Microbial Ecology (2019) 78:232–242
https://doi.org/10.1007/s00248-018-1279-9

HOST MICROBE INTERACTIONS

Detecting Associations Between Ciliated Protists and Prokaryotes with Culture-Independent Single-Cell Microbiomics: a Proof-of-Concept Study

Alessia Rossi[1] · Alessio Bellone[1] · Sergei I. Fokin[1,2,3] · Vittorio Boscaro[1,4] · Claudia Vannini[1]

Received: 16 August 2018 / Accepted: 22 October 2018 / Published online: 8 November 2018

Abstract

Symbioses between prokaryotes and microbial eukaryotes, particularly ciliated protists, have been studied for a long time. Nevertheless, researchers have focused only on a few host genera and species, mainly due to difficulties in cultivating the hosts, and usually have considered a single symbiont at a time. Here, we present a pilot study using a single-cell microbiomic approach to circumvent these issues. Unicellular ciliate isolation followed by simultaneous amplification of eukaryotic and prokaryotic markers was used. Our preliminary test gave reliable and satisfactory results both on samples collected from different habitats (marine and freshwater) and on ciliates belonging to different taxonomic groups. Results suggest that, as already assessed for many macro-organisms like plants and metazoans, ciliated protists harbor distinct microbiomes. The applied approach detected new potential symbionts as well as new hosts for previously described ones, with relatively low time and cost effort and without culturing. When further developed, single-cell microbiomics for ciliates could be applied to a large number of studies aiming to unravel the evolutionary and ecological meaning of these symbiotic systems.

Keywords Microbiota · Microbiomics · Symbiosis · SSU rRNA gene · Bacterial symbionts · Ciliates

Introduction

Microbial associations are exceptionally common and widespread [1–4], and those between eukaryotic hosts and the prokaryotic organisms they harbor are the most intensely studied. Historically, the focus has been put on "symbioses", broadly intended as relationships involving protracted physical contact between the partners [5, 6], regardless of the consequences of the relationship (e.g., mutualism, commensalism, or parasitism). Attention has now shifted to the study not just of one or a few microbial symbionts, but of the entire prokaryotic communities associated with eukaryotic hosts—the "microbiomes" [7]—which include both stable and temporary members (from "true symbionts" to food organisms). Almost invariably, the investigated hosts are large metazoans or plants. And yet, symbioses between prokaryotes and unicellular microbial eukaryotes (protists) are just as common [e.g., 1, 8–12]. Associations between bacteria and ciliates (phylum Ciliophora), in particular, boast a long history of studies which dates back to the nineteenth century [13, 14] and has significantly grown during the last decades [e.g., 15–21]. Even so, the bulk of the literature deals only with a few ciliate host species and genera, such as *Paramecium*, *Euplotes*, *Spirostomum*, and *Metopus* [e.g., 22– 25]. Among more than 1500 described ciliate genera [26], only about 30 have been screened for the presence of prokaryotic symbionts [27]. A molecular characterization of the

The nucleotide sequence data reported are available in the ENA database under the accession numbers LT985649-LT985676 and study number PRJEB25414.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00248-018-1279-9) contains supplementary material, which is available to authorized users.

✉ Claudia Vannini
claudia.vannini@unipi.it

[1] Department of Biology, University of Pisa, Pisa, Italy

[2] Department of Invertebrate Zoology, St.-Petersburg State University, St. Petersburg, Russia

[3] St. Petersburg Branch of the S.I. Vavilov Institute of History of Science and Technology, Russian Academy of Sciences, St. Petersburg, Russia

[4] Department of Botany, University of British Columbia, Vancouver, Canada

associated bacteria has been achieved even less frequently. In the few cases when considerable data exist for a ciliate-harbored prokaryotic symbiont, the focus is usually on a single bacterial species. However, it is known that complex consortia can be found associated with each individual ciliate host [28, 29], true microbiomes within microbes. While detection and characterization of microbiomes associated with multicellular eukaryotic hosts have considerably advanced in the last decades [3, 4, 30, 31], the same topic is almost completely unexplored for unicellular eukaryotic hosts.

Ultimately, the source of almost all these limitations is the scarcity and unreliability of cultivation-independent methods. Ciliate-prokaryote associations have been studied so far mainly in hosts that are easy to maintain in standard laboratory conditions. Among ciliate taxa found in any natural sample, few if any can be reliably established as monoclonal cultures with the stability and abundance required by the full-cycle rRNA approach [32]; moreover, those few do not necessarily represent well the original community. It is therefore reasonable to assume that a huge amount of yet-undiscovered microbial associations, involving uncultivable or difficult-to-cultivate ciliate hosts, is present in the natural environment. The ecological meaning of ciliate-prokaryote associations has been elucidated only in a few instances [1, 27], always using cultivation-dependent approaches [33–38]. Field investigations are either completely lacking or performed without a precise identification of the involved partner [39]. Coupling reliable characterizations of the hosts to environmental surveys has been extremely difficult up to now. Hence, an assessment of the distribution and potential adaptive value of ciliate-prokaryote associations in the natural environment is currently impossible. As a consequence, nothing is known concerning the epidemiology of even the most studied symbioses: virtually no quantitative data are available on geographic distributions or habitat preferences, nor on the prevalence of infection in host natural populations. Such basic data are essential to unravel any potential role of these associations in the ecology and evolution of both hosts and symbionts.

A promising approach to address such issues is the application of single-cell "omic" techniques to specimens freshly isolated from samples. Single-cell genomics [40] and transcriptomics [41] have been already successfully performed on ciliates. Prokaryotic gene cloning from single-cell isolates has been occasionally used in the context of field studies [28, 29, 42, 43], but it proved not always sufficient to characterize all the associated bacteria of potential interest [28]. Single-cell ciliate microbiomics, the characterization of prokaryotic communities harbored by a single host cell using high-throughput sequencing techniques, could represent the next step.

Here, we present a pilot single-cell microbiomic study, suggesting that this approach can be easily and efficiently used for field research on microbial associations. Based on ciliate single-cell PCR followed by Illumina sequencing of small subunit (SSU) rRNA gene fragments, the method described does not require host cultivation and allows immediate processing of environmental samples for an accurate characterization of both hosts and associated microbiomes. We tested the approach on ciliates from freshwater and marine environments, of various sizes and belonging to different taxonomic groups. Obtained results encourage its use for rapid and successful surveys of microbial associations between ciliates and prokaryotes in the environment, bringing us closer to fully understand their extent, distribution, and diversity.

Materials and Methods

Sampling and Ciliate Isolation

Sediment-water samples (45 ml each) were collected in the same week, between the end of February and the beginning of March 2017, from two freshwater sandy ponds (P1_1, P3_2) near the mouth of the river Serchio (Pisa, Italy) and from two small rocky tidal pools (L1_4, L2_1) along the Ligurian Sea shore in Livorno (Italy). About 30 ml of medium from each sample were transferred into Petri dishes and observed with a Wild Heerbrugg optical stereo microscope (× 400). As is often the case in freshly collected samples from such habitats [44, 45], ciliate diversity was rather high, while population abundances were low. Twenty-eight ciliate specimens of several morphotypes were individually isolated by glass micropipette (see Table 1). At least one specimen per observed morphotype was processed from each sample: twelve from sample P1_1, ten from sample P3_2, three from sample L1_4, and three from sample L2_1. Isolated ciliate cells were washed to minimize the presence of prokaryotes not tightly attached to the host cell. Freshwater specimens were rinsed one by one three times with sterile mineral water in separate wells, and marine specimens were rinsed with artificial sterile marine water (33‰ salinity). Each single cell was then washed three more times with sterile distilled water and stored in a 0.2-ml tube in 70% (*v*/*v*) ethanol. This procedure, followed by storage at − 20 °C, was performed within 48 h from sample collection to reduce the risk of contamination from the lab. The portion of each sample (15 ml) not used to harvest ciliates was fixed in 70% (*v*/*v*) ethanol and divided into three aliquots used in our survey as controls, for the purpose of characterizing the background environmental microbial communities.

Table 1 Ciliate identification. NCBI Blastn-based affiliations of isolated ciliate cells based on SSU rRNA genes

Environment	Sample	Specimen ID	Blastn best hit	Class	Accession number	Identity (%)
Freshwater	P1_1	9	*Euplotes aediculatus*	Spirotrichea	AF508756	99.8
		25	*Euplotes daidaleos*	Spirotrichea	KF887346	100.0
		36	*Oxytricha ferruginea*	Spirotrichea	AF370027	99.3
		22	*Oxytricha granulifera*	Spirotrichea	X53486	99.2
		16	*Stylonychia mytilus*	Spirotrichea	AJ310499	100.0
		39	*Halteria* sp.	Spirotrichea	LN869934	99.8
		13	*Spirostomum minus*	Heterotrichea	HG939543	99.5
		14	*Spirostomum minus*	Heterotrichea	HG939543	100.0
		15	*Spirostomum minus*	Heterotrichea	HG939543	100.0
		19	*Paramecium* sp.	Oligohymenophorea	FJ875142	99.5
		12	*Frontonia*-like ciliate	Oligohymenophorea	LN870026	95.7
		37	*Metopus contortus*	Armophorea	KY432957	98.4
	P3_2	76	*Euplotes daidaleos*	Spirotrichea	KF887346	100.0
		79	*Euplotes daidaleos*	Spirotrichea	KF887346	99.9
		66	*Paruroleptus lepisma*	Spirotrichea	AF164132	100.0
		69	*Pseudouroleptus caudatus*	Spirotrichea	KF591597	99.2
		43	*Urocentrum turbo*	Oligohymenophorea	AF255357	99.9
		52	*Urocentrum turbo*	Oligohymenophorea	AF255357	99.7
		45	*Caenomorpha medusula*	Armophorea	MF828615	100.0
		71	*Metopus laminarius*	Armophorea	KF607088	99.3
		73	*Trithigmostoma steini*	Phyllopharyngea	X71134	98.3
		48	*Loxodes striatus*	Karyorelictea	AM946031	100.0
Marine	LIV1_4	L_4	*Aspidisca leptaspis*	Spirotrichea	EU880597	96.8
		L_6	*Diophrys scutum*	Spirotrichea	HQ413691	99.9
		L_1	*Hartmannula derouxi*	Phyllopharyngea	AY378113	98.8
	LIV2_1	L_9	*Euplotes magnicirratus*	Spirotrichea	AJ305250	99.9
		L_14	*Diophrys scutum*	Spirotrichea	HM154532	100.0
		L_15	*Frontonia ocularis*	Oligohymenophorea	FJ868198	99.7

Eukaryotic and Prokaryotic SSU rRNA Gene Amplification and Sequencing

Ethanol was removed from tubes containing single ciliate cells using a SpeedVac SVC100 (SAVANT). As a first step, simultaneous PCR amplifications of eukaryotic and prokaryotic SSU rRNA genes were performed directly on individually isolated cells in the same tube where each cell was stored (no DNA extraction was performed). The Takara ExTaq (Takara Biochemicals) reaction solution, including primers 18S F9 Euk [46] and 18S R1513 [47] for ciliates, and primers 8F [48] and UNI-b-rev [32] for prokaryotes, was pipetted on top of the cell [45]. In order to identify the hosts, amplicons were purified with the Eurogold Cycle-Pure Kit (Euroclone) and diluted 1:100, then subjected to two semi-nested amplifications, one with eukaryotic primers 18S F9 and 18S R1052 [49], the other with eukaryotic primers 18S F783 [49] and 18S R1513 [47]. Resulting amplicons were further purified and Sanger sequenced using multiple appropriate internal primers [49, 50] by GATC Biotech (Cologne, Germany). In order to characterize the prokaryotic microbiomes of ciliates, a nested PCR was performed on amplicons obtained in the first step. This amplification used the KAPA HiFi HotStart Ready Mix with the prokaryotic primer set for the V3–V4 regions of the SSU rRNA gene suggested by Klindworth and colleagues [51]. The Illumina overhang adapter sequences added to the forward and reverse primers were 5′-TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG-3′ and 5-GTCTCGTG GGCTCGGAGATGTGTATAAGAGACAG-3′, respectively (Illumina protocol, Part # 15044223, Rev. B).

In order to characterize background prokaryotic communities, including both free-living and host-associated taxa, total genomic DNA was extracted from 0.25 g of each of the three control aliquots per sample using the PowerSoil DNA Isolation Kit (MoBio). Extracted DNA was used as template for amplification with the KAPA HiFi HotStart Ready Mix and the prokaryotic primer set for the V3–V4 regions of the SSU rRNA gene as described above.

Prokaryotic amplicons from single host cells and controls were barcoded, pooled, and sequenced by BMR Genomics (Padova, Italy) on the Illumina MiSeq platform (2 × 300 paired-end sequencing with MiSeq Reagent Kit v3) by BMR Genomics (Padova, Italy).

Sequence Analysis

Eukaryotic SSU rRNA gene sequences obtained by Sanger sequencing were analyzed with NCBI Blast [52] for putative identification of the ciliate hosts.

Raw reads of prokaryotic V3–V4 regions obtained by Illumina MiSeq were analyzed using the Quantitative Insights Into Microbial Ecology version 2 (QIIME2, https://qiime2.org) software package (v. 2017.2, [53]). Reads were initially truncated at base 290 to remove the lower-quality last 10 base calls. Then, quality filtering, primer trimming, and pair-end read merging were performed with DADA2 [54] (default settings: sequences with any N character discarded; sequences truncated at any base with a quality score of 2 or lower; maximum expected error allowed: 2; chimera removal de novo). Unique reliable sequences ("sequence variants," each representing one or more identical sequences) were aligned using MAFFT [55], and highly variable positions were masked. A phylogenetic tree was inferred with FastTree [56]. Three long-branching sequence variants were manually inspected and removed as host contaminants (NCBI Blast best hits corresponded to ciliates of the genera *Trithigmostoma*, *Hartmannula*, and *Sterkiella*). A fourth long-branching sequence variant, represented by only two sequences in a single library, was removed because it was clearly chimeric. Taxonomic classification was performed using the Greengenes database [57] release 13.8. Following Werner and colleagues [58], the regions of interest were extracted from SSU rRNA representative sequences (99% similarity clustered Operational Taxonomic Unit) and used to train a Naive Bayes classifier. Sequence variants identified as mitochondria or chloroplasts were removed before further data processing (e.g., bar plots and heatmap building), which was also performed on QIIME2. No remaining sequence was assigned to eukaryotes or eukaryotic organelles.

Statistical Analysis

Before performing statistical analyses, 4569 sequences (the number of merged, quality-filtered reads in the smallest library) were randomly sampled from each library. Four measures of Alpha-diversity were calculated on QIIME2: sequence variant number, Faith's Phylogenetic Diversity (qualitative index using phylogenetic information), and Shannon's (quantitative, non-phylogeny-based index) for richness and Pielou's Evenness for evenness. Rarefaction curves were also inferred. Comparisons among different communities were performed using the Kruskal-Wallis non-parametric test. Beta-diversity analyses were performed using Permanova and multivariate PCoA, testing various metrics: Bray-Curtis and Jaccard for quantitative and qualitative data, respectively, and Uni-Frac distances, both weighted and unweighted, to assess the impact of phylogeny.

Results

Ciliate Hosts Identification

Partial eukaryotic SSU rRNA gene sequences were obtained from each of the 28 single-cell ciliate specimens (minimum length 858 bp, maximum length 1855 bp). The sequences always included the V4 region, recognized as the most variable and information-rich region of the SSU rRNA gene for ciliates [59]. Ciliate identification was then performed using NCBI Blastn (Table 1). Ciliates belonging to 17 different genera, distributed in six different classes [26], were identified. Obtained sequences have been deposited to the ENA database under the accession numbers LT985649-LT985676.

Ciliate Prokaryotic Microbiomes

The final dataset contained 567,919 sequences, with 14,198 ± 7281 mean sequences per library. The library with most reads was obtained from a marine ciliate specimen (L_4, likely belonging to the genus *Aspidisca*; 40,898 sequences), the one with fewest from a freshwater control (P1_1_1; 4569 sequences). Raw reads have been deposited to the ENA database (study number PRJEB25414). Obtained sequences were grouped in 3575 different sequence variants. The average numbers of sequence variants were 24.3 ± 16.9 (samples P1_1 and P3_2) and 79.3 ± 66.5 (samples L1_4 and L2_1) in freshwater and marine ciliate microbiomes, respectively, and 469.8 ± 127.6 (samples P1_1 and P3_2) and 314.3 ± 146.5 (samples L1_4 and L2_1) for freshwater and marine prokaryotic environmental communities in controls. The difference in richness (number of sequence variants) between ciliate microbiomes and environmental communities (controls) in the same habitat, with microbiomes being considerably poorer, was significant in libraries of marine samples ($p = 0.01$) and highly significant in libraries of freshwater samples ($p < 0.01$). Other richness indexes confirmed the pattern, with differences always being highly significant (Online Resource 1). Similarly, evenness was significantly lower in microbiomes than in environmental communities. Differences in richness and evenness measures among controls were not significant or barely significant ($p = 0.05$).

Four different clusters, i.e., microbiomes associated with freshwater ciliates, microbiomes associated with marine ciliates, communities of freshwater controls, and communities of

marine controls, are visible in the arrangement of libraries in PCoA graphs (Fig. 1). The microbiome of the marine ciliate L_1 (likely belonging to the genus *Hartmannula*) is the only conspicuous outlier, clustering with the microbiomes of freshwater ciliates. The presence in the same library of a single eukaryotic sequence variant (later removed during quality filtering, see the "Materials and Methods" section), identical to the one independently obtained by Sanger sequencing from the *Hartmannula* host, excludes the possibility of contamination or mislabeling. Permanova tests of the differences between groups are highly significant (p value < 0.01) regardless of the employed metric, with a single exception (the separation of microbiomes from marine ciliates and marine control communities according to the weighted Uni-Frac distances, for which $p = 0.011$). Results of Permanova tests are reported in Online Resource 2.

Plateaus in rarefaction curves confirm that sequencing depth was sufficient to sample all sequence variants in the libraries (data not shown). In all prokaryotic communities, the most represented phyla were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* (Online Resource 3). The number of detected phyla mirrors the observed richness trends, being higher in control communities and lower in ciliate microbiomes.

Detection of Potential Prokaryotic Symbionts

Heatmaps of the 100 most abundant prokaryotic taxa (identified at the least inclusive level allowed by the reference database, roughly corresponding to the genus rank) were built for freshwater and marine environments (Fig. 2). In freshwater libraries (Fig. 2a), it is immediately apparent that a group of bacterial taxa abundant in ciliate microbiomes is absent or scarcely represented in controls. Other bacterial genera are well-represented both in freshwater ciliates and controls. Finally, many prokaryotic taxa are abundant in controls, but absent or scarcely represented in ciliate microbiomes. A somewhat similar situation applies to marine libraries, although a larger fraction of the taxa is present and abundant both in ciliate microbiomes and environmental control communities.

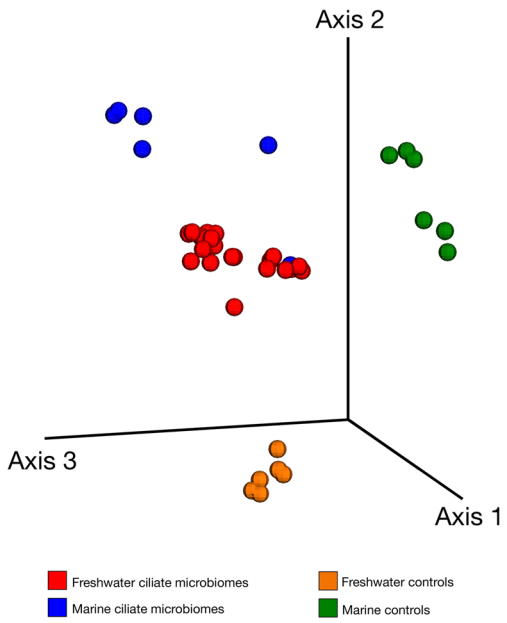

Fig. 1 Principal component analysis. PCoA graph of prokaryotic communities from controls and single-cell ciliate specimens (unweighted Uni-Frac distances). Similar results were produced using different metrics (weighted Uni-Frac, Bray-Curtis, and Jaccard)

The same three patterns can be appreciated in taxa bar plots (Fig. 3). Several well-known prokaryotic symbionts of ciliates are retrieved with high relative abundances (> 5% of the sequences) among taxa detected in ciliate microbiomes. For example, members of the family *Rickettsiaceae* (*Proteobacteria*), which includes only obligate intracellular symbionts and has often been reported in ciliates [25, 60–62], are abundant (> 5%, up to 42%) in some ciliates (e.g., cells 66 and 73, assigned to *Paruroleptus* and *Trithigmostoma*, respectively), but rare (< 0.1%) or undetectable in controls (Fig. 3). Bacteria of the genus *Polynucleobacter* (*Proteobacteria*), well studied as symbionts of freshwater ciliates, are completely absent from marine libraries, and their average relative abundance in freshwater controls is only 0.13 ± 0.11%, compared to up to 18.6% in freshwater ciliate microbiomes (cells 25, 76, and 79, assigned to the genus *Euplotes*; cells 12, 22, and 37, likely belonging to the genera *Frontonia*, *Oxytricha*, and *Metopus*, respectively). A taxon belonging to the family *Neisseriaceae* (*Proteobacteria*), which includes many commensal and parasitic bacteria, was only detected in the microbiome of ciliate 13 (likely affiliated to the genus *Spirostomum*), with a high relative abundance (20.13%). Bacteria of the genera *Wautersiella* (*Bacteroidetes*), *Corynebacterium* (*Actinobacteria*), and *Ochrobactrum* (*Proteobacteria*), all usually found in the microbiomes of humans and other mammals, were undetectable in controls but showed relative abundances up to 38.5%, 18.2%, and 22.2%, respectively, in several freshwater ciliate microbiomes (Fig. 3). Other prokaryotes were, on the contrary, present only in controls, like several taxa belonging to the order *Bacteroidales* (*Bacteroidetes*) (Fig. 3). Taxa retrieved with high relative abundances both in controls (> 1%) and in microbiomes (> 5%) included marine gammaproteobacteria of the genus *Glaciecola* (*Proteobacteria*) [63] (Fig. 3) and taxa of the order *Sphingobacteriales* (*Bacteroidetes*).

Genus-level taxa with the same abundance pattern as the known symbionts (>5 % in any microbiome library, < 0.1% in every control library) account for up to 66% of the total prokaryotic taxa in the microbiome of analyzed ciliates. This value varies broadly both for freshwater ciliates (0 to 66%) and marine ciliates (0 to 60%). When inspecting the number of putative symbiotic genus-level taxa within each prokaryotic phylum, most of the genera with this characteristic belong to the phylum *Proteobacteria* (21), followed by *Actinobacteria* (9), *Firmicutes* (8), and *Bacteroidetes* (4).

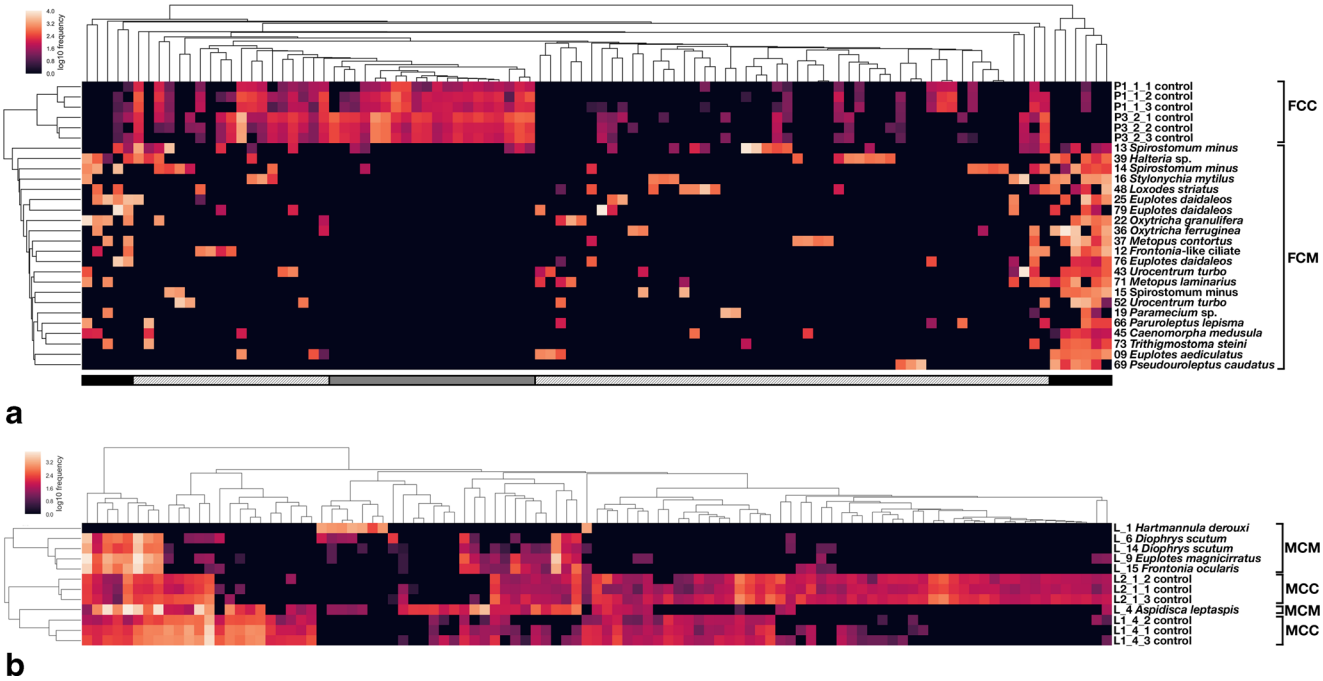

Fig. 2 Heatmaps of the 100 most abundant freshwater and marine prokaryotic taxa. **a** Heatmap showing the relative abundances (percentage of sequences) of freshwater prokaryotic taxa identified at the least inclusive taxonomic level in the Greengenes taxonomy. As shown by the trees, rows and columns are arranged according to UPGMA clustering for readability. Bar under the heatmap indicates the three different patterns observed: bacterial taxa abundant in ciliate microbiomes, but absent or scarcely represented in controls (black); bacterial taxa well represented both in freshwater ciliates and controls (pale gray); and bacterial taxa abundant in controls, but absent or scarcely represented in ciliate microbiomes (dark gray). FCM: freshwater ciliate microbiomes. FCC: freshwater control communities. **b** Heatmap showing the relative abundances (percentage of sequences) of marine prokaryotic taxa identified at the least inclusive taxonomic level in the Greengenes taxonomy. As shown by the trees, rows and columns are arranged according to UPGMA clustering for readability. MCM: marine ciliate microbiomes. MCC: marine control communities

Discussion

The described approach provided reliable and satisfactory results on all ciliate specimens, regardless of their original environment and taxonomic affiliation. Rich and distinctive V3–V4 libraries were obtained with relatively little time investment, and microbiome profiles (lists of prokaryotes associated with various degrees with eukaryotic cells) could be unambiguously linked to molecularly identified hosts.

Although the sampling effort needs to be improved in order to obtain more reliable data, this preliminary work indicates that prokaryotic microbiomes of ciliate cells are different from total prokaryotic communities in the same site and habitat. As expected, background environmental communities have higher biodiversity, measured either as taxa richness or taxa evenness, which is also influenced by the presence of a community of active protistan grazers [64–67]. PCoA analyses and Permanova tests show that microbiomes of ciliates from the same habitat form a well-defined cluster, suggesting similar selective pressures on ciliate-associated prokaryote communities. These data suggest that, as already assessed for many macro-organisms like plants or metazoans [3, 4, 30, 31], ciliated protists possess a specific microbiome, distinguishable from the microbial community of the surrounding environment.

In our survey, differences between ciliate microbiomes and environmental communities were far less pronounced in marine than in freshwater samples. We found instead consistent differences between microbiomes of freshwater and marine ciliates, with habitat clearly surpassing other factors in affecting microbiome composition. However, these trends need to be tested with specifically designed studies and with a larger sampling effort, especially for the marine habitat.

Inspecting relative abundances of bacterial taxa in ciliate microbiomes and controls allowed to easily spot previously known bacterial symbionts of ciliates. All of them were present with relative abundances lower than 0.1% in control communities and higher than 5% in the microbiomes of their known hosts. Only bacteria of the genus *Polynucleobacter* appeared with slightly higher relative abundance values also in control communities (between 0.11 and 0.26%), and this can be explained by the fact that the genus comprises free-living as well as symbiotic strains [68]. Therefore, bacterial taxa showing similar abundance patterns can be reasonably regarded as putative symbionts, although more studies are certainly needed to refine these rough estimates. We are aware of no comparable data from single ciliate cells, but a recent meta-analysis screening of available libraries for a bona fide ciliate symbiont sequence also reported values well below 0.1% in reads from environmental libraries [69].

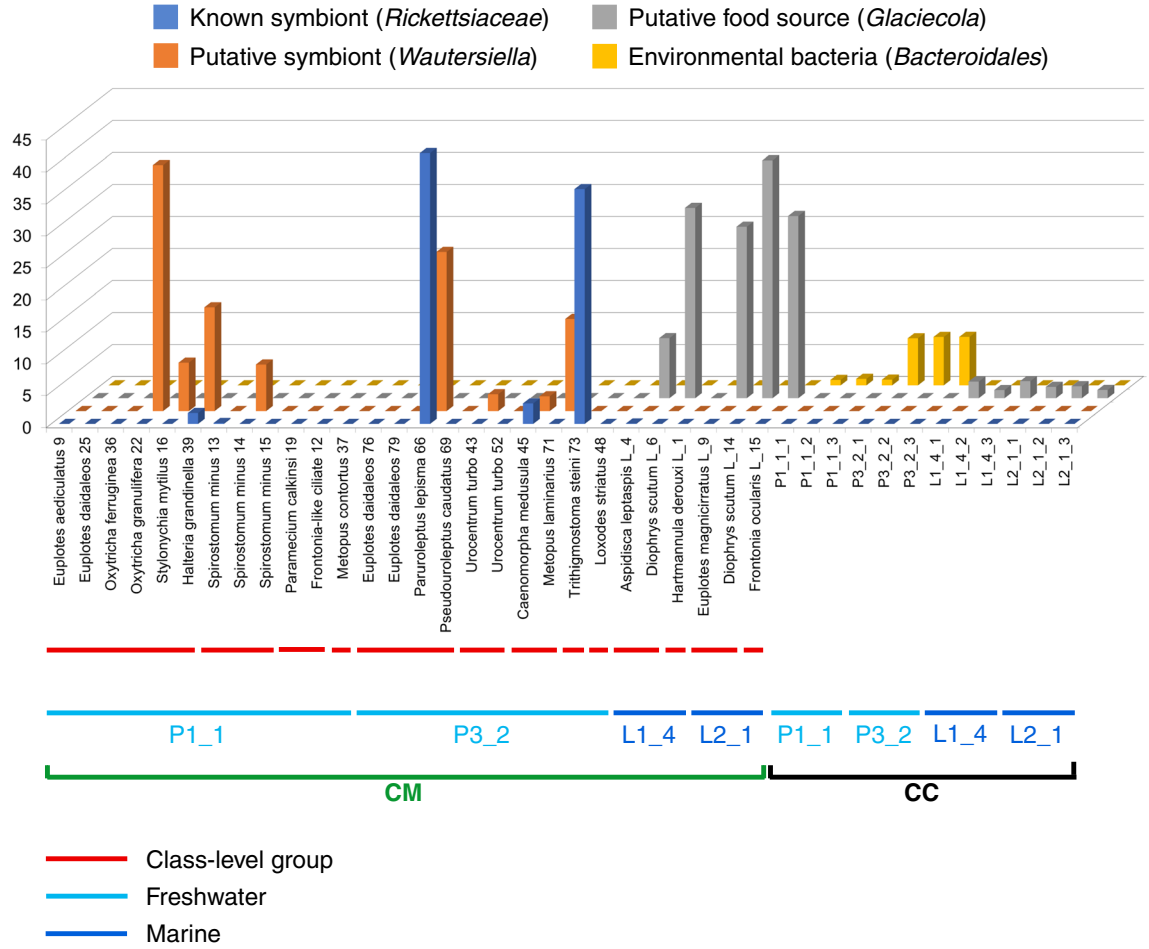

Fig. 3 Different patterns of taxa relative abundances. Histograms showing three different patterns of abundances when comparing ciliate microbiomes (CM) and control communities (CC). Known symbiont: bacteria of the family *Rickettsiaceae* (*Alphaproteobacteria*), previously reported as symbionts of ciliates, show a high relative abundance (> 5%) in two ciliate microbiomes, while they are absent or negligible (< 0.1%) in control communities. Putative food source: bacteria of the genus *Glaciecola* (*Gammaproteobacteria*) are present in libraries from marine samples; their relative abundance is high both in marine ciliate microbiomes (> 5%) and in marine control communities (> 1%). This pattern is consistent with that of a food organism. Putative symbiont: bacteria of the genus *Wautersiella* (*Flavobacteria*) display the same pattern shown by known symbionts; therefore, they could be regarded as potential candidate symbionts of ciliates. Environmental bacteria: bacteria of the order *Bacteroidales* (*Bacteroidetes*) are present and abundant only in freshwater control communities. They are undetectable in ciliate microbiomes, indicating they are not associated with these protists. *Y* axes represents relative abundances, expressed as sequence percentages in the various libraries

Using this criterion, known prokaryotic symbionts have in fact been detected here for the first time in association with unexpected hosts. For example, *Polynucleobacter* bacteria have been since reported as symbionts only in the genus *Euplotes* [22, 70], but were retrieved with high abundances (> 5%) also in the microbiomes of ciliates affiliated to the genera *Frontonia*, *Oxytricha*, and *Metopus*. Similarly, obligate intracellular bacteria of the family *Rickettsiaceae* were previously characterized in the ciliate genera *Euplotes*, *Paramecium*, *Spirostomum*, *Diophrys*, and *Pseudomicrothorax* [25, 60, 61]. Here, they have been additionally detected in microbiomes of ciliates affiliated to the genus *Paruroleptus*, which was never screened before for the presence of symbionts, and to the genus *Trithigmostoma*, in which macronuclear, unidentified symbiotic bacteria have been reported once [27]. It has to be mentioned that methanogenic archaea, well known as symbionts of anaerobic and microaerofilic ciliates [71], have been retrieved only in the microbiome of cell 37, assigned to the genus *Metopus* (one of the known hosts), and only in low relative abundances (one genus-level taxon at 1.94% and a second at 4.14%, both belonging to the *Euryarchaeota* lineage). They were not retrieved in other specimens belonging to putative hosts (i.e., a second *Metopus* and a specimen of *Caenomorpha*). The choice of amplification primers, targeting only a small fraction of archaea (426/160,767 according to Ribosomal Database Project), could have played a crucial role [72]. On the other hand, a previous investigation on the prokaryotes associated with a new species of the genus *Metopus* also failed in detecting methanogens [43], even if performed using archaeal-specific primers.

In addition to previously known symbionts, several novel prokaryotic taxa potentially associated with ciliates were here detected for the first time. Interesting examples include a taxon belonging to the family *Neisseriaceae* and bacteria of the genera *Wautersiella* and *Corynebacterium*. Protists in general, and ciliates in particular, have been already shown to harbor symbionts belonging to groups that also include pathogenic bacteria [61, 73–76]; the detection of a bacterial family including important mammal pathogens [77] and of genera mostly collected from human patients [78, 79] is definitely worth further investigations.

Obviously, prokaryotic taxa that are not strictly associated with ciliates are also bound to appear in single-cell libraries. Most commonly, they will be contaminants from the surrounding environment, or ingested organisms in food vacuoles. In either case, the involved taxa should also display high abundances in the total environmental community, except for ciliates with more selective feeding behaviors that might selectively prey on specific prokaryotes. Although food selection in filter-feeding ciliates is not entirely understood [80–83], the most important factor is generally considered to be prey size [84, 85]. Hence, in most situations, a comparative assessment of relative abundances will highlight the most promising symbiont candidates. Within our data collection, on the basis of results obtained for known symbionts, taxa with a relative abundance higher than 5% in host-associated libraries and lower than 0.1% in controls may be reasonably considered putative symbionts. Anything represented by 1% or more of control sequences should be treated carefully, even when present in high abundance in one or more ciliates. Borderline cases can certainly occur and should be tackled on a case-by-case scenario. Additionally, despite the depth of screening allowed by high-throughput sequencing techniques, the possibility that the used approach was not always sufficient to detect all bacteria associated with any single ciliate cell cannot be completely ruled out.

As might be expected, most of the prokaryotic genera with abundance profiles suggesting a closer relationship with the ciliate belong to phyla (*Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*) that are the most represented both in the investigated environment and in the literature on symbionts of ciliates. In each ciliate-associated microbiome, the percentage of genus-like taxa that might represent true symbionts based on their abundance is very variable. This fits the expectation that many, but by no means all, ciliates harbor symbiotic bacteria [27]. It can be expected that, when one or few populations of stable symbionts are actually present, they will constitute a major part of the single-cell library. Conversely, in their absence, libraries will include a more diverse assemblage of prokaryotes with low abundances, only loosely associated with the eukaryotic cell.

Overall, the results of this pilot study provide an optimistic picture of the feasibility of single-cell microbiomics for ciliates. The method detected known symbiotic taxa in previously characterized as well as novel hosts and provided evidence for putative new symbionts that deserve attention. Studies applying the methodology to larger sample numbers should be able to address questions that are outside the possibilities of culture-dependent methods. To begin with, data on the environmental distribution of symbioses in ciliate populations could be collected, whereas information was previously confined to the few ciliates from each environment that could be cultivated. The temporal dynamics of symbionts in natural populations could be monitored. Once enough data is collected, correlations with abiotic parameters might also become apparent. As a consequence, the varying effect of symbionts on host fitness depending on the environment, currently only hypothesized based on lab observations [33, 35, 36], could be tested by properly designed studies in the field.

Acknowledgements This work was supported by the University of Pisa (565-60%2016, 565-60%2017, PRA_2018_63) and by the Italian Ministry of University and Research (565-FFABR 2017). The authors wish to thank Simone Gabrielli for the help with graphic artworks and Irene Barbagli for the help in sampling. The authors are grateful to the Migliarino San Rossore Massaciuccoli Regional Park for giving permission for sampling.

References

1. Gast RJ, Sanders RW, Caron DA (2009) Ecological strategies of protists and their symbiotic relationships with prokaryotic microbes. Trends Microbiol. 17:563–569
2. Dziallas C, Allgaier M, Monaghan MT, Grossart HP (2012) Act together – implications of symbioses in aquatic ciliates. Front Microbiol 3:288. https://doi.org/10.3389/fmicb.2012.00288
3. McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Lošo T, Douglas AE, Dubilier N, Eberl G, Fukami T, Gilbert SF, Hentschel U, King N, Kjelleberg S, Knoll AH, Kremer N, Mazmanian SK, Metcalf JL, Nealson K, Pierce NE, Rawls JF, Reid A, Ruby EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ (2013) Animals in a bacterial world, a new imperative for the life sciences. Proc Nat Acad Sci 110:3229–3236
4. Turner TR, James EK, Poole PS (2013) The plant microbiome. Genome Biol. 14:209. https://doi.org/10.1186/gb-2013-14-6-209
5. de Bary A (1879) Die Erscheinung der Symbiose, ed Trübner KJ (Verlag von Karl,Strassburg)
6. Margulis L, Fester R (1991) Symbiosis as a source of evolutionary innovation: speciation and morphogenesis, Cambridge (Mass), MIT press
7. Shropshire JD, Bordenstein SR (2016) Speciation by symbiosis: the microbiome and behavior. mBio 7:e01785–e01715. https://doi.org/10.1128/mBio.01785-15.
8. Desai MS, Strassert JFH, Meuser K, Hertel H, Ikeda-Ohtsubo W, Radek R, Brune A (2010) Strict cospeciation of devescovinid flagellates and *Bacteroidales* ectosymbionts in the gut of dry-wood termites (Kalotermitidae). Environ Microbiol 12:2120–2132
9. Edgcomb VP (2016) Marine protist associations and environmental impacts across trophic levels in the twilight zone and below. Curr. Opin. Microbiol. 31:169–175
10. Görtz HD (2006) Symbiotic associations between ciliates and prokaryotes. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH,

Stackebrandt E (eds) The prokaryotes. Springer, New York, pp 364–402
11. Schulz F, Lagkouvardos I, Wascher F, Aistleitner K, Kostanjšek R, Horn M (2014) Life in an unusual intracellular niche: a bacterial symbiont infecting the nucleus of amoebae. ISME J 8:1634–1644
12. Yubuki N, Edgcomb VP, Bernhard JM, Leander BS (2009) Ultrastructure and molecular phylogeny of *Calkinsia aureus*: cellular identity of a novel clade of deep-sea euglenozoans with epibiotic bacteria. BMC Microbiol. 9:16. https://doi.org/10.1186/1471-2180-9-16
13. Claparéde E, Lachmann J (1858-1861) Etudes sur les infusoires et les rhizopodes, vol 1-2. Kessmann, Geneva
14. Müller J (1856) Einige Beobschtungen an Infusorien. Monatsber Preuss Akad Wissensch, pp 389–393
15. Boscaro V, Felletti M, Vannini C, Ackerman MS, Chain PS, Malfatti S, Vergez LM, Shin M, Doak TG, Lynch M, Petroni G (2013) *Polynucleobacter necessarius*, a model for genome reduction in both free-living and symbiotic bacteria. Proc Nat Acad Sci 110(46):18590–18595
16. Bright M, Espada-Hinojosa S, Lagkouvardos I, Volland JM (2014) The giant ciliate *Zoothamnium niveum* and its thiotrophic epibiont "*Candidatus* Thiobios zoothamnicoli": a model system to study interspecies cooperation. Front Microbiol 5:145. https://doi.org/10.3389/fmicb.2014.00145
17. Filker S, Kaiser M, Rosselló-Mora R, Dunthorn M, Lax G, Stoeck T (2014) "*Candidatus* Haloectosymbiotes riaformosensis" (*Halobacteriaceae*), an archaeal ectosymbiont of the hypersaline ciliate *Platynematumsalinarum*. Syst Appl Microbiol 37:244–251
18. Fokin SI, Görtz HD (2009) Diversity of *Holospora*-bacteria in *Paramecium* and their characterization. In: Fujishima M (ed) Endosymbionts in *Paramecium*, microbiology monographs, 12. Springer-Verlag, Heidelberg, pp 161–199
19. Petroni G, Spring S, Schleifer KH, Verni F, Rosati G (2000) Defensive extrusive ectosymbionts of *Euplotidium* (Ciliophora) that contain microtubule-like structures are bacteria related to *Verrucomicrobia*. Proc Nat Acad Sci U S A 97:1813–1817
20. Seah BKB, Schwaha T, Volland JM, Huettel B, Dubilier N, Gruber-Vodicka HR (2017) Specificity in diversity: single origin of a widespread ciliate-bacteria symbiosis. P Roy Soc B-Biol Sci 284: 20170764
21. Zaila KE, Doak TG, Ellerbrock H, Tung CH, Martins ML, Kolbin D, Yao MC, Cassidy-Hanley DM, Clark TG, Chang WJ (2017) Diversity and universality of endosymbiotic rickettsia in the fish parasite *Ichthyophthirius multifiliis*. Front Microbiol 8:189. https://doi.org/10.3389/fmicb.2017.00189
22. Boscaro V, Kolisko M, Felletti M, Vannini C, Lynn DH, Keeling PJ (2017) Parallel genome reduction in symbionts descended from closely related free-living bacteria. Nat Ecol Evol 1:1160–1167
23. Hirakata Y, Oshiki M, Kuroda K, Hatamoto M, Kubota K, Yamaguchi T, Harada H, Araki N (2015) Identification and detection of prokaryotic symbionts in the ciliate *Metopus* from anaerobic granular sludge. Microbes Environ 30:335–338
24. Lanzoni O, Fokin SI, Lebedeva N, Migunova A, Petroni G, Potekhin A (2016) Rare freshwater ciliate *Paramecium chlorelligerum* Kahl, 1935 and its macronuclear symbiotic bacterium "*Candidatus* Holospora parva". PLoS One 11:e0167928. https://doi.org/10.1371/journal.pone.0167928
25. Schrallhammer M, Ferrantini F, Vannini C, Galati S, Schweikert M, Görtz HD, Verni F, Petroni G (2013) "Candidatus Megaira polyxenophila" gen. nov. spec. nov.: considerations on evolutionary history, host range and shift of early divergent rickettsiae. PLoS One 8:e72581. https://doi.org/10.1371/journal.pone.0072581
26. Lynn DH (2008) The ciliated protozoa: characterization, classification, and guide to the literature. Springer Science and Business Media B.V.
27. Fokin SI (2012) Frequency and biodiversity of symbionts in representatives of the main classes of Ciliophora. Europ J Protistol 48: 138–148
28. Edgcomb VP, Leadbetter ER, Bourland W, Beaudoin D, Bernhard JM (2011) Structured multiple endosymbiosis of bacteria and archaea in a ciliate from marine sulfidic sediments: a survival mechanism in low oxygen, sulfidic sediments? Front Microbiol 2:article 55. https://doi.org/10.3389/fmicb.2011.00055
29. Gong J, Qing Y, Zou S, Fu R, Su L, Zhang X, Zhang Q (2016) Protist-bacteria associations: *Gammaproteobacteria* and *Alphaproteobacteria* are prevalent as digestion-resistant bacteria in ciliated protozoa. Front Microbiol 7:498. https://doi.org/10.3389/fmicb.2016.00498
30. Bevins CL, Salzman LH (2011) The potter's wheel: the host's role in sculpting its microbiota. Cell Mol Life Sci 68(22):3675–3685
31. Liu H, Carvalhais LC, Crawford M, Singh E, Dennis PG, Pieterse CMJ, Schenk PM (2017) Inner plant values: diversity, colonization and benefits from endophytic bacteria. Front Microbiol 8:2552. https://doi.org/10.3389/fmicb.2017.02552.
32. Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143–169
33. Bella C, Koheler L, Grosser K, Berendonk TU, Petroni G, Schrallhammer M (2016) Fitness impact of obligate intranuclear bacterial symbionts depends on host growth phase. Front Microbiol 7:article 2084. https://doi.org/10.3389/fmicb.2016.02084.
34. Duncan AB, Fellous S, Kaltz O (2011) Reverse evolution: selection against costly resistance in disease-free microcosm populations of *Paramecium caudatum*. Evolution 65:3462–3474
35. Dusi E, Krenek S, Schrallhammer M, Sachse R, Rauch G, Kaltz O, Berendonk TU (2014) Vertically transmitted symbiont reduces host fitness along temperature gradient. J Evol Biol 27:796–800
36. Fenchel T, Finlay BJ (1991) Endosymbiotic methanogenic bacteria in anaerobic ciliates: significance for the growth efficiency of the host. J Protozool 38:18–22
37. Görtz HD, Fokin SI (2009) Diversity of endosymbiotic bacteria in *Paramecium*. In: Fujishima M (ed) Endosymbionts in *Paramecium*, Microbiology Monographs 12, Chapter 6. Springer-Verlag, Heidelberg, pp 132–160
38. Vannini C, Sigona C, Hahn MWH, Petroni G, Fujishima M (2017) High degree of specificity in the association between symbiotic betaproteobacteria and the host *Euplotes*. Europ J Protistol 59: 124–132
39. Orsi W, Charvet S, Vd'ačnỳ P, Bernhard JM, Edgcomb VP (2012) Prevalence of partnerships between bacteria and ciliates in oxygen-depleted marine water columns. Front Microbiol 3:341. https://doi.org/10.3389/fmicb.2012.00341
40. Maurer-Alcalá XX, Knight R, Katz LA (2018) Exploration of the germline genome of the ciliate *Chilodonella uncinata* through single-cell omics (transcriptomics and genomics). mBio 9: e01836–e01817. https://doi.org/10.1128/mBio.01836-17.
41. Kolisko M, Boscaro V, Burki F, Lynn DH, Keeling PJ (2014) Single-cell transcriptomics for microbial eukaryotes. Curr. Biol. 24:R1081–R1082
42. Foster RA, Collier JL, Carpenter EJ (2006) Reverse transcription PCR amplification of cyanobacterial symbiont 16S rRNA sequences from single non-photosyntetic eukaryotic marine planktonic host cell. J. Phycol. 42:243–250
43. Omar A, Zhang Q, Zou S, Gong J (2017) Morphology and phylogeny of the soil ciliate *Metopus yantaiensis* n. sp. (Ciliophora, Metopida), with identification of the intracellular bacteria. J. Eukaryot. Microbiol. 64:792–805
44. Fenchel T, Esteban GF, Finlay BJ (1997) Local versus global diversity of microorganisms: cryptic diversity of ciliated protozoa. Oikos 80:220–225

45. Rossi A, Boscaro V, Carducci D, Serra V, Modeo L, Verni F, Fokin SI, Petroni G (2016) Ciliate communities and hidden biodiversity in freshwater biotopes of the Pistoia province (Tuscany, Italy). Europ J Protistol 53:11–19
46. Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified 16S-like rRNA coding regions. Gene 71:491–499
47. Petroni G, Dini F, Verni F, Rosati G (2002) A molecular approach to the tangled intrageneric relationships underlying phylogeny in *Euplotes* (Ciliophora, Spirotrichea). Mol Phylogenet Evol 22: 118–130
48. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, New York, pp 115–147
49. Modeo L, Rosati G, Andreoli I, Gabrielli S, Verni F, Petroni G (2006) Molecular systematics and ultrastructural characterization of a forgotten species: *Chattonidium setense* (Ciliophora, Heterotrichea). Proc Jpn Acad Ser B Phys Biol Sci 82:359–374
50. Andreoli I, Mangini L, Ferrantini F, Santangelo G, Verni F, Petroni G (2009) Molecular phylogeny of unculturable Karyorelictea (Alveolata, Ciliophora). Zool Scripta 38:651–662
51. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing based diversity studies. Nucleic Acids Res. 41(1):e1. https://doi.org/10.1093/nar/gks808
52. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402
53. Caporaso JG, Kuczynsky J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Met 7:335–336
54. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13(7):581–583
55. Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30:772–780
56. Price MN, Dehal PS, Arkin AP (2010) FastTree 2 – approximately maximum-likelihood trees for large alignments. PLoS One 5: e9490. https://doi.org/10.1371/journal.pone.0009490
57. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J 6:610–618
58. Werner JJ, Koren O, Hugenholtz P, DeSantis TZ, Walters WA, Caporaso JG, Angenent LT, Knight R, Ley RE (2012) Impact of training sets on classification of high-throughput bacterial 16S rRNA gene surveys. ISME J 6:94–103
59. Stoeck T, Bass D, Nebel M, Christen R, Jones MD, Breiner HW, Richards TA (2010) Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Mol. Ecol. 19:21–31
60. Ferrantini F, Fokin SI, Modeo L, Andreoli I, Dini F, Görtz HD, Verni F, Petroni G (2009) "*Candidatus* Cryptoprodotis polytropus," a novel *Rickettsia*-like organism in the ciliated protist *Pseudomicrothorax dubius* (Ciliophora, Nassophorea). J. Eukaryot. Microbiol. 56:119–129
61. Vannini C, Boscaro V, Ferrantini F, Benken KA, Mironov TI, Schweikert M, Görtz HD, Fokin SI, Sabaneyeva EV, Petroni G (2014) Flagellar movement in two bacteria of the family *Rickettsiaceae*: a re-evaluation of motility in an evolutionary perspective. PLoS One 9:e87718. https://doi.org/10.1371/journal.pone.0087718
62. Vannini C, Petroni G, Verni F, Rosati G (2005) A bacterium belonging to the *Rickettsiaceae* family inhabits the cytoplasm of the marine ciliate *Diophrys appendiculata* (Ciliophora, Hypotrichia). Microb Ecol 49:434–442
63. Shivaji S, Reddy GS (2014) Phylogenetic analyses of the genus *Glaciecola*: emended description of the genus *Glaciecola*, transfer of *Glaciecola mesophila, G. agarilytica*, *G. aquimarina*, *G. arctica*, *G. chathamensis*, *G. polaris* and *G. psychrophila* to the genus *Paraglaciecola* gen. Nov. as *Paraglaciecola mesophila* comb. nov., *P. agarilytica* comb. nov., *P. aquimarina* comb. nov., *P. arctica* comb. nov., *P. chathamensis* comb. nov., *P. polaris* comb. nov. and *P. psychrophila* comb. nov., and description of *Paraglaciecola oceanifecundans* sp. nov., isolated from the Southern Ocean. Int. J. Syst. Evol. Microbiol. 64:3264–3275
64. Šimek K, Kojecká P, Nedoma J, Hartman P, Vrba J, Dolan JR (1999) Shifts in bacterial community composition associated with different microzooplankton size fraction in a eutrophic reservoir. Limol Oceanogr 44:1634–1644
65. Hahn MW, Höfle MG (2001) Grazing of protozoa and its effect on aquatic populations of bacteria. FEMS Microbiol. Ecol. 35:113–121
66. Jürgens K, Matz C (2002) Predation as shaping force for the phenotypic and genotypic composition of planktonic bacteria. Antonie Van Leeuwenhoek 81:413–434
67. Matz C, McDougald D, Moreno AM, Yung PY, Yildiz FH, Kjelleberg S (2005) Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio colerae*. Proc Natl Acad Sci 102:16819–16824
68. Hahn MW, Scheuer T, Jezberova J Koll U, Jezbera J, Šimek K, Vannini C, Petroni G, Wu QL (2012) The passive yet successful way of planktonic life: genomic and experimental analysis of the ecology of a free-living *Polynucleobacter* population. PLoS One 7: e32772. https://doi.org/10.1371/journal.pone.0032772
69. Castelli M, serra V, Senra M, Basuri CK, Soares CAG, Fokin SI, Modeo L, Petroni G (2018) The hidden world of Rickettsiales symbionts: "*Candidatus* Spectrorickettsia obscura", a novel bacterium found in Brazilian and Indian Paramecium caudatum. Microb Ecol. https://doi.org/10.1007/s00248-018-1243-8
70. Vannini C, Ferrantini F, Ristori A, Verni F, Petroni G (2012) Betaproteobacterial symbionts of the ciliate *Euplotes*: origin and tangled evolutionary path of an obligate microbial association. Environ Microbiol 14:2553–2563
71. van Hoek AHAM, van Alen TA, Sprakel VSI, Leunissen JAM, Brigge T, Vogels GD, Hackstein JHP (2000) Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. Mol. Biol. Evol. 17:251–258
72. Eloe-Fadrosh EA, Ivanova NN, Woyke T, Kyrpides NC (2016) Metagenomics uncovers gaps in amplicon-based detection of microbial diversity. Nat Microbiol 1:15032
73. Boscaro V, Vannini C, Fokin SI, Verni F, Petroni G (2012) Characterization of "*Candidatus* Nebulobacter yamunensis" from the cytoplasm of *Euplotes aediculatus* (Ciliophora, Spirotrichea) and emended description of the family *Francisellaceae*. System and Appl Microbiol 35:432–440
74. Schrallhammer M, Schweikert M, Vallesi A, Verni F, Petroni G (2011) Detection of a novel subspecies of *Francisella noatunensis* as endosymbiont of the ciliate Euplotes raikovi. Microb. Ecol. 61: 455–464
75. Martínez-Pérez ME, Macek M, Castro Galván MT (2004) Do protozoa control the elimination of *Vibrio colerae* in brackish water? Internat Rev Hydrobiol 89:215–227
76. Sun S, Noorlan P, McDougald D (2018, 1017) Dual role of mechanisms involved in resistance to predation by protozoa and

virulence to humans. Front Microbiol 9. https://doi.org/10.3389/fmicb.2018.01017
77. Garrity GM, Brenner DJ, Krieg NR, Staley JT (2005) The family *Neisseriaceae*. In: BERGEY'S MANUAL OF systematic bacteriology Second Edition, Volume Two The Proteobacteria, Bergey's Manual Trust, pp 775–863.
78. Giordano C, Falleni M, Capria AL, Caracciolo F, Petrini M, Barnini S (2016) First report of *Wautersiella falsenii* genomovar 2 isolated from the respiratory tract of an immunosuppressed man. IDCases 4: 27–29
79. Hosseini Dehkordi SH, Lee S, Aponte J, Stavropoulos C (2017) Corynebacterium striatum as an unusual case of endocarditis in an intravenous drug user: case report and review of the literature. Infect Dis Clin Pract 25:301–304
80. Eisenman H, Letsiou I, Feuchtinger A, Beisker W, Mannweiler E, Hutzler P, Arnz P (2001) Interception of small particles by flocculent structures, sessile ciliates, and the basic layer of a wastewater biofilm. Appl Environ Microbiol 67:4286–4292
81. Thurman J, Parry JD, Hill PJ, Laybourn-Parry J (2010) The filter feeders *Colpidium striatum* and *Tetrahymena piriformis* display selective feeding behaviours in the presence of mixed, equally-sized, bacterial prey. Protist 161:577–588
82. Bautista-Reyes F, Macek M (2012) Ciliate food vacuole content and bacterial community composition in the warm-monomictic crater Lake Alchichica, Mexico. FEMS Microbiol Ecol 79:85–97
83. Tuorto SJ, Taghon GL (2014) Rates of benthic bacterivory of marine ciliates as a function of prey concentration. J Exp Mar Bio Ecol 460:129–134
84. Fenchel T (1980) Suspension feeding in ciliated protozoa: functional response and particle size selection. Microb. Ecol. 6:1–11
85. Montagnes DJS, Barbosa AB, Boenigk J, Davidson K, Jürgens K, Macek M, Parry JD, Roberts EC, Šimek K (2008) Selective feeding behaviour of key free-living protists: avenue for continued study. Aquat. Microb. Ecol. 53:83–98