

# ***Deianiraea*, an extracellular bacterium associated with the ciliate *Paramecium*, suggests an alternative scenario for the evolution of *Rickettsiales***

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## **Abstract**

*Rickettsiales* are a lineage of obligate intracellular *Alphaproteobacteria*, encompassing important human pathogens, manipulators of host reproduction, and mutualists. Here we report the discovery of a novel *Rickettsiales* bacterium associated with *Paramecium*, displaying a unique extracellular lifestyle, including the ability to replicate outside host cells. Genomic analyses show that the bacterium possesses a higher capability to synthesise amino acids, compared to all investigated *Rickettsiales*. Considering these observations, phylogenetic and phylogenomic reconstructions, and re-evaluating the different means of interaction of *Rickettsiales* bacteria with eukaryotic cells, we propose an alternative scenario for the evolution of intracellularity in *Rickettsiales*. According to our reconstruction, the *Rickettsiales* ancestor would have been an extracellular and metabolically versatile bacterium, while obligate intracellularity would have evolved later, in parallel and independently, in different sub-lineages. The proposed new scenario could impact on the open debate on the lifestyle of the last common ancestor of mitochondria within *Alphaproteobacteria*.

## **Introduction**

The “Bergey’s Manual of Systematics of Archaea and Bacteria” states that *Rickettsiales* (*Alphaproteobacteria*) “multiply only inside [eukaryotic] host cells” [1]. Obligate intracellularity indeed represents the main common feature of this bacterial order, considering that their host range, metabolic capabilities, and spectrum of effects on the hosts can vary greatly [2-5]. Indeed *Rickettsiales* encompass highly diverse representatives, including human pathogens [6-8], reproductive parasites of arthropods [3], mutualists of nematodes [3, 9] or arthropods [10], and several bacteria associated with unicellular eukaryotes, producing undisclosed consequences on their hosts [5]. Additionally, *Rickettsiales* are well known to evolutionary biologists, since several studies suggested them as the sister group of mitochondria [11-13], although this point is highly debated [14, 15].

A common trait of most *Rickettsiales* is the ability to perform horizontal transmission, testified by lack of congruence in host and symbiont phylogenies [2, 6, 7, 16-21] and experimental host transfers [20, 22- 24]. Thus, *Rickettsiales* are “host-dependent”, but, with few exceptions [25], not “host-restricted”, because they are not exclusively vertically transmitted. The life cycle of most *Rickettsiales* can be ideally subdivided into two phases. The main phase is intracellular, during which all life functions, including replication, are exerted. During the other, facultative, phase, the bacteria can survive temporarily in the extracellular environment and can enter new host cells [24, 26-28]. According to our best knowledge, these “transmission forms”, sometimes displaying altered morphology [29-31], have never been reported to replicate.

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Until recently, a *sensu lato* definition of *Rickettsiales* was employed, however recent studies [13, 32-36] repeatedly indicated that this group is paraphyletic. Thus, in this work we will follow the *sensu stricto* definition by Szokoli and co-authors [37] of *Rickettsiales*, i.e. including only the three families *Rickettsiaceae*, *Anaplasmataceae*, “*Candidatus* (*Ca.*) *Midichloriaceae*” (from now on, *Midichloriaceae*), and placing the other *sensu lato* members in the evolutionary independent order *Holosporales*. While *Rickettsiales sensu stricto* form a strongly supported monophyletic group (e.g. [13, 17, 35, 37, 38]), the members of the three families can be distinguished by multiple features, including the host cell entrance mechanism and the presence or absence of a host-derived membrane envelope in the intracellular phase [5, 26, 27]. Regardless of these differences, the transition to an obligate intracellular state has always been assumed to have occurred only once in *Rickettsiales* evolution, before the separation of the three families [7, 24, 39, 40]. Thus, this trait has been considered as directly inherited from their common ancestor.

Here we present the first report of an *Rickettsiales* bacterium, “*Ca.* *Deianiraea vastatrix*” (from now on *Deianiraea vastatrix*; Taxonomic Description in Supplementary Material 1), displaying an unprecedented extracellular and putatively parasitic lifestyle in the interaction with the ciliate protist *Paramecium primaurelia*. According to the obtained novel morphological, phylogenetic and genomic data, we re-evaluated traditional hypotheses on the evolution of *Rickettsiales*, which always implied an “intracellularity early”, dating back to the common ancestor. Thus, based on the here presented findings, we propose an alternative scenario, named “intracellularity late”, under which we suggest that intracellularity could have evolved in parallel and independently along multiple *Rickettsiales* sub-lineages from an extracellular, yet host-associated, ancestor.

## Materials and methods

### *Paramecium* strain origin and maintenance

The monoclonal *Paramecium* strain CyL4-1 was isolated on 29 September 2014 from a water sample (9‰ salinity) collected from a wastewater stream in Larnaca, Cyprus (34.91°N, 33.60°E). It was initially maintained in a lettuce infusion inoculated with *Enterobacter aerogenes* (gradually adapted to 0‰). In order to maintain it in the laboratory, a transfer procedure was performed every 2-3 days, i.e., single viable cells of *Paramecium* were picked up and transferred into a Petri dish containing 250 µl of fresh lettuce infusion inoculated with *E. aerogenes* and supplemented with 0.8 µg/ml β-sitosterol (Merck, Darmstadt, Germany).

For living cell observations, paramecia were analysed using differential interference contrast (DIC) with a Leica 6000 microscope (Leica Microsystems, Wetzlar, Germany) equipped with a digital camera DFC 500.

### Transmission electron microscopy (TEM) and atomic force microscopy (AFM)

For TEM, *Paramecium* cells were processed and observed as described previously [41], except that 0.1 M phosphate buffer was used for fixation.

For AFM, living cells were placed on a cover slip and air-dried. The surface topography was registered by means of NTEGRA AURA microscope in a semi-contact mode.

### *Deianiraea* shift assays

To check the ability of *Deianiraea* to colonise the cell surface of other *Paramecium* strains and affect them, cells of the strain CyL4-1, preliminary confirmed to be abnormally shaped, unable to move, and densely covered by *Deianiraea* bacteria, were placed in Petri dishes containing 250 µl of lettuce infusion inoculated with food bacteria, ~50 *Paramecium* cells for each dish. The *Deianiraea*-free CyL4-1\* sub-replicate of the original strain plus six additional

naïve strains were then selected (Supplementary Material 2), and each added in duplicate to these Petri dishes, 25-30 cells per dish. As a control, the target cells were transferred in dishes containing only lettuce infusion inoculated with food bacteria. Cells were fed once a week with 50 µl of fresh medium inoculated with food bacteria. The dishes were checked every 4 days for the presence of living motile cells and for the signs of the developing colonisation by *Deianiraea* for a period of 40 days.

### **Molecular characterisation of *Paramecium* and *Deianiraea***

About 80-100 CyL4-1 cells were individually picked up, washed through several passages in sterile water and fixed with 70% ethanol. DNA was extracted with the Nucleospin Plant II kit (Macherey-Nagel), following the protocol for mycelium. All PCR reactions were performed as previously described [42]. For the molecular characterisation of *Paramecium*, 18S rRNA gene, ITS and COI gene were amplified and directly sequenced [42] (Supplementary Material 3).

Characterisation of *Deianiraea* was performed by PCR amplification of the almost complete 16S rRNA gene with universal bacterial primers and sequencing with internal primers, as previously described [43].

### **Fluorescence in situ hybridisation (FISH)**

*Paramecium* cells were processed and observed as described previously [41], with the following modifications: fixation was performed in 4% paraformaldehyde in PBS, and the hybridisation buffer contained 15% formamide. Specific probes for *Deianiraea* were newly designed, and their specificity was assessed by search on the Ribosomal Database Project (RDP) [44] and on SILVA [45] (Supplementary Material 4).

### **16S rRNA phylogenetic analysis**

Maximum likelihood (ML) and Bayesian inference (BI) phylogenetic analyses on the partial 16S rRNA gene sequence of *Deianiraea* were performed with ARB [46], as described previously [47]. The sequences of 33 other *Rickettsiales sensu stricto* [37] sequences were selected, plus 10 other *Alphaproteobacteria* as outgroup. 16S rRNA gene identity values were calculated on the alignment employed for phylogeny (Supplementary Material 5 and 6).

### **16S rRNA gene amplicon database screening**

With ARB, the whole set of sequences belonging to each of the four *Rickettsiales* families *sensu stricto* were selected from the SSURef NR99 128 Silva database [45]. After filtering out chimeras with DECIPHER [48] and manual inspection, the sequences were grouped in clusters with >93% identity. The final set of *Rickettsiales* included a member for each cluster (the sequence with the lower average divergence from the same cluster) (Supplementary Material 7). Thus, the sequences from this set were queried on the integrated microbial NGS (IMNGS) platform [49], selecting >200 bp hits with 90% minimal identity. For each family, the retrieved hits were merged, filtering out those shared by two or more families. Operational taxonomic units (OTUs) for each family were retrieved with UCLUST [50] with 97% identity, and assigned to the respective prevalent environmental origin according to IMNGS categories.

### **Genome sequencing and assembly**

The sequencing and assembly procedure is described in detail in the Supplementary Material 8-12. DNA was extracted from ~100 cells of *Paramecium* covered by *Deianiraea* as described above. In order to reach sufficient DNA quantity, the extract was subjected to whole-genome amplification (WGA) with the REPLI-g Single Cell Kit (Qiagen). The WGA product was processed through a Nextera XT library and sequenced on an Illumina HiSeq X machine by Admera Health (South Plainfield, NJ, USA), producing 37,100,964 pairs of 150 bp reads.

After a preliminary assembly using SPAdes 3.6 [51], the blobology pipeline was applied [52] to separate contigs belonging to *Deianiraea* from those from *Paramecium* and other free-living bacteria. Namely, contigs with coverage  $\geq 100x$  and best megablast hit with *Bacteria* or no hit were selected. This set of contigs was manually checked and revised. Thus, the reads mapping on the final selection of contigs were reassembled separately, obtaining a total of 12 contigs. According to the putative connections among contigs in the assembly graph, PCR assays were performed to join all the contigs (Supplementary Material 13) into a single circular chromosome (1,205,153 bp).

### **Genomic analyses**

The *Deianiraea* genome was annotated using Prokka [53], followed by manual curation. Secreted proteins and transmembrane domains were predicted with SignalP [54] and TMHMM [55].

Presence of insertion sequences and prophages were predicted with IS finder [56] and PHAST [57], respectively, and comparing the obtained results with the annotation. The origin of the identified mobile elements was investigated by inspecting blastp hits on the whole NCBI nr protein, and on selected subsections of nr based on taxonomy.

### **Phylogenomic analyses**

A previously published, curated dataset of 120 highly conserved orthologs was employed [36]. A representative taxon selection was chosen among *Rickettsiales sensu stricto*, other *Alphaproteobacteria*, and, as outgroup, other *Proteobacteria* (100 total organisms). The amino acid sequences of *Deianiraea* orthologs were identified by reciprocal blastp hits followed by careful manual inspection.

Then, they were added to the original alignment [36] with MAFFT [58] by keeping the originally aligned positions, and the site masking by Parks and co-authors [36] was employed (34,747 final positions).

Thus, after substitution model selection with ProtTest [59], ML and BI phylogenies were estimated for each set, respectively, with RaxML [60] with 1000 pseudoreplicates, and with MrBayes [61] with three runs each with one cold and three heated chains, iterating until convergence (i.e. average standard deviation of split frequencies below 0.01).

### **Metabolic prediction and comparison with other *Rickettsiales***

Metabolic pathways reconstruction was performed employing Pathway Tools [62], and KEGG [63], followed by manual inspection.

Cluster of orthology groups (COGs) were identified using the NCBI pipeline [64] for *Deianiraea* and other 11 representative *Rickettsiales sensu stricto*. The repertoire of COGs and functional categories was directly compared among those organisms, to identify *Deianiraea* peculiar traits.

The biosynthetic pathways for amino acids of *Deianiraea* were manually reconstructed in detail (Supplementary Materials 13-15).

### **Horizontal gene transfer (HGT) tests**

In order to evaluate if the *Deianiraea* genes assigned to the COGs absent in all other *Rickettsiales* were a result of HGT, blastp hits on the whole NCBI nr protein database and on selected subsections based on taxonomy were inspected. “Informative HGT-candidate genes” (i.e. those with identity with best hits  $> 50\%$ , and with at least 5% difference in the identities

with the best hits on whole nr and on *Alphaproteobacteria*-only) were selected for phylogenetic analyses. Briefly, orthologs were identified on selected genomes, and processed as described above (“Phylogenomic analyses”), in order to perform single-gene ML phylogenies.

For each amino acid biosynthetic pathway peculiar of *Deianiraea* (Supplementary Material 13), the ML phylogenetic analyses were performed on concatenated alignments, and compared with the respective organismal phylogeny. Additionally, compositional variations were tested respect to the whole *Deianiraea* gene set as described previously [40].

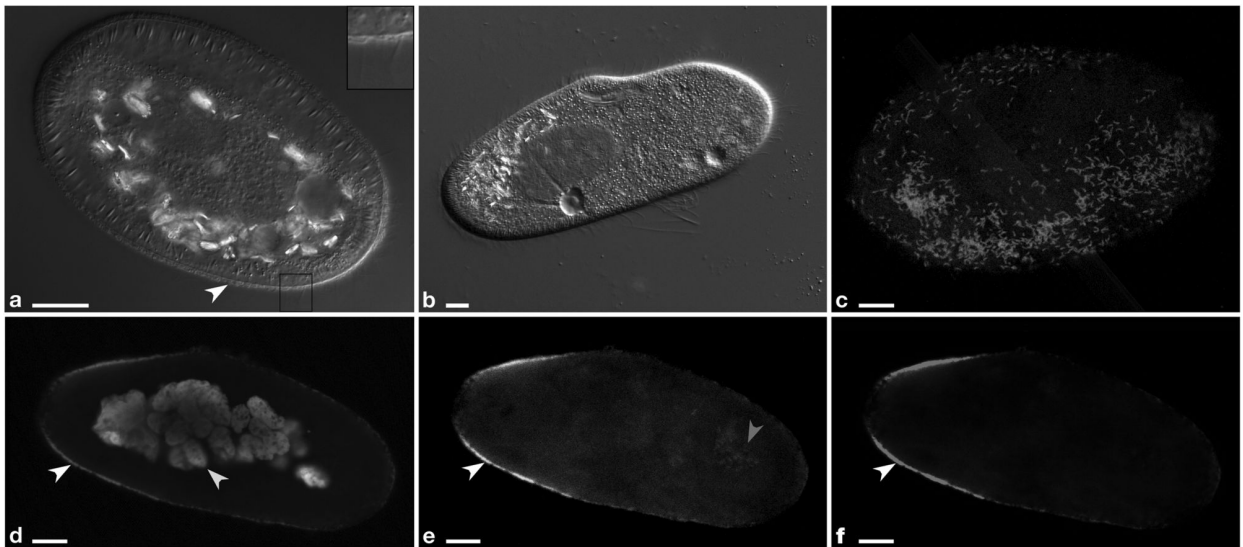
## Results

### A novel putative bacterial parasite of *Paramecium*

A monoclonal culture of *P. primaurelia* cells (strain CyL4-1) was obtained from a sample of wastewater collected in Larnaca—Cyprus. After one month of laboratory cultivation, many dead cells were registered. Microscopic observations revealed an abnormal phenotype: shortened ovoid- rounded shape and immotility caused by a nearly complete loss of cilia, which in paramecia fully cover the cell and are employed for locomotion and feeding (Fig. 1a, b; Supplementary Material 16d). The deciliated areas of the cell surface were covered by a dense layer of tightly packed intermingled bacteria (Figs. 1a and 2a; Supplementary Material 16c), in a fashion reminiscent of the arrangement of episymbiotic bacteria covering the surface of ciliates from oxygen-depleted environments [65, 66]. The thickness of the overlay looked roughly proportional to the level of degeneration in the shape of the ciliates. These bacteria appeared morphologically uniform, presenting the typical double-membrane cell structure of Gram-negatives, and were slightly curved rods (1.6-1.7 x 0.1-0.2  $\mu\text{m}$ ). Bacterial cells appeared to be narrowing on one end, with a sharp apical tip, which sometimes took directly contact with the external side of the *Paramecium* cell membrane (Fig. 2b, c). The bacterial cytoplasm was electron-dense, containing numerous ribosomes. Within the bacterial population, a small portion of dividing cells was regularly observed (Fig. 2d). In *Paramecium*, neither the intracellular ciliary basal bodies were altered in number and structure, nor the external cell membrane was damaged in its integrity. However, other features potentially linked to the pathological state were observed (Supplementary Material 16a, b). Moreover, no intracellular bacteria were ever observed aside from those in food vacuoles.

The extracellular bacteria attached to the *Paramecium* surface propagated rapidly within the culture, spreading the abnormal phenotype to other ciliate cells. Survival of the ciliate population was possible only by operating regular (every 2-3 days) transfers of *Paramecium* cells into fresh medium containing food bacteria, thereby diluting the load of epibiotic bacteria. Untreated (i.e. non-transferred) host cells invariably died within maximum 4 days. This procedure allowed laboratory maintenance for nearly 11 months of sub-replicates of the original *Paramecium* cultures harbouring the bacteria, never demonstrating any sign of bacterial presence inside the *Paramecium* cells (except within food vacuoles) in DIC, FISH with specific probes, or TEM observations.

The observed behaviour led us to hypothesise a probable parasitic effect of the surface-attached bacteria. To investigate this aspect, we obtained a sub-replicate culture (substrain CyL4-1\*), in which, through prolonged regular transferring, we managed to completely remove the surface-attached bacteria. This sub-strain was successfully maintained in culture with no sign of the altered phenotype. Strain CyL4-1\* and six other laboratory *Paramecium* strains were tested for susceptibility to the putative parasitic bacteria in experimental shift assays. Sub-strain CyL4-1\* cells died within the 4th day of the experiment, while two others were affected, showing the same phenotype as in the original CyL4-1 strain albeit more slowly than CyL4-1\*. The remaining four strains were fully untouched (Supplementary Material 2).



**Fig. 1** Microscopy images of *P. primaurelia* CyL4-1 covered by extracellular *Deianiraea* bacteria (**a, c-f**) and of control uninfected host cells (**b**). **a** Differential interference contrast (DIC) of a heavily infected CyL4-1 cell, displaying an atypical shortened and ovoid shape. The *Paramecium* cell surface is covered by a bacterial multilayer (white arrowhead). As shown in the enlarged inset on the right (corresponding to the square in the main picture) the ciliature is highly reduced, with only few remnants, **b** DIC picture of control uninfected host cells, **c** Fluorescence in situ hybridisation microscopy image of the surface of a *Deianiraea*-covered CyL4-1 cell. *Deianiraea* cells are visible by the green signal of the FITC (fluorescein isothiocyanate)-labelled universal bacterial probe EUB338 [103], **d-f** Multichannel microscopy images showing a transversal plan from a FISH experiment on a CyL4-1 cell at an advanced stage of invasion by the putative parasite. In (**d**) the blue signal from DAPI (4',6-diamidino-2-phenylindole) highlights the *Paramecium* fragmented macronucleus in autogamy (green arrowhead) and the bacterial cells. In (**e**) the green signal of FITC-labelled EUB338 is shown; in (**f**) the red signal of the *Deianiraea*-specific probe Deia\_416 labelled with Cy3. In (**d-f**), the *Deianiraea* cells covering the external surface of the host cell on its outline are fluorescently stained (white arrowheads). In (**e**), food bacteria in a digestive vacuole are also visible (red arrowhead), without any corresponding signal from the specific probe in (**f**). Bars stand for 10  $\mu$ m

### ***Deianiraeaceae*, a new family of order *Rickettsiales***

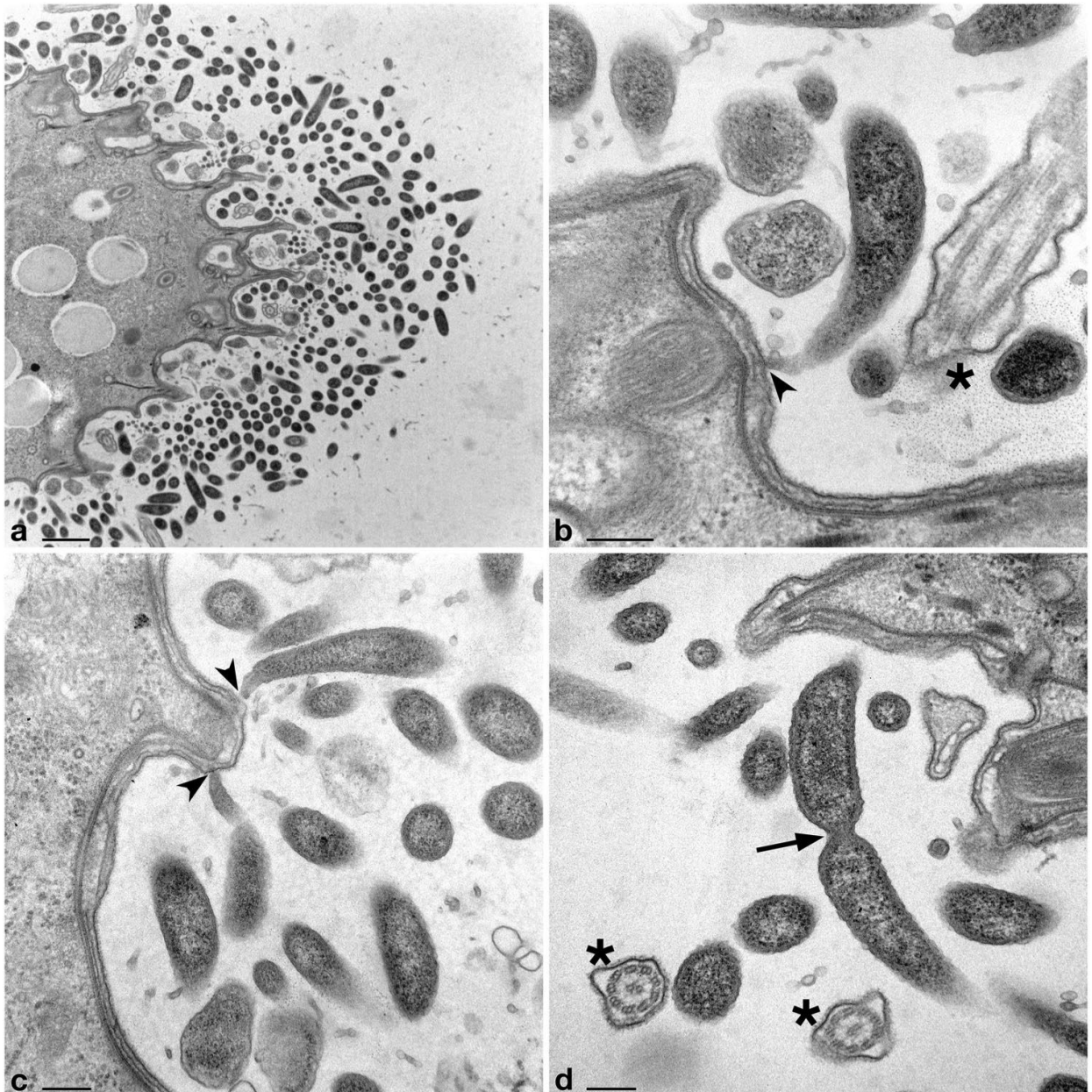
A single almost full-length 16S rRNA gene sequence (1431 bp) was obtained from CyL4-1 strain by PCR with universal bacterial primers followed by direct sequencing. The sequence showed the highest similarities with uncultured sequences of *Rickettsiales*-related bacteria, with best blastn hit against a bacterium from nephridia of the earthworm *Eiseniella neapolitana* (82.7% identity; JX644354 [67]). The use of newly designed specific probes in FISH experiments demonstrated that the characterised sequence belonged to the extracellular, putatively parasitic, bacterium (named *Deianiraea vastatrix*, Taxonomic description in Supplementary Material 1) and clearly showed that it constituted the entire external layers around the *Paramecium* cells (Fig. 1c-f; Supplementary Materials 17, 18). Conversely, *Deianiraea* was absent in food vacuoles, and never found inside the ciliate cells, confirming the extracellularity of this bacterium.

Phylogenetic analyses show that *Deianiraea* is a member of *Rickettsiales sensu stricto*, and belongs to a novel divergent family-level clade with 12 other uncharacterised database sequences. This clade is fully supported in the analyses (100 ML; 1.00 BI; Fig. 3; Supplementary Material 19) and will be referred from now on as *Deianiraeaceae* (Taxonomic Description in Supplementary Material 1). Within *Rickettsiales*, *Deianiraeaceae* formed a strongly supported group together with the families *Anaplasmataceae* and *Midichloriaceae* (97 ML; 1.00 BI). Although the precise relationships among those three lineages were unresolved, they were all equally supported. Overall, the 16S rRNA gene sequence identity within *Deianiraeaceae* and compared to other *Rickettsiales* are, respectively, 77.4–100% and 75.9–86.0% (Supplementary Material 6).

The sequences of other representatives of *Deianiraeaceae* belong to uncharacterised bacteria, mostly from aquatic environments, in several cases associated with diverse eukaryotes [67–71].

Other members of the clade were found in association to terrestrial animals, including, notably, on human skin of dermatitis-affected subjects [72, 73] (Fig. 3).

In order to evaluate the distribution and abundance of members of the novel family, a screening was performed on the IMNGS platform [49]. The family *Rickettsiaceae* displayed the highest total number of retrieved OTUs, followed by *Deianiraeaceae*, *Midichloriaceae*, and *Anaplasmataceae* (Fig. 3; Supplementary Material 20). In terms of environmental origin, *Rickettsiaceae* and *Anaplasmataceae* displayed similar patterns, with soil as the prevalent source (>20% OTUs), and several other OTUs from different water origins. On the contrary, *Midichloriaceae* and *Deianiraeaceae* OTUs derived mostly from aquatic environments, with the prevalence of seawater or marine for *Midichloriaceae* and freshwater for *Deianiraeaceae*.



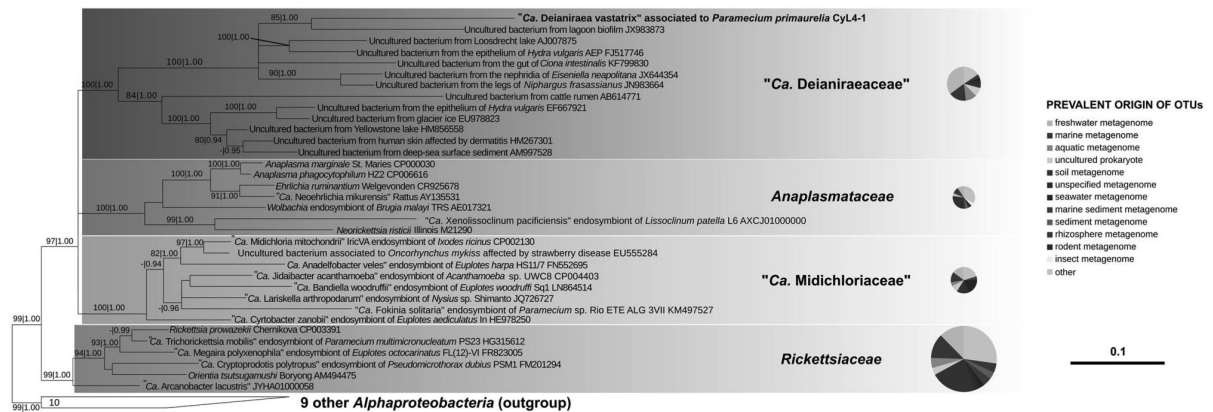
**Fig. 2** Transmission electron microscopy images of *Deianiraea* bacteria on the surface of *P. primaurelia* CyL4- 1 cells, **a** Portion of *Paramecium* cell surrounded by a huge number of extracellular *Deianiraea* bacteria, forming multiple layers. Bacterial cells appear electron dense, irregularly arranged, and sectioned in variably oriented planes, **b-d** Details of *Deianiraea* cells at higher magnification. In **(b)** and **(c)** longitudinal sections of some *Deianiraea* cells are shown. The bacterial side proximal to the *Paramecium* cell progressively narrows, ending up in a sharp and slightly curved apical tip, which takes direct contact with the external side of the *Paramecium* cell membrane (black arrowheads). In **(d)** a dividing *Deianiraea* cell, with the division septum highlighted by a black arrow. The few residual *Paramecium* cilia are evidenced by black asterisks in **(b, d)** sectioned from variable angles. Scale bars stand for, respectively, 1  $\mu\text{m}$  (**a**) and 200 nm (**b-d**)

## Genome features

The genome of *Deianiraea* is formed by a single circular replicon, ~1.2 Mbp long. Its size and GC content (32.9%) are consistent with the range of known *Rickettsiales*. The overall coding percentage is -92.2%, the highest among sequenced *Rickettsiales* (Supplementary Material 21). The genome includes 1129 ORFs, 34 tRNAs, and a single rRNA operon (split with 16S rRNA gene in one locus and 23S-5S rRNA genes in another, as in all *Rickettsiales*).

A limited number of mobile elements or their remnants was found: three transposases (two complete and one partial), two putative incomplete prophages (17 total phage genes), plus 13 other phage genes scattered in the genome (Supplementary Materials 22 and 23). The best blastp hits of the respective CDSs on NCBI nr proteins were quite divergent (Supplementary Materials 22 and 23), preventing a clear identification of their evolutionary relatedness to mobile sequences from other organisms.

Phylogenomic analyses were performed on a previously determined set of highly conserved protein coding genes [36]. The retrieved results confirmed 16S rRNA gene analysis, in particular the affiliation of *Deianiraea* to *Rickettsiales sensu stricto* and the monophyly of the group composed by *Anaplasmataceae*, *Midichloriaceae* and *Deianiraea* (Fig. 4; Supplementary Material 24). In addition, unlike the tritomy shown in the 16S phylogeny, these phylogenomic analyses indicated a sister relationship of *Deianiraea* to *Anaplasmataceae*. Moreover, none of the analysis retrieved a sister relationship of *Holosporales* with respect to *Rickettsiales sensu stricto* and, in turn (Supplementary Material 24), *Holosporales* did not appear monophyletic, in agreement with recent phylogenomic trees [36].



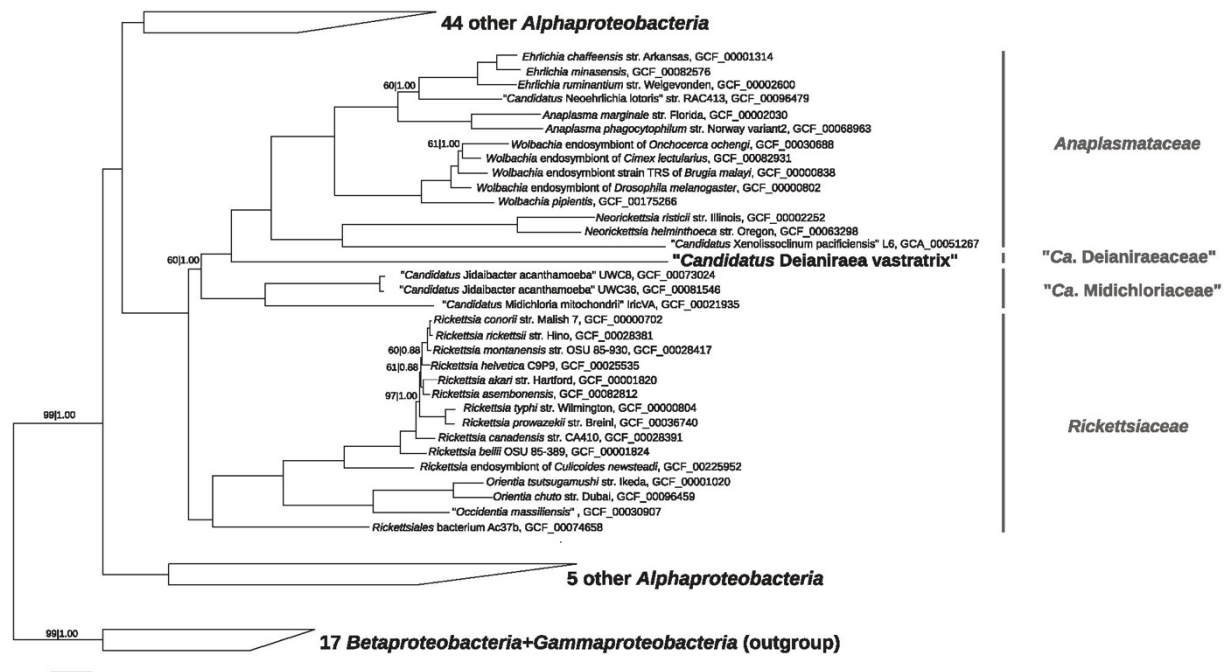
**Fig. 3** Bayesian phylogenetic tree on 1398 16S rRNA gene positions on 34 bacteria of the order *Rickettsiales* with the GTR+I+G substitution model. Posterior probabilities (after 1,000,000 generations) and maximum likelihood bootstrap values (with 1000 pseudo-replicates) of each internal tree nodes are shown on branches (values below 0.85/70 were omitted; full trees shown in Supplementary material 19). The newly characterised *Deianiraea*, putative parasite of *P. primaurelia* CyL4-I is evidenced in bold. The four *Rickettsiales* families (*Rickettsiaceae*, *Anaplasmataceae*, *Midichloriaceae*, and the herein newly proposed *Deianiraeaceae*) are highlighted by coloured boxes. The outgroup, composed by other 10 *Alphaproteobacteria*, is shown collapsed as a trapezoidal shape. The scale bar stands for estimated 0.10 sequence divergence. On the right side, for each family the pie chart shows the proportion of prevalent environmental origins (according to the IMNGS categories) of the OTUs obtained after IMNGS database queries with the representative sequence set of the family. The relative areas of the charts are proportional to the total amount of OTUs of the respective families. Only environmental origins that are the prevalent for at least 3% of total OTUs assigned to one family were displayed

## Predicted *Deianiraea* metabolic features

Most of the *Deianiraea* ORFs (916) found a COG assignment, in total 749 distinct COGs were identified, of which 108 corresponding to more than a single ORF (Supplementary Material 25). Most of the predicted metabolic and cellular features, including number and proportion of different functional categories, are similar to other *Rickettsiales* (Supplementary Materials 26 and 27).



In terms of carbohydrate and energetic metabolism, *Deianiraea* is predicted to perform complete gluconeogenesis and the non-oxidative pentose-phosphate pathway (except transaldolase). The gene sets for pyruvate dehydrogenase, Krebs cycle and oxidative phosphorylation are present (the same repertoire of other *Rickettsiales*, with the exception cytochrome *c* and its oxidase and reductase, which are missing in *Deianiraea*). A *tic* ATP/ADP translocase, homologue to those of other *Rickettsiales*, was identified as well.



**Fig. 4** Bayesian phylogenomic tree of representative *Rickettsiales* and other *Alphaproteobacteria*. Inference was based on the concatenated protein alignment (34,747 characters) from 120 highly conserved orthologs [36] with the LG+I+G+F evolutionary model. Maximum-likelihood bootstrap values (with 1000 pseudo-replicates) and posterior probabilities (after 100,000 generations) of internal tree nodes are shown on branches (fully supported values of 100/1.00 were omitted). The newly characterised *Deianiraea* putative parasite of *P. primaurelia* CyL4-1 is evidenced in bold. The representatives of each of the four families of *Rickettsiales* (*Rickettsiaceae*, *Anaplasmataceae*, *Midichloriaceae*, and *Deianiraeaceae*) are evidenced in red on the right side. The three clades including other 49 total *Alphaproteobacteria* (two clades) and the outgroup (composed by 17 other *Proteobacteria*), are shown collapsed as trapezoidal shapes (full trees are shown in Supplementary material 24c, d). The scale bar stands for estimated 0.1 sequence divergence

*Deianiraea* is also equipped with biosynthetic pathways for the main structural components of cell inner membrane, cell wall and outer membrane. These traits are shared with many other *Rickettsiales*, with the significant exception of *Anaplasmataceae* and *Orientia*, which lack lipopolysaccharide (LPS) and, partially or completely, peptidoglycan. No capsule synthesis is present in *Deianiraea*, as compared to “*Ca. Jidaibacter acanthamoeba*” [24, 74].

In compliance with other *Rickettsiales*, there is an overall paucity of other biosynthetic pathways in *Deianiraea*. In particular, the potential for nucleotide synthesis is scarce, and only some cofactors can be produced (biotin, hem, ubiquinone, folate and Fe-S clusters). As in other *Rickettsiales*, the activity of multiple membrane transporters may complement the metabolic deficiencies of *Deianiraea*. Contrarily to other recently sequenced *Rickettsiales* genomes [24, 40, 75], no flagellar or chemotaxis genes were found.

A total of 84 COGs (~11.2%) of *Deianiraea* resulted absent in the other analysed *Rickettsiales* (Supplementary Material 28). Among these COGs, the prominent peculiarities were genes involved in the biosynthesis of amino acids and in cell adhesion. Notably, consistently with microscopy observations (Fig. 2), *Deianiraea* is also the only *Rickettsiales* member possessing a bacterial cytoskeletal component, namely a bactofilin family protein possibly involved in asymmetrical cell division and cell polarity [76].

### **Amino acid metabolism**

*Deianiraea* has the predicted capability to synthesise the vast majority (16 out of 20) of proteinogenic amino acids, a striking difference with respect to all known *Rickettsiales* (Fig. 5; Supplementary Materials 13-15). Most importantly, the biosynthesis of eight of those amino acids is a unique competence of *Deianiraea* among *Rickettsiales*, raising the total number of amino acids that can be synthesised by at least one *Rickettsiales* member from 10 to 18. *Deianiraea* is by far the richest *Rickettsiales* in terms of amino acids biosynthesis, with a total of 56 genes devoted to amino acid production, while no other member of the order outreaches 30 genes (Fig. 5) [11, 24, 40, 75, 77].

### **Interaction with *Paramecium***

*Deianiraea* is equipped with several secretion systems, which may mediate the interaction with *Paramecium*. These include most of the *Rickettsiales* typical set [78], namely Sec and Tat translocons, and type IV secretion system, the latter overall compliant with the canonical *rvh* set [79] (no VirB7 homologue was found, a possible false negative due to the short length of this gene and its high sequence divergence [80]).

On the other side, no components of secretion systems widely present in other *Rickettsiales*, namely the Apr/Hly type I secretion system proteins and Sca-like type V system autotransporter proteins [76], were found.

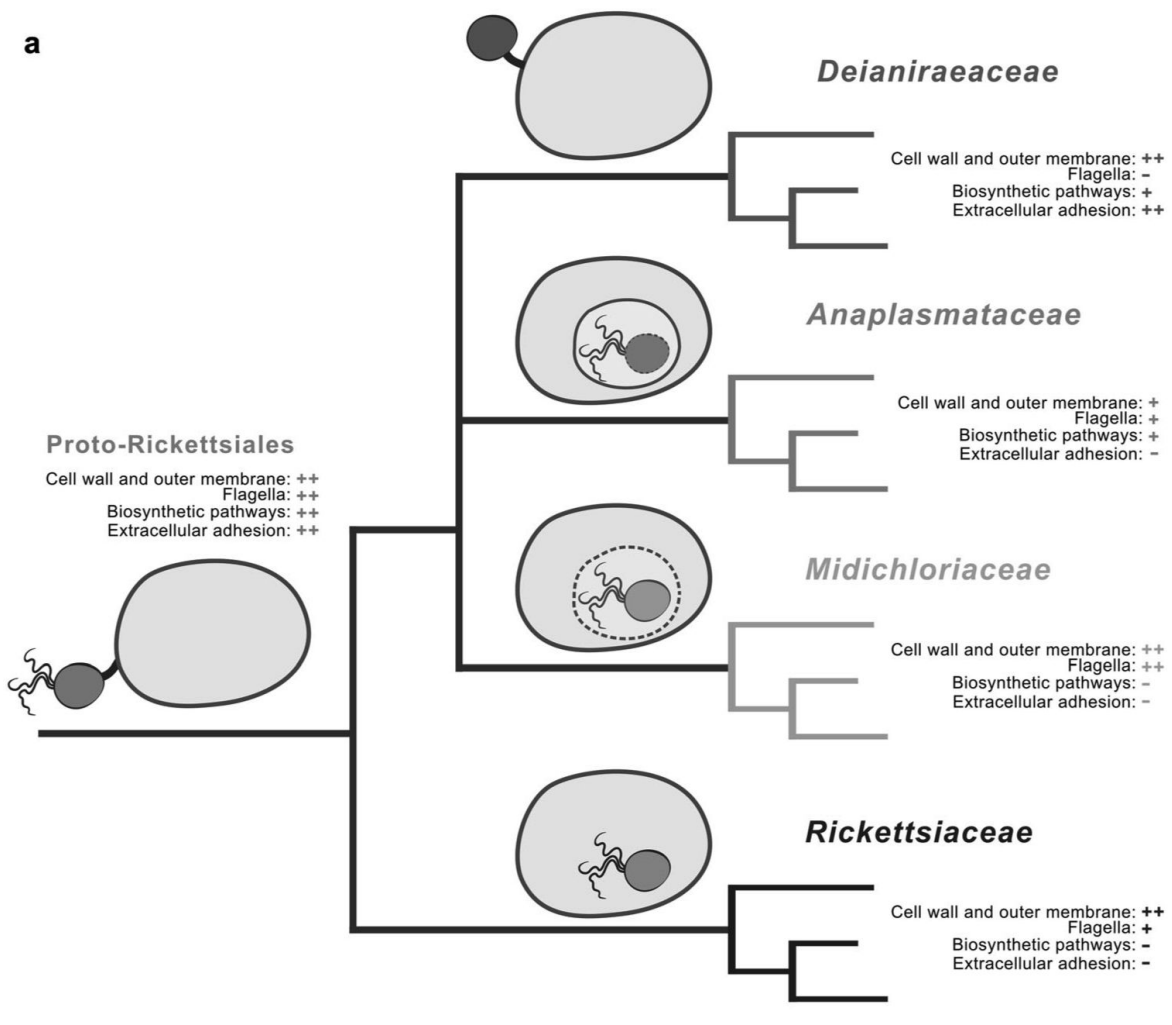
Additionally, the core gene set for type II secretion system [81], was identified. This structure has an equivalent complete counterpart among *Rickettsiales* only in “*Ca. Jidaibacter*”. Moreover, two ORFs, arranged in a putative operon, were recognised as secreted effectors (COG3210) of a two-partner type V secretion system, and include hemagglutinin repeats (Supplementary Material 25). Proteins of this family are involved in cell adhesion and toxicity towards eukaryotes or other bacteria, and frequently occur in operons [82]. The two proteins share extremely low (<32%) sequence identities with homologues from other bacteria, preventing to infer their function more precisely. In close proximity (but reverse oriented), the gene for the corresponding outer-membrane transporter was found (COG2831, Supplementary Material 25). No homologues of adhesins or invasins typical of other *Rickettsiales* were found.

Fifty-three ORFs were predicted to possess a signal peptide and no transmembrane helix, a group that may include secreted effectors (Supplementary Material 29). Interestingly, some of them present eukaryotic-like domains potentially involved in interaction with *Paramecium*, such as ankyrin and tetratricopeptide repeats. Additionally, three MORN repeat proteins (COG4642) were identified in the *Deianiraea*-exclusive set (Supplementary Material 28). This domain is also potentially involved in host cell adhesion and invasion [83].

### **HGT evaluation**

The possibility that the 98 proteins assigned to the 84 *Deianiraea*-only COGs were the result of HGT was investigated by inspecting sequence identity of blastp hits across multiple taxonomic ranges (Supplementary

Materials 15 and 28), and, in selected cases (six genes), phylogenetic analyses. In all cases, the high sequence divergence allowed us to exclude a recent HGT event from a known donor, preventing to robustly trace the recent evolutionary history of those *Deianiraea* genes (Supplementary Material 30). On the other side, some of the genes (21) were found to have putative homologues in some *Rickettsiales* (other than those employed in the COG classification).



**b**

	Ala	Glu	Gln	Gly	Ser	Cys	Asp	Asn	Pro	Lys	Thr	Met	His	Leu	Val	Ile	Tyr	Phe	Trp	Arg	TOTAL genes
<i>Rickettsiaceae</i>	1	1	1	1	0	0	1	3	3	8	4	2	1	2	2	2	1	1	1	3	29
<i>Anaplasmataceae</i>	1	1	1	1	0	0	1	3	1	8	1	2	0	2	2	2	2	2	1	8	30
<i>Midichloriaceae</i>	1	1	1	1	0	0	1	3	0	6	1	1	0	1	1	1	1	1	0	1	16
<i>Deianiraeaceae</i>	1	1	1	1	0	1	1	3	3	9	2	8	8	9	5	9	8	8	5	2	56
<b>Proto-Rickettsiales</b>	✓	✓	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗	✓	

**Fig. 5** Reconstruction of ancestral *Rickettsiales* features according to the "intracellularly late" hypothesis. **a** An idealised evolutionary tree of the four *Rickettsiales* families, is shown. The drawings and notes summarise selected features of each family on the right, and the inferred features of the ancestor at the root of the tree. In the drawings, flagella are shown if present in at least one member of the family, while vacuolar membrane is shown as a solid line if present for all members of the family (*Anaplasmataceae*), or a dashed line if present only for some members (*Midichloriaceae*). "Cell wall and outer membrane" and "Biosynthetic pathways" indicate the degree of completeness of, respectively, peptidoglycan and lipopolysaccharide biosynthesis or the synthesis of amino acids, cofactors, and nucleotides: "++" stands for complete in most known representatives, "+" for (at most) rich in most known representatives, "-" for (at most) scarce in most known representatives. Solid and dashed bacterial outlines in the drawing correspond to different degrees of "Cell wall and outer membrane". "Flagella" and "Extracellular replication" indicate, respectively, the presence of flagella/flagellar genes or of an extracellular replication respect to the host cells (alike *Deianiraea*): "+ ++" stands for present in most known representatives, "++" for present in some known representatives, "-" for absent in all known representatives. **b** Summary of the amino acids biosynthetic abilities in the *Rickettsiales* lineages. Columns stand for single amino acids, rows for organisms or families. Filled circles indicate that the corresponding biosynthetic pathway is present in the respective organism or family (presence in a single family member is the sufficient condition). The number in each circle indicate the total number of distinct ortholog genes involved in the pathway found in the respective organism or family (Supplementary material 14). The pathway presence in the Proto-Rickettsiales ancestor was inferred based on the presence in at least one current *Rickettsiales*. Total numbers of genes per organism or family are indicated on the right, and differ from the sum of the rows due to the presence of genes shared by multiple pathways (Supplementary material 14)

The genes assigned to *Deianiraea*-only COGs involved in amino acids biosynthetic pathways were subjected to additional and more detailed analyses including phylogenies on concatenated protein sequences, statistical tests based on GC content and codon adaptation index (CAI), and

careful comparative analyses of the inferred pathways (Supplementary Materials 13-15). Overall, because of high sequence variation with respect to any other organism and compositional consistence with respect to the other *Deianiraea* ORFs, no clear recent HGT was identified (Supplementary Material 15), supporting the inference that these genes could have been vertically inherited from the *Rickettsiales* ancestor.

## Discussion

The order *Rickettsiales* encompasses a wide range of human pathogens, mutualist symbionts, parasites, and manipulators of the host reproduction [2, 7-10]. In this plethora of lifestyles, the intracellular localisation has always been marked as a constant, since this condition is necessary for their cell division. Parsimoniously, such trait has always been considered as inherited from the ancestor of the order [7, 24, 39, 40].

Herein, we present *Deianiraea vastatrix*, the first extracellular *Rickettsiales* bacterium. This bacterium has been observed attached to the surface of the unicellular eukaryote *P. primaurelia*, but never entering the host cell (Figs. 1 and 2). *Deianiraea* replicates in this extracellular location, covering the *Paramecium* cells, and being the probable cause of observed morphological alterations and lethal effects. On the other side, it is not yet determined whether in other conditions or interacting with other organisms this bacterium could be able to invade host cells and become facultatively intracellular, thus it cannot be formally defined as “strictly extracellular”. The extracellular interaction of *Deianiraea* and *Paramecium* is reminiscent of the one between the phylogenetically unrelated *Vampirovibrio chlorellavorus* (*Melainabacteria*) and *Chlorella* unicellular algae [84]. However, *Vampirovibrio* has a much wider metabolic repertoire, including lytic enzymes putatively involved in digestion of host cells.

On the other side, the molecular bases of the interaction between *Deianiraea* and its host are not disclosed, but several genes in the genome of this bacterium provide hints on the possible underlying mechanisms, namely the presence of secretion systems and adhesion molecules. A distinctive two-partner type V secretion system likely enables *Deianiraea* to release effectors bearing hemagglutinin repeats, with potential adhesive and toxic effects [82]. A type II secretion system, rare in *Rickettsiales*, may be involved in adhesion and motility as well, considering that its components are homologous of pili [85]. Additionally, given the limited biosynthetic repertoire of *Deianiraea*, it probably acquires the necessary small precursor molecules directly from the *Paramecium* host, exploiting a relatively wide set of membrane transporters, as other *Rickettsiales*. The transport of small molecules and secreted proteins could be exerted extracellularly (e.g. interacting with proteins on the surface of eukaryotic cells), and, in addition, could work in a hypothetical, though never observed, intracellular phase.

In synthesis, *Deianiraea* presents a unique extracellular lifestyle, distinct from all other *Rickettsiales*. This novel finding raises the question whether *Deianiraea* has reverted to extracellularity from an obligate intracellular ancestor or extracellularity was the ancestral state of the last common ancestor of the order. The two opposed scenarios may be called “intracellularity early” and “intracellularity late”. The traditional “intracellularity early” hypothesis could be seen as more parsimonious, however it implies the reversal of *Deianiraea* to extracellularity. At present, it seems difficult to evaluate how many and which changes and gains-of- function would have been necessary for such a complex and, to our knowledge unprecedented, evolutionary process. On the other side, the alternative “intracellularity late” hypothesis can be newly inferred based on the novel and distinctive extracellular lifestyle of *Deianiraea*, and, as detailed below, may find additional support considering its phylogenetic position within *Rickettsiales*, its biosynthetic peculiarities, but also the diversity of “intracellularity” among all other *Rickettsiales*.

From a phylogenetic point of view (Figs. 3 and 4), *Deianiraea* is a member of a new family clade of *Rickettsiales*, named *Deianiraeaceae*, with an estimated diversity comparable to the other three families of *Rickettsiales*. While no information is available on other *Deianiraeaceae* bacteria, in many cases their origin, namely the skin of aquatic animals or humans [68-70, 72], allows us to wonder whether they may be extracellular as well, and whether this trait could have been directly inherited from the ancestor.

Moreover, *Deianiraea* displays genes encoding for the biosynthetic pathways of 16 amino acids, a repertoire much larger than that of any other known *Rickettsiales* (Fig. 5) [11, 24, 40, 77]. Due to the absence of the *Deianiraea*-only amino acids biosynthesis gene sets in all other sequenced *Rickettsiales*, to the low level of identity with available orthologs and to the consequently limited phylogenetic signal, it is difficult to evaluate whether they are ancestral or the result of an ancient HGT event. In the future, the sequencing of additional phylogenetically related genomes may allow to obtain a more complete picture. At present, considering also the compositional homogeneity with the rest of the genome, we can assert that there is no evidence of recent HGT for any of the pathways analysed (Supplementary Material 15). This scenario is consistent with the expectations in the case they were indeed inherited vertically from the last common ancestor of *Rickettsiales* (Proto-*Rickettsiales*), which could have been more biosynthetically capable than previously thought (up to 18 amino acids) (Fig. 5).

While all known *Rickettsiales*, except *Deianiraea*, are intracellular, most of them are able to perform horizontal transmission. The process and molecular recognition machinery used to enter host cells, as well as the consequent intracellular behaviour and location, are not conserved among the *Rickettsiales* lineages, and can differ even within the same family [26, 27, 86]. *Rickettsiaceae* enter host cells by inducing phagocytosis, and typically escape the phagosomal membrane, residing freely in the host cytoplasm and exploiting host cytoskeleton for movement [26, 86-88]. *Anaplasmataceae* hijack the phagocytic process, modifying the host vacuole for their survival and proliferation, exploiting the intra-vacuolar niche [27, 89-92]. *Midichloriaceae* are more diverse, encompassing both bacteria enclosed in host-derived membranes and others dwelling free in the cytoplasm [24, 40, 93]. Such observed differences could be indicative of an independent parallel evolution towards the strictly obligate intracellular lifestyle.

Following the “intracellularity late” hypothesis, we inferred the main features of the last common ancestor of *Rickettsiales* (Proto-*Rickettsiales*), based both on previous knowledge and on novel findings (Fig. 5). The Proto-*Rickettsiales* would have been extracellular, though probably displaying a pre-adaptation to the interaction with eukaryotic cells, such as type IV secretion systems [80] or ATP/ADP translocases [94], which could have been instrumental in the successive parallel evolution towards strictly intracellular lineages. Possibly, the Proto-*Rickettsiales* could have been already capable of temporary invasion of host cells, as a facultative intracellular bacterium. Considering that most *Rickettsiales* (including *Deianiraea*) are found in aquatic environments [5] (Fig. 5), the Proto-*Rickettsiales* was likely an aquatic bacterium as well [7, 17, 19, 95-97]. It probably had a rather high metabolic and functional complexity, consistent with the extracellular and likely eukaryotic-associated lifestyle (Fig. 5), which could be reflected in the diversity of the extant descendants, as well as their generally conserved ability to colonise new hosts. The Proto-*Rickettsiales* could have been rich in biosynthetic pathways, in particular for amino acids (present work), but also for cofactors and nucleotides (as in multiple current *Anaplasmataceae* [77]), and was possibly capable to survive in low-oxygen conditions [40]. It could have been provided with a robust structure made by functional peptidoglycan and LPS (present work [24, 40, 98]), could synthesise capsule [24], possessed pili (present work and Schulz et al. [24]), flagella [24, 40, 75, 99, 100], and could be able to perform chemotaxis [75]. During the course of evolution, the association of its descendants to eukaryotic cells would have become tighter and tighter, with a parallel reduction

in genome size and functional complexity, finally leading, in most known lineages, to loss of capability to reproduce extracellularly, and to consequent adaptation to the obligate strict intracellular state. Such “intracellularity late” scenario would have additional consequences on evolutionary inferences on mitochondrial origin. Indeed, there is an open debate on whether mitochondria and *Rickettsiales* are sister groups [11-13] or not [14, 15]. Assuming that the Proto-*Rickettsiales* was in fact an extracellular bacterium, even in case mitochondria and *Rickettsiales* truly shared a common ancestor, it would become evident that such ancestor should have been extracellular as well. Consequently, their intracellular lifestyles would have been acquired independently, much similarly to what would have happened if *Rickettsiales* and mitochondria were unrelated [15]. Thus, the hypothetical common ancestor of mitochondria and *Rickettsiales* could be envisioned as a bacterium with a degree of metabolic independence, and possibly equipped with the machinery to interact with other microorganisms.

In recent years, an increasing amount of research was produced on neglected members of the order *Rickettsiales* (e.g. [24, 38, 41, 74, 75, 101, 102]), which, contrarily to those thoroughly studied in the past, are not of direct medical or veterinary concern. Such investigations represented a major contribution to understand the phylogenetic and genomic diversity of *Rickettsiales* and are gradually reshaping our interpretation of their evolution. In this framework, the discovery of *Deianiraea* has the potential to represent a tipping point for the evolutionary views on *Rickettsiales*, providing the first supporting evidence for the novel idea that obligate intracellularity in this order would have evolved multiple times in parallel and independently from an extracellular ancestor. Obviously, with the available data it is still premature to conclude which hypothesis is preferable between the “intracellularity early” and “intracellularity late” in *Rickettsiales*. In this regard, future research on other under-investigated lineages of *Rickettsiales*, in particular *Deianiraeaceae*, may definitely lead to a more complete understanding of the processes that have led to the evolution and diversification of extant *Rickettsiales*.

#### **Data availability**

Sequence data have been deposited in GenBank with the accession codes: *P. primaurelia* CyL4-1 partial 18S-ITS1-5.8S-ITS2-28S—MH185950, *P. primaurelia* CyL4-1 partial cytochrome oxidase subunit I gene—MH 188082, *Deianiraea vastatrix* CyL4-1 partial 16S rRNA gene—MH 197138; *Deianiraea vastatrix* CyL4-1 genome—CP028925.

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**Author contributions** NL isolated the host cells and performed live experiments. ES performed TEM and fluorescence microscopy. KB performed AFM. OL, AP and GP performed molecular experiments and probe design. MC, OL and GP performed phylogeny. MC and OL performed IMNGS analyses. MC, AMF and DS performed genome assembly and analyses. MC, AMF, SG and DS performed metabolic and evolutionary analyses. General interpretation of the results and manuscript writing were performed by MC, DS and GP, and contributed by CB, LM, ES and AP. All the authors contributed to the interpretation and writing of the specific results, and to the discussions. GP planned and coordinated the whole project, with DS planning and coordinating the genomic analyses, and ES coordinating the microscopy.

#### **Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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