Symbionts of the ciliate *Euplotes*: diversity, patterns, and potential as models for bacteria-eukaryote endosymbioses

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ABSTRACT

Endosymbioses between bacteria and eukaryotes are enormously important in ecology and evolution, and as such are intensely studied. Despite this, the range of investigated hosts is narrow in the context of the whole eukaryotic tree of life: most of the information pertains to animal hosts while most of the diversity is found in unicellular protists. A prominent case study is the ciliate *Euplotes*, which has repeatedly taken up the bacterium *Polynucleobacter* from the environment, triggering its transformation into obligate endosymbiont. This repeated origin makes the relationship an excellent model to understand recent symbioses, but *Euplotes* may host bacteria other than *Polynucleobacter*, and a more detailed knowledge of these additional interactions is needed in order to correctly interpret the system. Here we present the first systematic survey of *Euplotes* endosymbionts, adopting a classical as well as a metagenomic approach, and review the state of knowledge. The emerging picture is indeed quite complex, with some *Euplotes* harboring rich, stable prokaryotic communities not unlike those of multicellular animals. We provide insights into the distribution, evolution, and diversity of these symbionts (including the establishment of six novel bacterial taxa), and outline differences and similarities with the most well-understood group of eukaryotic hosts: insects.

Keywords

Devosia; *Euplotes*; *Francisellaceae*; *Holosporales*; Prokaryote-eukaryote symbioses; *Rickettsiales*

Background

Endosymbiosis is defined as a highly-interconnected relationship between two organisms of different species, one of which (the endosymbiont) lives inside the other (the host), and is a widespread and important phenomenon deeply affecting ecology and evolution [McFall-Ngai et al 2013, Müller et al 2016]. Symbiotic events were involved in several milestones of the history of life, including the origin of mitochondria and plastids [Dyall et al 2004], the ability of animals to digest plant material [Buchner 1965], and the building of coral reefs [Baker 2003]. Because of their ubiquity and importance, bacteria-eukaryote symbioses are the subject of a vast literature. However, nearly all model systems are focussed on a single type of hosts: insects [Buchner 1965, Baumann 2005, McCutcheon & Moran 2012]. Studies on insect symbioses over several decades, especially mutualisms in hosts restricted to nutritionally poor foods (plant sap, vertebrate blood, wood), have provided important insights into the endosymbiosis process. Nevertheless, to understand universal rules we need to expand the range of investigations to a variety of hosts and ecological contexts, and identify suitable systems among all eukaryotic lineages [e.g. Spribille et al 2016, Nowack & Weber 2018].

One such model that has been developing over recent years is *Euplotes*, a speciose genus of unicellular ciliates found in many aquatic environments [Boscaro et al 2019]. All *Euplotes* species in the "clade B" group [Syberg-Olsen et al 2016] harbor endocytoplasmic bacteria that are both obligate (they cannot survive outside their host) and essential (the host survival and reproduction depend on them) [Heckmann et al 1983, Vannini et al 2005, 2012]. The most common of these bacteria belong to the genus *Polynucleobacter* [Heckmann & Schmidt 1987], and are coopted from an abundant free-living pool in the water column [Jezberová et al 2010]. Extant symbiotic *Polynucleobacter* arose multiple times independently and relatively recently [Boscaro et al 2017], evolving from very similar ancestors and experiencing similar selective pressures. They therefore represent the end products of a natural evolutionary experiment rerunning the evolutionary transition from free-living to obligate endosymbiont. Access to free-living strains closely related to each symbiotic lineage has allowed to address questions that cannot be answered in systems where symbiosis originated only once, in the distant past.

But endosymbioses between *Euplotes* and prokaryotes are by no means limited to *Polynucleobacter*. A minority of populations in clade B depends on different bacteria, members of the genera *Devosia* [Boscaro et al 2018] and "*Candidatus* Protistobacter" [Vannini et al 2012, 2013]. Either of them may be a remnant of the original symbiotic event, indeed co-evolving with its host but being replaced in most cases by *Polynucleobacter*. In addition to these essential symbionts, many *Euplotes* also harbor "accessory" bacteria that are probably not required for host survival and whose role, if any, is unknown [Heckmann et al 1983, Vannini et al 2010, Boscaro et al 2012a, 2013a, Schrallhammer et al 2013, Vannini et al 2014]. Finally, bacterial symbioses in species of *Euplotes* outside clade B are considerably less studied, but have been occasionally reported [Vannini et al 2004, Schrallhammer et al 2011, Vallesi et al in press].

Here, we provide the first detailed survey of the diversity of bacteria harbored by *Euplotes*. We examined a large number of *Euplotes* strains, integrating the standard "Full-Cycle rRNA Approach" [Amann et al 1991], that involves characterization of the 16S rRNA gene and validation with fluorescent *in situ* hybridization (FISH), with metagenomic screening to enhance the completeness of the survey. Further developing *Euplotes* as a useful model for the study of endosymbiosis requires an understanding of all the components of the system. This is the most comprehensive attempt to date to achieve this goal. We report and discuss previously unknown

symbiotic taxa (25 bacterial strains including six new species and three new genera), their host distribution, and their features of interest in order to understand the intricacies of these multi-partner relationships.

Methods

Overview and source of *Euplotes*. Novel data is provided for 17 *Euplotes* monoclonal strains (14) or populations (3), each coming from a different sampling site and representing in total eight morphospecies. Two freshwater strains were never previously characterized: Eae4 and Eae6, both assigned to *Euplotes aediculatus* based on their 18S rRNA gene sequences. Both were collected in Tuscany (Italy) and maintained as reported elsewhere (e.g. [Boscaro et al 2017]). Other *Euplotes* were previously identified at the species level (see Supplementary Table S1 for a complete list), often together with some of their symbionts (see Supplementary Table S2). We describe additional bacteria harbored by these *Euplotes* strains and populations, using FISH experiments to validate 16S rRNA gene sequences of putative symbionts obtained by PCR amplification and Sanger sequencing [Amann et al 1991] and later integrated with metagenomic screening. In most instances, live stocks were available. In three cases (*Euplotes eurystomus* EM, *Euplotes octocarinatus* FL(12)-VI, and *Euplotes woodruffi* POH1), metagenomic screening could be conducted on old DNA extractions, but no live cell was available for FISH. Only one strain, *Euplotes enigma* MaS2, died before either DNA of sufficient quality for metagenomics or fixed cells for FISH could be collected; the detection of bacterial symbionts in this strain is based on 16S rRNA gene amplification, cloning, and Sanger sequencing.

Molecular methods. DNA extractions, Illumina library preparations and MiSeq sequencing were performed as reported previously [Boscaro et al 2017] for 13 of the *Euplotes* strains and populations (Eae1-6, Eda1, Eoc1/2, Ewo1, POH1, Fsp1.4, Na2, and LIV5). Archived extracted DNA from other *Euplotes* was obtained as described in the corresponding reference papers (Supplementary Table S1). Accessory symbionts in population EMP and strain MaS2 were characterized through alphaproteobacterial-specific PCR amplification of the 16S rRNA gene and cloning as described in Vannini et al 2012, with the exception of *Caedimonas* in EMP, whose 16S rRNA gene sequence was amplified with primers 16S_F35Caedcar [Schrallhammer et al 2018] and 1492R (modified from [Lane 1991]) and sequenced directly.

Metagenomic screening. Raw metagenomic reads were trimmed as reported previously [Boscaro et al 2017] and screened for 16S rRNA gene sequences with PhyloFlash v3.3b1 [Gruber-Vodicka et al 2019]. Full-length 16S and 18S rRNA gene sequences were then extracted from the targeted PhyloFlash assembly. A total metagenome assembly was also carried out in SPAdes v3.12.0 using default settings [Bankevich et al 2012]. The resulting assembly graph was inspected using Bandage [Wick et al 2015] and the assembly was checked by BlobTools [Laetsch & Blaxter 2017] to confirm multiple closely related symbionts present in a single host. Only fully assembled 16S rRNA were considered. Sequences from putative symbionts (e.g. belonging to groups of exclusively intracellular bacteria or related to previously described protist symbionts) had usually far higher coverage than those from common environmental contaminants living in the cultures.

Data availability. 18S and 16S rRNA gene sequences are deposited in the GenBank/EMBL/ENA database (accession numbers: LR585330-53 and XXXX).

Oligonucleotide probe design and fluorescence *in situ* hybridization protocol. Species-specific probes were designed for the newly described symbiont species and for *Francisella adeliensis*. The probe-design tool from the ARB software package was used [Ludwig et al 2004], based on the SILVA 128 database [Quast et al 2013]. The specificity of each new probe was also tested *in silico* on the Ribosomal Database Project [Cole et al 2009]. The sequence of each probe is reported in Supplementary Table S2. Fluorescently-labeled oligonucleotides were synthesized by Eurofins Genomics (Ebersberg, Germany). Fluorescence *in situ* hybridization (FISH) was performed according to the protocol of Manz et al [1992], using two probes of different specificity and emission wavelength in each experiment, adding DAPI to visualize the ciliate nucleus and employing negative controls with no probes to test for autofluorescence. Hybridized ciliates were observed with a Zeiss Axioplan epifluorescence microscope equipped with a Nikon Digital Sight DS-U1 camera and pictures were captured by the ACT-2U software. At least 20 cells per host strain were observed in each experiment. Most FISH were performed or repeated at least a year after DNA was obtained for metagenomics libraries, and hence attest bacterial populations stable at this temporal scale.

Phylogenetic inference. 16S rRNA sequences were aligned with the linsi algorithm in MAFFT [Katoh & Standley 2013]. Character matrices were trimmed at both ends to remove columns with more than 50% missing data. Maximum Likelihood trees were inferred using IQ-TREE version 1.6.6 [Nguyen et al 2015], using the best-fitting model according to the Bayesian Information Criterion.

Results

Symbionts of *Euplotes aediculatus* (clade B). 16S rRNA sequences from Eae2, Eae3, and Eae5 libraries did not suggest the presence of putative symbionts beyond the previously reported *Polynucleobacter* (*Betaproteobacteria*, *Burkholderiales*) [Boscaro et al 2017], and the cytoplasmic signal from eubacterial and *Polynucleobacter*-specific fluorescent probes confirmed this. The same results were obtained for the newly analyzed strain Eae4.

In strains Eae1 and Eae6, harboring the essential symbionts *Polynucleobacter* [Boscaro et al 2017] and "*Ca.* Protistobacter", respectively, the metagenomic screening additionally detected "*Ca.* Nebulobacter yamunensis" (*Gammaproteobacteria*, *Thiotrichales*) and "*Ca.* Cyrtobacter zanobii" (*Alphaproteobacteria*, *Rickettsiales*). Species-specific oligonucleotide probes confirmed the presence of both bacteria in the cytoplasm of all inspected cells (Supplementary Figs. S1x-y). 16S rRNA sequences, bacterial shape and size, and abundance of symbionts matched those in the original descriptions [Boscaro et al 2012a, Boscaro et al 2013a].

Symbionts of *Euplotes daidaleos* (clade B). Strain Eda1 harbors *Polynucleobacter* [Boscaro et al 2017]. Additionally, a 16S rRNA gene sequence affiliated to "*Ca.* Finniella" (*Alphaproteobacteria*, *Holosporales*) was detected in the metagenome. The sequence shares 95.4% identity with that of "*Ca.* Finniella lucida" from the cercozoan *Orciraptor* [Hess et al 2016]. The species-specific oligonucleotide probe Fin_1025 was designed for FISH experiments,

confirming the presence of this bacterium in the cytoplasm of all inspected Eda1 cells. Relatively short (about 1.7 µm) rod-like bacteria were visible in some hosts, while in others a second, extremely elongated (up to more than 25 µm) form was present. The two morphotypes occasionally occurred in the same host cell (Fig. 1A).

Symbionts of *Euplotes eurystomus* (clade B). Strain EM, now extinct, was originally described as a host of "*Ca.* Protistobacter" [Vannini et al 2012]. The metagenomic screening on archived DNA revealed 16S rRNA gene sequences from "*Ca.* Protistobacter" as well as four additional putative symbionts: (i) "*Ca.* Megaira polyxenophila", a common symbiont found in many protists [Lanzoni et al 2019] (*Alphaproteobacteria*, *Rickettsiales*); (ii) an uncultured bacterium belonging to the family "*Ca.* Midichloriaceae" (*Alphaproteobacteria*, *Rickettsiales*; 94.3% - 95.2% identity with representatives of the genus "*Ca.* Cyrtobacter"); (iii) a bacterium sharing high sequence identity (99.7%) with the "*Ca.* Finniella" accessory symbiont of *E. daidaleos* Eda1; and (iv) a bacterium affiliated to "*Ca.* Endonucleariobacter rarus" (*Gammaproteobacteria*), an endosymbiont of the opisthokont *Nuclearia* [Dirren et al 2014] (96.5% - 97.3% identity). Live cells were not available for FISH experiments, but since all described *Rickettsiales* and *Holosporales* live intracellularly it is safe to assume that at least three of the four mentioned bacteria are indeed endosymbiotic.

Symbionts of *Euplotes octocarinatus* (clade B). The monoclonal strain Eoc1 harbored *Polynucleobacter* [Boscaro et al 2017]. Data presented here were obtained from the population Eoc1/2 that strain belonged to. At least five putative alphaproteobacterial accessory symbionts were predicted by the metagenomic screening: (i) a bacterium closely related to *Holospora*-like infectious symbionts (82.4% 16S rRNA identity to "*Ca.* Hafkinia simulans", accession: MH319377); (ii) a second *Holosporales* symbiont resembling the "Ca. Finniella" already mentioned for *E. daidaleos* Eda1 and *E. eurystomus* EM (99.0% -99.1% sequence identity); (iii) "*Ca.* Megaira polyxenophila"; and two representatives of the family "*Ca.* Midichloriaceae", one (iv) affiliated to the genus "*Ca.* Anadelfobacter" (95.4% identity to "*Ca.* Anadelfobacter veles", accession: FN552695), and the other (v) only distantly related to described bacteria (best BLAST hit: uncultured bacterium T47, 91.8% identity, accession: KU524857). The presence of "*Ca.* Megaira" in the cytoplasm of *Euplotes* was confirmed using the oligonucleotide probe MegPol436 [Schrallhammer et al 2013] (Supplementary Fig. S1x). Species-specific probes Fuji_838, Ana2_436, and EocBan_828 were designed and tested for the *Holospora*-related bacterium (Fig. 1B), "*Ca.* Anadelfobacter" (Fig. 1C), and the divergent "Ca. Midichloriaceae" bacterium (Fig. 1D), respectively. They gave positive signals in all inspected host cells, except for Fuji_838. This probe matched small coccoid bacteria preferentially distributed at the anterior end of the cell, and sometimes entirely absent. Probe Fin_1025, validated on the "*Ca.* Finniella" of Eda1, did not work on population Eoc1/2, despite several attempts at various formamide concentrations. It is possible that in this case the symbiont was lost in the time between DNA extraction and FISH experiments.

The extinct strain FL(12)-VI was reported to harbor "*Ca.* Megaira polyxenophila" [Schrallhammer et al 2013] as well as the essential symbiont "*Ca.* Protistobacter" [Vannini et al 2012]. Our metagenomic screening additionally found the same "*Ca.* Anadelfobacter" (99.9% 16S rRNA gene sequence identity) described in the conspecific Eoc1/2.

Symbionts of *Euplotes woodruffi* (clade B). Strain Ewo1 harbors *Polynucleobacter* [Boscaro et al 2017]. In the metagenomic screening two accessory alphaproteobacteria were also found: "*Ca.* Megaira venefica", originally described in *Paramecium* [Lanzoni et al 2019], and a bacterium associated with "*Ca.* Bandiella", belonging to "*Ca.* Midichloriaceae" and previously also observed in *E. woodruffi* [Senra et al 2016] (95.8% 16S rRNA gene identity, accession: LN864514). FISH probes MegVene_95 [Lanzoni et al 2019] (Supplementary Fig. S1x) and the newly designed BanNum_173 confirmed the localization of the bacteria in the cytoplasm of all host cells, in very high number in the case of "*Ca.* Bandiella" (Fig. 1E). A very similar "*Ca.* Bandiella" (99.8% sequence identity), but no other accessory symbiont, emerged from the metagenomic screening of the extinct *E. woodruffi* strain POH1, which harbored "*Ca.* Protistobacter" as its essential symbiont.

Symbionts of *Euplotes* sp. (clade B). Population EMP could not be unambiguously assigned to any known *Euplotes* morphospecies, but it is deeply nested within clade B and harbors *Polynucleobacter* [Vannini et al 2012]. Three accessory symbionts could be characterized by PCR amplification, cloning, and FISH experiments: (i) "*Ca.* Megaira polyxenophila" (Supplementary Fig. S1x); (ii) *Caedimonas* (formerly *Caedibacter*) *varicaedens* (*Alphaproteobacteria*, *Holosporales*), a "killer-symbiont" of *Paramecium* [Pond et al 1989, Schrallhammer et al 2018] never detected before in *Euplotes* (Supplementary Fig. S1y); and (iii) a bacterium in the family *Paracaedibacteraceae*, like "*Ca.* Finniella", but not closely related to any described symbiont (85.4% identity with "*Ca.* Finniella lucida", accession: KT343635). The species-specific Paraf_838 probe was designed and tested for the *Paracaedibacteraceae* bacterium, targeting numerous small cytoplasmic bacteria (Fig. 1F).

Symbionts of *Euplotes platystoma* (clade B). *Euplotes platystoma* (some strains of which were previously misclassified as *Euplotes harpa* [Lian et al 2018]) is more distantly related to all other clade B *Euplotes* species, and it is often sampled in low-salinity rather than freshwater environments. Strain Fsp1.4 harbors *Polynucleobacter* [Vannini et al 2005], while strain Na2 is unique in clade B for harboring a member of the genus *Devosia* (*Alphaproteobacteria*, *Rhizobiales*), "*Ca.* Devosia symbiotica", as the essential symbiont [Boscaro et al 2018]. Metagenomic screenings on these strains did not detect any additional 16S rRNA gene sequence that is likely to belong to accessory symbionts.

Symbionts in marine *Euplotes* species of clade A. Strain LIV5 of *Euplotes magnicirratus*, like all previously screened strains of this species, depends on "*Ca.* Devosia euplotis" for reproduction and long-term survival [Vannini et al 2004]. Our metagenomic screening also recovered *Francisella adeliensis* (*Gammaproteobacteria*, *Thiotrichales*), described as a symbiont of *Euplotes petzi* [Vallesi et al in press], which belongs to the distantly related clade E. The probe Franci_199 confirmed the presence of the bacterium in the cytoplasm of LIV5 cells, although in relatively low amount (Supplementary Fig. S1x).

The single strain of *Euplotes enigma* we had access to did not survive long enough to perform a thorough investigation of its symbionts. Through PCR amplification and cloning, however, a partial 16S rRNA gene sequence similar to those of symbiotic *Devosia* in other *Euplotes* (96.5% identity with "*Ca.* Devosia euplotis" and 97.1% identity with "*Ca.* Devosia symbiotica") was obtained.

Phylogenetic analysis. All symbiotic *Polynucleobacter* strains, including the newly described symbiont of *E. aediculatus* Eae4, fall within the PnecC clade, that originally coincided with the species *Polynucleobacter necessarius* [Hahn et al 2016a] (Fig. 2A). Their relationship with free-living strains cannot be resolved using the 16S rRNA gene. The new "*Ca.* Protistobacter" is the first reported in *E. aediculatus*, and clusters within the genus. The sister group status of *Polynucleobacter* and "*Ca.* Protistobacter" within the family *Burkholderiaceae* is not strongly supported.

Alphaproteobacterial symbionts belonging to *Rickettsiales* and *Holosporales* cluster within established families of obligate intracellular symbionts, in various relationships with existing genera (Fig. 2B). Three of the new strains are particularly long-branching and not reliably associated to described bacteria: one of the two "*Ca.* Midichloriaceae" bacteria in Eoc1/2, the *Holosporaceae* bacterium in the same host, and the *Paracaedibacteraceae* bacterium in *Euplotes* sp. EMP.

In *Gammaproteobacteria*, most *Euplotes* symbionts cluster in the related families *Francisellaceae* and *Fastidiosibacteraceae* (Fig. 2C). Finally, the partial sequence of *Devosia* obtained from *E. enigma* MaS2 belongs to a clade of symbionts together with the two previously described species found in *Euplotes*, although bootstrap support for the clade is low, as is the case for all sub-genus relationships in *Devosia* (Fig. 2D).

Discussion

Establishment of novel bacterial taxa. Defining bacterial "species" is notoriously tricky, and "genera" are even more artificial concepts. Due to the universal use of nucleotide sequences as standard data, most discrimination is based on nucleotide identity thresholds. When establishing new taxa, we applied a 94.5% 16S rRNA gene sequence identity threshold for genera and a 98.7% threshold for species [Yarza et al 2014], while also taking into account the support for taxa monophyly. *Polynucleobacter* is a slightly more complex case: symbiotic *Polynucleobacter* lineages are scattered in the clade that once corresponded to *P. necessarius*, but that has since been split into several species [Hahn 2016a], all extremely similar at the 16S rRNA gene sequence level but differing considerably in genomic gene content [Hahn et al 2016b]. Symbiotic *Polynucleobacter* are here classified only as *Polynucleobacter* sp.

Of the 25 newly detected symbiotic strains 11 belong to already established species: "*Ca.* Protistobacter heckmanni" (1), "*Ca.* Megaira polyxenophila" (3), "*Ca.* Megaira venefica" (1), "*Ca.* Cyrtobacter zanobii" (2), *Caedimonas varicaedens* (1), *Francisella adeliensis* (1), and "*Ca.* Nebulobacter yamunensis" (2). Of these, "*Ca.* Megaira venefica" and *Caedimonas varicaedens* were never previously reported in *Euplotes*. The strain of "*Ca.* Megaira polyxenophila" in *E. eurystomus* EM actually shares only 96.9% sequence identity with its conspecifics, but this is probably due to the low quality of this metagenomic sequence, and the phylogenetic analysis confirms its placement within this species.

Seven symbiotic strains were assigned to three new species in existing genera: "*Ca.* Anadelfobacter sociabilis" sp. nov. (in *E. octocarinatus* Eoc1/2 and FL(12)-VI), "*Ca.* Bandiella numerosa" sp. nov. (in *E. woodruffi* Ewo1 and POH1), and "*Ca.* Finniella dimorpha" sp. nov. (in *E. daidaleos* Eda1, *E. eurystomus* EM, and *E. octocarinatus* Eoc1/2, the latter being the most divergent). Three strains warranted the establishment of as many novel genera. This was the case for "*Ca.* Euplotella sexta" gen. nov., sp. nov., a "*Ca.* Midichloriaceae" symbiont in *E.*

octocarinatus Eoc1/2; "*Ca.* Fujishimia apicalis" gen. nov., sp. nov., the coccoid *Holosporaceae* bacterium infecting some cells of the same host population; and "*Ca.* Parafinniella ignota" gen. nov., sp. nov., the *Paracaedibacteraceae* bacterium harbored by *Euplotes* sp. EMP. Formal descriptions of the new taxa are provided in Supplementary Text S1.

Finally, three of the characterized putative symbionts belong to undescribed taxa that cannot be formally established in the absence of a successful FISH with a specific probe [Murray & Stackebrandt 1995]. The uncultured "*Ca.* Midichloriaceae" in *E. eurystomus* EM is probably a new species of "*Ca.* Cyrtobacter" according to sequence identity and phylogenetic position. Similarly, the gammaproteobacterial endosymbiont in the same host differs enough from "*Ca.* Endonucleariobacter rarus" to be considered a different species of the same genus. We have little information on the *Devosia* harbored by *E. enigma*, which is a very close relative of *Euplotes* symbionts "*Ca.* Devosia euplotis" and "*Ca.* Devosia symbiotica".

Taxonomy, distribution, and biology of the bacterial endosymbionts of *Euplotes*. A detailed synopsis of all known bacterial symbionts of *Euplotes*, with an in-depth review of the literature, can be found in the Supplementary Text S2. Their distribution pattern is shown in Fig. 3.

Characterizations of bacterial endosymbionts in *Euplotes* are not uncommon, but have until now been mostly anecdotal, with descriptions of individual taxa selected from larger prokaryotic communities, additionally biased by the narrowness of the employed screening methods and the situational interest of the researchers. In order for *Euplotes* to become a robust model system, more information on the identity and distribution of its intracellular bacteria is needed. We have here attempted to provide a comprehensive picture by including metagenomic data mining and by investigating old, partially characterized *Euplotes* strains alongside new ones.

Some features emerge as generalized. But for a single report [Rosati & Verni 1975], all known symbionts of *Euplotes* have been observed in the cytoplasm, either free or enclosed in a host-derived membrane, whereas other ciliates may have conspicuous ectosymbionts [Petroni et al 2000, Bright et al 2014, Seah et al 2017] or harbor bacteria in their nuclear apparatus [Vannini et al 2014, Potekhin et al 2018, Schrallhammer et al 2018]. One explanation for the rarity of bacteria in the *Euplotes* macronucleus might be its relatively small diameter, although impressive *Holospora* infections can take place in the tiny micronuclei of certain *Paramecium* [Görtz & Fujishima 1983]. Alternatively, the complex "replication band" structures in *Euplotes* and related ciliates, responsible for the duplication of DNA before cell division, might render the nucleus inhospitable. Another general feature is that no known symbiont of *Euplotes* is motile or possesses flagella, although more ultrastructural studies are needed to confirm this.

At least 15 genera and 20 species of bacteria have now confirmed representatives in *Euplotes*. However, they belong to relatively few large lineages. All *Euplotes* symbionts are *Proteobacteria*, and the vast majority is confined to the family *Burkholderiaceae* in *Betaproteobacteria* and the specialized intracellular orders *Rickettsiales* and *Holosporales* in *Alphaproteobacteria*.

In contrast to their limited phylogenetic affiliations, the accessory endosymbionts show an extensive range of distribution and co-distribution patterns. A single *Euplotes* can harbor from zero to six prokaryotic species stably coexisting in its cytoplasm (over several years in lab cultures). Most *Rickettsiales* and *Holosporales*, as well as *Francisella*, are found in different host species, but not in all strains or populations of those species. The essential symbionts are notably different: either *Polynucleobacter* or "*Ca.* Protistobacter" are always present in clade B *Euplotes* species (with the single exception of *E. platystoma* Na2, harboring "*Ca.* Devosia symbiotica"

instead), and "*Ca.* Devosia euplotis" is always present in the marine *E. magnicirratus*. No strong correlation with host taxonomy can be inferred for other bacteria. In clade B, in particular, accessory alpha- and gammaproteobacteria do not match the presence of either *Polynucleobacter* or "*Ca.* Protistobacter", suggesting little, if any, taxon-specific interaction with these betaproteobacteria. *Euplotes* harboring *Devosia* have not been intensely investigated yet, but they seem to be less rich in accessory symbionts. Finally, no clear pattern of co-occurrence among different accessory symbionts emerges, with an intriguing exception: "*Ca.* Cyrtobacter zanobii" and "*Ca.* Nebulobacter yamunensis" from *E. aediculatus* are always detected together. Should this observation stand the test of time, it would definitely be interesting to look at their genomes for signs of metabolic integrations as reported in co-occurring symbionts of insects [e.g. McCutcheon & Von Dohlen 2011].

Phylogenetic analyses can provide many indirect insights on the biology of these bacteria. It was through phylogenomics that the multiple establishments of symbiosis in *Polynucleobacter* was proven [Boscaro et al 2017]. Strains of "*Ca.* Megaira", "*Ca.* Bandiella", and *Francisella* in *Euplotes* are scattered in clades including symbionts of diverse hosts, sometimes from unlike environments. This provides strong evidence for horizontal transmission of these bacteria, by no means confined to ciliates. Details of the ecology of infectious bacteria in aquatic environments are largely unknown, and it would be important to assess if ciliates and other protists play a role in their spread, as arthropods do in terrestrial environments [Husnik 2018]. Horizontal transmission in culture has been observed only for "*Ca.* Bandiella woodruffi", but it did not lead to long-term establishment in secondarily infected *Euplotes* [Senra et al 2016].

It is tempting to conclude that at least the infectious taxa are probably parasitic. There is however no evidence for any harmful effect on the *Euplotes* hosts. The prevalence of most of these bacteria is close to 100% in isolated host strains, and the symbionts are usually present in high numbers (roughly correlating with the size of the bacteria) in each host cell, a footprint of well-adapted parasites or commensals. It cannot be excluded that some might even be beneficial to their hosts, but it would then become difficult to explain why they occur only in some strains of the host species, and not others in the same habitats. *Polynucleobacter*, "*Ca.* Protistobacter", and *Devosia* are certainly beneficial for the hosts, for reasons still unclear [Boscaro et al 2013b], and yet cannot be described as mutualists in the absence of long-term benefits for the bacterium.

Comparison with insect symbioses: are *Euplotes* endosymbioses suitable model systems?

Protists are hugely diverse and far less known than metazoans and plants, which makes them intriguing as well as challenging model systems that require specific expertise. *Euplotes* is becoming the most deeply- and widely- sampled protist when it comes to symbiotic interactions with bacteria. This window into the diversity and evolutionary history of *Euplotes* symbionts allows us to draw preliminary comparisons to insect symbioses, that have been studied with molecular methods for three decades [Unterman et al 1989] and note a few interesting similarities and differences. This is made particularly relevant by the prominent position held by ciliates, among protists, as model organisms for several fundamental processes shared with metazoans [Ruehle et al 2016], despite their extreme divergence in the evolutionary history of eukaryotes.

First, the narrow taxonomic diversity of *Euplotes* endosymbionts is strikingly mirrored by insect symbioses where clades such as *Wolbachia*, *Rickettsia* (both *Rickettsiales*), *Sodalis*, *Arsenophonus* (both *Gammaproteobacteria* in the family *Enterobacteriaceae*), and "*Candidatus* Cardinium" (*Bacteroidetes*) are extremely common symbionts due to their ability to infect

eukaryotic cells and spread horizontally among species [Moran et al 2008]. Within groups that are common symbionts of all eukaryotes, such as *Rickettsiales*, the total diversity of protist symbionts is much higher, likely reflecting the evolutionary time for these symbioses to originate and diversify in protists, the bacterivorous nature of the hosts, and their lack of complex immune systems.

Second, the most evolutionarily successful bacteria associated with arthropods and nematodes are reproductive manipulators that shift the sex ratios of their hosts to increase their chance of maternal transmission, including *Wolbachia*, *Rickettsia*, and "*Ca.* Cardinium". No such manipulation is needed in single-celled eukaryotes, but we predict that some of the ciliate symbionts are likely just parasites that are good at (i) staying in both daughter cells after the host divides, (ii) avoiding host defense against bacteria, and (iii) spreading horizontally by infectious stages (e.g. spores) or when their original host is eaten by a different protist. On the other hand, accessory mutualists in insects were shown to have a diverse array of functions, particularly nutritional and defensive [Oliver et al 2010]. Whether some of the numerous accessory symbionts in *Euplotes* confer protection from pathogens or provide nutrients to the host or co-symbionts remains to be elucidated, although we predict that nutritional symbioses will not be very common in bacterivorous organisms.

Third, this study shows that up to six different symbionts can co-occur in the cytoplasm of a single *Euplotes* species. Of course, this is not easily comparable to much larger, multicellular animals that often house different bacterial symbionts in distinct bacteriocyte cells, and yet less than ten different species of intracellular symbionts are known from the most-understood insects such as whiteflies from the *Bemisia tabaci* species complex or pea aphids [Oliver et al 2010]. In the case of whiteflies, five accessory symbionts ("*Candidatus* Hamiltonella", *Arsenophonus*, "*Ca.* Cardinium", *Wolbachia*, and *Rickettsia*) can even co-occur with an essential "*Candidatus* Portiera" symbiont in the same host cell [Gottlieb et al 2008] and either compete or cooperate in diverse metabolic interactions [Opatovski et al 2018]. Unlike in insects, it is difficult to sample the same protist species from multiple geographic locations, so drawing conclusions about prevalence and abundance across populations is premature. Nevertheless, some of the ciliate symbionts appear to be generalists infecting various protists and some appear to be species-specific, again drawing parallels with insect symbioses [Oliver et al 2010].

Our view of eukaryotic symbioses is biased by our model systems, that currently do not even come close to representing the possible range of eukaryotic host diversity. Due to the long history of research, increasing amount of data, and ease of laboratory culture of both the host and free-living relatives of some of the symbionts, we view *Euplotes* symbioses as a valuable model for understanding symbioses in single-celled eukaryotes and identify generalized features of bacteria-eukaryote symbioses.

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Figure Legends

Figure 1. Fluorescent *in situ* hybridizations with species-specific oligonucleotide probes for the six novel endosymbiotic taxa. **A**, "*Ca.* Finniella dimorpha" in *E. daidaleos* Eda1. **B**, "*Ca.* Fujishimia apicalis" in *E. octocarinatus* Eoc1/2. **C**, "*Ca.* Anadelfobacter sociabilis" in *E. octocarinatus* Eoc1/2. **D**, "*Ca.* Euplotella sexta" in *E. octocarinatus* Eoc1/2. **E**, "*Ca.* Bandiella numerosa" in *E. woodruffi* Ewo1 (the asterisk marks autofluorescence signal from an undigested alga). **F**, "*Ca.* Parafinniella ignota" in *Euplotes* sp. EMP. Grey outlines represent *Euplotes* cells and were drawn based on corresponding bright field pictures. Bars represent 10 μm.

Figure 2. Phylogenetic affiliations of bacterial endosymbionts of *Euplotes*. **A**, Phylogenetic tree of family *Burkholderiaceae* (*Betaproteobacteria*), including symbiotic *Polynucleobacter* forming a polyphyletic group in the otherwise free-living clade "PnecC", and the exclusively symbiotic genus "*Ca.* Protistobacter". **B**, Phylogenetic tree of orders *Rickettsiales* and *Holosporales* (*Alphaproteobacteria*), entirely composed of intracellular bacteria harbored by diverse hosts. **C**, Phylogenetic tree of the closely related families *Francisellaceae* and *Fastidiosibacteraceae*, including obligate and opportunistic endosymbionts as well as free-living bacteria. **D**, Phylogenetic tree of *Devosia* (*Alphaproteobacteria*) and closely related genera. All trees are based on 16S rRNA gene sequences and were built according to the Maximum Likelihood criterion. *Euplotes* endosymbionts are highlighted, and color-coded according to their host species. Sequence accession numbers are provided in brackets. Numbers in square brackets represent the number of sequences in collapsed nodes. Standard bootstrap supports, when at or above 70%, are provided close to the corresponding node. Bars stand for an estimated sequence divergence of 0.1.

Figure 3. Synopsis of all *Euplotes* strains and populations screened for the presence of bacterial endosymbionts with molecular techniques. On the left, a simplified phylogeny of the *Euplotes* species investigated is presented. On columns, symbionts are organized first by their characterization as "essential" or "accessory", and then by taxonomy. Asterisks mark bacterial

677 species found in hosts other than *Euplotes*. The "absence" status is employed for negative FISH
678 or negative metagenomic screening results. Black dots represent presences inferred by the
679 recovery of 16S rRNA gene sequences (through Sanger or high-throuput sequencing) but not
680 confirmed by FISH.