A new obligate bacterial symbiont colonizing the ciliate *Euplotes* in brackish and freshwater: '*Candidatus* Protistobacter heckmanni'

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ABSTRACT: A monophyletic group of freshwater and brackish ciliate species belonging to the genus *Euplotes* is known to harbor bacterial endosymbionts of the class *Betaproteobacteria* permanently in the natural environment. One or other of 2 different species of bacteria have been shown to be present: the obligate symbiont *Polynucleobacter necessarius* or a recently described betaproteobacterium. While the association between *P. necessarius* and its *Euplotes* hosts has been well studied in the past, knowledge about the relationship between the newly discovered symbiont and its *Euplotes* hosts is still very poor. Here we present the SSUrRNA gene characterization of both host and symbiont for an additional brackish-water population of *Euplotes* hosting the newly discovered symbiont. We also provide the symbiont genome size determination and ultrastructural description. Attempts to cultivate the new symbiont outside its host, using many different methods, failed. This result indicates that, as in the case of the symbiotic *P. necessarius*, we are probably dealing with an obligate symbiont, unable to grow outside the cytoplasm of its natural host. On the basis of the results obtained we propose the new symbiont as a candidate new species with the name of '*Candidatus* Protistobacter heckmanni', according to the current rules of prokaryotic nomenclature.

KEY WORDS: *Polynucleobacter* · *Euplotes* · 16S rRNA · 18S rRNA · Symbiosis · Coevolution

INTRODUCTION

Microbial symbiotic associations between bacteria and ciliated protists are a widespread and diversified phenomenon (Vannini et al. 2003, Fokin 2004, Görtz 2006). Although several prokaryotic symbionts have been characterized and described, especially during recent years (Irbis & Ushida 2004, Vannini et al. 2004a, 2005a, 2010, Schrallhammer et al. 2006, 2011, Shinzato et al. 2007, Eschbach et al. 2009, Ferrantini et al. 2009, Boscaro et al. 2012, 2013), knowledge about this topic is still very incomplete. In some cases, studies into the biological meaning of symbiosis have demonstrated the existence of parasitic as well as mutualistic relationships between the part-

ners involved (Kusch et al. 2002, Hori & Fujishima 2003, Vannini et al. 2004b, Fels & Kaltz, 2006). Nevertheless, in most cases there are no pointers to the kind of relationship involved, and information on the adaptive or ecological meaning of the associations is even scarcer.

The association between a well-defined monophyletic group of brackish and freshwater species of the genus *Euplotes* (Ciliophora, Spirotrichea) and prokaryotes belonging to the class *Betaproteobacteria* has been studied since 1975 (Heckmann 1975, Heckmann et al. 1983). The group of *Euplotes* hosts comprises 10 different species, namely *E. aediculatus*, *E. woodruffi*, *E. eurystomus*, *E. daidaleos*, *E. octocarinatus*, *E. patella*, *E. plumipes*, *E. harpa* and 2 fur-

ther *Euplotes* spp. (Heckmann et al. 1983, Vannini et al. 2005b, 2012). These *Euplotes* species are dependent on the presence of bacterial symbionts and always harbor as a permanent, obligate symbiont the betaproteobacterium *Polynucleobacter necessarius* or a different betaproteobacterium which has been recently discovered (Vannini et al. 2012). Up to now, *P. necessarius* and the new symbiont have never been found together inside any individual host, or inside ciliate cells of the same natural population. While the *Polynucleobacter-Euplotes* association is well studied, and several studies have characterized the organisms involved and their relationship, knowledge of the symbiosis between *Euplotes* and the newly discovered betaproteobacterial symbiont is still inadequate.

The betaproteobacterium *Polynucleobacter necessarius* has been described and characterized as an obligate and mutualistic symbiont of several *Euplotes* species (Heckmann et al. 1983, Vannini et al. 2005b, 2012). Although free-living populations of *P. necessarius* have also been described (Hahn 2003), the free-living and the symbiotic *P. necessarius* represent 2 distinct and separate subspecies (Vannini et al. 2007b, Hahn et al. 2009), never shifting from one lifestyle to the other. Ciliates hosting *P. necessarius* in their cytoplasm are never found in nature without their symbiont and are completely dependent on it for reproduction and survival (Heckmann 1975, Heckmann et al. 1983, Vannini et al. 2005b, 2007a). On the other hand, symbiotic *P. necessarius* are not able to grow outside their hosts (Vannini et al. 2007b). Thus, the *Polynucleobacter-Euplotes* association is obligate for both partners.

A new betaproteobacterial, cytoplasmic symbiont has been recently detected in a few host samples of *Euplotes* species that usually harbor *Polynucleobacter necessarius* (namely *E. eurystomus* EM, *E. octocarinatus* FL(12)-VI and *E. woodruffi* POH1). This new betaproteobacterial symbiont colonizes the ciliate hosts as an alternative to *P. necessarius.* It represents a completely new phylogenetic lineage, branching basally with respect to the genus *Polynucleobacter* (Vannini et al. 2012). *In vivo* experiments demonstrated that ciliate hosts deprived of the new symbiont show a significant reduction in the rate of division (Vannini et al. 2012). Hence, *Euplotes* always harbor *P. necessarius* or the new symbiont and these 2 bacteria play a similar role with respect to their ciliate host. Nothing is known about the degree of dependence of the new symbiont on its hosts. Phylogenetic studies indicate that substitution events took place during the evolutionary path of the sym-

biosis (Vannini et al. 2012), but at present it is not possible to determine which one of the 2 betaproteobacterial symbionts originally established the association with the common ancestor of the present-day *Euplotes* host species. It is evident that both bacteria have a key-role in the evolution, physiology and ecology of their protistan hosts. Present data suggest that these symbioses are and have been of great importance for the evolutionary history of the microorganisms involved and for their adaptive success in freshwater and brackish environments.

In the present paper we bring together multidisciplinary data that refines the characterization of the newly discovered symbiont and data elucidating the relationship between this symbiont and its hosts. We present the small subunit (SSU) rRNA gene characterization of both host and symbiont of an additional population colonizing a brackish-water pond (represented by strain OS52/1 of *Euplotes woodruffi*), the symbiont genome size determination and ultrastructural description. Data on cultivation attempts of the new symbiont outside its host are also presented in order to shed light on the kind of relationship that exists between the partners of this association. On the basis of the results obtained we propose the new symbiont as candidate new species with the name of *'Candidatus* Protistobacter heckmanni', according to the current rules of prokaryotic nomenclature (Murray & Schleifer 1994, Murray & Stackebrandt 1995).

MATERIALS AND METHODS

Ciliate isolation and culture conditions

Research was performed on 2 monoclonal strains of *Euplotes woodruffi* (OS52/1 and POH1) and 1 monoclonal strain of *E. octocarinatus* (FL(12)-VI), obtained from samples of natural populations. Ciliate host species determination was performed in the present study for strain OS52/1 (see below); strains POH1 and FL(12)-VI had been previously characterized and identified (Vannini et al. 2012). The presence of a symbiont in the cytoplasm of strain OS52/1 was shown in the present study (see below).

The strains studied were obtained by isolating ciliate cells with a micropipette from environmental samples. Samples were collected in Pisa, Italy (OS52/1), Pololu, Hawaii, USA (POH1) and Burnell, Florida, USA (FL(12)-VI). The monoclonal strain OS52/1, here presented for the first time, was isolated from a sample collected in a brackish-water pond near the mouth of Serchio River (Pisa, Italy). Ciliate cells were

grown and maintained in artificial seawater (5‰ salinity) at a temperature of 19°C and regularly fed a monoclonal strain of *Dunaliella tertiolecta*.

Morphological identification of ciliate host

Morphological identification of ciliate host (strain OS52/1) was performed combining several different approaches. The general morphology of the cells was studied both by *in vivo* observations, using phase-contrast and Nomarski differential interference contrast with a Leitz optical microscope, and by scanning electron microscopy (SEM) observations. For SEM, cells were fixed in 2% OsO_4 in distilled water, then placed on poly-L-lysine coated slides, dehydrated in ethanol, and, after critical point drying, coated with gold and observed with a JEOL/JSM-5410 electron microscope. Nuclear apparatus and other morphological features (like argyrome organization) were studied using the Feulgen staining procedure and silver nitrate impregnation (Corliss 1953).

rRNA gene characterization and fluorescence *in situ* hybridization (FISH)

Total genomic DNA extraction was performed on cells of strain OS52/1 using the protocol for mycelium DNA of NucleoSpin™ Plant DNA Extraction Kit (Macherey-Nagel). The obtained DNA was then used as template for SSU rRNA gene amplification. The 18S rRNA gene of the ciliate host was amplified using the forward primer 18S F9 Euk (5′-CTG GTT GAT CCT GCC AG-3′, Medlin et al. 1988) and the reverse primer 18S R1513 (5′-TGA TCC TTC YGC AGG TTC-3′, Petroni et al. 2002). Amplification was performed with annealing taking place at 57°C for 35 cycles and products were then directly sequenced with appropriate internal primers (Modeo et al. 2006). The 16S rRNA gene of the betaproteobacterial symbiont was amplified using the betaproteobacterial-specific primers 16S β F19 (5′-GAT CCT GGC TCA GAT TGA AC-3′; Vannini et al. 2005b) and 16S β R1517 (5′-TGA TCC AGC CGC ACC TTC-3′; Vannini et al. 2005b). PCR reactions were performed using a 'touchdown' PCR (Don et al. 1991), with annealing taking place at 63, 57 and 50°C. PCR products were then directly sequenced using internal primers (Vannini et al. 2004a). In order to confirm the validity of the obtained 16S rRNA gene sequence, fluorescence *in situ* hybridization (FISH) was performed with the probe Proti_445 (5′-ACC AGG ATC GTT TCG TTC C-3′) as described by Vannini et al. (2012). The eubacterial universal probe Eub338 (Amann et al. 1990) was also used in order to verify the possible presence of additional symbionts.

rRNA gene sequence analysis

SSU rRNA sequences were at first analyzed by NCBI BLAST (Altschul et al. 1997). A similarity analysis was then performed using the specific tool of the ARB program package (Ludwig et al. 2004), comparing the sequences obtained from *Euplotes* strain OS52/1 to related sequences present in a SSU eukaryotic database comprising more than 14 500 18S rRNA gene sequences, and in the bacterial SILVA_SSURef_114 database comprising 621 948 16S rRNA gene sequences (Pruesse et al. 2007). Sequences were aligned using the editor and alignment tools from the ARB program package (Ludwig et al. 2004) together with related sequences. The alignments were then corrected taking into account the base-pairing scheme in rRNA secondary structure.

In order to better elucidate phylogenetic relationships between the newly characterized symbiont of strain OS52/1 and related bacteria, a phylogenetic analysis was performed on a selection of 32 SSU rRNA gene sequences of betaproteobacterial organisms. The analyses were run using different analytical methods and different filter sets. The PHYML program (Guindon & Gascuel 2003) from the ARB program package was used for maximum likelihood tree reconstructions, with a GTR + G model selected by Modeltest (Guindon & Gascuel 2003, Posada 2008) under both Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). Neighbor joining and maximum parsimony analysis were performed by the DISTANCE program with Kimura correction and DNAPARS program from the PHYLIP package (Felsenstein 1989). DNAPARS, neighbor joining and PHYML were performed with a bootstrap analysis of 1000 replicates. A Bayesian inference analysis was performed using MrBayes (Huelsenbeck & Ronquist 2001) with the GTR + G model. Markov chain Monte Carlo analysis was performed with 2 sets of 4 chains. The length of chains was 1 000 000 generations, with sampling each 100 generations and the first 2500 being discarded as burn-in. A consensus tree was generated and posterior probabilities were calculated using the remaining trees. Three different filter sets were applied: filter pos_var_ssu:bacteria, provided with

the SILVA database, which removes the highly variables positions, and 2 filters retaining, respectively, only positions conserved in at least 50 and 10% of *Betaproteobacteria.*

Genome size determination

The genome size of '*Candidatus* Protistobacter heckmanni' hosted by strain OS52/1 of *Euplotes woodruffi* was determined by pulsed field gel electrophoresis (PFGE). Ciliate cells of strain OS52/1 were harvested and repeatedly washed in sterile culture medium. Cell pellets were then included in agarose plugs and treated following the protocol of Strous et al. (2006).

PFGE was performed in a contour-clamped homogeneous electric field system (CHEF-DRIII, Biorad) at a temperature of 11°C. TAE 1× pH 8 was used as buffer and the gel was made with 1% Seakem Gold Agarose (Cambrex). Electrophoresis was run by 3 resolution steps, differing in pulse time (5 to 60, 6 to 200 and 200 to 1000 s), angles (120°, 120° and 106°) and time (12, 34 and 44 h). Electric potential was kept at 4 V cm^{-2}. Fragment length was estimated by comparison with standard size DNA ladders (*Saccharomyces cerevisiae* and *Hansenula wingei*, PFGE Markers, Biorad).

Transmission electron microscopy

For TEM, ciliate cells of strains OS52/1, FL(12)-VI and POH1 were fixed in 2.5% glutaraldehyde and 1% OsO$_4$ in cacodylate buffer 0.05M pH 7.4. After fixation, cells were dehydrated in ethanol and embedded in Epon-Araldite mixture. Thin sections were stained with uranyl acetate and lead citrate. Observations were made with a JEOL 100S microscope.

Isolation attempts

Cultivation attempts were performed in order to verify the ability of '*Candidatus* Protistobacter heckmanni' to grow outside its host. The methods employed require the use of large volumes of highly concentrated ciliate cultures. As such cultures were obtained only for *Euplotes* strain POH1 (*E. woodruffi*), these experiments were performed using this strain. In order to minimize the possibility of contamination, starved ciliate cells were cleaned by filtration through a 100 µm pore-size membrane filter, then repeatedly washed in sterile culture medium, harvested and treated with chloramphenicol 0.2 mg ml^{-1} overnight (preliminary experiments and subsequent FISH observations with the specific probe Proti_445 showed that such treatment does not affect betaproteobacterial symbionts, data not shown). After this treatment, ciliates were washed and harvested again, then mechanically crushed by repeated passages through a needled syringe. The presence of '*Candidatus* Protistobacter heckmanni' in the obtained homogenate was checked by FISH with the probe Proti_445. The homogenate was then used as inoculum for the isolation experiments. Isolation attempts were performed on the following culture media: CASO (casein-peptone 15 g l^{-1}, soy-peptone 5 g l^{-1}, NaCl 5 g l^{-1}), and R2A (yeast extract 0.5 g l^{-1}, proteose peptone 0.5 g l^{-1}, casamino acid 0.5 g l^{-1}, glucose 0.5 g l^{-1}, soluble starch 0.5 g l^{-1}, Na-pyruvate 0.3 g l^{-1}, K$_2$HPO$_4$ 0.3 g l^{-1}, MgSO$_4$ × 7H$_2$O 0.05 g l^{-1}). A fraction of the obtained ciliate homogenate was sterilized by filtering through a 0.2 µm pore-size filter and also used as culture medium. Attempts to culture the endosymbiont on these 3 media were performed both on liquid and solid (15 g l^{-1} agar-added) media. Isolation experiments were also carried out using an acclimatization method (AM): a stepwise acclimatization to NSY (nutrient broth, soytone, yeast extract) 3 g l^{-1} starting from IBM (inorganic basal medium) (Hahn et al. 2004, Vannini et al. 2007b). The same method was also tried using sterilized ciliate homogenate as starting medium instead of IBM. All the culturing experiments were performed in parallel at both 19 and 30°C. Growth of the symbiont was checked at Days 1, 3, 7, 14 and 30 by FISH with the specific probe Proti_445 (Vannini et al. 2012).

RESULTS

Identification of host ciliate

Ciliate species of strain OS52/1 was determined using both molecular and morphological characterization. Ciliate cells showed an oval shape, dorso-ventral flattened, slightly enlarged and squared in the anterior part. Average dimensions were 120 × 90 µm (n = 25), with the cytostomal region occupying about four-fifths of the total body length; 70 to 80 adoral zone membranelles (AZM) were present (Fig. 1). The cirral pattern was constituted by 9
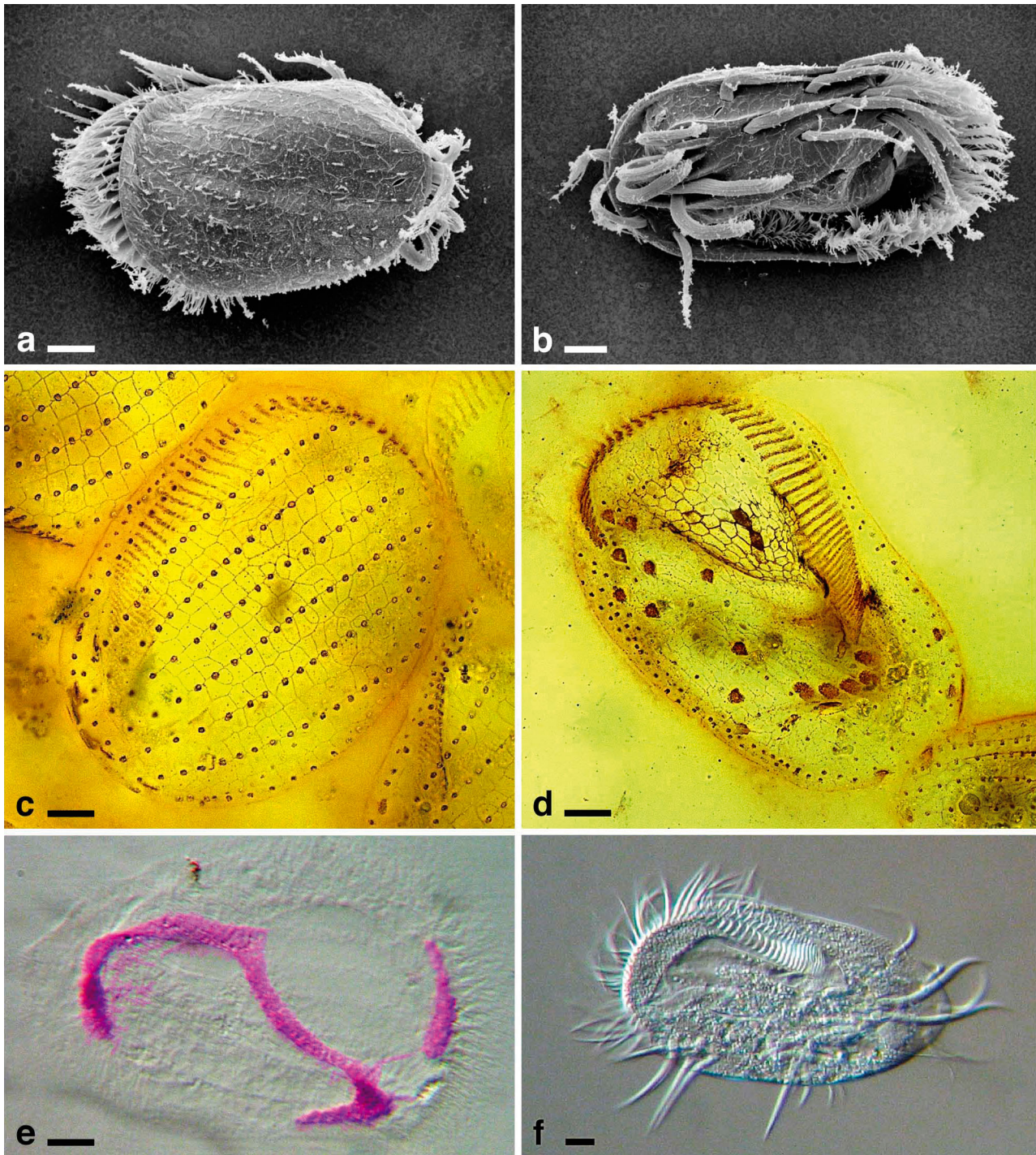
Fig. 1. *Euplotes woodruffi.* Morphology of *E. woodruffi* OS52/1 as seen by (a,b) scanning electron microscopy, (c,d) silver nitrate impregnation, (e) Feulgen staining and (f) *in vivo* observations. Scale bars: 10 µm

frontoventral, 5 transverse and 4 caudal cirri, with frontoventral cirri showing a 9 type I cirrus pattern (*Euplotes patella*-like, Fig. 1a–d). The nuclear apparatus consisted of 1 macronucleus and 1 micronucleus. The macronucleus was T-shaped,

with the left arm consistently longer (about twice as long) then the right arm (Fig. 1e). Cells presented a double argyrome, with organization resembling that of *E. eurystomus* (double-eurystomus type, Fig. 1c). An almost complete SSU rRNA

gene sequence was obtained (1832 bp, accession number HF548208). This sequence gave as the best hit on NCBI BLAST the *E. woodruffi* sequence JQ801447 (Dai et al. 2013) with a similarity value of 100%. This result was confirmed by the similarity analysis performed on ARB: the sequence showed a range of similarity values between 99.7 and 100% with respect to 18S rRNA gene sequences of previously characterized *E. woodruffi* and *E. parawoodruffi*.

Morphology and localization of symbionts

Rod-shaped bacteria, showing the same ultrastructural appearance, were observed in the cytoplasm of all the examined ciliate host strains (OS52/1, POH1,

FL(12)-VI). Symbionts were 0.4 to 0.8 μm wide and 1.5 to 3 μm long and they were always surrounded by a vacuolar membrane (symbiosome), which was distinct and clearly visible. The cytoplasm was very often very electron-dense at the periphery of the cell, but this feature was not always present (Fig. 2). Ribosomes of the host were sometimes visible on the external surface of the symbiosome (Fig. 2d). The presence of nucleoids was never observed. In some cases, dividing bacteria were observed, showing that they are able to divide inside their host (not shown).

Observations carried out both by FISH experiments and by TEM, showed that bacteria were evenly distributed inside the cytoplasm of the ciliate host, being present near the plasma membrane as well as in other areas of the host cell (Fig. 3). FISH experiments performed with the specific probe
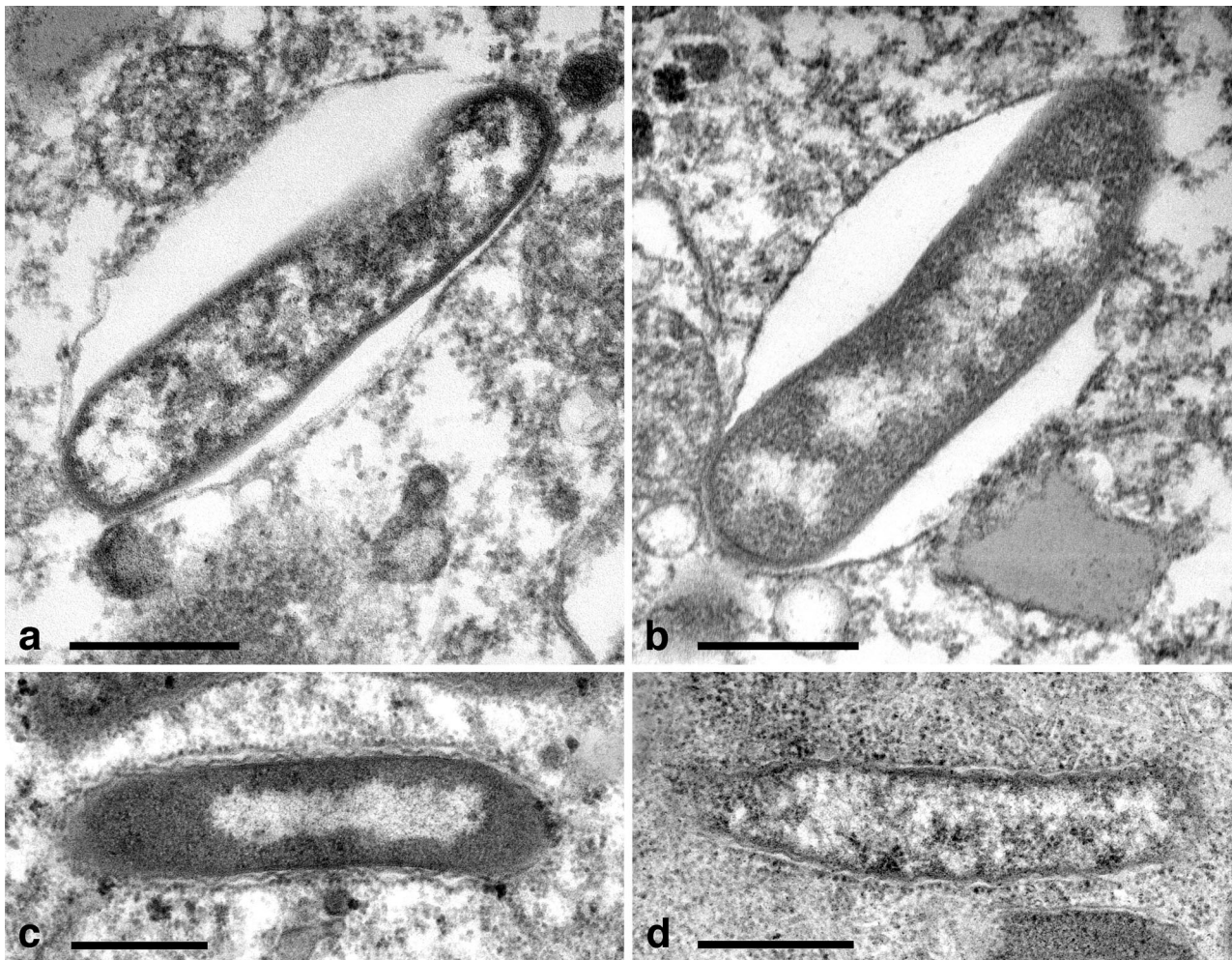

Fig. 2. Transmission electron micrographs of the symbiont '*Candidatus* Protistobacter heckmanni' in (a,b) *Euplotes woodruffi* POH1, (c) *E. octocarinatus* FL(12)-VI and (d) *E. woodruffi* OS52/1. The bacterial cytoplasm can appear homogeneously distributed (a,d) or more electron-dense at the periphery of the cell (b,c). Scale bars: 0.5 μm
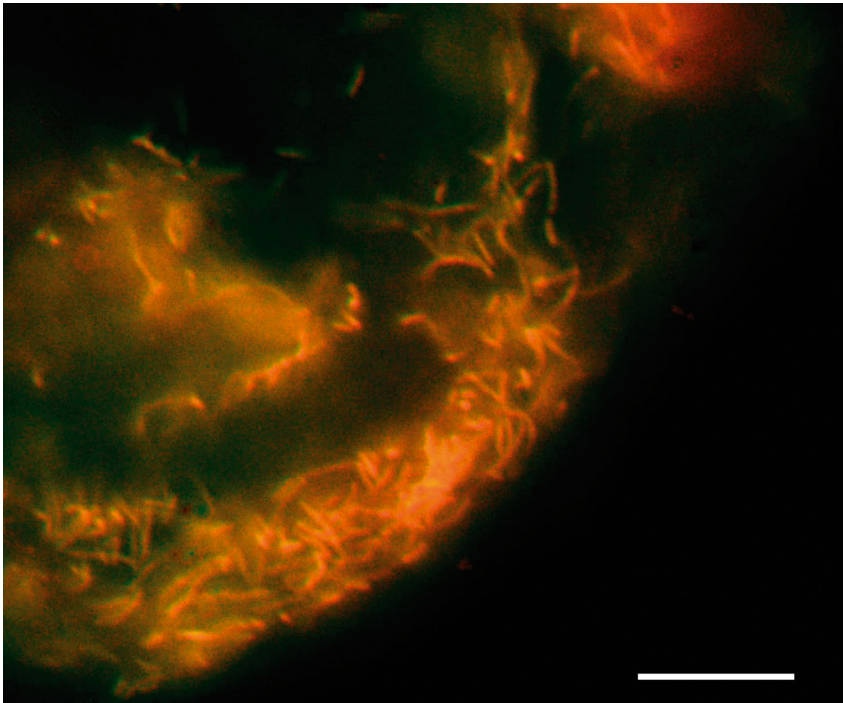
Fig. 3. Results of fluorescence *in situ* hybridization (FISH) performed using the specific probe Proti_445 (red signal) and the eubacterial universal probe Eub 338 I (green signal). The complete overlap of the 2 signals show the presence of only 1 species of bacterial symbiont. Scale bar: 10 µm

The retrieved 16S rRNA gene sequence showed a range of similarity values between 92.4 and 93.4 % with respect to sequences of bacteria of the *Polynucleobacter* genus, and between 99.1 and 99.8 % with respect to the previously determined sequences of the new symbiont (Vannini et al. 2012). In particular, it showed a 2-bp difference when compared to the sequence of the symbiont from *Euplotes woodruffi* POH1 (FR873711), a 14-bp difference in comparison with the sequence of the symbiont from *E. octocarinatus* FL(12)-VI (FR873667-FR873 669) and an 11-bp difference in comparison with the sequence of the symbiont from *E. eurystomus* EM (FR873710). All the constructed phylogenetic trees showed that the symbiont from *E. woodruffi* OS52/1 belonged to the clade constituted by the recently described symbiont of *Euplotes.* This phylogenetic clade was always very well supported and represented a clear, independent monophyletic group. With the exception of 2 maximum parsimony trees, this group always branched basally with respect to the genera *Polynucleobacter*, *Ralstonia* and *Cupriavidus* (Fig. 4). Within this cluster, the newly characterized symbiont was permanently associated with the symbiont of *E. woodruffi* POH1.

Proti_445 together with eubacterial universal probe Eub_338I demonstrated that no other symbionts are present inside the ciliate (Fig. 3). The use of the specific probe Proti_445 never showed the presence of the symbionts outside the host cells (data not shown). According to FISH observations, the amount of symbiotic bacteria inside the host cytoplasm was apparently not very variable.

Genome size, 16S rRNA gene characterization and phylogeny of symbionts

The genome size of bacterial symbiont from *Euplotes woodruffi* strain OS52/1, as determined by PFGE, is 2.7 Mb. An almost complete 16S rRNA gene sequence of this organism was also obtained (1473 bp) and deposited in the DDBJ/EMBL/Gen-Bank databases under the accession number HF54 8207. This sequence gave as best hits on NCBI BLAST analysis the already deposited sequences of symbionts from strains POH1, EM and FL(12)-VI. The second best hit was represented by the sequence of *Pandorea* sp. EU090895, with a similarity value of 94 %.

Isolation attempts

All attempts to grow the symbionts outside their ciliate host failed both on solid and liquid CASO, and ciliate homogenate. No positive signal from the specific probe Proti_445 was obtained on samples at any time during the experiments, nor at any of the different temperatures tested. The same negative result was obtained from isolation attempts performed using AM on IBM and ciliate homogenate. Only 1 positive signal was observed for 1 sample in liquid R2A at Day 1 (30°C). The same isolation tube always gave negative FISH results when checked on Days 3, 7, 14 and 30. All the other replicates from isolation attempts in R2A medium always gave negative results.
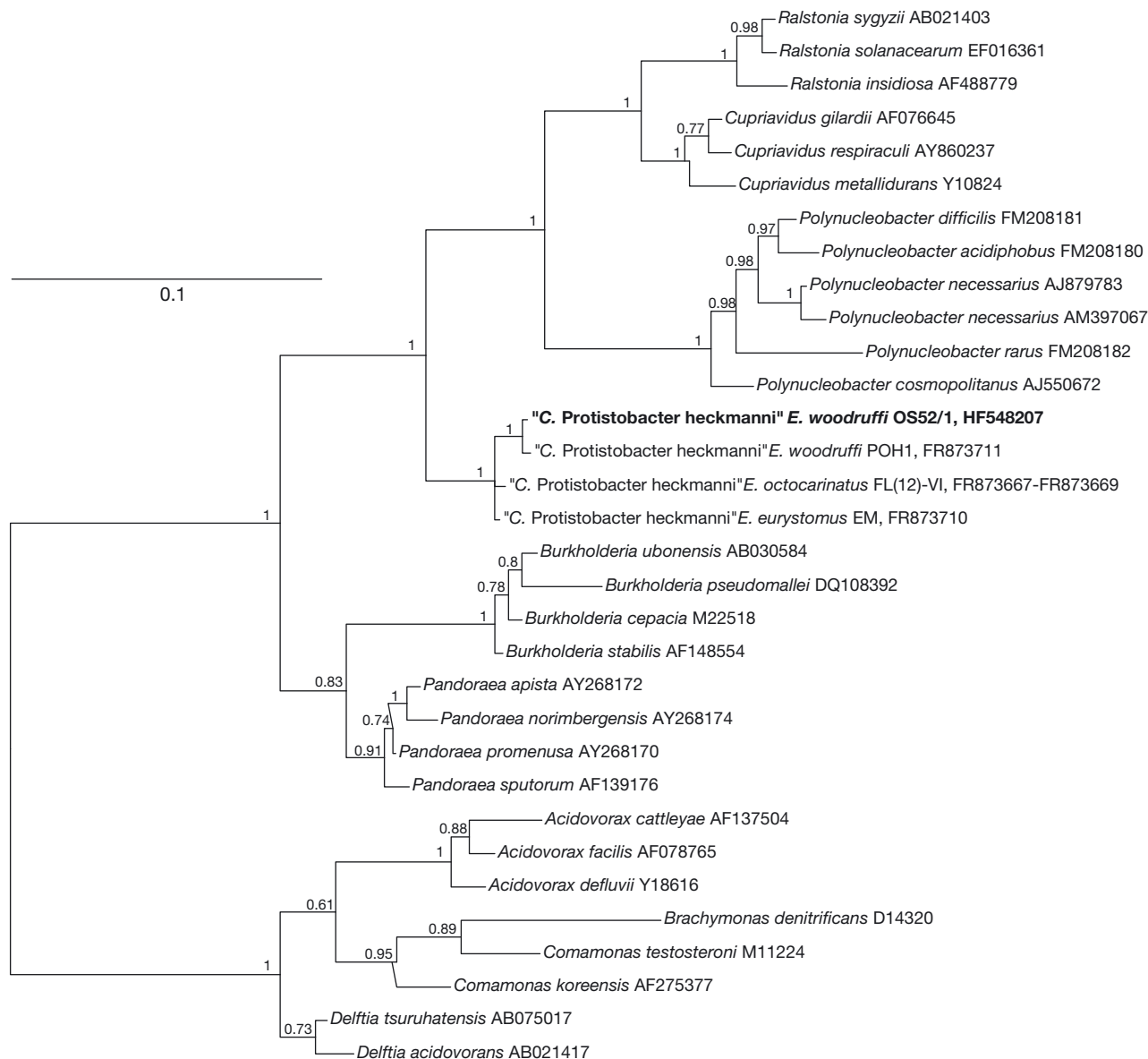
Fig. 4. Bayesian phylogenetic tree calculated on the basis of comparative analysis of 16S rRNA gene sequences. Accession number is reported for each sequence. The newly determined sequence of the symbiont from strain OS 52/1 is in **bold**. Numbers at bifurcations represent posterior probability values. Scale bar: 10% estimated sequence divergence

DISCUSSION

Both molecular and morphological data strongly indicate that ciliates of strain OS52/1 belong to the species *Euplotes woodruffi* (Gaw 1939). Although their adaptation to brackish-water environment and the different lengths of the 2 arms of the T-shaped macronucleus suggest they could belong to the species *E. parawoodruffi* (Song & Bradbury 1997), this last species has been recently recognized as a junior synonym of *E. woodruffi* (Dai et al. 2013).

16S rRNA gene similarity data as well as phylogenetic analysis clearly show that the bacterial endosymbiont colonizing the cytoplasm of ciliate cells of strain OS52/1 is very similar to the one already described in the same host species (strain POH1, *Euplotes woodruffi*) and in 2 additional *Euplotes* species (represented by monoclonal strain FL(12)-VI, *E. octocarinatus* and polyclonal strain EM, *E. eurystomus*) by Vannini et al. (2012). We propose for this species the provisional name '*Candidatus* Protistobacter heckmanni'.

The 2 16S rRNA gene sequences of '*Candidatus* Protistobacter heckmanni' from strains POH1 from the Pacific Ocean, and OS52/1, sampled in Italy, of the same host species (*Euplotes woodruffi*) are almost identical (2-bp difference) and are consistently associated in the phylogenetic trees. This indicates that, at least in the species *E. woodruffi*, the symbiosis, once established, has been constantly maintained during the evolutionary path of the host. Unfortunately, the number of studied *Euplotes* strains hosting '*Candidatus* P. heckmanni' is still too low to assess whether coevolution between the group of host species and the bacterial symbiont actually took place.

Morphological data at the ultrastructural level are here provided for the first time on this newly discovered betaproteobacterial symbiont. It is notable that nucleoids, a typical morphological feature of the symbiotic *Polynucleobacter necessarius*, were never observed in '*Candidatus* Protistobacter heckmanni'. However '*Candidatus* Protistobacter heckmanni'- sometimes shares with the symbiotic *P. necessarius* the presence of a consistently electron-dense cytoplasm near the periphery of the cell. The presence of a distinct, clearly visible symbiosomal membrane and of host ribosomes on the external surface of the symbiosome could indicate high metabolic activity connected with the presence of the symbionts.

Even if reduced when compared to other bacteria belonging to the *Burkholderiaceae* family, the genome size of '*Candidatus* Protistobacter heckmanni' (around 2.7 Mb) is significantly higher than that of the symbiotic *Polynucleobacter necessarius* assessed by the same method (between 1.7 and 1.8 Mb, Vannini et al. 2007b). This datum indicates a deeper genome-reduction process in the species of the genus *Polynucleobacter*. On the other hand, *in vivo* experiments performed on aposymbiotic ciliates show that ciliated hosts are completely dependent for survival on the bacterial symbiont in the *P. necessarius-Euplotes* symbiosis (Heckmann 1975, Heckmann et al. 1983, Vannini et al. 2005b, 2007a), while they can survive for a certain period of time when deprived of '*Candidatus* P. heckmanni' (Vannini et al. 2012). Thus, available data support the existence of a stronger interdependent relationship between symbiotic *P. necessarius* and *Euplotes* than between the same host species and '*Candidatus* P. heckmanni'.

Attempts to cultivate '*Candidatus* Protistobacter heckmanni' outside its hosts were performed on different culture media and at different temperatures, testing both conditions which give successful results for phylogenetically related bacteria (like *Ralstonia* and *Cupriavidus*) and conditions which work for free-living representatives of the species *Polynucleobacter necessarius* (Hahn et al. 2004). The observation of 1 single cell in the R2A medium on Day 1 of the experiment has to be considered only as evidence of longer survival by 1 cell, and not as a proof of the growth of this bacterium, as no other positive signal was observed in the following days. Therefore, '*Candidatus* P. heckmanni' probably represents, like the symbiotic strains of *P. necessarius*, an obligate symbiont, unable to grow outside the cytoplasm of its natural host. It should be mentioned that the stable, independent phylogenetic cluster of this symbiotic bacterium does not include, up to now, any sequence of potentially free-living bacteria, not even from any uncultured organism. It is then reasonable to hypothesize that this phylogenetic lineage originated by the establishment of the obligate symbiotic life-style.

On the basis of the collected data we propose the establishment of a candidate new genus and species with the name of '*Candidatus* Protistobacter heckmanni', according to the current rules of prokaryotic nomenclature (Murray & Schleifer 1994, Murray & Stackebrandt 1995).

Description of '*Candidatus* Protistobacter heckmanni'

Protistobacter heckmanni (Pro.tis.to.bac'ter heck. man.ni; N. L. adj. *protisto*, which precedes; N. L. masc. n. *bacter*, rod; N. L. masc. n. *Protistobacter*, preceding rod; N. L. adj. *heckmanni*, dedicated to Professor Klaus Heckmann, who first studied the symbiosis between *Euplotes* and betaproteobacterial symbionts).

Rod-shaped bacterium, 0.4 to 0.8 µm wide and up to 3 µm long, always surrounded by a vacuolar membrane (symbiosome), cytoplasm often electron-dense at the periphery of the cell. Gram negative cell-wall organization. Basis of assignment: 16S rRNA gene sequence (accession number: HF548207) and positive match with the specific FISH oligonucleotide probe Proti_445 (5'-ACC AGG ATC GTT TCG TTC C-3', Vannini et al. 2012). Up to now identified in the cytoplasm of the ciliates *Euplotes eurystomus* EM, *E. octocarinatus* FL(12)-VI and *E. woodruffi* POH1 and OS52/1. Uncultured thus far.

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