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## Single cell genomics of a rare environmental alphaproteobacterium provides unique insights into Rickettsiaceae evolution

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### Abstract

The bacterial family Rickettsiaceae includes a group of well-known etiological agents of many human and vertebrate diseases, including epidemic typhus-causing pathogen *Rickettsia prowazekii*. Due to their medical relevance, rickettsiae have attracted a great deal of attention and their host-pathogen interactions have been thoroughly investigated. All known members display obligate intracellular lifestyles, and the best-studied genera, *Rickettsia* and *Orientia*, include species that are hosted by terrestrial arthropods. Their obligate intracellular lifestyle and host adaptation is reflected in the small size of their genomes, a general feature shared with all other families of the Rickettsiales. Yet, despite that the Rickettsiaceae and other Rickettsiales families have been extensively studied for decades, many details of the origin and evolution of their obligate host-association remain elusive. Here we report the discovery and single cell sequencing of ‘*Candidatus Arcanobacter lacustris*’, a rare environmental alphaproteobacterium that was sampled from Damariscotta Lake that represents a deeply rooting sister lineage of the Rickettsiaceae. Intriguingly, phylogenomic and comparative analysis of the partial ‘*Candidatus Arcanobacter lacustris*’ genome revealed the presence chemotaxis genes and vertically inherited flagellar genes, a novelty in sequenced Rickettsiaceae, as well as several host-associated features. This finding suggests that the ancestor of the Rickettsiaceae might have had a facultative

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intracellular lifestyle. Our study underlines the efficacy of single cell genomics for studying microbial diversity and evolution in general, and for rare microbial cells in particular.

## Keywords

alphaproteobacteria; endosymbiosis; host adaptation; Rickettsiales; Rickettsiaceae; single cell genomics

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## Introduction

The Rickettsiales are an order within the Alphaproteobacteria that comprise obligate intracellular endosymbionts of arthropods and mammals. They include the causative agents of many mild to severe diseases in humans and other animals, for example epidemic- and scrub typhus (Andersson *et al.*, 1998; Cho *et al.*, 2007), ehrlichiosis (Dunning Hotopp *et al.*, 2006) heartwater (Collins *et al.*, 2005) and anaplasmosis (Brayton *et al.*, 2005). Their genomes are typically small ( ~ 2.1 Mb) due to reductive evolution and have an AT-rich composition (Darby *et al.*, 2007). Among the four taxonomic families that are recognized within the Rickettsiales (Anaplasmataceae, Rickettsiaceae, *Ca.* Midichloriaceae and Holosporaceae), the most well known are the Rickettsiaceae, for they are human pathogens. They include *Rickettsia prowazekii* (epidemic typhus), *Rickettsia rickettsia* (Rocky Mountain spotted fever), *Rickettsia typhi* (murine typhus) and *Orientia tsutsugamushi* (scrub typhus) all of which are transmitted by hematophagous arthropods. Due to the wide variety of diseases they are responsible for, genome sequencing efforts have so far focused on the pathogenic *Rickettsia* and *Orientia* genera, while other genera that are not carried by hematophagous hosts, also referred to as “Neglected Rickettsiaceae” (Schrallhammer *et al.*, 2013) have largely been ignored (Merhej and Raoult, 2011). Currently, genomic sequences are available for 57 *Rickettsia* and 2 *Orientia* strains, but none for other genera of Rickettsiaceae even though 157 small subunit (SSU) rRNA sequences of uncultured members are available for them in the SILVA database ((Quast *et al.*, 2012); release 119). Obviously, this apparent unbalance in taxon sampling is detrimental for our understanding of their evolution and emerging pathogenicity. With the advent of methodologies that allow the characterization of genomic sequences without the need for cultivation, the opportunity has arisen to explore the Rickettsiaceae diversity in an unbiased manner and gain a significantly better understanding of their evolution. In this work, we have used such a cultivation-independent approach and obtained and sequenced a single-cell amplified genome (SAG) from a novel alphaproteobacterium sampled from Damariscotta Lake (Martinez-Garcia *et al.*, 2012). This alphaproteobacterium, for which we propose the name ‘*Candidatus* Arcanobacter lacustris’ (from here on referred to as *A. lacustris*), represents a novel Rickettsiales lineage with a deep sister relationship to the Rickettsiaceae. By applying comparative and phylogenomic analyses, we provide a unique insight into the evolution of the Rickettsiaceae, and about the nature and abundance of this novel and rare alphaproteobacterial lineage.

## Materials and methods

### Single cell sorting, lysis and whole genome amplification

In an attempt to explore alphaproteobacterial diversity, a single-cell genomics pipeline was applied on an environmental freshwater sample obtained from Damariscotta Lake (USA) (April 28th 2009) as described by (Martinez-Garcia *et al.*, 2012). In brief, the single cell was obtained with fluorescent activated cell sorting (FACS) of the sample and subsequently lysed. Extracted DNA was subjected to real time multiple displacement amplification (rtMDA). The MDA product was then verified by SSU rRNA qPCR. A second round of MDA (Swan *et al.*, 2011) was required to obtain sufficient quantity of genomic DNA for Illumina sequencing. All above steps were carried out at the Bigelow Laboratory Single Cell Genomics Centre (Maine, USA).

### Whole genome sequencing, assembly and annotation

A short-insert paired-end TruSeq library (Illumina) was prepared according to the manufacturer's prescriptions. The library, with insert size of approximately 400 bp, was sequenced on an Illumina HiSeq2000 instrument, yielding a total of 9,171,854 read pairs, with each read being 100 bp long. Raw reads are deposited at NCBI's SRA under study number SRP055079. Before assembly, the quality of the sequence data was assessed with FastQC 0.10.1 (Andrews), and showed an average Phred Score per read of 38. The reads were assembled *de novo* using SPAdes 2.4 (Nurk *et al.*, 2013) in single cell mode ('--sc') with default k-mer sizes (21, 33, 55), including read error correction and mismatch- and indel-correction steps. Resulting contigs were filtered in three steps. First, all contigs smaller than 200 bp or with an average k-mer coverage lower than 10 were removed. Second, ORFs were predicted with Prodigal 2.50 (Hyatt *et al.*, 2010) and classified at Domain level by MEGAN 4.7 (Huson *et al.*, 2011, 4) based on a BLASTP search (Altschul *et al.*, 1990) versus NCBI's non-redundant database (nr). All contigs in which less than a third of the predicted ORFs were classified as bacterial were removed. Finally, contigs were checked for contig edges that were inverted repeats relative to each other. These repeats are the result of 'self-priming', a known MDA artefact (Lasken and Stockwell, 2007). In case such repeats were detected, the 5' end of the contig was trimmed accordingly. The remaining contigs were annotated using Prokka 1.11 (Seemann, 2014) using the '--rfam' flag and RNAmmer (Lagesen *et al.*, 2007) as RNA predictor. In addition, Prokka was modified to allow prediction of partial ORFs on contig edges. Eukaryotic domains in predicted proteins were identified using the lists of eukaryotic-like domains available at [effectors.org](http://effectors.org) (Jehl *et al.*, 2011). Putative type IV secretion system components and effectors were identified by a BLASTP search of the SecReT4 (Bi *et al.*, 2013, 4) 'T4SS components', 'Experimentally verified effectors' and 'T4SS effectors' databases. The genome has been deposited as a whole genome shotgun project at DDBJ/EMBL/GenBank under the accession JYHA00000000. The version described in this paper is version JYHA01000000.

### Estimation of genome completeness and genome size

Genome completeness and consequently genome size were estimated as follows. HMM profiles of the 129 Rickettsiales panorthologous marker genes (see Supplementary Methods) were constructed with HMMER3 (Eddy, 1998) and used to search the predicted proteome.

In earlier work (Rinke *et al.*, 2013), an “unweighted” completeness estimate would be obtained by dividing the number of present marker genes by the total number of marker genes. Thus, each present marker gene would contribute equally to the completeness estimate, thereby assuming that all marker genes are spread evenly in the genome. A better, “weighted” estimate is obtained when weighing each marker gene with the median genomic distance to the closest marker gene in the dataset (unpublished observation). For example, ribosomal protein genes, which are generally organized in operons, have a lesser individual weight than other genes that are generally more evenly spread out in the genome. Here, for each of the 20 Rickettsiales genomes (see Figure 1; except for draft genomes *Holospora undulata* and *Ca. Odysseella thessalonicensis*) we measured the distance of all present 129 Rickettsiales panorthologous markers in one genome to its closest upstream and downstream neighbors, normalized distances by the genome size, and used the normalized median distance recorded for each marker as a weight to estimate how clustered this marker generally is. Thereby, marker genes that tend to be located near other marker genes get a lower weight than marker genes that are relatively isolated. The weights were normalized again by dividing them by the sum of all weights, so that a genome containing all markers would have a completeness estimate of 1. Finally, the completeness estimate for *A. lacustris* was obtained by taking the sum of all normalized weights of marker genes found in this genome. The genome size was then estimated by dividing the total assembly length by the completeness estimate.

### Phylogenetic analyses

Unless otherwise stated, all alignments were made with MAFFT 7.050b (Katoh and Standley, 2013) using the local pair option (mafft-linsi) and trimmed with trimAl 1.4 (Capella-Gutiérrez *et al.*, 2009), removing sites for which more than half of the taxa contained a gap. Maximum likelihood (ML) phylogenies were inferred with RAxML 7.2.8 (Stamatakis, 2006), using 100 rapid bootstraps under the  $\Gamma$  model for rate heterogeneity among sites with the GTR substitution matrix for nucleotide alignments and the LG substitution matrix (Le and Gascuel, 2008) for protein alignments. Bayesian phylogenies (BI) were inferred with PhyloBayes MPI 1.4f (Lartillot *et al.*, 2013) using 4 MCMC chains under the CAT-Poisson model. The log likelihood, total tree length,  $\alpha$ -parameter and number of categories of all trees per chain were traced and visually inspected to choose the burn-in cutoff. Whenever convergence (maxdiff < 0.1) between chains was not fully achieved, the topology of individual chain trees were examined to ensure that they were congruent for critical nodes. Trees were drawn with FigTree 1.4 (Andrew Rambaut, <http://tree.bio.ed.ac.uk/software/figtree/>). Additional details regarding the phylogenetic analyses that were carried out in the present study are available in the Supplementary Methods.

### Genome content comparison

The occurrence of KEGG Pathways (Kanehisa *et al.*, 2014) in *A. lacustris* and members of the Rickettsiaceae were compared as follows: first, the prodigal predicted (partial) protein sequences from *A. lacustris* and the protein RefSeq accessions from *Rickettsia prowazekii* Madrid E, *Rickettsia belli* RML369 and *Orientia tsutsugamushi* Ikeda were used in a BLASTP search (E-value cutoff:  $10^{-5}$ ) of NCBI nr. The BLASTP results were then imported into MEGAN-5.1.5 and the occurrence of all KEGG Pathways were visually compared.

The rickCOGs (see Supplementary Methods) served as a basis to compare the genome content of *A. lacustris* with the Anaplasmataceae, Rickettsiaceae, Holosporaceae and *Ca. Midichloria mitochondrii*; see Figure 2 for family attribution). A Venn diagram was constructed using the R package VennDiagram (Chen and Boutros, 2011) to visualize how many rickCOGs were unique for *A. lacustris* or each of the Rickettsiales family and how many were common between two or more families. Each rickCOG was considered as unique to a given family if it contained a sequence from at least one taxon that belonged to that family and contained no sequences from other families. A rickCOG was considered as common between two or more families if it contained sequences from at least one taxon of each of those families and contained no sequences from the other remaining families. Outgroup taxa (see Figure 2) were not considered in this analysis.

To annotate the rickCOGs that were unique for *A. lacustris* and uniquely shared with specific Rickettsiales families, a BLAST analysis was performed. rickCOGs were aligned with MAFFT and resulting alignments were used as query to search nr with PSIBLAST (E-value cutoff:  $10^{-10}$ ) (Altschul *et al.*, 1997). If a rickCOG only contained a single sequence, it was used directly as query to search nr with BLASTP (E-value cutoff:  $10^{-5}$ ). The protein identity of the rickCOG was then called based on the best PSI-BLAST or BLASTP hit.

### Estimation of environmental abundance

The metagenomic datasets of the freshwater lakes Damariscotta (spring and summer), Mendota (spring and summer), Sparkling (spring and summer) (Martinez-Garcia *et al.*, 2012), Ekoln, Erken and Vattern (Zaremba-Niedzwiedzka *et al.*, 2013) were used to estimate the relative abundance of *A. lacustris* and ten LD12 SAGs in freshwater lakes (Zaremba-Niedzwiedzka *et al.*, 2013) by recruiting the metagenomic reads to their contigs with NUCmer (Kurtz *et al.*, 2004). Detected reads were filtered based on identity ( $\geq 80\%$ ), alignment length ( $\geq 100$  bp and  $\geq 90\%$  of read length). Obtained read counts were corrected for genome completeness estimates (calculated for the LD12 SAGs as described above by using 139 well conserved bacterial marker genes (Rinke *et al.*, 2013)), and subsequently used to calculate abundance relative to total metagenome size. Abundances of the LD12 SAGs relative to *A. lacustris* were calculated per metagenome by dividing their completeness-corrected read counts.

Small subunit rRNA amplicon datasets were screened using an approach described by (Lagkouvardos *et al.*, 2014). In brief, all raw SSU rRNA amplicon sequence data from environmental samples in the databases SRA (June 2013) (Kodama *et al.*, 2012) and VAMPS (“Not Normalized”) (September 2014) (Huse *et al.*, 2010); <http://vampls.mbl.edu/>) were extracted and organized by sample in independent datasets, retaining sample-associated meta-data. The databases were searched using BLAST and the full-length 16S rRNA gene sequence of *A. lacustris* and LD12 (accession no. Z99997.1) as query. The detected amplicon reads were filtered with respect to size ( $\geq 200$  nucleotides), alignment length ( $\geq 80\%$  of read length), and identity ( $\geq 95\%$ ). For those datasets that contained *A. lacustris* hits, abundances relative to amplicon dataset size were calculated. For detected datasets that contained hits for both organisms, the relative abundance of LD12 compared to *A. lacustris* was calculated by dividing the LD12 hit count with the *A. lacustris* hit count.

## Results

### General features of the single cell amplified genome

With the aim of exploring genetic diversity of environmental Alphaproteobacteria, we applied a single-cell genomics pipeline on an environmental freshwater sample obtained from Damariscotta Lake (USA; sampled in April 2009). Because single-cell sequence data is difficult to assemble due to extreme sequence depth bias, contamination sensitivity, potential chimeric sequences and other MDA artifacts, we employed SPAdes (Nurk *et al.*, 2013), an assembler that is specifically designed to handle such data and checked the resulting assembly thoroughly for contamination and MDA artifacts.

The resulting assembly of the single cell amplified genome (SAG) consists of 151 contigs comprising a total size of 822,563 bp with an average GC-content of 32.6 % and coding density of 88.4% (Table 1). A total of 882 protein-coding genes were identified, of which 45 were annotated as repeat-containing proteins (transposases, ankyrin repeat proteins, integrases, etc.). Based on a weighted single-copy gene count, we estimate a completeness of 48% and consequently predict a genome size of 1.7 Mb. SAGs are generally incomplete and a completeness estimate of 50% is considered typical. Compared to other members of the Rickettsiales, *A. lacustris* has a relatively large genome, an average GC-content and high coding density (Figure 1).

### Phylogenetic relationship to other Rickettsiales

To assess the phylogenetic relationships relative to other Rickettsiales, phylogenomic analyses based on Rickettsiales panorthologs and on SSU rDNA were performed. For the phylogenomic analysis, 12,197 Rickettsiales-specific clusters of orthologous groups (rickCOGs) were constructed from all proteins encoded in 20 representative Rickettsiales and 4 outgroup Alphaproteobacteria. From these, 129 panorthologs were extracted that were present in exactly one copy per genome in all genomes excluding *A. lacustris*. Out of these, 64 were present in *A. lacustris* (Supplementary Table 1). To assess the effect of missing data and tree reconstruction method, maximum likelihood (ML) and Bayesian inference (BI) phylogenies were inferred from the '64-panortholog' dataset and the '129-panortholog' dataset. A deep sister relationship to the Rickettsiaceae was retrieved with strong support (bootstrap support [BS]: 94, posterior probability [PP]: 0.99), robust to tree reconstruction methods and missing data (Figure 2A; Supplementary Figure 1). In addition, a phylogenetic analysis of 64 SSU rRNA sequences from Rickettsiales and outgroup Alphaproteobacteria was performed. A deep sister relationship to the Rickettsiaceae was also retrieved in this analysis (BS: 99) (Figure 2B; Supplementary Figure 2). Despite the inclusion of more Rickettsiales taxa in the analysis, no significant affiliation with any of the established families was observed, indicating that *A. lacustris* represents a novel clade.

### Genome content comparisons

The unique phylogenetic placement of *A. lacustris* as a sister clade to Rickettsiaceae allowed us to investigate the evolution of the latter family in more detail. To this end, we inferred candidate genes lost by the last Rickettsiaceae common ancestor (LRCA) by searching for KEGG pathways (Kanehisa *et al.*, 2014) for which component genes were present in *A.*

*lacustris* but completely absent in Rickettsiaceae members *R. prowazekii*, *R. bellii* and *O. tsutsugamushi*. Pathways with the majority of the genes missing in Rickettsiaceae were the flagellar assembly (eighteen genes) and bacterial chemotaxis (nine genes). Other identified pathways and protein complexes include threonine metabolism (three genes; *thrA*, *thrB* and *thrC*), glycolysis (two genes; *pgi* and *eno*), pantothenate and CoA biosynthesis (two genes: *coaD* and *coaX*), terpenoid backbone synthesis (*thiB*, *thiP* and *thiQ*), the thiamine ABC transporter (three genes: *tbpA*, *thiP* and *thiQ*) and an antibiotics ABC-2 transporter (two genes: *yadH* and *yadG*).

We then compared the genome content with other Rickettsiales families by utilizing the rickCOGs that were also used for the phylogenomic analyses. All *A. lacustris* predicted proteins (886) belonged to a total of 723 ortholog clusters. Among these, 299 (41%) were unique to *A. lacustris* (Figure 3), including 143 (20%) that did not have detectable homologs (E-value  $10^{-6}$ ) in the NCBI nr database. Those for which homologs could be detected included eight ortholog clusters putatively involved in heme metabolism and fourteen putatively mobile elements (Supplementary Table 2). Twenty-seven ortholog clusters were uniquely shared with the Rickettsiaceae and included six putatively involved in toxin-antitoxin systems that were encoded by three gene pairs that each putatively contained one toxin and one antitoxin gene. A total of sixteen ortholog clusters were uniquely shared with the Anaplasmataceae and include a DNA primase and a putative phage-related ATPase. The flagellar hook protein FlgK and a PAP2 superfamily protein were uniquely shared with 'Midichloriaceae'. A remarkable high number (41) of ortholog clusters were shared with the 'Holosporaceae'. These included seven chemotaxis proteins.

### Evolutionary origins of flagellar and chemotaxis proteins

To assess whether the flagellar and chemotaxis genes were lost in the LRCA or independently acquired, we sought to investigate their evolutionary origins.

In addition to the eighteen flagellar genes identified above, four more were identified. The flagellar genes are often located in pairs or on their own, with exception of two clusters: one containing *fliW*, *flgL*, *flgK*, *flgJ*, *flgI*, *motA* and *motB* and one containing *fliC*, *fliD*, *fliS*, *fliL* and *fliM*. All key components of the flagellum (hook, filament, basal body and motor) are represented. Phylogenetic analyses were performed on a set of 14 conserved flagellar genes (Liu and Ochman, 2007)(Sassera *et al.*, 2011) of which eight were present in *A. lacustris*. The dataset was expanded with newly sequenced alphaproteobacterial representatives and ML and BI phylogenies were inferred for both concatenated '14 core flagella' and '8 core flagella' datasets. Resulting trees were congruent with the expected species phylogeny in which *A. lacustris* groups with Rickettsiales at or near the root of the Alphaproteobacteria (Figure 4; Supplementary Figure 3). This strongly suggests that the flagellar genes were vertically inherited. Two chains of the '8-core flagella' Bayesian phylogenies did not display full species tree congruence, but *A. lacustris* clustered with Rickettsiales with high confidence, supporting the idea that these genes were vertically inherited.

Chemotaxis genes are very rare in sequenced Rickettsiales and have only been detected in one species whose host is a protist, *Ca. Odysseella thessalonicensis*. In *A. lacustris*, the genes

are distributed between a *cheAWYBR* cluster, a *cheZY* cluster and two genes encoding for methyl-accepting chemotaxis proteins (*mcp*, and *pctC*). Proteins encoded by these genes are most similar to homologs in the Rhodospirillales, except for *pctC* that shows more similarity to homologs in Caulobacterales and Rhizobiales.

### Genes indicating a host-associated or intracellular lifestyle

Since *A. lacustris* is a member of the Rickettsiales, which comprises obligate intracellular bacteria, we screened its genome content for any indication of host-associated lifestyle. Indeed, we identified several gene families that are indicative of such a lifestyle:

First, an ATP/ADP translocase homolog was identified. This transporter allows host-adapted bacteria to parasitize their hosts energy by exchanging their cytoplasmic ADP with host ATP (Krause *et al.*, 1985). Although not all homologs of this enzyme necessarily transport ATP (Audia and Winkler, 2006), all are only found in intracellular bacteria and chloroplasts and represent a reliable marker for intracellular lifestyle. We sought to predict the substrate of this homolog with a phylogenetic analysis including all functionally characterized homologs (Audia and Winkler, 2006; Vahling *et al.*, 2010; Schmitz-Esser *et al.*, 2008; Linka *et al.*, 2003). In the resulting tree, the *A. lacustris* homolog branched at the base of three duplication events (PP: 0.77) in the Rickettsiaceae and was not closely related to any of the other homologs, including those that were characterized (Supplementary Figure 4) leaving us unable to predict its function with confidence.

Second, eight genes putatively encoding components of a *virB* type IV secretion system (T4SS) were identified, distributed over three loci. The loci consist of (i) *virB8*, *virB9*, *virB10*, *virB11* and *virD4*, (ii) *virB4* and *virB2*, and (iii) a second *virB8*. *virB* type T4SSs in Rickettsiales are thought to be of the ‘effector translocator’ type involved in host interaction and this hypothesis was recently experimentally enforced in *Ehrlichia chaffeensis* and *Anaplasma marginale* (Liu *et al.*, 2012; Lockwood *et al.*, 2011). We investigated whether the T4SS genes in *A. lacustris* were related to these systems or horizontally acquired with a phylogenetic analysis. The resulting tree, which was based on a concatenation of five well-conserved genes (*virB4*, *virB8*, *virB9*, *virB10*, *virB11*), suggests that the T4SS genes were vertically inherited and thus suggests that the T4SS in *A. lacustris* is likely to be of the ‘effector translocator’ type as well (Supplementary Figure 5).

Lastly, we were able to predict a number of candidate effector proteins. By searching the SecReT4 (experimentally verified) effectors database (Bi *et al.*, 2013, 4), we found 18 putative T4SS effectors (Supplementary Table 3). These included several ankyrin repeat proteins, phosphoglucomutases and Fic-family proteins. Because many effectors have been shown to contain domains that are typically found in eukaryotes, we searched for additional putative effectors with eukaryotic-like domains by using the Effective database (Jehl *et al.*, 2011). Hits ( $Z$ -score  $\geq 4$ ) included three proteins containing a glycosyltransferase domain (pfam: ‘Gly\_transf\_sug’, acc: PF04488), one containing a PhoPQ-activated pathogenicity-related protein domain (pfam: ‘PhoPQ\_related’ acc: PF10142) and one containing a galactosyl transferase domain (pfam: ‘Glyco\_transf\_34’, acc: PF05637).



## Environmental abundance and diversity

To estimate the abundance of this novel Rickettsiales lineage in freshwater environment, reads from metagenomic datasets of six lakes, including the spring metagenome of Damariscotta Lake from which the single cell was sampled (Martinez-Garcia *et al.*, 2012), were recruited to *A. lacustris* contigs. Only 0.004-0.014 % of the metagenomic reads could be recruited (Table 2). Interestingly, it was not the metagenome of lake Damariscotta (spring), but that of lake Erken that had the highest relative abundance of recruited reads. Compared to LD12, another freshwater alphaproteobacterium for which SAGs are available, *A. lacustris* is generally 100-1000 times less abundant in these metagenomic datasets (Figure 5).

Broadening the scope of the analysis, public available SSU amplicon databases of SRA (Kodama *et al.*, 2012) and VAMPS (Huse *et al.*, 2010) were screened for reads with high sequence similarity to *A. lacustris* SSU. Hits were identified in eleven SRA datasets, where they constituted between ~0.002 and ~0.2 % of the total dataset and in three VAMPS datasets, where hits constituted between ~0.0005 and ~0.007 % of the total dataset (Table 2). In contrast, when the same screen was performed with LD12 SSU, 115 SRA datasets and seven VAMPS datasets were hit. In amplicon datasets where both *A. lacustris* and LD12 hits were found, LD12 was approximately between 100 and 1000 times more abundant, with exception of “Rare biosphere at the North Falmouth Fire Station”, Damariscotta (spring) and Sparkling (spring) datasets (Figure 5). Yet, the lower fold difference in these datasets can be explained by a lower LD12 hit count and not a higher *A. lacustris* count.

To get a rough idea of the phylogenetic diversity that can be found in the novel lineage that *A. lacustris* represents, reads that were identified in the screens above and additional (partial) SSU sequences with high similarity found in the NCBI-nt database were incorporated in the SSU phylogeny used earlier in this study. In the resulting tree, *A. lacustris* formed a monophyletic group with 8 OTUs (BS: 92) containing only reads originating from freshwater environments (Figure 6; Supplementary Figure 6). Interestingly, a clade of 28 reads originating from the North Falmouth water distribution system were the closest relatives of *A. lacustris* with moderate support (BS: 36). In addition, other closely related reads originated were associated with freshwater environments as well (Supplementary Table 4).

## Discussion

In this work we sought to obtain novel insights into the evolution of Rickettsiaceae by using a methodology that is cultivation-independent and by targeting environmental Alphaproteobacteria as opposed to targeting those that are medically or agriculturally relevant. We have identified *A. lacustris*, an environmental alphaproteobacterium isolated from the freshwater Damariscotta Lake. Below we discuss its inferred lifestyle, its implications for our understanding of Rickettsiaceae evolution and its apparent extreme rarity in the sampled biosphere.

## Lifestyle

We were able to isolate and identify *A. lacustris* from a freshwater sample because it was a “free” single cell within the sample at the time of sorting. Even though this could indicate a free-living lifestyle, it does not necessarily have to be the case. One can think of several other possible scenarios that lead to a “free” single cell at the time of sorting: (i) *A. lacustris* is facultative intracellular, and the microbe was captured during a free-living stadium of its lifecycle, (ii) it is obligate intracellular but was released after it caused its host cell to lyse, similar to other Rickettsiales (Hackstadt, 1996) or (iii) it was inside a host cell at the time of sampling, but was released when the host cell lysed due to mechanical processing of the sample (i.e. filtration or cell sorting). In summary, from this knowledge alone we cannot distinguish between an obligate free-living, facultative intracellular or obligate intracellular lifestyle.

We therefore inspected its genomic content to get more insights. Several factors were found that are supportive of an intracellular lifestyle. The presence of an ATP/ADP translocase homolog is the strongest indicator, as homologs of this protein have been found in intracellular bacteria only (Schmitz-Esser *et al.*, 2004). Additionally, the presence of several components of a *virB*-type T4SS and a number of putative effectors suggest a host-associated lifestyle. We suggest that the T4SS is of the effector secretion type, as it shares a recent common ancestor with the *vir*-type T4SS of *E. chaffeensis* and *A. marginale* for which it has been recently shown that they are capable of secreting effector proteins into host cells (Lockwood *et al.*, 2011)(Liu *et al.*, 2012). Conversely, we found an array of flagellar and chemotaxis genes that could indicate a (partially) free-living lifestyle. For example, both systems could support *A. lacustris* in locating and targeting nutrients and/or new host cells. However, as these systems are also encoded by the obligate intracellular symbiont *Ca. Odysseella thessalonicensis*, these systems do not necessarily indicate a free-living lifestyle.

Taken together, we suggest either a facultative or obligate intracellular lifestyle for *A. lacustris*. The identity of the host remains obscure, but the relative high amount of uniquely shared protein families with *Ca. Odysseella thessalonicensis* (hosted by the amoebae *Acanthamoeba*) and *Holospira undulata* (hosted by the ciliate *Paramecium*), hints toward a protist host.

## Evolution

From our phylogenomic and phylogenetic analyses, we observed that *A. lacustris* was not closely affiliated with any of the currently established Rickettsiales families. Rather, it represented a previously unexplored branch, basal to the Rickettsiaceae. Although this topology has been observed before for *Ca. Midichloria mitochondrii*, (Sassera *et al.*, 2011), our analyses strongly showed that it affiliated with the Anaplasmataceae instead (Figure 2). This affiliation has been observed by other studies as well (Driscoll *et al.*, 2013; Montagna *et al.*, 2013). The phylogenetic novelty of *A. lacustris* is further underlined by the large fraction (143 out of 299) of *A. lacustris* unique protein families that, not only have no detected homologs in other Rickettsiales families, but also none in NCBI's non-redundant database. This phylogenetic placement provided us with a unique opportunity to obtain novel insights

into the evolution of the Rickettsiaceae. The most significant insight is the presence of an array of flagellar and chemotaxis genes, which are completely absent in all current Rickettsiaceae genomes. As we showed that flagellar genes were vertically inherited, this implies that they were lost in the last common ancestor of the Rickettsiaceae, likely due to the heavy genome reduction forces that are characteristic of all Rickettsiaceae. In line with this hypothesis *A. lacustris* is estimated to have a relatively large genome size compared to most Rickettsiales (~1.7 Mb vs ~1.0-1.5 Mb) (Figure 1).

The presence of flagellar genes follows the current trend in which an increasing amount of Rickettsiales members are found to encode flagellar genes or have been observed to synthesize actual flagella: *Ca. Midichloria mitochondrii* (Sassera *et al.*, 2011) (Mariconti *et al.*, 2012), *Ca. Odysella thessalonicensis*, (Georgiades *et al.*, 2011), *Lyticum*, (Boscaro *et al.*, 2013) and *Trichorickettsia* and *Gigarickettsia* (Vannini *et al.*, 2014) are examples that contradict the previous assumption that flagella were lost in all Rickettsiales and fortifies the suggestion that flagella were present in the last common Rickettsiales ancestor.

The Rickettsiales have traditionally been put forward as candidates for the closest relatives of mitochondria (Fitzpatrick *et al.*, 2006; Williams *et al.*, 2007). However, there is still no consensus about exact phylogenetic placement of mitochondria. This is mainly caused by the tree reconstruction artifacts long branch attraction (LBA) and compositional bias: the Rickettsiales, SAR11 and mitochondria all share a fast evolutionary rate and have AT-rich genomes (Grote *et al.*, 2012; Wang and Wu, 2015). Indeed, the affiliation of SAR11 with mitochondria was convincingly shown to be a compositional bias artifact (Rodríguez-Ezpeleta and Embley, 2012; Viklund *et al.*, 2012; Brindefalk *et al.*, 2011). The availability of *A. lacustris*, which breaks the relatively long branch leading to the Rickettsiaceae, reduces the LBA problem and may help future studies to identify the closest extant relatives of mitochondria with more confidence.

## Rarity

After exploring the diversity and abundance of *A. lacustris* in metagenomic datasets, it seemed that this bacterium and relatives are rare in the sampled biosphere. Compared to the free-living freshwater alphaproteobacterium LD12, it is about 100-1000 times less abundant. Even in both available metagenomes of Damariscotta Lake, the source of this bacterium, only up to 15 reads (0.008%) could be identified as an *A. lacustris* relative. However, this apparent rarity might be an artifact: many environmental samples go through a size filtration step prior to DNA extraction and follow up metagenomic sequencing, effectively filtering out a large fraction of eukaryotic cells and thus putative *A. lacustris* host cells. Because of this and the inferred intracellular lifestyle, we cannot rule out the possibility that *A. lacustris* is more abundant in the biosphere than we can observe in current metagenomic and SSU amplicon datasets.

## Conclusion

In conclusion, we present here the existence of a novel Rickettsiales member that represents a lineage that is a deep sister relative to the Rickettsiaceae. This lineage appears to be ultra-rare in the sampled biosphere and occupies a freshwater niche. We predict a facultative or

obligate intracellular lifestyle, perhaps in association with a protist and finally, observe the presence of chemotaxis genes and vertically inherited flagellar genes. This study has highlighted the power of single cell genomics with regard to exploring the biological dark matter and its implications for understanding microbial evolution. It would very likely have not been possible to discover *A. lacustris* and characterize its genome with standard cultivation-based and metagenomics approaches, because of its seemingly extreme rarity and symbiotic lifestyle. Single cell genomics and other cultivation-independent techniques will be of great value for future studies that aim to gain insight into the evolution of the Rickettsiaceae and other uncultivable or hard to cultivate microbial lineages.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>PP</b>	posterior probability
<b>BS</b>	bootstrap support
<b>SAG</b>	single-cell amplified genome
<b>ML</b>	maximum likelihood
<b>BI</b>	Bayesian inference

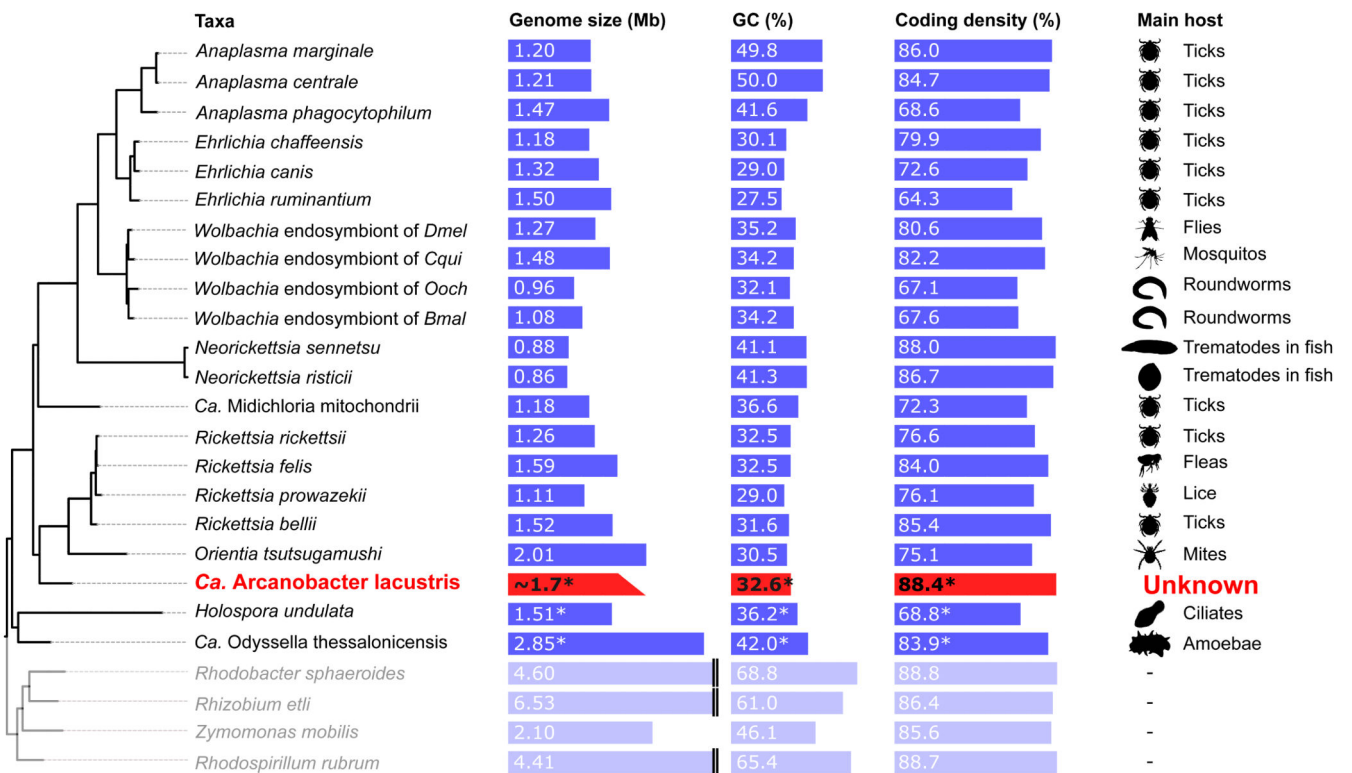
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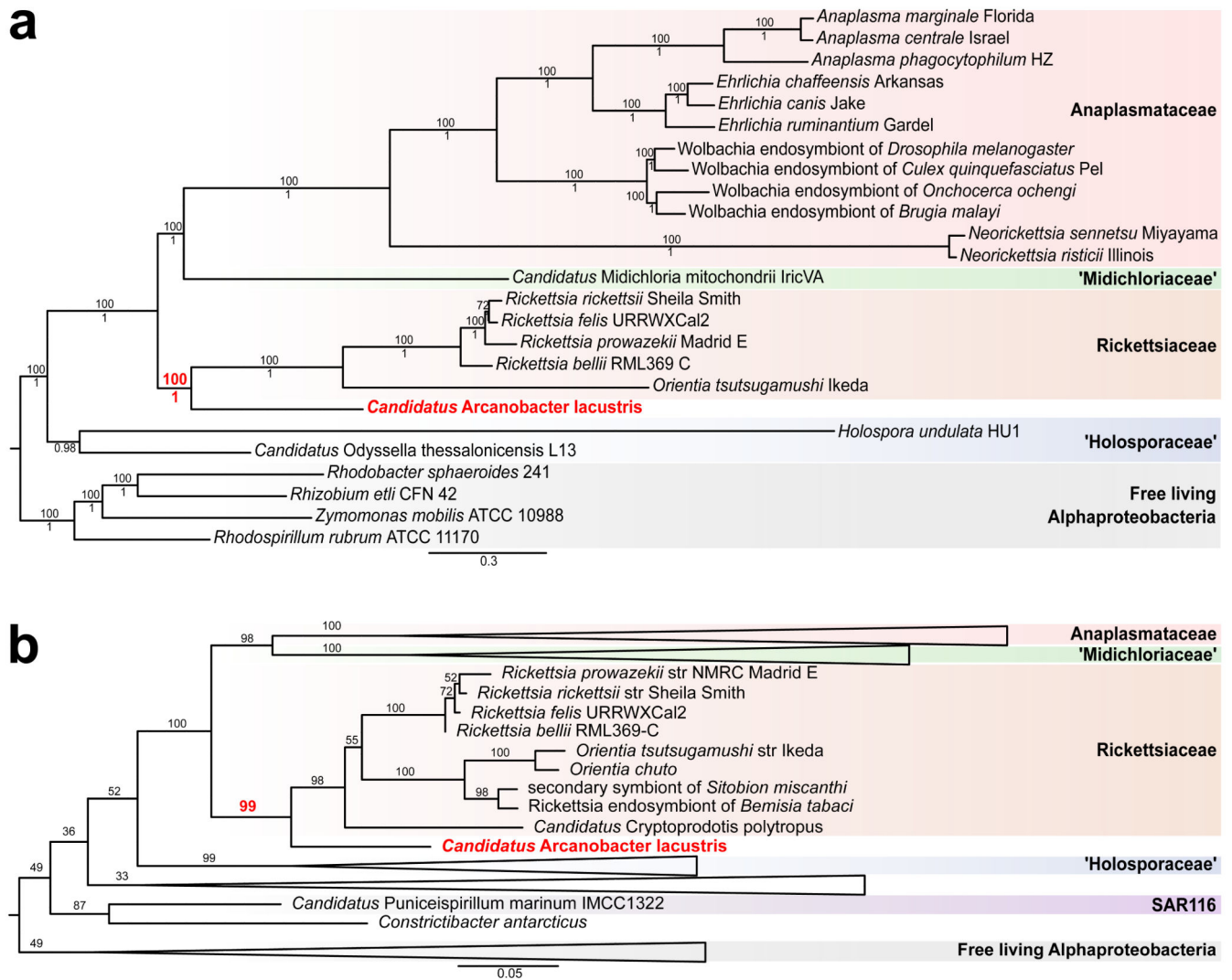


**Figure 1. A comparison of genome characteristics and host species of Rickettsiales for which genomic sequences are available**

The widths of the bars that represent the genome size, GC% and coding density are scaled.

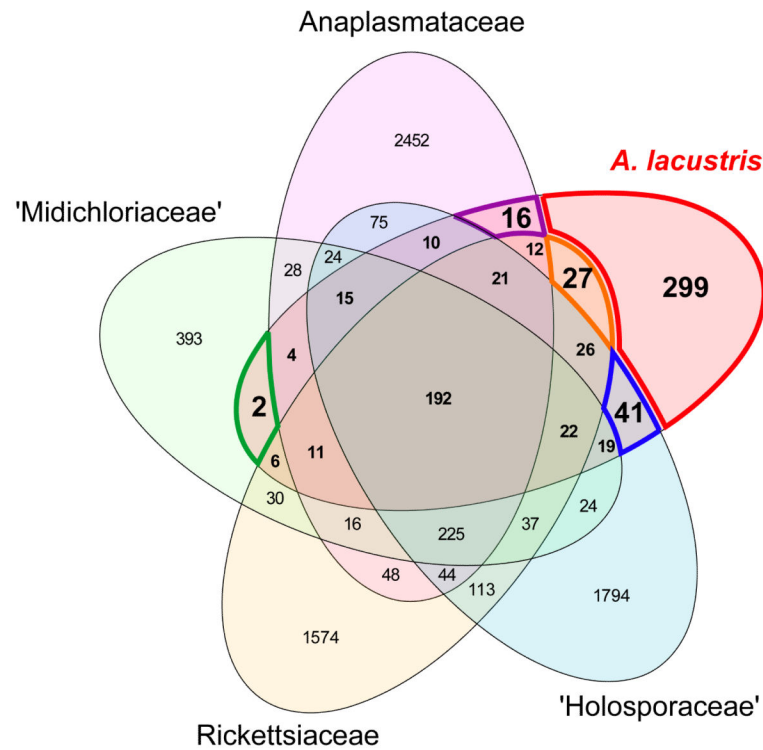
Asterisks (\*) indicate draft genomes. The tree shown on the left is the same as the PhyloBayes genome tree (see figure 2).



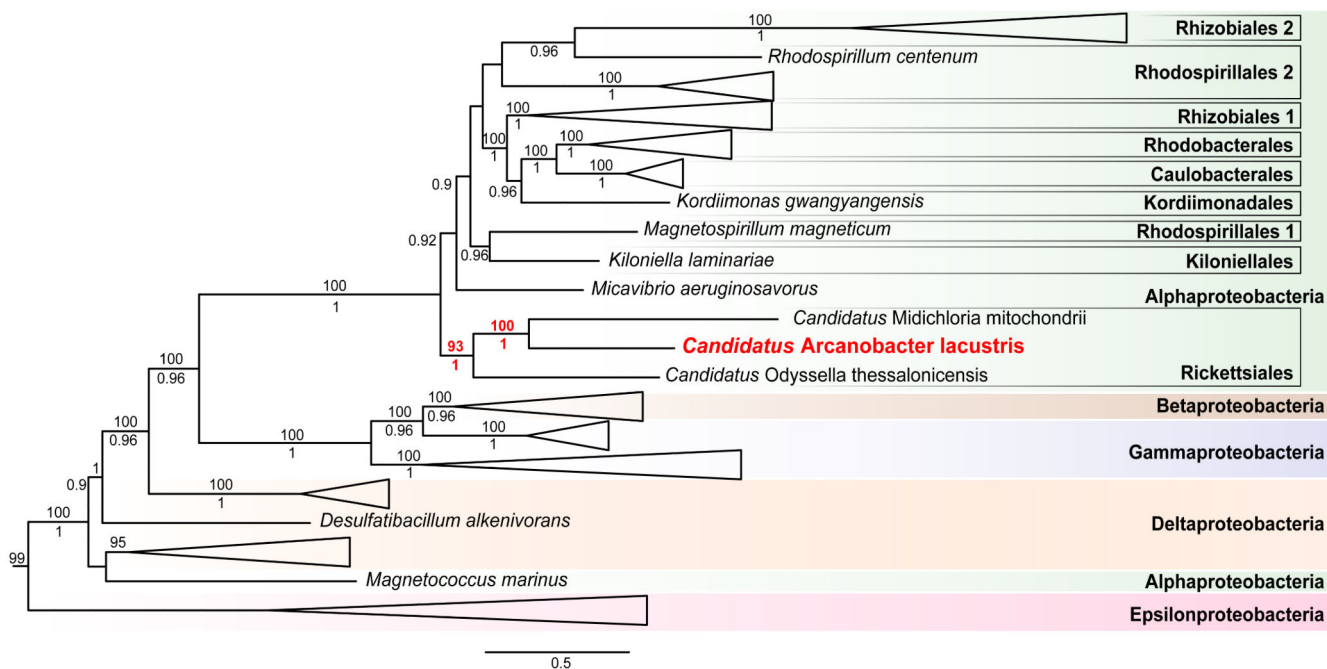


**Figure 2. Phylogenetic placement of *A. lacustris***

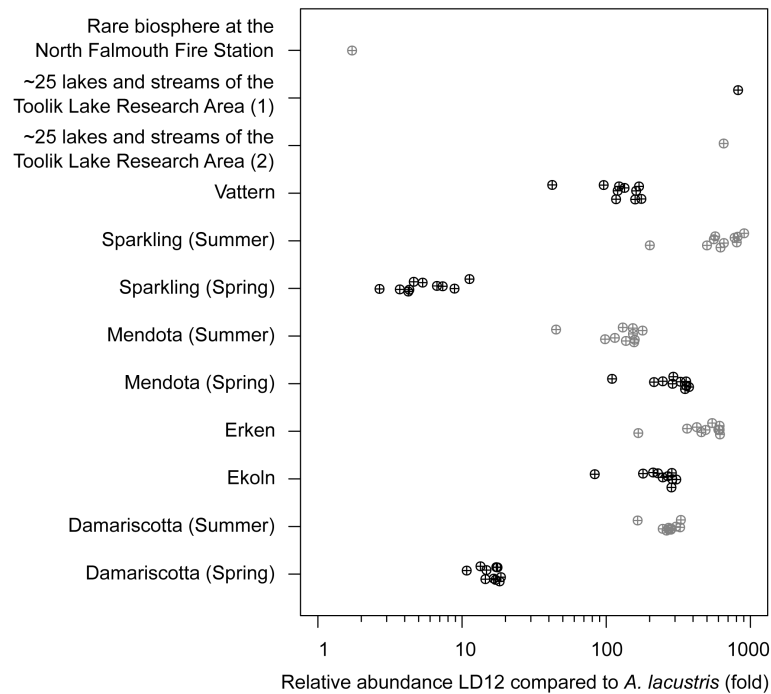
*A. lacustris* and branch support values on branches of interest have been marked with bold red. **(a)** Phylogenetic tree based on a concatenated alignment of 64 Rickettsiales panorthologs. Tree topology and posterior probability branch support values (shown below branches) were inferred using PhyloBayes (model: CAT-Poisson). Bootstrap branch support values (shown above branches) were calculated with RAxML (model:  $\Gamma$ +LG). Separate full RAxML and PhyloBayes phylogenetic trees are shown in Supplementary Figure 1. **(b)** Phylogenetic tree based on SSU rRNA gene. Tree topology and bootstrap support values (shown above branches) were inferred with RAxML. Major taxonomic groups have been collapsed to improve readability. Full phylogenetic tree is shown in Supplementary Figure 2.



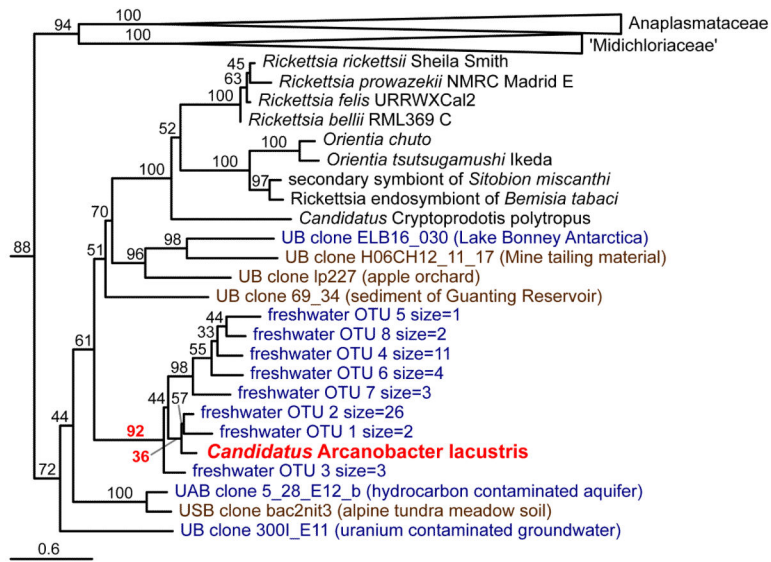
**Figure 3. Five-set Venn diagram depicting gene content comparison between *A. lacustris* and other Rickettsiales families based on Rickettsiales specific clusters of orthologous groups**  
 The borders of intersections of interest are marked and colored: *A. lacustris* unique genes (red), genes uniquely shared between *A. lacustris* and either Anaplasmataceae (purple), Rickettsiaceae (orange), 'Holosporaceae' (blue) or 'Midichloriaceae' (green).



**Figure 4. Phylogenetic analysis based on concatenated alignment of 14 conserved flagellar genes *A. lacustris* and branch support values on branches of interest have been marked with bold red.** Tree topology and posterior probability branch support values (shown below branches) were inferred with PhyloBayes (model: CAT-Poisson). Posterior probability support values lower than 0.70 were omitted. Bootstrap branch support values (shown above branches) were calculated with RAxML (model:  $\Gamma$ +LG). Separate full PhyloBayes and RAxML trees are shown in Supplementary Figure 3. Major taxonomic groups have been collapsed and outgroup has been trimmed to improve readability.



**Figure 5. Relative abundance of alphaproteobacterium LD12 compared to *A. lacustris***  
 The relative abundances were assessed for public lake metagenomes Vattern, Sparkling (Summer), Sparkling (Spring), Mendota (Summer), Mendota (Spring), Erken, Ekoln, Damariscotta (Summer) and Damariscotta (Spring) and public SSU amplicon datasets Rare biosphere at the North Falmouth Fire Station (VAMPS accession: RARE\_NFF\_Bv6v4), ~25 lakes and streams of the Toolik Lake Research Area sequencing run 1 & 2 (SRA accessions: SRR305966, SRR305967). Each point represents the relative abundance of a LD12 SAG (described in (Zaremba-Niedzwiedzka *et al.*, 2013)) for lake metagenomes and the relative abundance of the LD12 SSU sequence (accession no. Z99997.1) for the amplicon datasets. Points are jittered and colored alternating black and dark grey per dataset to improve readability.



**Figure 6. Phylogenetic tree based on SSU rRNA gene including *A. lacustris* related reads identified SRA and VAMPS SSU amplicon datasets and related sequences in NCBI-nt**  
 The *A. lacustris* leaf and branch support values on branches of interest have been marked with bold red. For NCBI-nt sequences, their environmental origin is stated between parentheses. Names of reads and NCBI-nt sequences have been colored based on the type of environment where the sample was taken (freshwater: blue, soil: brown). Internal branches with bootstrap support less than 30 were collapsed. Major taxonomic groups have been collapsed. Outgroup has been omitted to improve readability. Full phylogenetic tree is shown in Supplementary Figure 6.

**Table 1***A. lacustris* draft genome features

<b>Feature</b>	<b>Value</b>
Number of contigs	151
N50 (bp)	12 448
Total size (bp)	822 563
GC (%)	32.6
ORFs	882
rRNA	1 <sup><i>l</i></sup>
tRNA	10
Coding density (%)	88.4
Average intergenic space length (bp)	99
Completeness estimate (%)	48
Genome size estimate (Mb)	1.7
Transposases	26
Integrases	9
Ankyrin repeat proteins	5
Other repeat type proteins	5

<sup>*l*</sup>The SSU rRNA gene

**Table 2**  
Relative abundance of *A. lacustris* related reads in metagenomic and SSU amplicon datasets.  
The project titles and type of environments of the SRA and VAMPS datasets can be found in Supplementary Table 5.

Dataset	Type	Size (n)	Recruited reads (n)	Correct for completeness (n)	Relative abundance (%)
Damariscotta (Spring)	454 metagenome	343,495	14	29	0.008
Damariscotta (Summer)	454 metagenome	399,994	15	31	0.008
Mendota (Spring)	454 metagenome	484,350	17	35	0.007
Mendota (Summer)	454 metagenome	562,100	11	23	0.004
Sparkling (Spring)	454 metagenome	137,643	4	8	0.006
Sparkling (Summer)	454 metagenome	53,174	1	2	0.004
Ekoln	454 metagenome	324,764	18	37	0.011
Erken	454 metagenome	665,775	46	95	0.014
Vattern	454 metagenome	332,583	18	37	0.011
SRR305966 <sup>/</sup>	SSU amplicon (SRA)	528,037	13	-	0.002
SRR305967 <sup>/</sup>	SSU amplicon (SRA)	435,378	8	-	0.002
ERR204530 <sup>/</sup>	SSU amplicon (SRA)	1,998	4	-	0.2
ERR204548 <sup>/</sup>	SSU amplicon (SRA)	1,154	1	-	0.087
ERR204555 <sup>/</sup>	SSU amplicon (SRA)	866	1	-	0.115
ERR204613 <sup>/</sup>	SSU amplicon (SRA)	604	1	-	0.166
ERR204649 <sup>/</sup>	SSU amplicon (SRA)	2,139	1	-	0.047
ERR204651 <sup>/</sup>	SSU amplicon (SRA)	673	1	-	0.149
ERR204690 <sup>/</sup>	SSU amplicon (SRA)	3,946	1	-	0.025
ERR204702 <sup>/</sup>	SSU amplicon (SRA)	919	2	-	0.218
ERR204743 <sup>/</sup>	SSU amplicon (SRA)	3,074	1	-	0.033
RARE_MSFF_Bv6v4 <sup>/</sup>	SSU amplicon (VAMPS)	216,537	7	-	0.003
RARE_NFF_Bv6v4 <sup>/</sup>	SSU amplicon (VAMPS)	307,126	22	-	0.007
RARE_WHF_Bv6v4 <sup>/</sup>	SSU amplicon (VAMPS)	440,607	2	-	0.0005

<sup>/</sup> Study titles and environment type can be found in Supplementary Table 5