

Supplementary Information Summary

The Supplementary Information contains the **Supplementary Text**, which describes the source of *A. lacustris*' name, the **Supplementary Methods**, describing the details of all performed phylogenetic analyses, and also the
5 **Supplementary Figures S1-S6** and **Supplementary Tables S1-S6**, which provide the full, uncollapsed phylogenies for all phylogenetic analyses performed in this study.

Supplementary Text

10 The name '*Candidatus* Arcanobacter lacustris' has been derived from the Latin words "arcanus", meaning mysterious or secret, and "lacustris", adjective from "lacus", the lake. The name reflects the mysterious nature of this bacterium (the uncertainty in lifestyle and low abundance) and the type of environment the single cell was isolated from.

15 Supplementary Methods

Detailed phylogenetic analyses

Panorthologs

The genome and corresponding protein coding sequences (CDSs) from 20 Rickettsiales and 4 non-Rickettsiales
20 Alphaproteobacteria were downloaded from NCBI RefSeq (accession numbers: [Supplementary Table 6](#)). Sequences from these 24 genomes and from *A. lacustris* were clustered in ortholog clusters as described in ([Guy et al., 2013](#)), These clusters are referred to as 'rickCOGs'.

From the 12,197 resulting clusters, 64 panorthologs (i.e. genes present in exactly one copy in all taxa) were
25 extracted ('64-panorthologs' dataset). In addition, a further 65 rickCOGs were extracted, which are panorthologs in all taxa but missing in *A. lacustris* and added to the previous dataset ('129-panorthologs' dataset).

All panorthologs were subsequently aligned individually and trimmed as described above. All 129 clusters were annotated by using the aligned panorthologs as a query in a PSI-BLAST search versus Swiss-Prot. Alignments
30 were concatenated into a final alignment consisting of 40,298 and 16,095 sites for the '129-panorthologs' and the '64-panorthologs' datasets, respectively. Genome phylogenies were then inferred with maximum likelihood and Bayesian methods as described above. Finally, for the '64-panorthologs' dataset, maximum-likelihood support for the Bayesian phylogeny was inferred by running 100 rapid bootstraps with RAxML.

35 Small subunit ribosomal DNA

From the SILVA database (Quast *et al.*, 2012), 64 representative Rickettsiales and free-living Alphaproteobacteria SSU rRNA sequences were retrieved. Sequences were selected so that as many Rickettsiales taxonomic groups as possible were represented by named strains and met the following criteria: sequence length \geq 1200 bp, sequence quality \geq 90, alignment quality \geq 90 and pintail quality \geq 90. The *A. lacustris* SSU rDNA sequence, predicted by RNAmmer 1.2, was added and the resulting dataset was aligned with SINA v1.2.11 (Pruesse *et al.*, 2012) using all sequences classified as Alphaproteobacteria as reference, trimmed and used to infer maximum likelihood phylogeny.

Exploration of *A. lacustris* phylogenetic diversity

45 The *A. lacustris* SSU sequence was used as a query in a standard BLASTN (Altschul *et al.*, 1990) search of the NCBI-nt database. Based on a subsequent distance tree analysis, the twelve most closely related sequences were retrieved and added to the SSU dataset. Finally, 65 sequence reads retrieved from the SRA (34) and VAMPS (31) databases (see Materials and Methods) with \geq 95% sequence identity were clustered into 10 OTUs with UCLUST 1.2.22 (Edgar, 2010), using a 97% sequence identity threshold. Seed sequences were extracted and subsequently checked for primer adapter sequences with BLASTN search of the NCBI UniVec databases. Primer adapters were clipped if necessary and the clipped sequences were added to the dataset. To this initial dataset consisting of twelve most closely related BLASTN hits, seven SRA reads and three VAMPS reads, the 64 Rickettsiales used in the SSU rDNA phylogeny were added. The dataset was then aligned with SINA v1.2.11, using all sequences classified as Alphaproteobacteria in the SILVA database as reference. The alignment was then trimmed and an initial maximum likelihood phylogeny was inferred. From the obtained phylogeny, five BLASTN hits that were too distant from *A. lacustris* were manually removed. The remaining sequences were then realigned with SINA, trimmed, and a maximum likelihood phylogeny was inferred. All internal branches with \leq 30 bootstrap support were collapsed with Newick-Utilities 1.6 (Junier & Zdobnov, 2010).

60 Flagella

Protein sequences of 14 core flagellar genes in 63 bacteria (Sassera *et al.*, 2011) were complemented with orthologs from *A. lacustris* and several newly sequenced Alphaproteobacteria (*Holospira undulata* HU1, *Candidatus Odysseella thessalonicensis* L13, *Kordiimonas gwangyangensis* DSM 19435, *Micavibrio aeruginosavorus* ARL-13, *Polymorphum gilvum* SL003B-26A1, *Kiloniella laminariae* DSM 19542 and *Magnetococcus marinus* MC-1; for accession numbers see Supplementary Table 1). Orthologs were identified in three steps. First, a PSI-BLAST search against the proteomes of *A. lacustris* and the new Alphaproteobacteria was done using the gene alignments from Sassera *et al.* as a query. All core flagellar genes had hits to all new taxa had hits, except *A. lacustris* (8 hits out of 14 genes), and *Holospira* (2 hits out of 14 genes). Because only two genes

(*fliI* and *motB*) had hits in *Holospora*, *Holospora* was omitted from any further analyses. Hits were added to the
70 base set and single gene phylogenies were inferred with FastTreeMP 2.1 (Price *et al.*, 2010). Finally, orthologs
were distinguished from distant paralogs by manual inspection of the trees. In cases where orthologs in the new
Alphaproteobacteria could not be clearly identified from the single gene trees, identification was done based on
gene synteny and NCBI RefSeq annotation.

The final dataset comprised 70 taxa, with *Polymorphum gilvum* and *Bradyrhizobium japonicum* having
75 each two complete sets of genes. Two concatenated alignments were prepared: one contained all 14 core flagella
(with six flagella missing in *A. lacustris*), and another only contained the eight core flagella that were present in *A.*
lacustris. Both alignments were subsequently trimmed and used to infer maximum likelihood and Bayesian
phylogenies.

80 ATP/ADP-translocase

The ATP/ADP translocase experimentally characterized in *Rickettsia prowazekii* Madrid E (Tlc1) (Audia &
Winkler, 2006) was used as a query for a BLASTP search to identify homologs in *A. lacustris* and for a PSI-
BLAST search with one iteration to identify homologs in the nr database. PSI-BLAST hits from a selected set of
taxa were extracted and pooled with the *A. lacustris* homolog. Taxa selection was done so that the number of
85 sequences was significantly reduced, but all diverse major taxonomic groups (Rickettsiales, Rhizobiales,
Chlamydiales, Deltaproteobacteria, and Gammaproteobacteria) and their subclades were still represented.
Sequences were aligned and the alignment was visually inspected with Seaview (Gouy *et al.*, 2010). Poorly
aligned and highly diverging sequences (based on their branch lengths in a neighbor joining tree) were manually
removed. The remaining alignment was subsequently trimmed and used to calculate a BI phylogeny.

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T4SS

Protein sequences from components of type IVA classified T4SS in *R. prowazekii* Madrid E, *R. bellii* RML369-C,
O. tsutsugamushi Ikeda, *N. risticii* Illinois, *Wolbachia* endosymbiont of *Drosophila melanogaster*, *Wolbachia*
endosymbiont TRS of *Brugia malayi*, *E. canis* Jake, *E. chaffeensis* Arkansas, *A. phagocytophilum* HZ, *A.*
95 *marginale* Florida, *A. tumefaciens* C58 plasmid Ti, *X. citri* plasmid pXcB, *B. henselae* Houston-1 and *L.*
pneumophila Lens were downloaded from the SecReT4 database (Bi *et al.*, 2013)(accession numbers:
Supplementary Table 1). Taxon selection was guided by Figure 2 of (Gillespie *et al.*, 2010). Gene clusters were
created for *virB4*, *virB8*, *virB9*, *virB10* and *virB11* based on SecReT4 annotation, complemented with
corresponding homologs in *A. lacustris* and used to infer single gene ML phylogenies. Paralogs were distinguished
100 from orthologs by manual inspection of the resulting trees and evaluation of gene synteny and removed. The
resulting orthologous groups were then used to prepare a trimmed concatenated alignment from which ML
phylogenies were inferred.

Supplementary References

- 105 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990). Basic local alignment search tool. *J Mol Biol* **215**:403–410.
- Audia JP, Winkler HH. (2006). Study of the Five Rickettsia prowazekii Proteins Annotated as ATP/ADP Translocases (Tlc): Only Tlc1 Transports ATP/ADP, While Tlc4 and Tlc5 Transport Other Ribonucleotides. *J Bacteriol* **188**:6261–6268.
- 110 Bi D, Liu L, Tai C, Deng Z, Rajakumar K, Ou H-Y. (2013). SecReT4: a web-based bacterial type IV secretion system resource. *Nucleic Acids Res* **41**:D660–D665.
- Edgar RC. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**:2460–2461.
- Gillespie JJ, Brayton KA, Williams KP, Diaz MAQ, Brown WC, Azad AF, *et al.* (2010). Phylogenomics Reveals a Diverse Rickettsiales Type IV Secretion System. *Infect Immun* **78**:1809–1823.
- 115 Gouy M, Guindon S, Gascuel O. (2010). SeaView Version 4: A Multiplatform Graphical User Interface for Sequence Alignment and Phylogenetic Tree Building. *Mol Biol Evol* **27**:221–224.
- Guy L, Nystedt B, Toft C, Zaremba-Niedzwiedzka K, Berglund EC, Granberg F, *et al.* (2013). A Gene Transfer Agent and a Dynamic Repertoire of Secretion Systems Hold the Keys to the Explosive Radiation of the Emerging Pathogen Bartonella. *PLoS Genet* **9**:e1003393.
- 120 Junier T, Zdobnov EM. (2010). The Newick utilities: high-throughput phylogenetic tree processing in the Unix shell. *Bioinformatics* **26**:1669–1670.
- Price MN, Dehal PS, Arkin AP. (2010). FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. *PLoS ONE* **5**:e9490.
- 125 Pruesse E, Peplies J, Glöckner FO. (2012). SINA: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* **28**:1823–1829.
- Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, *et al.* (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41**:D590–D596.
- Sassera D, Lo N, Epis S, D’Auria G, Montagna M, Comandatore F, *et al.* (2011). Phylogenomic Evidence for the Presence of a Flagellum and cbb3 Oxidase in the Free-Living Mitochondrial Ancestor. *Mol Biol Evol* **28**:3285–3296.
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Supplementary Table Titles

Supplementary Table S1

135 Annotation of the 129 Rickettsiales panorthologous marker genes that were used for the phylogenomic analyses and completeness estimate and occurrence in the *A. lacustris* single cell draft genome. *Provided as Excel file.*

Supplementary Table S2

140 Annotated rickCOGs either unique to *A. lacustris* or uniquely shared with Rickettsiaceae, Anaplasmataceae, 'Midichloriaceae' or 'Holosporaceae'. Ortholog clusters mentioned in the main text are given in bold italics and colored according to putative function: mobile element (green), heme metabolism (brown), toxin-antitoxin systems (purple), chemotaxis (blue) and phage related proteins (black). *Provided as Excel file*

Supplementary Table S3

145 List of putative T4SS effector proteins in *A. lacustris* based on BLASTP versus SecReT4 effectors and experimentally verified effectors databases. *Provided as Excel file*

Supplementary Table S4

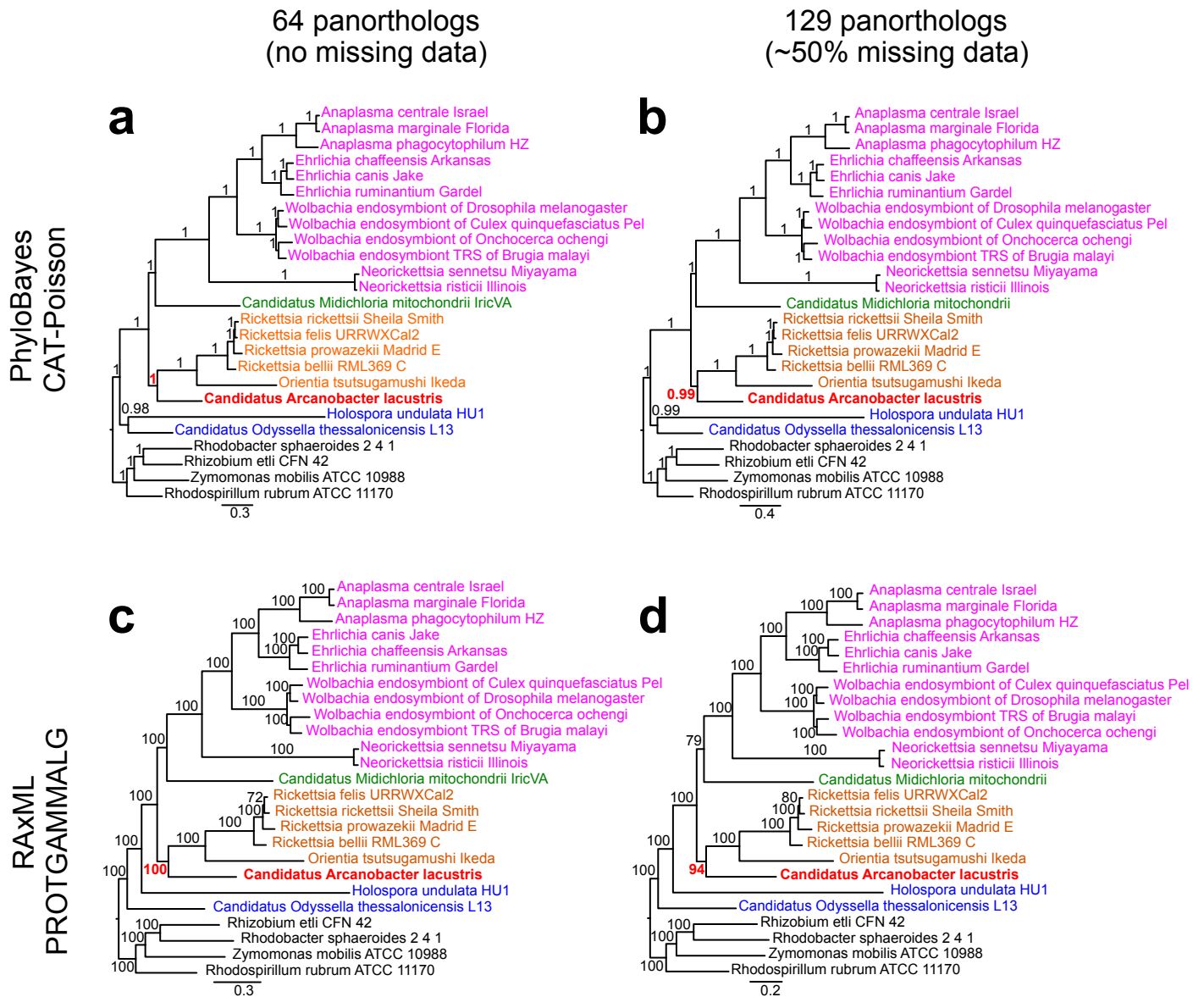
150 Accessions, databases and types of environment for all *A. lacustris* related sequences that were incorporated in Figure 6 and Supplementary Figure 6. *Provided as Excel file*

Supplementary Table S5

155 Accession numbers, project titles and type of environment for the SSU amplicon datasets available in SRA and VAMPS that contained reads with high identity to *A. lacustris* SSU. *Provided as Excel file*

Supplementary Table S6

Accession numbers of publicly available genomes used in this study. *Provided as Excel file*



Supplementary Figure S1

Robustness of phylogenetic placement of *A. lacustris* to tree inference method and missing data in concatenated panorthologs alignments. Phylogenies were either inferred with PhyloBayes (model: CAT-Poisson) (**a,b**) or RAxML (model: Γ +LG) (**c,d**) from either the concatenated alignment of 64 panorthologs (no missing data) (**a,c**) or the concatenated alignment of 129 panorthologs (~50% missing data) (**b,d**). Taxa are colored according to their taxonomic affiliation at the family level: Anaplasmataceae: pink, 'Midichloriaceae': green, Rickettsiaceae: orange and 'Holosporaceae': blue. Outgroup taxa are in black. *A. lacustris* and branch support values of interest are shown in bold red.

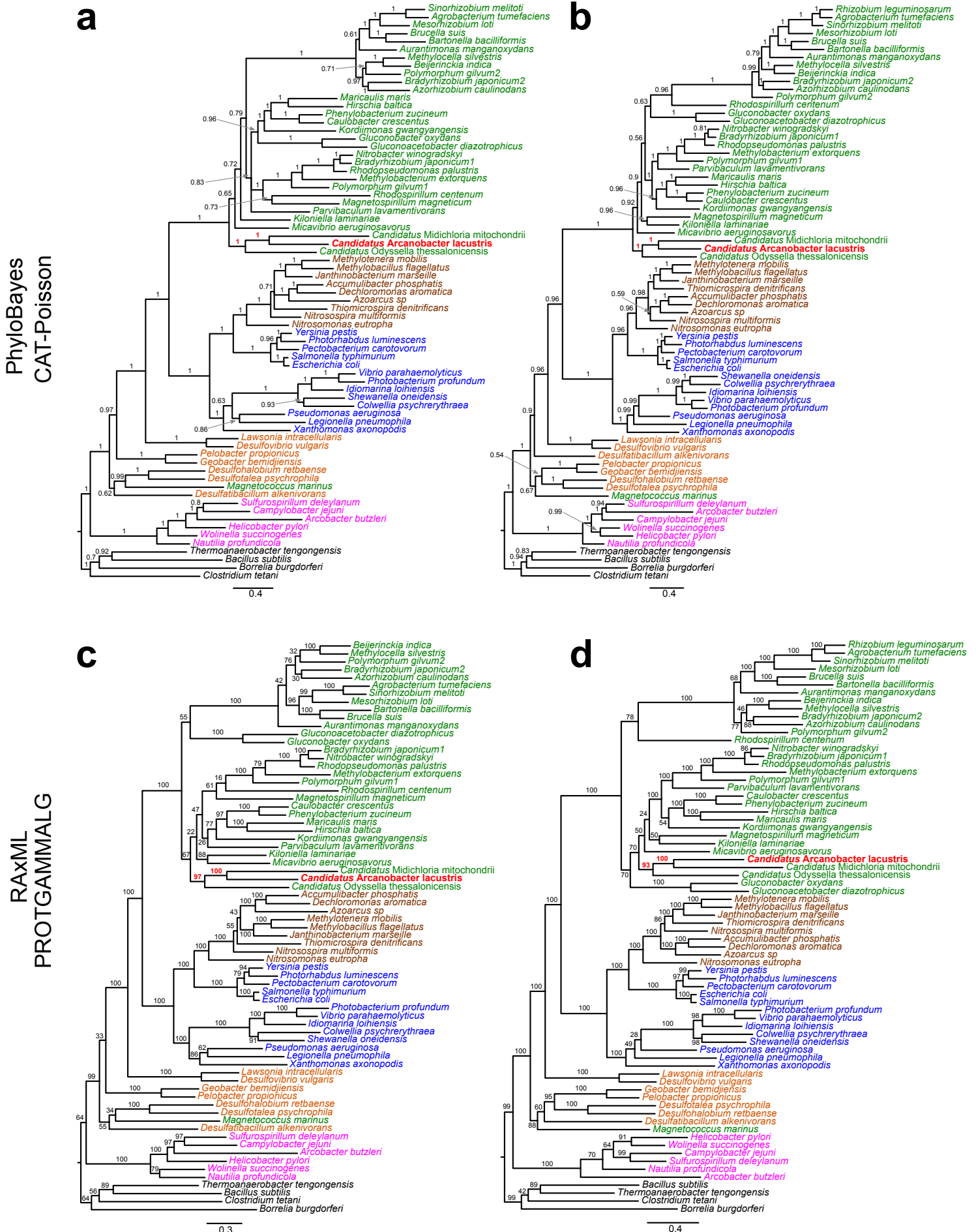


Supplementary Figure S2

Phylogenetic tree based on SSU rRNA gene. Tree topology and bootstrap support values were inferred with RAXML (model: Γ +LG). This is the full tree of the phylogenetic analysis shown in Figure 2b. The *A. lacustris* leaf and branch support values on branches of interest have been marked with bold red. Other taxa names were colored as follows: Anaplasmataceae: pink, 'Midichloriaceae': green, Rickettsiaceae: orange, 'Holosporaceae': blue, SAR116: purple, 'Free-living Alphaproteobacteria': grey.

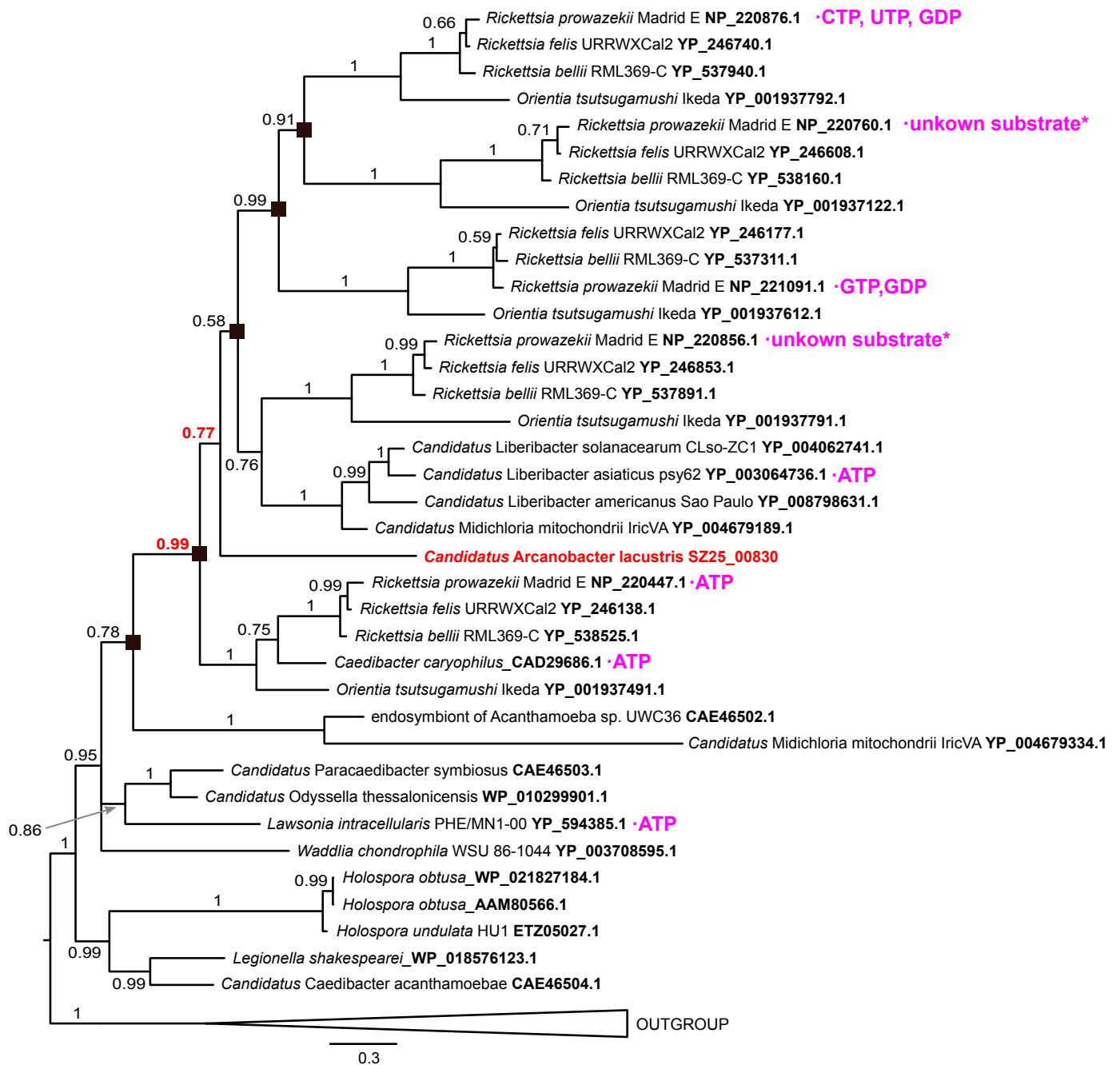
8 core flagella
(no missing data)

14 core flagella
(~57% missing data)



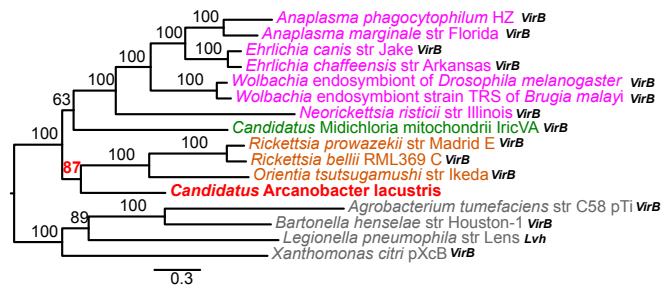
Supplementary Figure S3

Robustness of phylogenetic analyses to tree inference method and missing data in concatenated core flagellar alignments. Phylogenies were either inferred with PhyloBayes (model: CAT-Poisson) (a,b) or RAXML (model: Γ +LG) (c,d) from either the concatenated alignment of 8 core flagella (a,c) or the concatenated alignment of 14 core flagella (b,d). Taxa are colored according to their taxonomic affiliation at the class level: Alphaproteobacteria: green, Betaproteobacteria: brown, Gammaproteobacteria: blue, Deltaproteobacteria: orange and Epsilonbacteria: pink. Outgroup taxa are in black. *A. lacustris* and branch support values of interest are shown in bold red.



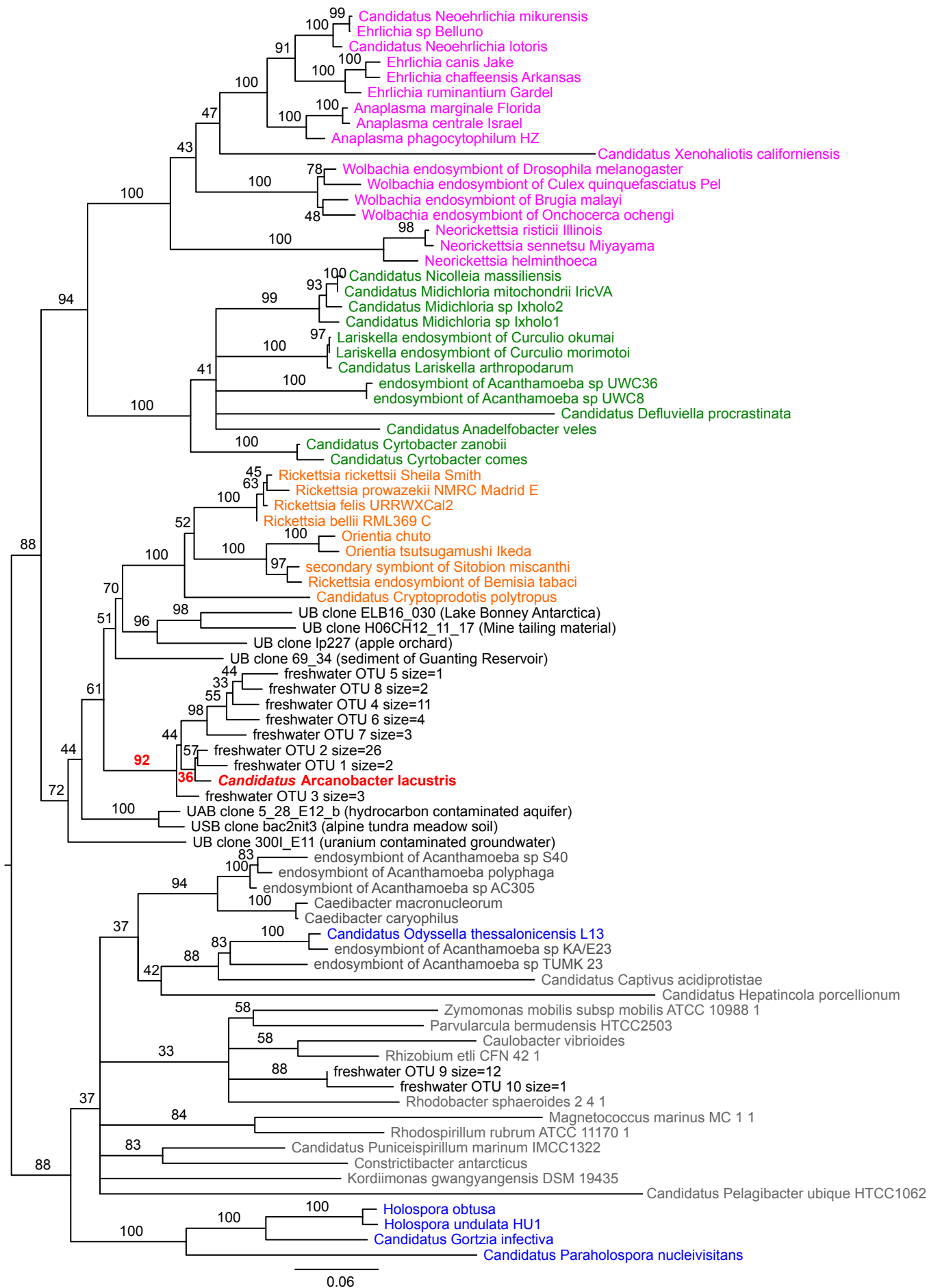
Supplementary Figure S4

Phylogenetic analysis of bacterial ATP,ADP-translocase proteins. Tree was inferred with PhyloBayes (model: CAT-Poisson). Inferred gene duplication events are indicated with black squares on the respective nodes. Accession numbers are given in bold. For proteins that have been functionally characterized, the substrate is given in bold pink. (*) Substrates for these proteins are not known, but shown not to be ATP, ADP, AMP, CTP, CMP, GTP, GDP, GMP, UTP or UMP. *A. lacustris* and branch support values of interest are shown in bold red. All Chlamydiales homologs are used as outgroup and have been collapsed to improve readability.



Supplementary Figure S5

Phylogenetic analysis based on the concatenated alignment of conserved T4SS proteins VirB4, VirB8, VirB9, VirB10 and VirB11. Tree was inferred with RAxML (model: Γ +LG). Taxa are colored according to their taxonomic affiliation at the family level: Anaplasmataceae: pink, 'Midichloriaceae': green and Rickettsiaceae: orange. Outgroup taxa are in grey. Names of the taxa specific T4SS, based on SecReT4 classification, are given in bold italics. *A. lacustris* and branch support values of interest are shown in bold red.



Supplementary Figure S6

Phylogenetic tree based on SSU rRNA gene including *A. lacustris* related reads identified in the lake metagenomes, SRA and VAMPS SSU amplicon datasets and related sequences in NCBI-nt. This is the full tree of the phylogenetic analysis shown in Figure 6. Internal branches with bootstrap support less than 30 were collapsed. Taxa are colored according to their taxonomic affiliation at the family level; Anaplasmataceae: pink, 'Midichloriaceae': green, Rickettsiaceae: orange and 'Holosporaceae': blue. Outgroup taxa are grey. The *A. lacustris* leaf and branch support values on branch of interests have been marked with bold red. For NCBI-nt sequences, their environmental origin is stated between parentheses.