Microbial Ecology (2023) 85:307–316
https://doi.org/10.1007/s00248-021-01938-x

HOST MICROBE INTERACTIONS

Single-cell Microbiomics Unveils Distribution and Patterns of Microbial Symbioses in the Natural Environment

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Received: 20 August 2021 / Accepted: 5 December 2021 / Published online: 20 January 2022

Abstract

Protist-bacteria associations are extremely common. Among them, those involving ciliates of the genus *Euplotes* are emerging as models for symbioses between prokaryotes and eukaryotes, and a great deal of information is available from cultured representatives of this system. Even so, as for most known microbial symbioses, data on natural populations is lacking, and their ecology remains largely unexplored; how well lab cultures represent actual diversity is untested. Here, we describe a survey on natural populations of *Euplotes* based on a single-cell microbiomic approach, focusing on taxa that include known endosymbionts of this ciliate. The results reveal an unexpected variability in symbiotic communities, with individual hosts of the same population harboring different sets of bacterial endosymbionts. Co-occurring *Euplotes* individuals of the same population can even have different essential symbionts, *Polynucleobacter* and "*Candidatus* Protistobacter," which might suggest that replacement events could be more frequent in nature than previously hypothesized. Accessory symbionts are even more variable: some showed a strong affinity for one host species, some for a sampling site, and two ("*Candidatus* Cyrtobacter" and "*Candidatus* Anadelfobacter") displayed an unusual pattern of competitive exclusion. These data represent the first insight into the prevalence and patterns of bacterial symbionts in natural populations of free-living protists.

Keywords Protist-bacteria symbiosis · Protist microbiome · *Bandiella* · *Devosia* · *Francisella* · Protist microbiota

Introduction

Symbioses between bacteria and unicellular eukaryotes (protists) are an extraordinarily common yet understudied phenomenon [1]. Among protists, ciliates are particularly prone to establishing symbiotic associations with prokaryotes due to several distinctive characteristics such as bacterivorous feeding behavior, large size, and a variety of intracellular compartments, which offer different microhabitats for bacterial colonization [2]. Associations between bacteria and ciliates constitute a traditional field of research in ciliatology [3], and in the last decades, the use of molecular and "-omics" approaches has renewed the interest in this topic. Many new bacterial symbionts of ciliates have been described (e.g. [4–7]) and characterized from the points of view of genomics and phylogeny [6–11], life cycle [12], and relationship with the host [13–16].

Euplotes is one of the most extensively studied ciliates in this regard. Species in this genus are common inhabitants of most aquatic environments; ancestrally marine, they have successfully invaded freshwater and soil habitats [17] and can be easily collected from the wild and maintained in the laboratory. Therefore, *Euplotes* has been used as a model system for genetics, molecular biology, cell biology, ecology, and symbiosis [5, 18–20]. Indeed, a considerable amount of data on very different and multifaceted bacterial symbioses in *Euplotes* is available. Within the genus, a monophyletic clade ("clade B" Syberg-Olsen et al. [17]) depends on endosymbiotic bacteria for reproduction and survival [9, 21, 22]. These essential symbionts have been recruited many times during the evolutionary history of the hosts, depicting a complex and intriguing picture of loss and gain [8, 9]. Up to now, three bacteria have been described as essential symbionts of clade B *Euplotes*, namely *Polynucleobacter*, "*Candidatus* Protistobacter" (both *Betaproteobacteria*), and "*Candidatus* Devosia" (*Alphaproteobacteria*) [9, 15, 23]. In addition to the essential symbionts, a variety

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of accessory bacteria have been described within *Euplotes* species of clade B, mostly belonging to the orders *Rickettsiales* and *Holosporales* of *Alphaproteobacteria*, or to *Gammaproteobacteria* [5]. Multiple accessory symbionts can coexist, with up to six different bacteria stably sharing the cytoplasm of a single host strain [5]. Essential symbionts, as well as some whose relationship with the host still has to be clarified, are also harbored by *Euplotes* belonging to other phylogenetic clades [11, 13, 24].

Up to now, all studies on the symbionts of *Euplotes* and other ciliates have been performed on laboratory cultures, which are essential for testing hypotheses on the physiology of the associations, but whose representativeness of the real situation in the natural environment has not been assessed. Field research is completely lacking, and nothing is known about the ecology of these symbiotic systems, including even basic data like distribution, prevalence, or co-occurrence patterns. The extreme abundance instability of protist populations in the natural environment, coupled with the lack of suitable and reliable methods, has hindered such investigations.

Here, we report the first survey of bacterial symbiont frequencies in natural populations of a ciliate, using a sensitive single-cell metabarcoding approach for simultaneous identification of both hosts and symbionts [25]. Detection and documentation of all bacteria associated with single individuals of *Euplotes* in their natural habitat provide a reliable snapshot of the natural diversity at the level of individual cells in populations. Here, we analyze these data to (i) assess the prevalence of bacterial symbionts in natural populations of *Euplotes*, (ii) document the natural diversity of symbionts and specific patterns of association between host and symbiont species in natural populations, and (iii) identify patterns of symbiotic consortia inside the same host cell in nature.

Methods

Sampling and Ciliate Cell Isolation

Samples were collected over two weeks in autumn 2018 in two different areas along the Tuscany coast within the Migliarino San Rossore Massaciuccoli Regional Park. One site (SR2A) was located in the San Rossore estate, along a small ditch connected to the mouth of river Arno, and four other sites were located in coastal ponds next to Marina di Vecchiano (MdV; sites MdV1A, MdV1D, MdV3A, MdV3B), near the mouth of river Serchio (Fig. 1). Microhabitats in both areas were extremely variable, subjected to wide fluctuations in water level (up to complete drying during the warm season; water temperature values ranging during the year from 1℃ up to more than 33 °C) and salinity (due to frequent coastal storms; water salinity ranging during one year from 0 to 13‰). Values of environmental parameters measured at the time of sampling are reported in Online resource 1. A total volume of about 45 mL (water and sediments) was collected from each site and immediately transported to the lab. After gentle mixing, a 30 mL aliquot from each sample was used for ciliate collection. *Euplotes* were detected by microscopical observation, individually washed (three steps in sterile, artificial brackish water, followed by two additional, fast steps in sterile distilled water), and then stored in 70% v/v ethanol inside a 0.2 mL tube at − 20 °C. Different sterile glass micropipettes were employed for each ciliate cell during isolation and for each washing step. The remaining sample volume of 15 mL was fixed in 70% (v/ v) ethanol and divided into three aliquots used as controls in order to characterize the background environmental microbial communities. The whole procedure was performed within 48 h from sample collection to reduce the risk of contamination from the lab.

Amplification and Sequencing of SSU rRNA Genes of Hosts and Associated Bacteria

A simultaneous amplification of eukaryotic 18S rRNA gene and bacterial 16S rRNA gene was carried out using the following primers: 18S F9 Euk (5′-CTGGTTGATCCTGCCAG-3′), 18S R1513 (5′-TGATCCTTCYGCAGGTTC-3′), 8F (5′-AGRGTTYGATYMTGGCTCAG-3′), and UNIb-rev (5′-GACGGGCGGTGTGTRCAA-3′). Amplification was performed directly on each individually stored ciliate cell, without performing DNA extractions, as described in Rossi et al. [25]. Amplicons were purified with the Eurogold Cycle-Pure Kit (Euroclone) and diluted 1:100; aliquots were then processed differently for host and bacteria characterization. For ciliate host identification, two semi-nested amplifications were performed, products were further purified, and Sanger sequenced using multiple appropriate internal primers by GATC Biotech (Cologne, Germany) [25].

In parallel, the characterization of host-associated bacteria was carried out with a metabarcoding approach, starting with a nested PCR using the KAPA HiFi HotStart Ready Mix with a prokaryotic primer set for the V3–V4 regions of the SSU rRNA gene: the forward primer S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and the reverse primer S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) [26]. Illumina overhang adapter sequences added to the primers were 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′ and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′, respectively (Illumina protocol, Part # 15,044,223, Rev. B). Amplification cycles ($n = 25$) were performed with an annealing temperature of 55℃.

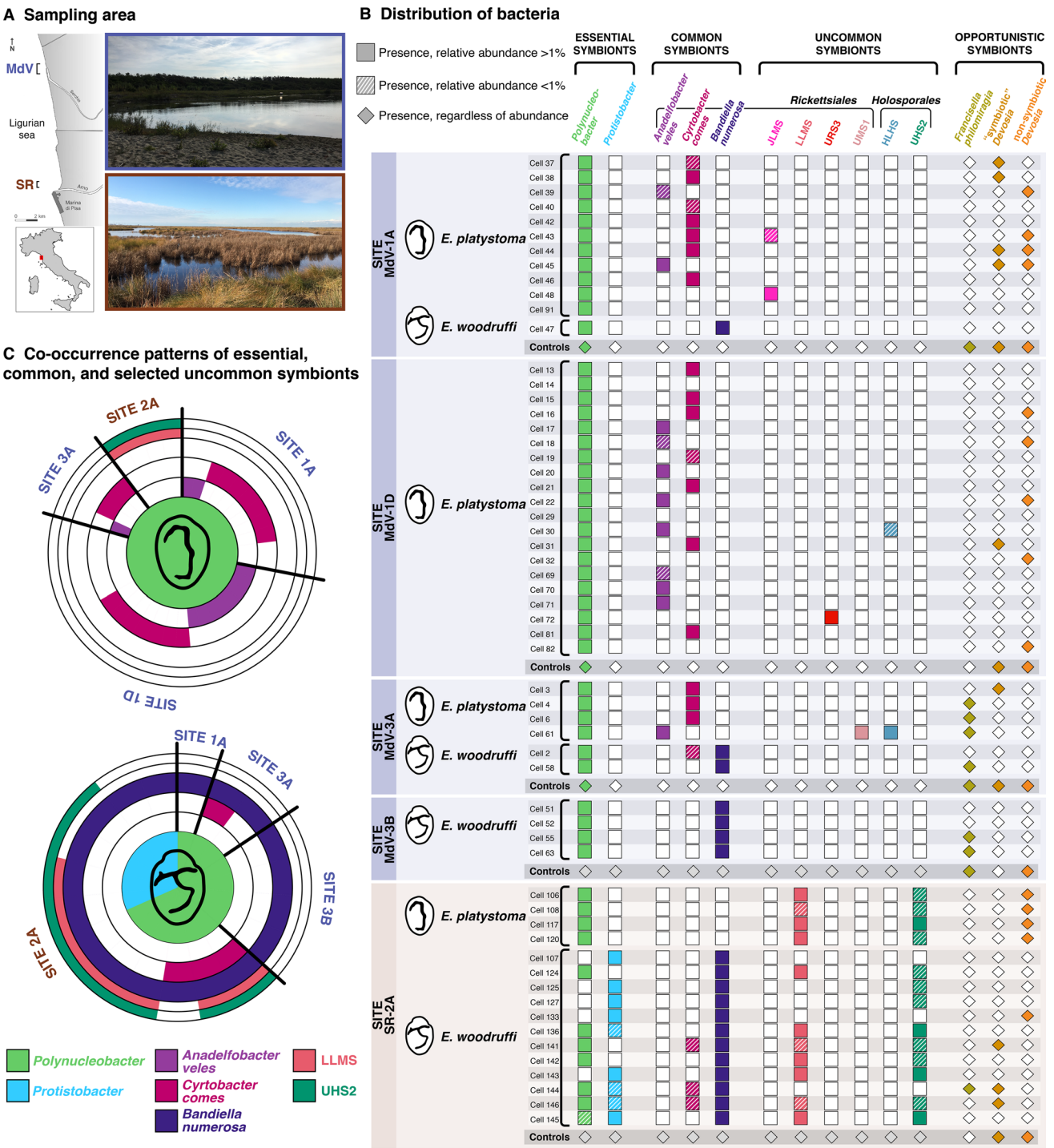

Fig. 1 Overview of sampling and summary of symbiont distribution. **A**, Localization of the two investigated areas, Marina di Vecchiano (MdV, in blue) and San Rossore (SR, in brown). **B**, Presence of targeted bacterial taxa in processed *Euplotes* single cells, grouped by site and host species. Hatched squares represent relative abundances below 1% for essential, common, and uncommon symbionts. The presence of bacteria is tracked both in *Euplotes* specimens and environmental controls, regardless of relative abundance. **C**, Synopsis of symbiont patterns arranged by host species. Individual cells are represented by wedges so that co-occurring symbionts are found along the same radius. From the inside out, circles depict essential symbionts, "*Ca.* Anadelfobacter veles," "*Ca.* Cyrtobacter comes," "*Ca.* Bandiella numerosa," and the bacteria provisionally named LLMS and UHS2

In order to characterize environmental prokaryotic communities, control aliquots were centrifuged at 10,000 *g* to pellet microbial organisms with the sediment; the supernatant was then removed, and total genomic DNA was extracted from 0.25 g of each pellet using the PowerSoil DNA Isolation Kit (MoBio). Extracted DNA was processed by the two amplification steps described above for bacterial community characterization.

Prokaryotic amplicons from single host cells as well as environmental controls were barcoded, pooled, and sequenced by IGA Technology (Udine, Italy) on the Illumina MiSeq platform (2 × 300 paired-end sequencing).

Sequence Analysis

Gene sequences of eukaryotic 18S rRNA were inspected with NCBI Blast [27] for putative identification of the ciliate hosts, using a species identity cutoff of 99%.

Raw reads of bacterial V3–V4 regions were analyzed using the Quantitative Insights Into Microbial Ecology version 2 (QIIME2, https://qiime2.org) software package [28]. In order to remove the lower-quality ending base calls, forward and reverse reads were truncated at base 290 and 260, respectively. Quality filtering, primer trimming, pair-end read merging, and clustering of reads in ASVs were performed with DADA2 [29], using default settings. Taxonomic classification was performed using the SILVA reference database, release 132 [30]. Following Werner et al. [31], the regions of interest were extracted from SSU rRNA reference sequences (99% similarity clustered database) and used to train a Naive Bayes classifier. ASVs identified as mitochondria or chloroplasts were removed before further data processing.

Data Mining for Bacterial Symbiont Identification and Detection

To identify putative symbionts in our dataset, first, we collected ASVs assigned by the Bayes classifier to *Polynucleobacter*, "*Ca*. Protistobacter," or within *Rickettsiales*, *Holosporales*, *Francisellaceae*, *Devosiaceae*, and *Verrucomicrobia*, which collectively include all known symbionts of *Euplotes*. ASVs assigned to *Rickettsiales*, *Holosporales*, *Francisellaceae*, and *Devosiaceae* (no ASV was classified as *Verrucomicrobia*) were added to previously curated alignments of reference full-length SSU rRNA gene sequences made with MAFFT [32]. Phylogenetic trees were inferred with IQ-TREE [33] using the –m TEST option to select the best-fitting substitution model, and running 100 standard non-parametric bootstrap replicates. Clades of closely related sequences, approximately corresponding to bacterial species, were manually inspected and reclassified (Fig. 2 and Online resource 2). Automated assignments made by the Bayes classifier were at this point disregarded or corrected. The relative abundances of named and unnamed taxa obtained this way were then assessed in host-derived libraries and environmental controls to detect symbionts. We considered likely symbionts of *Euplotes* only those taxa displaying a much higher relative abundance in host-derived libraries than in environmental controls (at least 100-fold difference, and with a minimum 1% abundance in at least one *Euplotes* library). Representative ASVs for putative bacterial taxa with provisional names mentioned here have been deposited in the European Nucleotide Archive (ENA) database under accession numbers OU452359-OU452364.

Results

Ciliate Host Identification

A total of 62 ciliate cells collected from ephemeral brackish water environments (Fig. 1A) were successfully processed both for ciliate host identification and for characterization of associated prokaryotes (up to 20 ciliate cells per morphospecies in the same site). For each ciliate cell, an almost complete 18S rRNA gene sequence was obtained (1311–1886 bp, with one 769 bp-long outlier, Online resource 3). The two most frequently retrieved *Euplotes* species were *Euplotes platystoma* (39 cells) and *Euplotes woodruffi* (19 cells), both belonging to clade B and known to host essential bacterial symbionts. Data analyses and discussions are therefore focused on these two species.

Distribution of Bacterial Symbionts in *Euplotes* Hosts

Ciliate-associated libraries averaged $1.35\ 10^5 \pm 3.57\ 10^4$ (SD) raw read pairs, $6.75\ 10^4 \pm 2.07\ 10^4$ (SD) merged sequences after quality control, and 102 ± 59.3 (SD) unique amplicon sequence variants (ASVs) (Online resource 4). Instead of taking into account the entire prokaryotic community associated with the hosts (Online resource 5), we focused only on bacterial lineages already known to include symbionts of *Euplotes*. Within these groups, we manually clustered ASVs into promising species-like taxa (Fig. 2, Online resource 2). Among them, we applied the previously defined abundance criteria and detected 11 which were mostly absent from environmental controls and relatively abundant in at least some *Euplotes*-derived libraries (Online resource 6), suggesting that signals from potential free-living forms and even symbionts are negligible compared to the overall background community. These 11 species, 5 of which were previously known as *Euplotes* symbionts, are here subdivided into three categories for convenience: (i) symbionts known from previous literature to be essential [2,

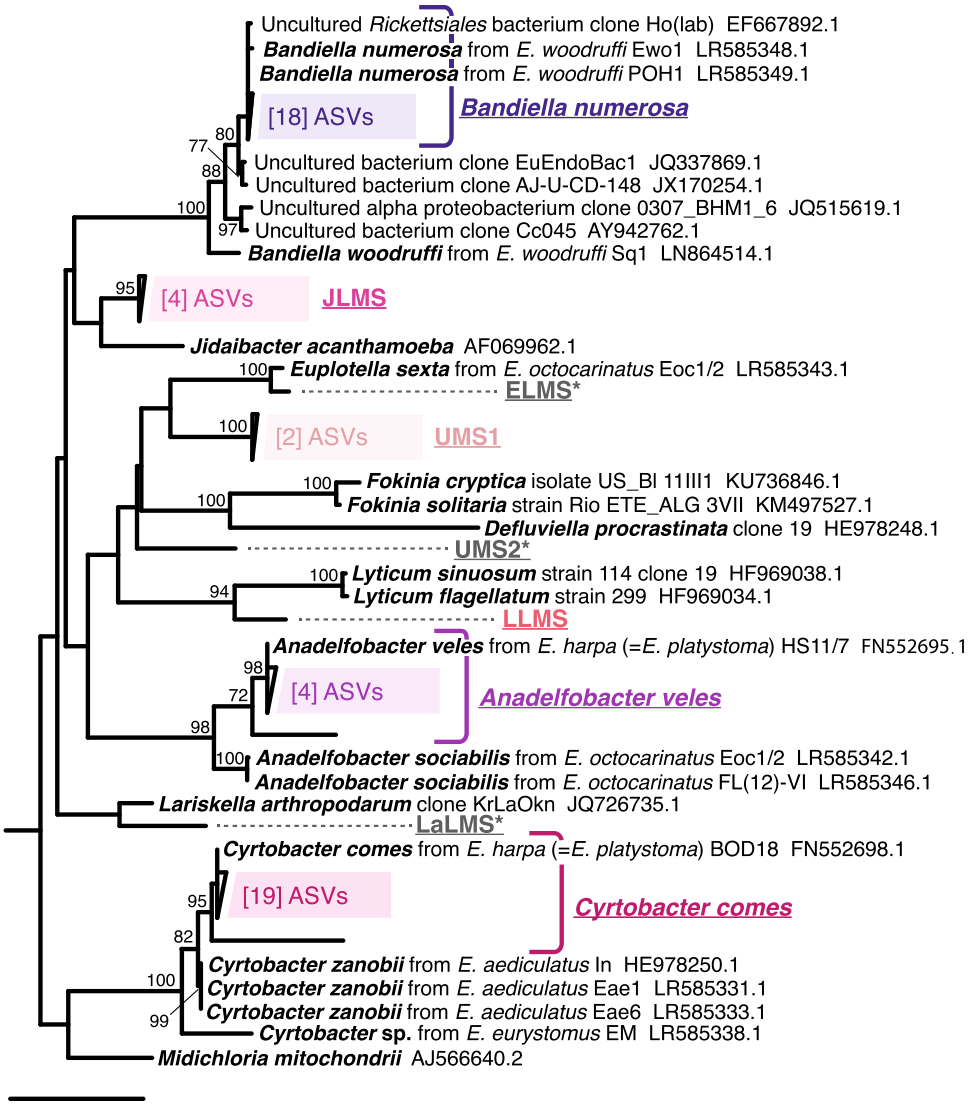

Fig. 2 Maximum-likelihood tree of family "*Candidatus* Midichloriaceae" used to cluster ASVs into species-like assemblages and refine their classification. Reference sequences are in black and associated with accession numbers. ASV names are not shown (for a complete list, see Online resources 2 and 6), and clades of related ASVs are collapsed, with ASVs numbers per cluster reported in square brackets. On the right, underlined, manually identified species-like assemblages are shown, together with their final identification. Short alphanumerical codes were used to provisionally name undescribed species. Colored taxa were considered putative symbionts of *Euplotes* and are discussed in the text (see also Fig. 1); taxa in gray and marked by an asterisk were not. The bar stands for an estimated sequence divergence of 0.1

8, 23], (ii) common accessory symbionts found in multiple sites, and (iii) uncommon accessory symbionts, found in only one site or less than five host cells.

Essential Symbionts

The two betaproteobacterial essential symbionts of clade B *Euplotes* species, namely *Polynucleobacter* and "*Ca.* Protistobacter," were the most common and abundant host-associated bacteria observed in our survey: every *E. platystoma* and *E. woodruffi* cell harbored one or the other. *Polynucleobacter* was the most prevalent, being detected in 52 out of 58 cells; the remaining 6 cells contained "*Ca.* Protistobacter" and belonged to a single population of *E. woodruffi* (Fig. 1B). Relative abundances were generally high, with *Polynucleobacter* averaging 56.9% of the sequences in host cells and "*Ca.* Protistobacter" averaging 14.9%. "*Ca.* Protistobacter" was never detected in controls, while *Polynucleobacter* was present at very low abundances (average: 0.043%), possibly reflecting the presence of free-living strains in the environment [34]. Interestingly, both betaproteobacteria were found in the *E. woodruffi* population from site SR2A (Fig. 1B), although each individual cell harbored only one of the two symbionts (with the possible exception of four cells which displayed an additional low signal from the other essential symbiont: $< 0.5\%$ of the total sequences, or < 50 times the abundance of the predominant one).

Common Accessory Symbionts

All other potential symbionts belonged to two alphaproteobacterial orders of intracellular bacteria, *Rickettsiales* and *Holosporales* (Fig. 1B). Three "*Candidatus* Midichloriaceae" (*Rickettsiales*) species previously described were the most common in our survey. "*Candidatus* Anadelfobacter veles" was only found in *E. platystoma* (in 11 out

of 40 cells, average abundance: 1.46%), the host species it was originally described from (at the time identified as *Euplotes harpa* [35]). "*Candidatus* Cyrtobacter comes," also originally characterized in *E. platystoma* [35], was also more prevalent in that species (detected in 17 out of 39 cells, average abundance: 6.12%; versus in 4 out of 19 cells of *E. woodruffi*, with a lower 0.45% average abundance). "*Ca.* Anadelfobecter veles" and "*Ca.* Cyrtobacter comes" were never found in the same host cell (Fig. 1B, C). "*Ca.* Bandiella numerosa," originally described in *E. woodruffi* [5], was detected in every cell of that species surveyed here (average abundance: 13.53%) and never in *E. platystoma*. None of these midichloriaceae were ever detected in environmental controls.

Uncommon Accessory Symbionts

Six more species-like taxa of *Rickettsiales* and *Holosporales* were found more sporadically in *Euplotes* (and never in controls). They could not be ascribed to known bacterial species and were given provisional names: "JLMS", "LLMS", and "UMS1" belong to "*Ca.* Midichloriaceae," "URS3" to *Rickettsiaceae* (*Rickettsiales*), "HLHS" and "UHS2" to *Holosporaceae* (*Holosporales*). These taxa were generally present only in a few host cells, sometimes at low abundance (Fig. 1B), but LLMS and UHS2 were prevalent in *Euplotes* cells of both species in site SR2A and were not found elsewhere (Fig. 1B, C).

Distribution of Bacteria from "Opportunistic" Genera

Other *bona fide* non-alphaproteobacterial symbionts of *Euplotes* such as *Nebulobacter* [36] and *Pinguicoccus* [11] were not found at all in the surveyed populations. However, two bacterial genera sometimes associated with *Euplotes* [4, 13, 15, 24] deserve mention despite (or rather, due to) not fitting our abundance criteria for symbiont detection: *Francisella* and *Devosia*.

By far, the most abundant *Francisella* species in our survey was *Francisella philomiragia*, detected here in low abundance from a few cells (7 out of 58 *Euplotes* divided between both species, average abundance: 1.91%) and in many environmental controls (average abundance: 0.062%) (Fig. 1B).

Two *Devosia* species were described as symbionts of marine and freshwater *Euplotes* [13, 15] and form a phylogenetic clade putatively considered *Euplotes*-specific (Online resource 2). We found sequences belonging to this clade associated both to *Euplotes* cells (9, belonging to both species, average abundance: 0.235%) and environmental controls (average abundance: 0.041%) (Fig. 1B). *Devosia* sequences not belonging to this clade presented a similar profile, although they were more abundant in controls (14 cells in both host species; average abundance: 0.230% in hosts, 0.620% in controls). Whenever *Devosia* were detected associated with *Euplotes*, their abundance was considerably lower than that of the essential betaproteobacterium (from approximately 2 to 700 times so).

Discussion

Suitability of Microbiomic Methods on Unicellular Eukaryotes

Interpreting microbiomics data, especially those based on metabarcoding and relying on low DNA input, is often challenging. The molecular techniques employed here were previously tested on ciliates [25, 37–39], but on much lower numbers of cells and focusing on whole microbial communities' composition instead of target symbiotic bacterial species. The results were compatible with the existing knowledge, but details were hard to pin down due to the huge diversity within observed microbial communities and the high potential for procedural artifacts. The analysis of this survey's data was designed to avoid two main pitfalls: first, by using a well-known host model with partially predictable outcomes (the presence of essential symbionts), we added a strong layer of control on top of routine environmental library collection; second, by focusing on symbiotic bacterial groups, we could largely reduce the problem of differentiating between "symbionts," "food," and "loosely host-associated bacteria," admittedly sacrificing the possibility to detect new symbiotic lineages in *Euplotes*.

The unfailing detection of predicted essential symbionts, the recovering of several previously known species of *Euplotes* symbionts, and the absence of signal from most of them in environmental controls all corroborate the conclusion that ours and previous attempts to describe bacterial communities within unicellular eukaryotes provide accurate depictions of the communities associated with individual cells.

Diversity of Bacterial Symbiont Communities in Natural Populations of *Euplotes*

The most striking observation from our data is that members of the same natural host population can harbor different communities of bacterial symbionts. Differences in bacterial symbiont profiles within the same population are well documented in insects, for which field campaigns have provided reliable data across both spatial and temporal scales [40, 41], but are virtually unknown in protists. Our survey shows for the first time that laboratory strains, either descending from a single isolated cell or maintained long enough as

to amount to the same thing, are not representative of the natural population from which they were derived, especially when it comes to "accessory" symbionts. This calls into question what conclusions based only on laboratory strains can be drawn on symbiont frequency, prevalence, and geographic distribution, topics that in protist systems are plagued by a scarcity of data to begin with.

Our data also show that the diversity of accessory symbionts in *Euplotes*, especially *Rickettsiales* and *Holosporales*, has not been exhaustively characterized yet. At the same time, it is noteworthy that the three most common accessory symbionts detected here had been previously described and in the same host species. Even though we cannot claim yet that our knowledge on the diversity of clade B *Euplotes* symbionts is comprehensive, we can probably speculate that such an understanding is within reasonable reach and that several of the most ecologically relevant symbionts have been characterized.

Concerning *Rickettsiales*, the common presence in *Euplotes* of several bacterial symbionts belonging to two of the three families of the order ("*Ca*. Midichloriaceae" and *Rickettsiaceae*) is consistent with previous results [5, 35]. Members of the third family, *Anaplasmataceae*, were not detected here (Online resource 2) and are indeed conspicuously absent from symbiont screenings in all protists [1]. All three *Rickettsiales* families were originally described as parasites of terrestrial arthropods [42, 43], but *Anaplasmataceae* seems to be the only one unable to colonize either unicellular eukaryotes or aquatic environments. Considering the phylogenetic tree of the order (e.g., [10]), with *Anaplasmataceae* sister lineage of "*Ca.* Midichloriaceae" and *Rickettsiaceae* more distantly related, terrestrial *Rickettsiales* can be assumed to be derived, rather than ancestral.

Patterns of Symbiont Distribution in *Euplotes*

We know from previous studies on the evolutionary history of *Polynucleobacter* and "*Ca.* Protistobacter" that at least the former can replace the latter (as well as different strains of its own species) "often" over evolutionary times [8, 9]. *Polynucleobacter* and "*Ca.* Protistobacter" have never been found inside the same cytoplasm despite theoretical expectations that such co-occurrence should be observable in a transitional step [9]. Libraries from a few *Euplotes* cells collected here did include reads from both, which would be consistent with the hypothetical presence of two essential symbionts in very different amounts in some host cells. Nevertheless, relative abundances for the less dominant symbiont were so low that they might also be explained by tiny cross-contaminations coupled with deep sequencing. On the other hand, the presence of both essential symbionts inside different individuals of the same *E. woodruffi* population (site SR2A) is strongly supported. This could only be observed by looking at individual host cells or by analyzing large numbers of clonal cultures originated from the same population, neither of which is commonly done. How common the replacement of *Polynucleobacter* by "*Ca.* Protistobacter" is in absolute terms is unknown, but their coexistence in the same host population is consistent with an ongoing takeover. We cannot, however, rule out the possibility that *Polynucleobacter*- and "*Ca.* Protistobacter"-carrying *Euplotes* belong to different strains with undistinguishable 18S rRNA gene sequences. Should more studies like this find similar situations in multiple target populations, especially if using time-series data, it would suggest that replacements of essential symbionts in *Euplotes* happen over a much shorter timespan than expected (i.e., years and decades, not millennia or millions of years).

Some of the accessory symbionts also seem to be specific to, or at least show a very strong affinity for, one host species. "*Ca.* Bandiella numerosa" was detected in every *E. woodruffi* cell here, and congeneric bacteria were found in most laboratory strains of the same host species [5], questioning if this symbiont is indeed "accessory" or if it might play a more important role. Notably, however, while "*Ca.* Bandiella numerosa" may be exclusively present in *E. woodruffi*, extremely close bacterial relatives were found in hosts as different as marine corals [44] and placozoans [45]. Other symbionts did not show a preference between *Euplotes* species and were instead tied to a specific location. Midichloriaceae and holosporaceae as a whole are found in a variety of unrelated eukaryotes, and their phylogeny does not match that of their hosts, so it is reasonable to assume that those with a broader host range, like LLMS and UHS2, are the rule rather than the exception.

Another clear pattern, here made more striking by the fact that it was shown both among and within populations, is the apparent competitive exclusion between "*Ca.* Anadelfobacter veles" and "*Ca.* Cyrtobacter comes". Both were originally described from *E. platystoma*, but from different strains [35], and in single-cell sampling, these midichloriaceae never seem to share the same cytoplasm, despite being common symbionts. This suggests some strong selection against their co-occurrence. Interaction dynamics between accessory bacterial symbionts of eukaryotes is a largely unexplored field. Most of the studies on this topic have been performed on accessory symbionts of aphids, for some of which competitive interactions have been shown, leading to drops in abundance, lowering of essential symbiont density, and weakening of functions useful to the host [46, 47]. Negative correlations between two species of accessory bacterial symbionts have been reported both in aphids and in the chestnut weevil, but the reasons behind this pattern remain to be clarified [40, 48]. All the previous findings in insects agree that competition is driven by many different factors, including benefits and costs tradeoffs, way of

transmission, environmental pressure, number of symbionts, and genotypes of both bacteria and hosts [41, 46, 47]. We do not know what the competition between "*Ca.* Anadelfobacter veles" and "*Ca.* Cyrtobacter comes" stems from, but their occasional absence from *E. platystoma* cells and strains makes it unlikely to be host-driven and suggests in turn that it might be actively triggered by one or both of the bacteria.

Opportunistic Symbionts

While the symbiotic status of the aforementioned bacteria is quite certain, either because of their known effect on the host, their affiliation to exclusively intracellular lineages, and/or their absence from our environmental controls, two taxa previously characterized as symbionts are probably best described as opportunistic inhabitants of *Euplotes* cytoplasm. This is not surprising in the case of *Francisella* because the entire genus is generally considered to be opportunistic and facultatively intracellular [49]. A handful of *Francisella* species have been recovered from marine *Euplotes* [4, 24], usually without any reported effect on the host; *Francisella philomiragia*, found here in brackish *Euplotes*, could be added to that list. At the same time, its presence in the environmental background community suggests that its specificity as a symbiont is at best tenuous.

The situation in *Devosia* is more complex. The genus is large and diverse and includes free-living as well as host-associated species [50]. However, the few well-characterized *Devosia* lineages in *Euplotes* did have a strong effect on their host, taking the role usually performed by *Polynucleobacter* in one *E. platystoma* strain [15], and being equally essential in the unrelated, marine *Euplotes magnicirratus* [13]. The phylogenetic relationships between *Devosia* species found in *Euplotes* suggested the existence of a *Euplotes*-specific clade [15]. Here, however, *Devosia* displayed a distribution pattern not dissimilar from the opportunistic *Francisella*, both within the putative *Euplotes*-specific clade and in the rest of the genus. The topic needs to be further explored, but it is possible that *Devosia* is also, generally speaking, opportunistic, and that previously reported cases (at least the one in *E. platystoma*) represent rare instances of an opportunist accidentally replacing another symbiont, much as free-living *Polynucleobacter* strains often do to other betaproteobacteria in clade B *Euplotes* species.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-021-01938-x.

Acknowledgements The authors wish to thank Simone Gabrielli and Davide Stano for support in sampling and for help with graphic artworks. The authors are grateful to the Migliarino San Rossore Massaciuccoli Regional Park for giving permission for sampling and to Francesca Logli in particular for her assistance.

Author contribution Vittorio Boscaro and Claudia Vannini conceived and designed the project, analyzed the data, and wrote the first draft of the paper. Field and lab work were performed by Vittoria Manassero and Claudia Vannini. Patrick Keeling and Claudia Vannini supervised the work. All authors contributed to the final draft.

Funding This work was supported by the University of Pisa (565–60% 2018, 565–60% 2019, 565–60% 2020, PRA_2018_63) and by the Gordon and Betty Moore Foundation (https://doi.org/10.37807/GBMF9201).

Data availability Sanger nucleotide sequences and metabarcoding raw reads have been deposited in the European Nucleotide Archive (ENA) database under accession numbers OU070010-OU070076 and project number PRJEB44318, respectively.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

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