred by using ProtTest 3.4.2, sorting models according to AIC and BIC). COGs of the available Midichloriaceae genomes were annotated using the NCBI COG pipeline (Galperin et al. 2015). The identified COGs were compared and graphically reported in a Venn diagram using the R software (R Core Team 2015). Metabolic pathway analysis was performed using the BioCyc and Pathway Tools suites (Karp et al. 2015; Caspi et al. 2016). Presence of insertion sequences, prophages, secretion systems, and secreted proteins was predicted using, respectively, ISSaga (Varani et al. 2011), PHAST (Zhou et al. 2011), TXSScan (MacSyFinder-based; Abby et al. 2014), SignalP (Emanuelsson et al. 2007), and TMHMM (Krogh et al. 2001) (with default parameters) and comparing the obtained results with the manually curated annotation.
positive in green, negative in purple. Position 1 was arbitrarily set as the strand in green; tRNAs in orange and rRNAs in fuchsia; the GC skew, sequences (CDSs) on the plus strand in light blue; CDSs on the minus strand in light blue; CDSs on the minus strand in light blue.

Circles, from the outermost to the innermost show, respectively, coding regions (CDSs) on the plus strand in light blue; CDSs on the minus strand in green; tRNAs in orange and rRNAs in fuchsia; the GC skew, positive in green, negative in purple. Position 1 was arbitrarily set as the start of the dnaA gene.

**Results and Discussion**

The complete genome of *F. solitaria* (fig. 1) is contained in a single circular chromosome. With a size of 837,348 bp, this is the smallest reported genome in the order *Rickettsiales* (table 1 and supplementary table S1, Supplementary Material online). On the other hand, although *F. solitaria* is long in both trees, again consistent with the previously published 16S rRNA gene trees (Martijn et al. 2015; Tully et al. 2016). The published, but such sequences were estimated by the authors to be incomplete (Martijn et al. 2015; Tully et al. 2016). The main genomic characteristics of *F. solitaria*, including GC content and average gene length, are encompassed within the range of *Rickettsiales* diversity. The genome contains just two insertion sequences and a paucity of phagic sequences, again characteristics similar to many other *Rickettsiales* genomes (table 1 and supplementary table S1, Supplementary Material online). On the other hand, although *F. solitaria* presents the smallest genome in the *Rickettsiales* order, it does not show features reported for highly reduced genomes, such as very high coding density (often >92%) or overlapping genes (Mira et al. 2001; McCutcheon and Moran 2011). Therefore, *F. solitaria* should be considered at a similar stage of genome reduction as the other *Rickettsiales*, and not at the more extreme stage of reduction found in other endosymbiont lineages.

Phylogenomic analyses were performed from a data set of 13 *Rickettsiales* genomes and 4 outgroup genomes (2 *Holosporales*, 1 *Rhodospirillales*, and 1 *Caulobacterales*). Two sets of genes were retrieved, one encompassing 75 orthologs present in all taxa, another including 24 highly conserved proteins (Lang et al. 2013). The two resulting trees present identical topologies (fig. 2 and supplementary fig. S1, Supplementary Material online). They are in agreement with previous 16S rRNA gene phylogenies (Montagna et al. 2013; Senra et al. 2016; Szokoli et al. 2016b) and to recent phylogenomic studies (Driscoll et al. 2013; Wang and Wu 2014b, 2015) in placing the *Midichloriaceae* as sister group of the *Anaplasmataceae*, and in positioning *F. solitaria* within the *Midichloriaceae* as early diverging respect to the *Midichloria* and *Jidaibacter* clade. The branch leading to *F. solitaria* is long in both trees, again consistent with the previously published 16S rRNA gene trees. This result provides additional evidence to strengthen the hypothesis that *F. solitaria* could be subjected to an accelerated rate of molecular evolution in relation to other *Rickettsiales* (Szokoli et al. 2016b). It is tempting to speculate a correlation between the hypothesized fast evolving nature of *F. solitaria* and its reduced genome size, possibly indicating an instance of the general trend of fast evolution and high genome reduction of bacterial symbionts, caused by genetic drift.

An ortholog search to compare the genome of *F. solitaria* with that of the three other available *Midichloriaceae* genomes revealed that *F. solitaria* possesses 32 COGs that are not present in other genomes of the family (Venn diagram representation in fig. 3). Some of these COGs are related to functions that are putatively connected with host interaction, but no clear complete pathway or structure was found (see supplementary table S2, Supplementary Material online). A total of 435 COGs are shared by all the considered *Midichloriaceae* (supplementary table S3, Supplementary Material online), while 208 orthologs are conserved in the three other available *Midichloriaceae* genomes, but absent in *F. solitaria* (supplementary table S4, Supplementary Material online).

A detailed inspection of the ortholog comparison, and of the *F. solitaria* genome repertoire, indicates that its predicted metabolic capabilities are a reduced version of other *Midichloriaceae*, consistent with the small genome size. From the energy metabolism point of view, the main difference is the complete absence of the Krebs cycle. This is, to our knowledge, a unique case among all *Rickettsiales*, even though other members of the order possess an incomplete Krebs cycle (Min et al. 2008). Nevertheless, some capability to perform oxidative phosphorylation is retained (NADH: quinone dehydrogenase, cytochrome bd ubiquinol oxidase, and ATP synthase complexes are present, but cytochrome c reductase and oxidase complexes are not), likely exploiting NADH derived from glycolysis. Genes for biosynthetic pathways are also scarce: just two amino acids (alanine and glycine), some cofactors (biotin, folate, ubiquinone, Fe-S clusters, and, partially, coenzyme A), and no nucleotides can be produced. Additionally, only partial methylethylthiol phosphate pathway for isoprenoid biosynthesis is present, while the
pentose phosphate pathway is likely absent (only one gene of the entire pathway appears to be present). The presence of two ATP/ADP translocases probably enables *F. solitaria* to scavenge energy and nucleotides directly from the host. Other *Midichloriaceae* are overall richer in such pathways, especially in TCA cycle, cytochrome c oxido-reductases, non-oxidative pentose phosphate pathway, amino acids (five more) (Sassera et al. 2011; Wang and Wu 2014a; Schulz et al. 2016). For what concerns cell structures, the genome sequence indicates that *F. solitaria* is able to synthesize membrane phospholipids and cell wall peptidoglycan. The capability of producing acetyl-CoA, thanks to the pyruvate dehydrogenase complex, is probably useful only for these biosynthetic pathways (since the Krebs cycle is missing), a situation possibly comparable to other endosymbionts of *Paramecium* (Dohra et al. 2014). On the other hand, lipopolysaccharide (LPS) biosynthesis appears to be fully missing, differently from other *Midichloriaceae*, but similarly to all

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**Table 1**

Genomic Characteristics of Selected Members of the Order *Rickettsiales*

| Organism                        | GC % | Genome Size (Mb) | Predicted IS Number | Coding Percentage | Family           |
|---------------------------------|------|------------------|---------------------|------------------|------------------|
| *Fokinia solitaria*             | 35.8 | 0.83             | 2                   | 88.54            | *Midichloriaceae*|
| *Neorickettsia sennetsu*        | 41.1 | 0.86             | 1                   | 85.20            | *Anaplasmataceae*|
| *Neorickettsia risticii*        | 41.3 | 0.88             | 1                   | 85.08            | *Anaplasmataceae*|
| *Neorickettsia helminthoeca*    | 41.7 | 0.88             | 1                   | 87.49            | *Anaplasmataceae*|
| *Wolbachia* wOv                 | 32.1 | 0.96             | 7                   | 72.42            | *Anaplasmataceae*|
| *Rickettsia prowazekii*         | 29   | 1.11             | 6                   | 77.12            | *Rickettsiaceae*  |
| *Rickettsia typhi*              | 28.9 | 1.11             | 0                   | 75.95            | *Rickettsiaceae*  |
| *Ehrlichia chaffeensis*         | 30.1 | 1.18             | 0                   | 77.84            | *Anaplasmataceae*|
| *Midichloria mitochondrii*      | 36.6 | 1.18             | 109                 | 79.17            | *Midichloriaceae*|
| *Wolbachia* wMel                | 35.2 | 1.24             | 138                 | 87.41            | *Anaplasmataceae*|
| *Rickettsia rickettsii*         | 32.5 | 1.27             | 7                   | 82.86            | *Rickettsiaceae*  |
| *Wolbachia* wNo                 | 34   | 1.30             | 136                 | 88.31            | *Anaplasmataceae*|
| *Rickettsia australis*          | 32.3 | 1.30             | 34                  | 83.40            | *Rickettsiaceae*  |
| *Anaplasma marginale*           | 49.8 | 1.47             | 0                   | 70.15            | *Anaplasmataceae*|
| *Anaplasma phagocytophilum*     | 41.6 | 1.50             | 0                   | 69.62            | *Anaplasmataceae*|
| *Rickettsia bellii*             | 31.6 | 1.52             | 47                  | 85.93            | *Rickettsiaceae*  |
| *Jidaibacter acanthamoeba* UWC8 | 34.8 | 1.62             | 19                  | 89.21            | *Midichloriaceae*|
| *Rickettsia endos. krodes scapularis* | 33.3 | 1.82         | 547                 | 90.56            | *Rickettsiaceae*  |
| *Orientia tsutsugamushi*        | 30.4 | 2.13             | 537                 | 79.15            | *Rickettsiaceae*  |
| *Jidaibacter acanthamoeba* UWC36 | 33.7 | 2.37             | 0                   | 80.31            | *Midichloriaceae*|

**Fig. 2.**—Phylogenomic tree showing the relationships between selected members of the order *Rickettsiales*, and four outgroup genomes. Tree was obtained from an alignment of 75 orthologous proteins analyzed with RAXML with 100 bootstraps. Bootstrap values are shown above each node. Scale bar stands for estimated sequence divergence.
members of the family Anaplasmataceae, where this trait is supposed to confer a specific advantage, probably helping to evade host defense mechanisms (Lin and Rikihisa 2007). This condition is, on the other hand, also found in mutualists such as Buchnera (Shigenobu et al. 2000), and could suggest a convergence in pathway loss in strongly reduced genomes.

A rich set of membrane transporters appears to be present in the genome of F. solitaria (such as ATP-binding cassette and major facilitator superfamily), and could complement metabolic deficiencies enabling direct uptake of small molecules from the host. The presence of multiple secretion systems (supplementary table S5, Supplementary Material online) and putative secreted proteins (supplementary table S6, Supplementary Material online), including an ankyrin repeat protein, likely enables F. solitaria to establish and regulate interactions with the host (Al-Khodor et al. 2010). Surprisingly, many of these annotated proteins are typically intracellular. On the other hand, among rickettsial species, there is increasing evidence for the surface localization of proteins that typically function in the bacterial cytoplasm or periplasm, suggesting “moonlighting” functions on cell surfaces of divergent rickettsial species (Gillespie et al. 2015).

Genes coding for flagellar proteins were not detected in the genome. While this result is in accordance with the electron microscopy observations of F. solitaria, it represents a difference with the other members of the family Midichloriaceae. All the three other available genomes, and even the partial genome of the symbiont of Trichoplax adhaerens (Driscoll et al. 2013) code for flagellar genes, even though our knowledge no direct detection of flagellar structures has been reported for these organisms. Moreover, genes that are typically involved in host cell invasion in several bacteria, such as pili or adhesins, are not present in the F. solitaria genome, a feature consistent with the available genome reports of the other Midichloriaceae.

In summary, the small genome of F. solitaria shows the absence of important genes in comparison with the other Midichloriaceae (i.e., Krebs cycle, cytochrome c reductase and oxidase, pentose phosphate pathway, LPS, flagella, synthesis of five amino acids), and the phylogenomic analyses confirm a fast-evolving genome. These results clearly indicate a stronger dependence on the host compared with other so far sequenced Midichloriaceae. It must be noted that some members of the two other Rickettsiales families also display reduction of metabolic capability in a variable and lineage-specific fashion, for example, Rickettsia spp. and Orientia tsutsugamushi are extremely scarce in biosynthetic pathways (Driscoll et al. 2017). In comparison to other metabolically depleted Rickettsiales genomes, we can observe in F. solitaria a different pathway specificity, which might be possibly related to differences in host organisms and interaction. However, the effect of F. solitaria on the host and the dynamics of transmission are unclear. The relatively higher genome reduction could be compatible with a recently evolved, vertically transmitted mutualist or, by contrast, with a parasite with some degree of host specificity. Some genomic traits, such as the absence of LPS biosynthesis and flagella, may support both hypotheses, while others are in favor of either of the two interpretations. For example, synthesized cofactors could provide a beneficial effect to the host Paramecium. By contrast, the previously observed autophagosomal lysis of F. solitaria by its host (Szokoli et al. 2016b) may indicate a parasitic behavior of the bacterium, which is supported by the presence of genes coding for ATP/ADP translocases. Further comparative and experimental analyses may help to elucidate the behavior of F. solitaria in Paramecium.

Supplementary Material
Supplementary data are available at Genome Biology and Evolution online.

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Literature Cited
Abby SS, Néron B, Ménager H, Touchon M, Rocha EPC. 2014. MacSyFinder: a program to mine genomes for molecular systems with an application to CRISPR-Cas systems. PLoS One 9(10):e110726.
Al-Khodr S, Price CT, Kalia A, Kwaik YA. 2010. Ankyrin-repeat containing proteins of microbes: a conserved structure with functional diversity. Trends Microbiol. 18(3):132–139.

Bankevich A, et al. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 19(5):455–477.

Bäumler A, Fang FC. 2013. Host specificity of bacterial pathogens. Cold Spring Harb Perspect Med. 3(12):a010041.

Bella C, et al. 2016. Fitness impact of obligate intranuclear bacterial symbionts depends on host growth phase. Front Microbiol. 7:2084.

Darriba D, Taboada GL, Doallo R, Posada D. 2011. ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 27(3):3760–3762.

Caspi R, et al. 2016. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res. 44(D1):D471–D480.

Castelli M, Sassera D, Petroni G. 2016. Biodiversity of ‘non-model’ Rickettsiales and their association with aquatic organisms. In: Thomas S, editor. Rickettsiales. Cham, Switzerland: Springer International Publishing. p. 59–91.

Comandatore F, et al. 2013. Phylogenomics and analysis of shared genes suggest a single transition to mutualism in Wolbachia of nematodes. Genome Biol Evol. 5(9):1668–1674.

Dagan T, Bekhman R, Graur D. 2006. The ‘domino theory’ of gene death: gradual and mass gene extinction events in three lineages of obligate symbiotic bacterial pathogens. Mol Biol Evol. 23(2):310–316.

Darby AC, Cho N-H, Fuxelius HH, Westberg J, Andersson SGE. 2007. Intracellular pathogens go extreme: genome evolution in the Rickettsiales. Trends Genet. 23(10):511–520.

Darriba D, Taboada GL, Doallo R, Posada D. 2011. ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 27(8):1164–1165.

Dohra H, Tanaka K, Suzuki T, Fujishima M, Suzuki H. 2014. Draft genome sequences of three Holospora species (Holospora abutusa, Holospora undulata, and Holospora elegans), endonuclear symbiotic bacteria of the ciliate Paramecium caudatum. FEMS Microbiol Lett. 359(1):16–18.

Dohra H, Tanaka K, Suzuki T, Fujishima M, Suzuki H. 2014. Draft genome sequences of three Holospora species (Holospora abutusa, Holospora undulata, and Holospora elegans), endonuclear symbiotic bacteria of the ciliate Paramecium caudatum. FEMS Microbiol Lett. 359(1):16–18.

Dohra H, Tanaka K, Suzuki T, Fujishima M, Suzuki H. 2014. Draft genome sequences of three Holospora species (Holospora abutusa, Holospora undulata, and Holospora elegans), endonuclear symbiotic bacteria of the ciliate Paramecium caudatum. FEMS Microbiol Lett. 359(1):16–18.

Driscoll T, Gillespie JJ, Nordberg EK, Azad AF, Sobral BW. 2013. Bacterial DNA sifted from the Hostome sequences of three Rickettsiales. Genome Biol. 14(4):R57.

Dunning Hotopp JC, et al. 2006. Comparative genomics of emerging pathogens. Microbiol Mol Biol Rev. 70(1):37–61.

Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc. 2(4):953–971.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32(5):1792–1797.

Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc. 2(4):953–971.

Epistasis S, et al. 2008. Midichloria mitochondrii is widespread in hard ticks (Ixodidae) and resides in the mitochondria of phylogenetically diverse species. Parasitology 135(4/5):485–494.

Fokin SI. 2008. Bacterial endocytobionts of ciliophora and their interactions with the host cell. Int Rev Cytopathol. 236:181–249.

Galperin MY, Makarova KS, Wolf YI, Koonin EV. 2015. Expanded microbial genome coverage and improved protein family annotation in the COG database. Nucleic Acids Res. 43(3):D261–D269.

Gillespie JJ, et al. 2015. Secretome of obligate intracellular Rickettsia. FEMS Microbiol Rev. 39(1):47–80.

Gustincich S, Manfioletti G, Del Sal G, Schneider C, Carninci P. 1991. A fast method for high-quality genomic DNA extraction from whole human blood. BioTechniques 11(3):298–302.

Kaltz O, Koella JC. 2003. Host growth conditions regulate the plasticity of horizontal and vertical transmission in Holospora undulata, a bacterial parasite of the protozoan Paramecium caudatum. Evolution 57(7):1535–1542.

Karp PD, et al. 2015. Computational metabolomics operations at BioCyc.org. Metabolites 5(2):291–310.

Krogh A, Larsson B, Von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. J Mol Biol. 305(3):567–580.

Kumar S, Jones M, Koutsouvolou G, Clarke M, Blaxter M. 2013. Blobology: exploring raw genome data for contaminants, symbionts and parasites using taxon-annotated GC-coverage plots. Front Genet. 4:237.

Kusch J, et al. 2002. Competitive advantages of Caedibacter-infected Paramecia. Protist 153(1):47–58.

Lang JM, Darling AE, Eisen JA. 2013. Phylogeny of bacterial and archaeal genomes using conserved genes: superubacteria and supermatrices. PLoS One 8(4):e62510.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9(4):357–359.

Li L, Stoeckert CJ, Roos DS. 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 13(9):2178–2189.

Lin M, Rikhisha Y. 2007. Degradation of p22phox and inhibition of superoxide generation by Ehrlichia chaffeensis in human monocyes. Cell Microbiol. 9(4):861–874.

Martin J, et al. 2015. Single-cell genomics of a rare environmental alphaproteobacterium provides unique insights into Rickettsiaaceae evolution. ISME J. 9(11):2373–2385.

McCutcheon JP, Moran NA. 2011. Extreme genome reduction in symbiotic bacteria. Nat Rev Microbiol. 10(1):13–26.

McCutcheon JP, Moran NA. 2007. Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proc Natl Acad Sci U S A 104(49):19392–19397.

Min CK, et al. 2008. Genome-based construction of the metabolic pathways of Orientia tsutsugamushi and comparative analysis within the Rickettsiales Order. Comp Funct Genomics 2008:1.

Mira A, Ochman H, Moran NA. 2001. Deletional bias and the evolution of bacterial genomes. Trends Genet. 17(10):589–596.

Montagna M, et al. 2013. ‘Candidatus Midichloriaeae’ fam. nov. (Rickettsiales), an ecologically widespread clade of intracellular alphaproteobacteria. Appl Environ Microbiol. 79(10):3241–3248.

Moran NA. 2002. Microbial minimalism. Cell 108(5):583–586.

Nikoh N, et al. 2014. Evolutionary origin of insect-Wolbachia nutritional mutualism. Proc Natl Acad Sci U S A 111(28):10257–10262.

Nunes A, Gomes JP. 2014. Evolution, phylogeny, and molecular epidemiology of Chlamydia. Infect Genet Evol. 23:49–64.

Nutu S, et al. 2013. Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. J Comput Biol. 20(10):714–737.

R Core Team 2015. R: a language and environment for statistical computing. R Foundation for Statistical Computing.

Sagan L. 1967. On the origin of mitosing cells. J Theor Biol. 14(3):225–256.

Sassera D, et al. 2011. Phylogenomic evidence for the presence of a flagellum and cbb3 oxidase in the free-living mitochondrial ancestor. Mol Biol Evol. 28(12):3285–3296.

Schulz F, et al. 2016. A Rickettsiales symbiont of amoebae with ancient features. Environ Microbiol. 18(8):2326–2342.
Schwartz RM, Dayhoff MO. 1978. Origins of prokaryotes, eukaryotes, mitochondria, and chloroplasts. Science 199(4327):395–403.
Schweikert M, Fujishima M, Göritz H-D. 2013. Symbiotic associations between ciliates and prokaryotes. In: Rosenberg E, et al., editors. The prokaryotes. Berlin, Germany: Springer-Verlag, p. 427–463.
Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30(14):2068–2069.
Sena MVX, et al. 2016. A house for two–double bacterial infection in Euplotes woodruffi Ssq1 (Ciliophora, Euplotia) sampled in Southeastern Brazil. Microb Ecol. 71(2):505–517.
Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids Buchnera sp. APS. Nature 407(6800):81–86.
Soldo AT, Godoy GA. 1973. Molecular complexity of Paramecium symbiont lambda deoxyribonucleic acid: evidence for the presence of a multicopy genome. J Mol Biol. 73(1):93–108.
Stamatakis A. 2015. Using RAxML to infer phylogenies. Curr Protoc Bioinformatics 51:6.14.1–14.
Szokoli F, Castelli M, et al. 2016a. Disentangling the taxonomy of Rickettsiales and description of two novel symbionts (“Candidatus Beleaia paramacronuclearis” and “Candidatus Fokinia cryptica”) sharing the cytoplasm of the ciliate protist Paramecium bisexual. Appl Environ Microbiol. 82(24):7236–7247.
Szokoli F, Sabaneyeva E, et al. 2016b. ’Candidatus Fokinia solitaria’, a novel ‘stand-alone’ symbiotic lineage of Midichloriaceae (Rickettsiales). PLoS One 11(1):e0145743.
Talavera G, Castresana J. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol. 56(4):564–577.
Thomas S, Alexander W, Gilligan FJ, Rikhisa Y. 2016. The Importance of Rickettsiales Infections. In: Thomas S, editor. Rickettsiales. Cham, Switzerland: Springer International Publishing. p. 3–21.
Tully BJ, Sachdeva R, Graham ED, Heidelberg JF. 2016. 290 metagenome-assembled genomes from the Mediterranean Sea: a resource for marine microbiology. Peer J. 5:e3558.
Vannini C, Ferrantini F, Ristori A, Verni F, Petroni G. 2012. Betaproteobacterial symbionts of the ciliate Euplotes: origin and tangled evolutionary path of an obligate microbial association. Environ Microbiol. 14(9):2553–2563.
Varani AV, Siguer P, Gourbeyre E, Chameau V, Chandler M. 2011. Issaga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. Genome Biol. 12(3):R30.
Vaughan JA, Tkach VV, Greiman SE. 2012. Neorickettsial endosymbionts of the digenea: diversity, transmission and distribution. Adv Parasitol. 79:253–297.
Wang Z, Wu M. 2015. An integrated phylogenomic approach toward pinpointing the origin of mitochondria. Sci Rep. 5(7949).
Wang Z, Wu M. 2014a. Complete genome sequence of the endosymbiont of Acanthamoeba Strain UW/C8, an amoeba endosymbiont belonging to the ‘Candidatus Midichloriaceae’ family in Rickettsiales. Genome Announc. 2(4):e00791-14.
Wang Z, Wu M. 2014b. Phyllogenomic reconstruction indicates mitochondrial ancestor was an energy parasite. PLoS One 9(10):e110685.
Wick RR, Schultz MB, Zobel J, Holt KE. 2015. Bandage: interactive visualization of de novo genome assemblies. Bioinformatics 31(20):3350–3352.
Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol. 13(6):e1005595.
Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. 2011. PHAST: a fast phage search tool. Nucleic Acids Res. 39(Web Server issue):W347–W352.

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