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# Symbiont replacement between bacteria of different classes reveals additional layers of complexity in the evolution of symbiosis in the ciliate *Euplotes*


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**Symbiosis is a diverse and complex phenomenon requiring diverse model systems. The obligate relationship between a monophyletic group of *Euplotes* species ("clade B") and the betaproteobacteria *Polynucleobacter* and "*Candidatus* Protistobacter" is among the best-studied in ciliates, and provides a framework to investigate symbiont replacements. Several other *Euplotes*-bacteria relationships exist but are less understood, such as the co-dependent symbiosis between *Euplotes magnicirratus* (which belongs to "clade A") and the alphaproteobacterium "*Candidatus* Devosia euplotis". Here we describe a new *Devosia* inhabiting the cytoplasm of a strain of *Euplotes harpa*, a clade B species that usually depends on *Polynucleobacter* for survival. The novel bacterial species, "*Candidatus* Devosia symbiotica", is closely related to the symbiont of *E. magnicirratus*, casting a different light on the history of bacteria colonizing ciliates of this genus. The two *Devosia* species may have become symbionts independently or as the result of a symbiont exchange between hosts, in either case replacing a previous essential bacterium in *E. harpa*. Alternatively, both may be remnants of an ancient symbiotic relationship between *Euplotes* and *Devosia*, in which case *Polynucleobacter* and "*Ca.* Protistobacter" are recent invaders. Either way, symbiont replacement between bacteria belonging to different classes must be evoked to explain this fascinating system.**



**Key words:** Symbiosis; fluorescence in situ hybridization; SSU rRNA gene; phylogeny; transmission electron microscopy.

## Introduction

The ciliate genus *Euplotes* (Spirotrichea, Euplotia) is species-rich and diverse (Borror and Hill 1995; Curds 1975; Petroni et al. 2002; Syberg-Olsen et al. 2016), and has been used as a model system on several topics (Beale 1990; Boscaro et al. 2013a, 2017; Di Giuseppe et al. 2011; Vallesi et al. 1995). Intracellular bacteria have been reported in *Euplotes* for more than half a century (Görtz 2006; Heckmann 1975; Heckmann et al. 1967;

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Rosati and Verni 1975), but molecular investigations on the identity of the symbionts are more recent (e.g. Boscaro et al. 2012, 2013b; Senra et al. 2016; Springer et al. 1996; Vannini et al. 2010, 2014). The most studied of these symbioses is the one between fresh- and brackish water *Euplotes* species of clade B (Syberg-Olsen et al. 2016) and the betaproteobacterium *Polynucleobacter* (*Burkholderiales*, *Burkholderiaceae*). Discovered by Heckmann and colleagues ("omikron particle"; Heckmann 1975; Heckmann and Schmidt 1987), the relationship between *Polynucleobacter* and its host is obligate for both partners (Heckmann et al. 1983; Vannini et al. 2005b) and was originally interpreted as a mutualism. The hypothesis that the bacterium compensates a metabolic deficiency arisen in the common ancestor of all host species (Heckmann et al. 1986; Vannini et al. 2007) has not been disproven, but genomic analyses cast doubts on the benefit of the relationship for *Polynucleobacter* (Boscaro et al. 2017). *Euplotes* and *Polynucleobacter* did not cospeciate. Symbiotic *Polynucleobacter* lineages evolved independently several times from a free-living pool, replacing previous bacterial strains in the cytoplasm of *Euplotes* (Boscaro et al. 2017; Vannini et al. 2012). A different essential betaproteobacterium, "*Candidatus* Protistobacter", has also been found in a smaller percentage of populations of the same host species (Vannini et al. 2012, 2013). In summary, *Euplotes* belonging to clade B are always associated with an essential prokaryotic partner for survival, but the evolutionary history of this symbiosis is complex and not fully elucidated.

Bacterial symbionts of other *Euplotes* species are less known. Several reports exist (e.g. Heckmann et al. 1967; Rosati et al. 1976; Rosati and Verni 1975), but few are backed up by molecular data. An *Euplotes raikovi* (clade C) strain hosting *Francisella noatunensis* has been described (Schrallhammer et al. 2011), but the nature of *Francisella* suggests that it might be considered an opportunistic infection. The relationship between *Euplotes magnicirratus*, a marine species of clade A, and its symbiont "*Candidatus* Devosia euplotis", seems to be more stable (Vannini et al. 2004a,b). *Devosia* is a genus of alphaproteobacteria (*Rhizobiales*, *Hyphomicrobiaceae*) originally established by removing *Pseudomonas riboflavina* from the polyphyletic genus *Pseudomonas* and renaming it *Devosia riboflavina* (Nakagawa et al. 1996). Numerous species of *Devosia* have been described since, mostly from soil habitats – e.g. heavily polluted sites (Ryu et al. 2008; Verma et al. 2009; Zhang et al. 2015), rhizospheres (Du et al. 2016; Mohd Nor et al. 2017), root nodules (Bautista et al. 2010; Rivas et al. 2003) – but also from diverse environments such as deep-sea sediments (Jia et al. 2014; Romanenko et al. 2013), alpine ice (Zhang et al. 2012), and the skin of medical leeches (Galatis et al. 2013). All *Devosia* species are easily cultivable under standard lab conditions – except for "*Ca.* Devosia euplotis". *E. magnicirratus* and "*Ca.* Devosia euplotis" are obligate partners, but no evidence has yet suggested a complicated evolutionary history such as that of the betaproteobacteria harbored by *Euplotes* species of clade B.

In this paper, we characterized a new species of *Devosia*, "*Candidatus* Devosia symbiotica", closely related to "*Ca.* Devosia euplotis". "*Ca.* Devosia symbiotica" is the sole symbiont in the investigated strain of *Euplotes harpa*, a species belonging to clade B that usually harbors *Polynucleobacter*. This discovery connects two symbiotic systems previously considered to have evolved independently and adds a piece to a fascinating puzzle that is gaining traction as a model for the study of symbiosis.

## Results

### Host Identification

The SSU rRNA gene sequence of *Euplotes* strain Na2 (1,885 bp long, acc. num.: LT898478) is 99.9-100% identical to six other available sequences of *E. harpa*. Five of the corresponding strains (FSP1.4, acc. num.: AJ811015; HS11/7, acc. num.: FN552694; FC1, acc. num.: AJ811016; Bod2, acc. num.: AJ305252; BOD18, acc. num.: FN552693) have been previously screened for symbionts, and they all contain *Polynucleobacter* (Vannini et al. 2005b, 2010, 2012).

### Characterization of the Bacterial Symbiont of *Euplotes harpa* Strain Na2

Preliminary fluorescence *in situ* hybridization (FISH) experiments using the "universal" eubacterial probe EUB338 showed the presence of a bacterial symbiont in the cytoplasm of strain Na2. No positive signal was obtained using the probe Poly_862, that targets both *Polynucleobacter* and "*Ca.* Protistobacter", the essential betaproteobacteria usually found in *Euplotes* species of clade B. The overlapping signals from the alphaproteobacterial probe ALF1b and the eubacterial probe EUB338 was consistent with the hypothesis that all symbiotic bacteria belong to *Alphaproteobacteria*. Further experiments with group-specific probes

targeting taxa previously detected in *Euplotes* and other ciliates (Dev_819, specific for the genus *Devosia*, positive signal; Rick_527, targeting the family *Rickettsiaceae*, negative signal) suggested the affiliation of the symbiotic bacterium to the genus *Devosia*.

Through PCR amplification with an alphaproteobacterial-specific forward primer and direct sequencing, a 1,398 bp long sequence of the symbiont's SSU rRNA gene was obtained. The most similar blastn (Altschul et al. 1990) hits assigned to named species were *Devosia psychrophila* (acc. num.: GU441678, 97.8% similarity) and "*Candidatus* Devosia euplotis" (acc. num.: AJ548825, 97.6% similarity). In order to complete the "Full Cycle rRNA Approach" (Amann et al. 1991) and confirm that the new sequence belonged to the cytoplasmic bacterium, the sequence-specific probe DevNa_1026 was designed and tested on fixed host cells with positive results (Fig. 1). A combined FISH using probes DevNa_1026 and EUB338 excluded the presence of other symbiotic bacteria in strain Na2 (Supplementary Material Fig. S1).

Bacterial cells appear as straight rods both in FISH imaging and transmission electron microscopy sections (Fig. 2). The bacteria are 1.5–2 μm long and surrounded by at least two membranes. The cytoplasm of the symbiotic bacteria is quite heterogeneous, with an irregularly-shaped electron-dense area surrounding a lighter core that might correspond to the nucleoid. In some dividing cells the lighter core is almost absent and the cytoplasm is uniformly electron-dense, while in others it is still clearly visible. It is possible that these morphotypes correspond to different stages of cytokinesis. No flagella were ever observed.

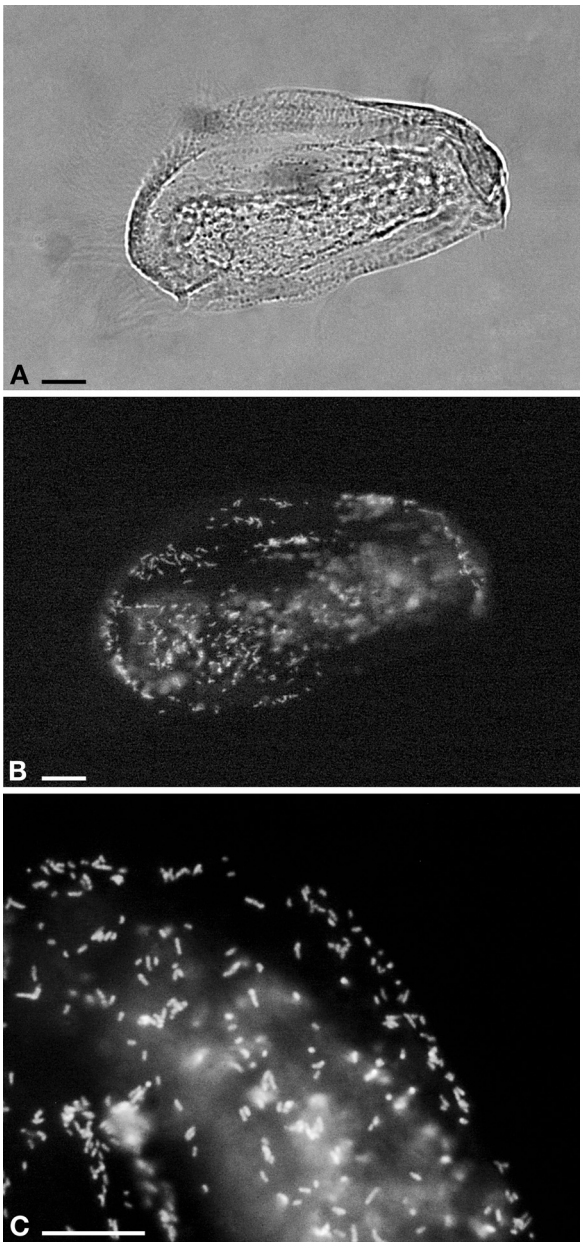
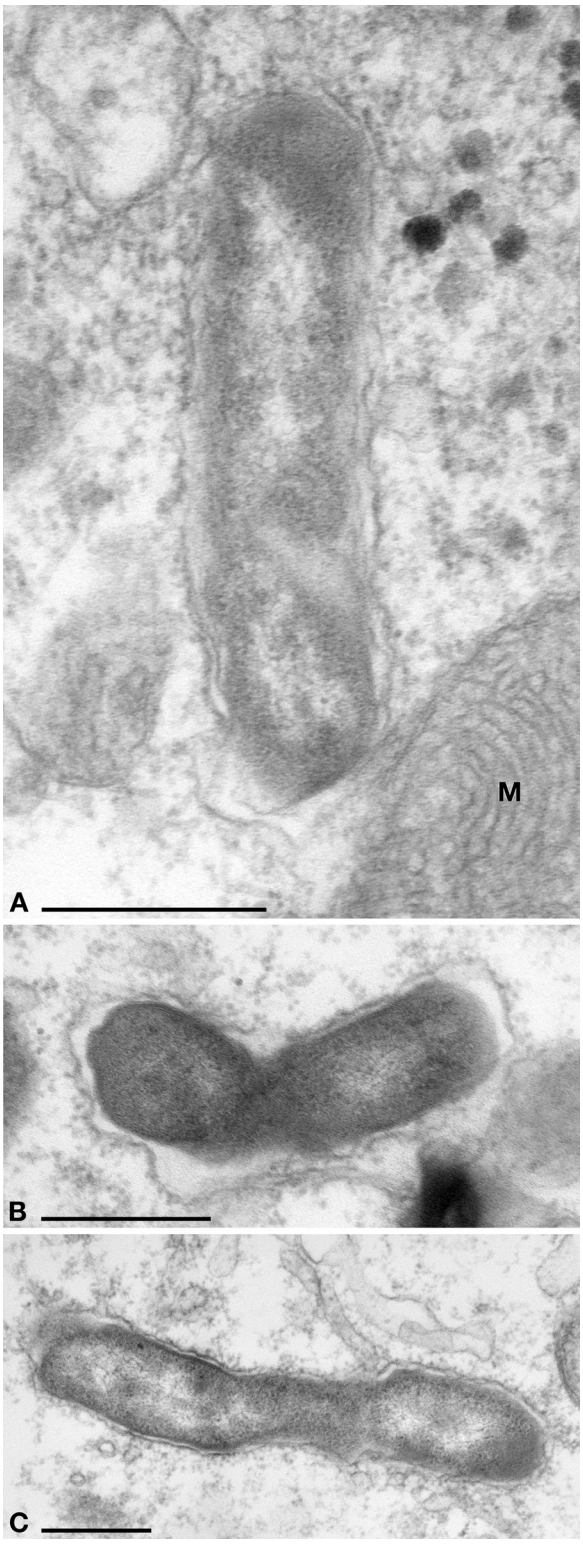



**Figure 1.** Fluorescence *in situ* hybridization using sequence-specific probe DevNa_1026. **A**,**B**, whole *Euplotes harpa* cell (**A**, bright field; **B**, epifluorescence signal). **C**, epifluorescence signal from the cytoplasm of a host cell at a higher magnification. Bars represent 10 μm.

## Phylogenetic Analysis

Maximum Likelihood and Bayesian Inference methods produced identical topologies for the phylogenetic tree of *Devosia* (Fig. 3). However, statistical supports for most nodes was low, as in other recent analyses (e.g. Du et al. 2016; Mohd Nor et al. 2017), suggesting that the SSU rRNA gene sequence does not carry enough information to reliably resolve all inter-specific relationships. The monophyly of the genus itself is uncertain, and ambiguities in the taxonomic status of some species and closely related genera exist (Park et al. 2016). Nevertheless, the newly characterized symbiont firmly clusters within the clade of *Devosia* species that includes the type species, *Devosia riboflavina*. According to both phylogenetic approaches, the new symbiont is the sister species of "*Candidatus* Devosia euplotis" detected from several strains of the marine *Euplotes magnicirratus* (Vannini et al. 2004a).




### Cultivation Attempts

Using common culture methods for non-symbiotic *Devosia* species (for examples, see Du et al. 2016; Mohd Nor et al. 2017; Park et al. 2016), attempts were made to establish cultures of the symbiotic bacteria, after they were isolated from ciliate cells. However, these attempts were unsuccessful.

### Symbiont Removal Attempts

Penicillin treatments were unsuccessful in producing symbiont-free host cells. Division rates were extremely low both in treated and non-treated Na2 cells (no more than one division was observed in any replicate), and the concentration of symbionts in the hosts' cytoplasm had not changed by the end of the treatment. Experiments with other antibiotics resulted in considerable differences in mortality between treated and not-treated ciliate populations, but similar results were also observed on the non-symbiotic *Euplotes* control, suggesting a toxicity effect on the ciliate themselves, and not on the bacterial endosymbionts.

## Discussion

### Establishment of *"Candidatus* Devosia symbiotica"

Strain Na2 of *Euplotes harpa* contains a single bacterial symbiont, which belongs to the genus *Devosia*. This bacterium could not be grown outside its host with methods used for non-symbiotic *Devosia* species, suggesting that its symbiosis with *Euplotes* is obligate. The molecular difference with related taxa (SSU rRNA similarity with the closest named species lower than 97.8%) warrants the establishment of a new species (Stackebrandt and Ebers 2006). The current inability to establish a pure culture of the novel bacterium requires the use of the "*Candidatus*" label (Murray and Stackebrandt 1995). We propose to establish the taxon "*Can-*

**Figure 2.** Transmission electron microscopy ultrathin sections of *Euplotes harpa* strain Na2, showing the ultrastructure of "*Candidatus* Devosia symbiotica". **A**, typical cell, in proximity of a host mitochondrion (M), displaying a non-flagellated rod shape; the cytoplasm is considerably heterogeneous. **B**, dividing cell with a largely homogeneous cytoplasm. **C**, dividing cell with a more differentiated cytoplasm, possibly indicative of a different stage of the division process. Bars represent 0.5 μm.

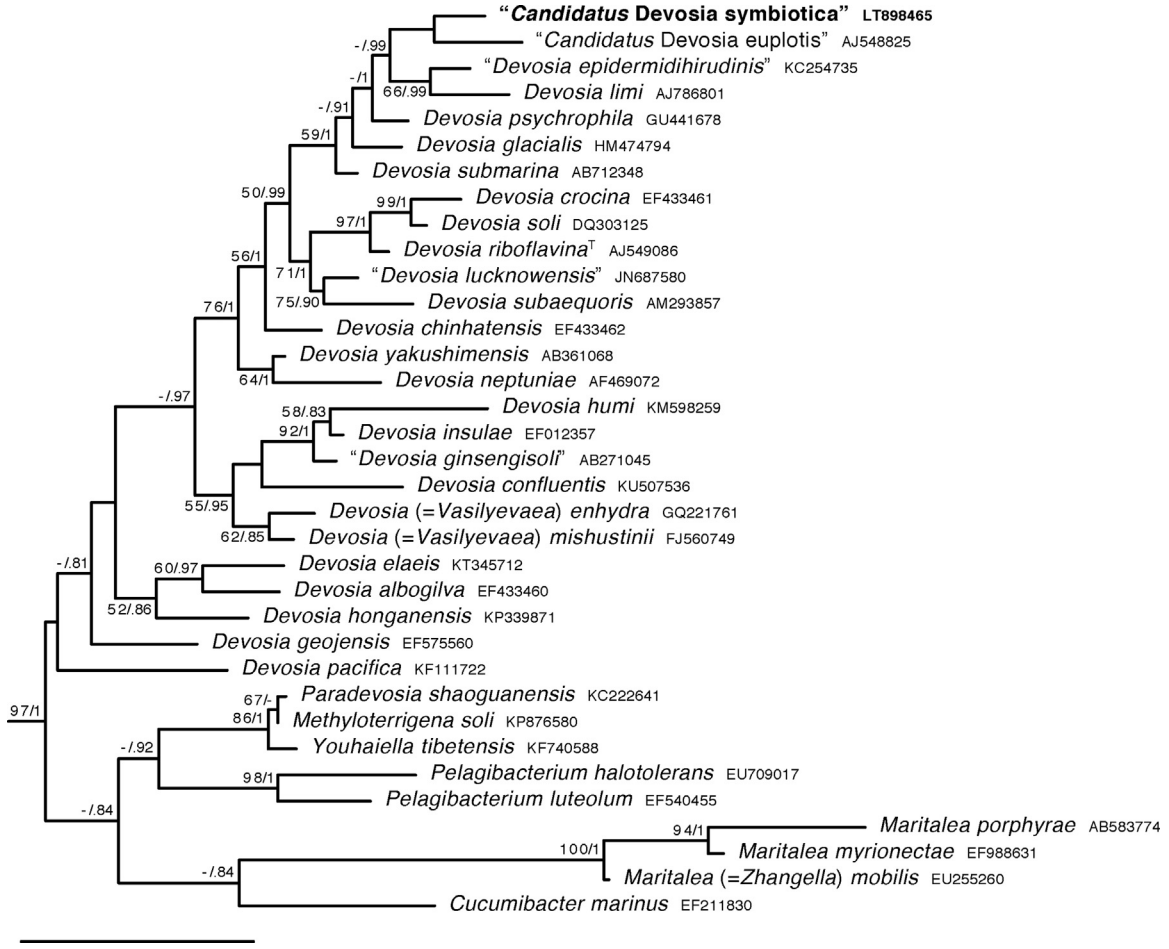



**Figure 3.** SSU rRNA gene Maximum Likelihood tree of *Devosia* and closely related genera. An identical topology was inferred by the Bayesian approach. The sequence obtained in this work is in bold. Numbers associated with nodes represent bootstrap values/posterior probability (values below 50/0.8 are not shown). The root was placed between the 35 ingroup sequences and more distantly related *Alphaproteobacteria* (not shown). *Devosia riboflavina*, the type species of the genus, is marked with a "T" superscript. The bar represents an estimated divergence of 5%.

*didatus* Devosia symbiotica" for this prokaryotic symbiont of *Euplotes harpa*.

## Description of "*Candidatus* Devosia symbiotica"

*Devosia symbiotica* (sym.bio'ti.ca L. fem. adj. *symbioticus*, symbiotic)

"*Ca.* Devosia symbiotica" is a rod-shaped, non-flagellated bacterium, 1.5–2 μm in length. It has a heterogeneously dense cytoplasm, with an irregular electron-dense area surrounding a central electron-lighter zone, sometimes non-detectable in dividing cells. It is an endosymbiont living in the cytoplasm of the ciliate *Euplotes harpa* (Ciliophora, Spirotrichea), identified from the *E. harpa* strain Na2, which was isolated from a brackish coastline puddle in Naples, Italy. Basis of assignment: SSU rRNA gene sequence (acc. num.: LT898465) and positive match with the FISH oligonucleotide probe DevNa_1026 (5′-GTG CCC AGG TCA CCG AAG TG-3′).

## Symbiotic Relationship Between "*Candidatus* Devosia symbiotica" and its Host

We were unable to test the degree of dependence of the *Euplotes* host on the bacterium. Na2 is a slow-growing strain with doubling time of several weeks. Attempts to remove the symbiont with

penicillin consistently failed, possibly because the extremely low rate of division of the holobiont provided no target for the beta-lactam antibiotic action. Other antibiotics seemed to have a toxic effect on a naturally non-symbiotic *Euplotes* strain, making them unsuitable replacements for penicillin.

It is however known that *Euplotes harpa* as a species depends on bacterial symbionts for survival (Vannini et al. 2005b). All previously investigated strains harbored the betaproteobacterium *Polynucleobacter*, the removal of which invariably resulted in the host's loss of the ability to divide and, eventually, its death. Moreover, comparative analyses have shown that the obligate symbiosis with bacteria is older than the divergence of *Euplotes harpa* from related species, dating back at least to the common ancestor of clade B (Syberg-Olsen et al. 2016; Vannini et al. 2012). It is thus likely that the role of essential symbiont played by *Polynucleobacter* in other *E. harpa* strains is taken on by "*Ca.* Devosia symbiotica" in strain Na2.

## The History of Symbiosis and Symbiont Replacement in *Euplotes*

While mutually obligate symbioses (which may or may not be mutualistic) are generally thought of as permanent and stable, there are many proven instances of symbiont replacements (Husnik and McCutcheon 2016; Koga and Moran 2014; Sudakaran et al. 2017; Toju et al. 2013; Vogel and Moran 2013). In *Euplotes* species of clade B, evidence for replacements is provided by the presence of two distinct betaproteobacteria as essential symbionts of different strains, *Polynucleobacter* and "*Ca.* Protistobacter" (Vannini et al. 2012). While the origin of the symbiosis itself had to be at least as old as the common ancestor of all the host species, the original symbiont might have been either *Polynucleobacter* (replaced several times by "*Ca.* Protistobacter") or "*Ca.* Protistobacter" (replaced in most strains by *Polynucleobacter*). The prevalence of *Polynucleobacter* made the first hypothesis appear more parsimonious, but the monophyly of "*Ca.* Protistobacter" and the fact that many *Polynucleobacter* strains are free-living argued for the second scenario (Vannini et al. 2012).

A recent phylogenomic analysis found that nine symbiotic strains of *Polynucleobacter* were polyphyletic, with regard to free-living strains, suggesting that symbiotic associations with *Euplotes* have evolved on at least eight separate occasions within this bacterial genus (Boscaro et al. 2017). *Polynucleobacter* colonized the cytoplasm of ciliates repeatedly over evolutionary time, replacing in each instance either a previous *Polynucleobacter* or a different essential bacterium. In this scenario, "*Ca.* Protistobacter" is the obvious candidate for the role of original symbiont, displaced by *Polynucleobacter* in many strains of each species or even entire species (there are currently no "*Ca.* Protistobacter" known, for example, from *E. harpa*, the most basal of the symbiont-bearing species in clade B). If this reconstruction is correct, "*Ca.* Devosia symbiotica" must have itself replaced a *Polynucleobacter* previously harbored by the ancestor of *E. harpa* strain Na2 (Fig. 4A).

While this interpretation fits the data, it implies that the mutually obligate symbiosis between the marine species *E. magnicirratus* (clade A) and "*Ca.* Devosia euplotis", the sister species of "*Ca.* Devosia symbiotica", is a coincidence. The alternative hypothesis, positing a single origin for the dependence of *Euplotes* species on essential bacteria, pushes back the establishment of the symbiotic system to the ancestor of both clades A and B (Fig. 4B). The original symbiont would have been a member of the genus *Devosia*, possibly co-speciating with its host. Freshwater betaproteobacteria would then have replaced the alphaproteobacterial symbiont in species of clade B, once in the case of "*Ca.* Protistobacter" and many times in the case of *Polynucleobacter*.

With the currently available information, the "two independent symbioses" and the "ancestral symbiosis" hypotheses seem equally plausible. It might then be useful to point out how either can be tested by future studies. Most species in clade A have not been screened for symbionts. Should members of *Devosia* be found in other representatives of this clade in addition to *E. magnicirratus*, then the ancestral symbiosis hypothesis would receive more support, especially if the phylogeny of those symbionts mirrored that of their hosts. Conversely, if many species in clade A are shown to lack symbionts entirely, then the ancestral symbiosis hypothesis would become less solid (since several complete losses of all "essential" symbionts would be required). Detecting "*Ca.* Protistobacter" in strains of *E. harpa*, the most basal species in clade B, might also conflict with the "ancestral symbiosis" hypothesis. In that case, parsimony would suggest that the symbiosis between *Euplotes* and "*Ca.* Protistobacter" dates back at least to the ancestor of clade B. In turn, this would strongly suggest that "*Ca.* Devosia symbiotica" simply replaced a previous betaproteobacterium (either *Polynucleobacter* or "*Ca.* Protistobacter") in Na2, and that two closely related *Devosia* species did indeed become

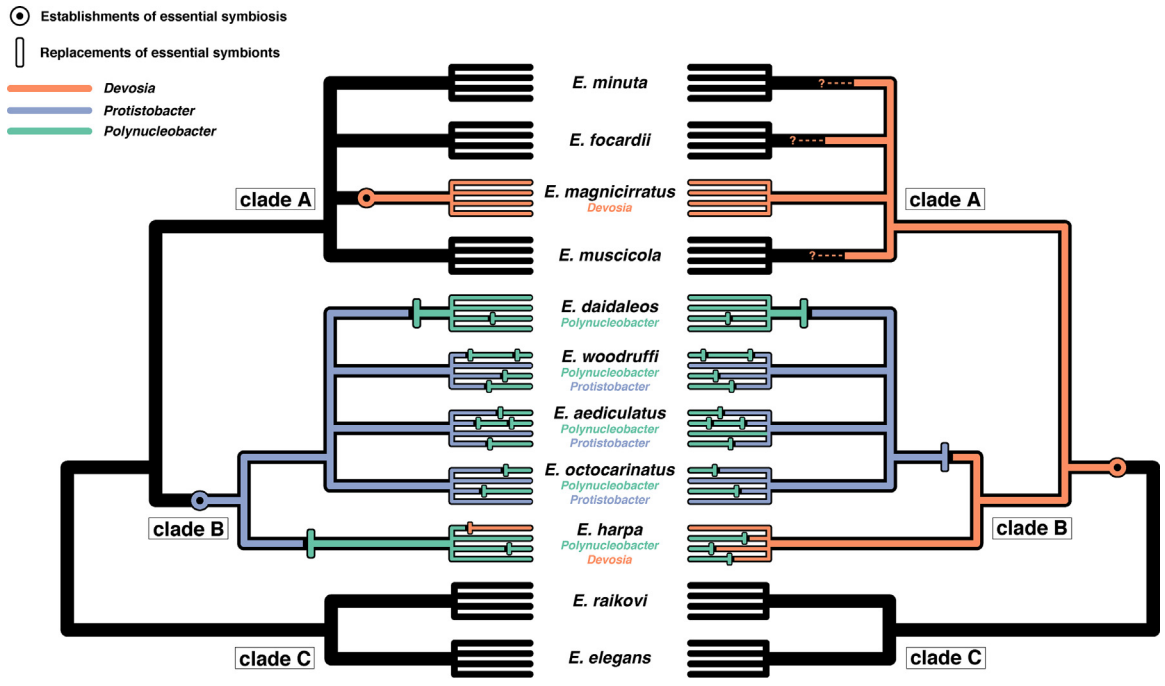



**Figure 4.** Alternative hypotheses describing the history of symbiont replacements in *Euplotes*. A simplified *Euplotes* phylogenetic tree based on previous phylogenetic analyses (e.g. Chen et al. 2013; Fotedar et al. 2016; Syberg-Olsen et al. 2016) is shown with thick black lines, using representative species of clades A, B, and C. Thinner lines represent strains of each species. Below the host species names, known essential symbionts are listed. The "independent-symbioses hypothesis" (**A**) assumes that two separate symbiotic events occurred, one in the ancestor of *Euplotes magnicirratus* and one in the ancestor of clade B species. These two ancestral *Euplotes* would have independently lost the ability to survive without symbiotic bacteria and established obligate relationships with a member of the genus *Devosia* and "*Candidatus* Protistobacter", respectively. The "ancestral symbiosis" hypothesis (**B**) assumes only one such event, in the ancestor of clades A and B, with the original symbiont belonging to the genus *Devosia*. "*Candidatus* Devosia symbiotica" replaced a betaproteobacterium in the ancestor of strain Na2 according to the first hypothesis, and is a remnant of the original symbiosis according to the second hypothesis. In both scenarios, *Polynucleobacter* strains have repeatedly replaced previous symbionts in most clade B *Euplotes* strains.

essential symbionts of *Euplotes* species independently (or through a symbiont transfer between two ciliates, which is however less likely due to the different environments of their hosts).

## Methods

**Host collection and identification:** The *Euplotes harpa* population was collected on February 15 2004 from the sediment-water interface in a brackish-water puddle located in a pebble beach close to the Stazione Zoologica Anton Dohrn (Naples, Italy). The monoclonal strain Na2 was established from a single isolated cell and maintained in 5‰ salinity water at 20 °C on a 12: 12 h light/dark cycle. The green alga *Dunaliella tertiolecta* was used as food source. In order to confirm species identification, total DNA was extracted from a pool of *Euplotes* cells using a protocol adapted from Wisotzkey et al. (1990) and sequencing the SSU rRNA gene of the ciliate as described elsewhere (Rosati et al. 2004).

**Molecular characterization of the bacterial symbiont:** The SSU rRNA sequence of the bacterial symbiont was obtained by PCR using extracted DNA and direct sequencing of the amplicon. The alphaproteobacterial forward primer 16S alfa F19a (Vannini et al. 2004a) and the eubacterial reverse primer 1492R (5′-GGN WAC CTT GTT ACG ACT T-3′, modified from Lane 1991) were used in a "touchdown" approach (annealing temperatures: 63-57-50 °C). Sequencing was performed using internal primers as previously described (Vannini et al. 2004a).

Fluorescence *in situ* hybridizations (FISH) were performed according to the protocol of Manz et al. 1992. Hybridized specimens were observed with a Zeiss Axioplan epifluorescence microscope, and images captured with a Nikon Digital Sight DS-U1 camera by the ACT-2U software. Probes tested included EUB338, targeting most eubacteria (Amann et al. 1990); ALF1b, targeting *Alphaproteobacteria* (Manz et al.

1992); Poly_862, targeting *Polynucleobacter* and "*Candidatus* Protistobacter" (Vannini et al. 2005b); Dev_819, targeting the genus *Devosia* (Vannini et al. 2004a); Rick_527, targeting the family Rickettsiaceae (Vannini et al. 2005a). The probe DevNa_1026 (5′-GTG CCC AGG TCA CCG AAG TG-3′) was designed to be sequence-specific for the new symbiotic bacterium. Probe specificity was tested *in silico* with the Probe Match tool of the Ribosomal Database Project (Cole et al. 2009), obtaining no exact match against bacterial sequences in the database.

**Phylogenetic analysis:** 35 SSU rRNA gene sequences from species of *Devosia* and closely related genera, plus 9 outgroup sequences from other *Alphaproteobacteria*, were used in the phylogenetic analysis. Sequences were aligned with mafft v7.310 ("linsi" algorithm; Katoh and Standley 2013) and trimmed at both ends to remove columns with missing information. The resulting character matrix (1,321 columns) was processed with IQ-TREE v1.5.3 (GTR + I + G4 model, 1,000 standard non-parametric bootstraps; Nguyen et al. 2015) to obtain the Maximum Likelihood topology, and MrBayes v3.2.5 (GTR + I + G4 model, 3 independent runs with 1 cold and 3 heated chains each, 1,000,000 generations sampled every 100 generations, 25% burnin; Ronquist et al. 2012) to obtain the Bayesian Inference topology.

**Transmission electron microscopy (TEM):** Ciliate cells were fixed in a 1:1 mixture of $OsO_4$ (aqueous solution 4%) and glutaraldehyde (5% in cacodylate buffer 0.05 M), dehydrated and embedded in Epoxy resin (Fluka, BioChemika). Ultrathin sections were prepared using a LKB 8800 Ultrotome III microtome, stained with uranyl acetate and lead citrate, and visualized using a JEOL 100SX electron microscope.

**Culturing attempts on the bacterial symbiont:** *Euplotes* cells were concentrated by centrifugation from 250 mL of filtered Na2 culture, briefly treated with streptomycin (400 mg/L) to remove external contaminants and mechanically lysed. The resulting 150 μL of cell lysate were diluted in 3 mL of sterile isosmotic water, and 50 μL aliquots were used as the inoculum in each treatment. Media LB (both liquid and with agar), R2 (both liquid and with agar), Marine Agar and Marine Broth (DIFCO) were tested to obtain cultures of the symbiotic bacterium. Tubes and plates were kept at 20 °C and 30 °C (3 replicates per treatment) and inspected for growth after 7 and 30 days.

**Symbiont removal attempts:** In the attempt to obtain aposymbiotic Na2 cells, penicillin treatments were performed following protocols that had been successful for *Polynucleobacter* (Heckmann et al. 1983; Vannini et al. 2007), "*Ca.* Protistobacter" (Vannini et al. 2012) and "*Ca.* Devosia euplotis" (Vannini et al. 2004b). Briefly, single *Euplotes* cells were isolated in separate wells and subjected to different penicillin concentrations. Untreated *Euplotes* cells from the same strain, as well as treated and untreated *Euplotes* cells from a strain without bacterial symbionts (*Euplotes curdsi* strain Min1) were used as controls. A different type of experiment was also performed, starting with 30 ciliate cells per well and assessing mortality over a 10-day period. The same experiments were also repeated with chloramphenicol, ampicillin and streptomycin instead of penicillin. Surviving treated cells at the end of each experiment were screened for the presence of symbionts with FISH.

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## Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.protis.2017.12.003.

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