

Supplementary Material

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

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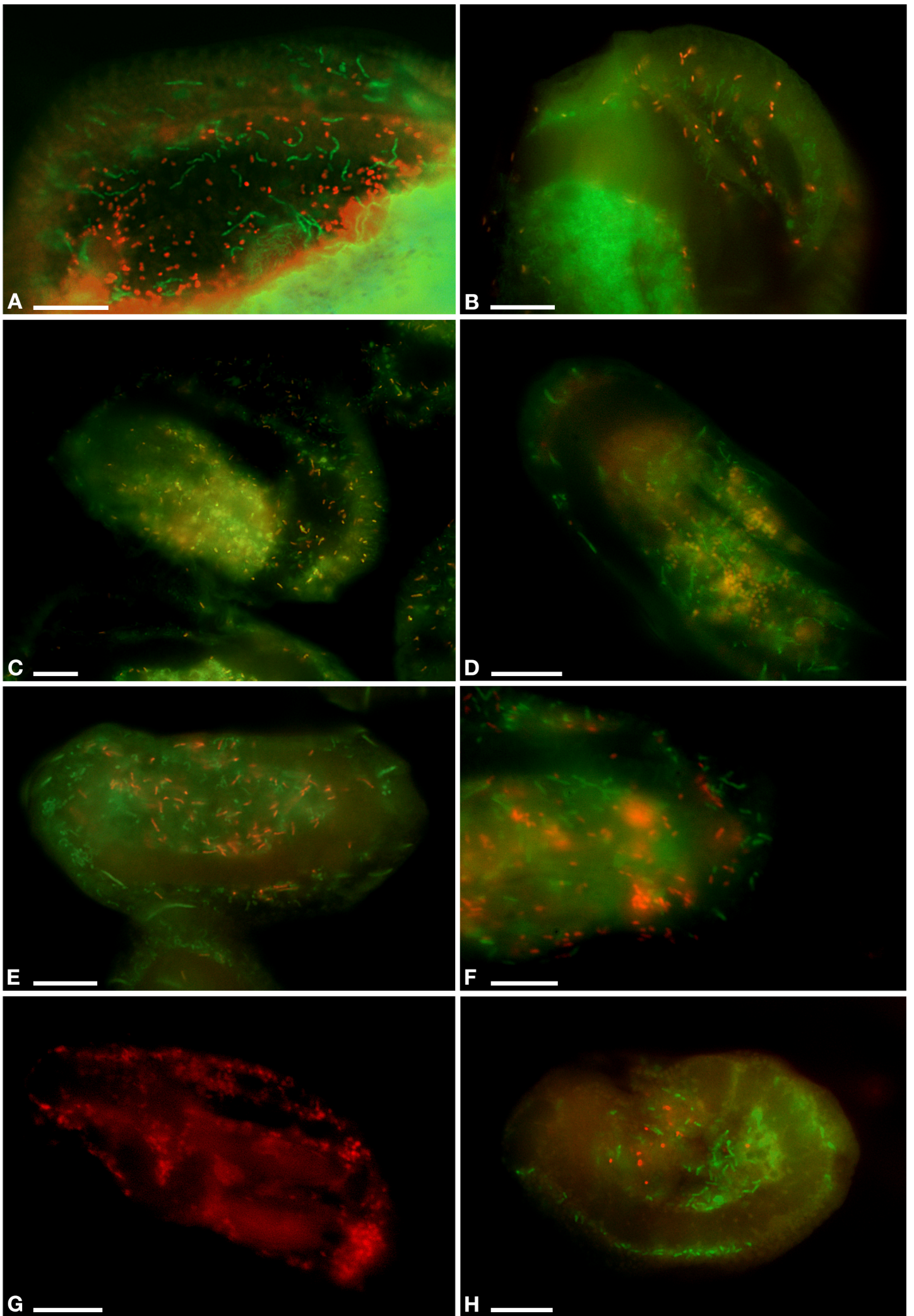
Table S1. List of *Euplotes* strains and populations analyzed in this paper.

Name	Type of culture	Species	Clade	Reference	18S rRNA gene sequence	Environment	Geographic origin	Metagenomic screening?	New FISH performed?
Eae1	Monoclonal strain	<i>E. aediculatus</i>	B	[17]	LT623907	Freshwater	Tuscany (Italy)	YES	YES
Eae2	Monoclonal strain	<i>E. aediculatus</i>	B	[17]	LT623908	Freshwater	Tuscany (Italy)	YES	*
Eae3	Monoclonal strain	<i>E. aediculatus</i>	B	[17]	LT623904	Freshwater	Tuscany (Italy)	YES	*
Eae4	Monoclonal strain	<i>E. aediculatus</i>	B	This publication	LR588889	Freshwater	Tuscany (Italy)	YES	YES
Eae5	Monoclonal strain	<i>E. aediculatus</i>	B	[17]	LT623902	Freshwater	Tuscany (Italy)	YES	*
Eae6	Monoclonal strain	<i>E. aediculatus</i>	B	This publication	LR588890	Freshwater	Tuscany (Italy)	YES	YES
Eda1	Monoclonal strain	<i>E. daidaleos</i>	B	[17]	LT623903	Freshwater	Tuscany (Italy)	YES	YES
EM	Population	<i>E. eurytomus</i>	B	[14]	FR873717	Freshwater	Lazio (Italy)	YES	NO
Eoc1/2	Population	<i>E. octocarinatus</i>	B	[17]	LT623905	Freshwater	Tuscany (Italy)	YES	YES
FL(12)-VI	Monoclonal strain	<i>E. octocarinatus</i>	B	[14]	N/A	N/A	Florida (USA)	YES	NO
Ewo1	Monoclonal strain	<i>E. woodruffi</i>	B	[17]	LT623906	Freshwater	Tuscany (Italy)	YES	YES
POH1	Monoclonal strain	<i>E. woodruffi</i>	B	[14]	FR873715	Brackish (low salinity)	Hawaii (USA)	YES	NO
EMP	Population	<i>Euplotes</i> sp.	B	[14]	FR873720	Freshwater	Lazio (Italy)	NO	YES (based on Sanger-sequenced 16S rRNA amplicons)
Fsp1.4	Monoclonal strain	<i>E. platystoma</i> (=harpa)	B	[13]	AJ811015	Brackish (low salinity)	Tuscany (Italy)	YES	*
Na2	Monoclonal strain	<i>E. platystoma</i> (=harpa)	B	[18]	LT898465	Brackish (low salinity)	Campania (Italy)	YES	*
LIV5	Monoclonal strain	<i>E. magnicirratu</i>	A	[25]	AJ549209	Marine	Ligurian Sea (Italy)	YES	YES
MaS2	Monoclonal strain	<i>E. enigma</i>	A	[10]	LT732572	Marine	British Columbia (Canada)	NO	NO

* No new FISH experiment required

Table S2. List of detected bacterial symbionts in the *Euplotes* strains and populations analyzed in this paper. Novel taxa are in bold.

Host	Symbiont	Classification	16S rRNA gene sequence	Reference	Tested FISH probe	FISH probe sequence	Known hosts
Eae1 (<i>E. aediculatus</i>)	<i>Polynucleobacter</i> sp. "Ca. <i>Nebulobacter yamunensis</i> "	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i> <i>Gammaproteobacteria</i> , <i>Thiotrichales</i> , <i>Fastidiosibacteraceae</i>	LT615228 (genome) LR585330	[17] This publication	Poly_862 [13] NebProb203 [21]	5'-GGCTGACTTCACGGCTTA-3' 5'-ATAGCGACTGCCCTAAAG-3'	Most <i>Euplotes</i> species in clade B [14] <i>E. aediculatus</i> [21, this publication]
Eae2 (<i>E. aediculatus</i>)	"Ca. <i>Cyrtobacter zanobii</i> "	<i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , "Ca. <i>Midichloriaceae</i> "	LR585331	This publication	Cyrii_142 [22]	5'-CGTTTCCAATAGTATTTTGGTAC-3'	<i>E. aediculatus</i> [22, this publication]
Eae3 (<i>E. aediculatus</i>)	<i>Polynucleobacter</i> sp.	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i>	LT606947 (genome)	[17]	Poly_862 [13]	5'-GGCTGACTTCACGGCTTA-3'	Most <i>Euplotes</i> species in clade B [14]
Eae4 (<i>E. aediculatus</i>)	<i>Polynucleobacter</i> sp.	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i>	LT606948 (genome)	[17]	Poly_862 [13]	5'-GGCTGACTTCACGGCTTA-3'	Most <i>Euplotes</i> species in clade B [14]
Eae5 (<i>E. aediculatus</i>)	<i>Polynucleobacter</i> sp.	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i>	LT606950 (genome)	[17]	Poly_862 [13]	5'-GGCTGACTTCACGGCTTA-3'	Most <i>Euplotes</i> species in clade B [14]
Eae6 (<i>E. aediculatus</i>)	"Ca. <i>Protistobacter heckmanni</i> " "Ca. <i>Nebulobacter yamunensis</i> " "Ca. <i>Cyrtobacter zanobii</i> "	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i> <i>Gammaproteobacteria</i> , <i>Thiotrichales</i> , <i>Fastidiosibacteraceae</i> <i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , "Ca. <i>Midichloriaceae</i> "	LR585335 LR585334 LR585333	This publication NebProb203 [21] This publication	Proti_445 [14] Cyrin_142 [22]	5'-ACCGAGATCGTTTGGTTC-3' 5'-ATAGCGACTGCCCTAAAG-3' 5'-CGTTTCCAATAGTATTTTGGTAC-3'	Several <i>Euplotes</i> species in clade B [14] <i>E. aediculatus</i> [21, this publication] <i>E. aediculatus</i> [22, this publication]
Eda1 (<i>E. doidaleos</i>)	<i>Polynucleobacter</i> sp.	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i>	LT606946 (genome)	[17]	Poly_862 [13]	5'-GGCTGACTTCACGGCTTA-3'	Most <i>Euplotes</i> species in clade B [14]
EM (<i>E. eurytomus</i>)	"Ca. <i>Finniella dimorpha</i> " sp. nov. "Ca. <i>Protistobacter heckmanni</i> " "Ca. <i>Megaira polyvenophila</i> " "Ca. <i>Cyrtobacter</i> sp."	<i>Alphaproteobacteria</i> , <i>Holospirales</i> , <i>Paracaulobacteraceae</i> <i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i> <i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , <i>Rickettsiaceae</i> <i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , "Ca. <i>Midichloriaceae</i> "	LR585336 FR873710 LR585337 LR585338	This publication [14] This publication This publication	Fin_1025 [This publication] Proti_445 [14] N/A N/A	5'-GTGAGCGCCAGCAAACT-3' 5'-ACCGAGATCGTTTGGTTC-3' N/A N/A	<i>E. doidaleos</i> , <i>E. eurytomus</i> , <i>E. octocarinatus</i> [This publication] Several <i>Euplotes</i> species in clade B [14] <i>Ciliates</i> [<i>Colpidium</i> , <i>Diaphrys</i> , <i>Euplotes</i> , <i>Ichthyophthirius</i> , <i>Paramecium</i> , <i>Spirostomum</i>], green algae [<i>Carteria</i> , <i>Mesostigma</i> , <i>Pleodorina</i> , <i>Volvox</i>], diatoms [<i>Hyalodiscus</i>] [42] <i>E. eurytomus</i> [This publication]
Eoc1/2 (<i>E. octocarinatus</i>)	"Ca. <i>Finniella dimorpha</i> " sp. nov. "Ca. <i>Endonucleobacter</i> sp."	<i>Alphaproteobacteria</i> , <i>Holospirales</i> , <i>Paracaulobacteraceae</i> <i>Gammaproteobacteria</i>	LR585339 LR585340	This publication This publication	N/A N/A	N/A N/A	<i>E. doidaleos</i> , <i>E. eurytomus</i> , <i>E. octocarinatus</i> [This publication] The species "Ca. <i>Endonucleobacter rarus</i> " has been found in the opisthokont <i>Nuclearia</i> [43]
	<i>Polynucleobacter</i> sp.	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i>	LT606951 (genome)	[17]	Poly_862 [13] PnevC-165-445 [63]	5'-GGCTGACTTCACGGCTTA-3', 5'-GAGCCGGTGTTCCTCC-3'	Most <i>Euplotes</i> species in clade B [14]
	"Ca. <i>Fujishimia apicalis</i> " gen. nov., sp. nov. "Ca. <i>Finniella dimorpha</i> " sp. nov.	<i>Alphaproteobacteria</i> , <i>Holospirales</i> , <i>Paracaulobacteraceae</i> <i>Alphaproteobacteria</i> , <i>Holospirales</i> , <i>Paracaulobacteraceae</i>	LR585344 LR585345	This publication This publication	Fuji_838 [This publication] N/A	5'-GCTCTCTAAAGAGTGC-3' N/A	<i>E. octocarinatus</i> [This publication] <i>E. doidaleos</i> , <i>E. eurytomus</i> , <i>E. octocarinatus</i> [This publication]
	"Ca. <i>Megaira polyvenophila</i> " "Ca. <i>Anaellobacter sociabilis</i> " sp. nov.	<i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , <i>Rickettsiaceae</i> <i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , "Ca. <i>Midichloriaceae</i> "	LR585341 LR585342	This publication This publication	MegP0436 [23] Ana2_436 [This publication]	5'-TTATCTTTCCAACTAAAG-3' 5'-CATTATTTCCTCACTAAAG-3'	<i>Ciliates</i> [<i>Colpidium</i> , <i>Diaphrys</i> , <i>Euplotes</i> , <i>Ichthyophthirius</i> , <i>Paramecium</i> , <i>Spirostomum</i>], green algae [<i>Carteria</i> , <i>Mesostigma</i> , <i>Pleodorina</i> , <i>Volvox</i>], diatoms [<i>Hyalodiscus</i>] [42] <i>E. octocarinatus</i> [This publication]
	"Ca. <i>Euplotella sexta</i> " gen. nov., sp. nov.	<i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , "Ca. <i>Midichloriaceae</i> "	LR585343	This publication	EocBan_828 [This publication]	5'-AACCTCGTCCCGACACC-3'	<i>E. octocarinatus</i> [This publication]
FL12)-VI (<i>E. octocarinatus</i>)	"Ca. <i>Protistobacter heckmanni</i> " "Ca. <i>Megaira polyvenophila</i> "	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i> <i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , <i>Rickettsiaceae</i>	FR873667-9 FR823004-7	[14] [23]	Proti_445 [14] MegP0436 [23]	5'-ACCGAGATCGTTTGGTTC-3' 5'-TTATCTTTCCAACTAAAG-3'	Several <i>Euplotes</i> species in clade B [14] <i>Ciliates</i> [<i>Colpidium</i> , <i>Diaphrys</i> , <i>Euplotes</i> , <i>Ichthyophthirius</i> , <i>Paramecium</i> , <i>Spirostomum</i>], green algae [<i>Carteria</i> , <i>Mesostigma</i> , <i>Pleodorina</i> , <i>Volvox</i>], diatoms [<i>Hyalodiscus</i>] [42]
Ewo1 (<i>E. woodruffi</i>)	<i>Polynucleobacter</i> sp. "Ca. <i>Megaira venefica</i> "	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i> <i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , <i>Rickettsiaceae</i>	LR585346 LR585347	This publication This publication	N/A MegVene95 [43]	N/A 5'-CGCTTGCCCACTACGAC-3'	<i>E. octocarinatus</i> [This publication] Most <i>Euplotes</i> species in clade B [14]
POH1 (<i>E. woodruffi</i>)	"Ca. <i>Bandiella numerosa</i> " sp. nov. "Ca. <i>Protistobacter heckmanni</i> "	<i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , "Ca. <i>Midichloriaceae</i> " <i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i>	LR585348 FR873711	This publication [14]	banNum_173 [This publication] Proti_445 [14]	5'-CCTCTCGGCAATATACAGTA-3' 5'-ACCGAGATCGTTTGGTTC-3'	<i>Ciliates</i> [<i>Euplotes</i> , <i>Ichthyophthirius</i> , <i>Paramecium</i>], cnidarians [<i>Hydra</i>] [43] Several <i>Euplotes</i> species in clade B [14]
	"Ca. <i>Bandiella numerosa</i> " sp. nov.	<i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , "Ca. <i>Midichloriaceae</i> "	LR585349	This publication	N/A	N/A	<i>E. woodruffi</i> , euglenids [<i>Eutreptiella</i>], cnidarians [<i>Hydra</i> , <i>Montastrea</i>], sponges [<i>Cymbastela</i>], echinoderms [<i>Apostichopus</i>] [This publication]
EMP (<i>Euplotes</i> sp.)	<i>Polynucleobacter</i> sp. "Ca. <i>Megaira polyvenophila</i> " <i>CaeDimonas varicadens</i> ("Caedibacter varicadens")	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i> <i>Alphaproteobacteria</i> , <i>Rickettsiales</i> , <i>Rickettsiaceae</i> <i>Alphaproteobacteria</i> , <i>Holospirales</i> , <i>CaeDimonadaceae</i>	N/A LR585350 LR585351	[14] This publication This publication	Poly_862 [13] Rick_697 [24] CC23a [64]	5'-GGCTGACTTCACGGCTTA-3' N/A 5'-TTCCACTTTGCTCTCTGG-3'	Most <i>Euplotes</i> species in clade B [14] <i>Ciliates</i> [<i>Colpidium</i> , <i>Diaphrys</i> , <i>Euplotes</i> , <i>Ichthyophthirius</i> , <i>Paramecium</i> , <i>Spirostomum</i>], green algae [<i>Carteria</i> , <i>Mesostigma</i> , <i>Pleodorina</i> , <i>Volvox</i>], diatoms [<i>Hyalodiscus</i>] [42] <i>E. woodruffi</i> , euglenids [<i>Eutreptiella</i>], cnidarians [<i>Hydra</i> , <i>Montastrea</i>], sponges [<i>Cymbastela</i>], echinoderms [<i>Apostichopus</i>] [This publication]
Fisp1.4 (<i>E. platystoma -harpa</i>)	"Ca. <i>Parafinniella ignota</i> " gen. nov., sp. nov.	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i>	FR873728-30	This publication	Paraf_838 [This publication]	5'-GATACAGAACTCCGCC-3'	<i>Euplotes</i> sp. [This publication]
Na2 (<i>E. platystoma -harpa</i>)	<i>Polynucleobacter</i> sp.	<i>Betaproteobacteria</i> , <i>Burkholderiales</i> , <i>Burkholderiaceae</i>	AJ585516	[13]	Poly_862 [13]	5'-GGCTGACTTCACGGCTTA-3'	Most <i>Euplotes</i> species in clade B [14]
LIV5 (<i>E. magnificiratus</i>)	"Ca. <i>Devosia symbiotica</i> " "Ca. <i>Devosia euplotis</i> " <i>Francisella adelensis</i>	<i>Alphaproteobacteria</i> , <i>Rhizobiales</i> , <i>Hyphomicrobiaceae</i> <i>Alphaproteobacteria</i> , <i>Rhizobiales</i> , <i>Hyphomicrobiaceae</i> <i>Gammaproteobacteria</i> , <i>Thiotrichales</i> , <i>Francisellaceae</i>	LT898465 AJ548823 LR585352	[18] [25] This publication	DevNa_1026 [18] DevEup993 [25] Franci_199 [This publication]	5'-GTGCCAGGTACCGGAAGTG-3' 5'-AAGTGCTCTGCTATGTC-3' 5'-CAGCAGCAAGTGCCAC-3'	<i>E. platystoma</i> [18] <i>E. magnificiratus</i> [25] <i>E. petzi</i> [27], <i>E. magnificiratus</i> [This publication] <i>E. enigma</i> [This publication]
Ma52 (<i>E. enigma</i>)	<i>Devosia</i> sp.	<i>Alphaproteobacteria</i> , <i>Rhizobiales</i> , <i>Hyphomicrobiaceae</i>	LR585353	This publication	N/A	N/A	



Supplementary Figure S1. Additional fluorescent *in situ* hybridizations. **A**, “*Ca. Protistobacter heckmanni*” (green, probe Proti_445) and “*Ca. Nebulobacter yamunensis*” (red, probe NebProb203) in *E. aediculatus* Eae6. **B**, “*Ca. Cyrtobacter zanobii*” (orange, combination of the species-specific red probe CyrIn_142 and the eubacterial green probe EUB338. Other bacteria are visible in green) in *E. aediculatus* Eae6. **C**, “*Ca. Cyrtobacter zanobii*” (orange, combination of the species-specific red probe CyrIn_142 and the eubacterial green probe EUB338. Other bacteria are visible in green) in *E. aediculatus* Eae1. **D**, “*Ca. Megaira polyxenophila*” (orange, combination of the specific red probe MegPol436 and the alphaproteobacterial green probe ALF1b. Other alphaproteobacteria are visible in green) in *E. octocarinatus* Eoc1/2. **E**, “*Ca. Anadelfobacter sociabilis*” (orange, combination of the species-specific red probe Ana2_436 and the eubacterial green probe EUB338. Other bacteria are visible in green) in *E. octocarinatus* Eoc1/2. **F**, “*Ca. Megaira polyxenophila*” (green, *Rickettsiaceae* probe Rick_697) and “*Ca. Parafinniella ignota*” (red, species-specific probe Paraf_838) in *Euplotes* sp. EMP. **G**, *Caedimonas varicaedens* (red, species-specific probe CC23a) in *Euplotes* sp. EMP. **H**, *Francisella adeliensis* (red, species-specific probe Franci_199) and “*Ca. Devosia euplotis*” (green, species-specific probe DevEup993). Red signals are produced by the Cy3 dye; green signals by the fluorescein, FITC, or Alexa488 dyes. Bars represent 10 μm .

Supplementary Text S1

Formal descriptions of new bacterial taxa belonging to the family “*Ca. Midichloriaceae*” (*Rickettsiales*, *Alphaproteobacteria*)

Description of “*Candidatus Anadelfobacter sociabilis*” sp. nov.

Anadelfobacter sociabilis (A.na.de.lfo.bac'ter so.ci.a'bi.lis; N.L. adj. *sociabilis*, sociable, referring to its co-occurrence with other endosymbionts)

Rod-shaped bacterium, about 1.9 µm in length. Endosymbiont living in the cytoplasm of the ciliate *Euplotes octocarinatus* (Ciliophora, Spirotrichea), identified in strains collected in Italy and the USA. Basis of assignment: 16S rRNA gene sequence (accession number: LR585342) and positive match with the oligonucleotide probe Ana2_436 (5'-CATTATTTTCCCCACTAAAAG-3').

Description of “*Candidatus Bandiella numerosa*” sp. nov.

Bandiella numerosa (Ban.di.el'la nu.me.ro'sa; N.L. adj. *numerosus*, abundant, referring to the high number of cells of this bacterium in each host cell)

Rod-shaped bacterium, about 1.7 µm in length. Endosymbiont observed in the cytoplasm of the ciliate *Euplotes woodruffi* (Ciliophora, Spirotrichea), identified in strains collected in Italy and the USA (Hawaii). Basis of assignment: 16S rRNA gene sequence (accession number: LR585348) and positive match with the oligonucleotide probe BanNum_173 (5'-CCTCTCGGCAATATACAGTA-3').

Description of “*Candidatus Euplotella sexta*” gen. nov., sp. nov.

Euplotella sexta (Eu.plo.tel'la sex.ta; N.L. fem. n. *Euplotella*, referring to the host, *Euplotes*; N.L. adj. *sextus*, sixth, since it was the sixth symbiont discovered in its *Euplotes* host population)

Rod-shaped bacterium, about 2.2 µm in length. Endosymbiont living in the cytoplasm of the ciliate *Euplotes octocarinatus* (Ciliophora, Spirotrichea), identified in a population collected in Italy. Basis of assignment: 16S rRNA gene sequence (accession number: LR585343) and positive match with the oligonucleotide probe EocBan_828 (5'-AACCTCGGTCCCGACACC-3').

Formal descriptions of new bacterial taxa belonging to the order *Holosporales* (*Alphaproteobacteria*)

Description of “*Candidatus Fujishimia apicalis*” gen. nov., sp. nov.

Fujishimia apicalis (Fu.ji.shi'mia a.pi.ca'lis; N.L. fem. n. *Fujishimia*, in honor of Professor Masahiro Fujishima, one of the pioneers in the study of *Holosporales*; N.L. adj. *apicalis*, apical, related to the most common position the bacterium occupies in the host cell)

Coccoid bacterium, about 0.55 µm in length. Endosymbiont living in the cytoplasm of the ciliate *Euplotes octocarinatus* (Ciliophora, Spirotrichea), identified in a population collected in Italy. Preferentially distributed in the marginal, especially apical, parts of the host cell. Not always observed in all *Euplotes* cells of the same culture. Basis of assignment: 16S rRNA gene sequence

(accession number: LR585344) and positive match with the oligonucleotide probe Fuji_838 (5'-GCTCTCCTAAAAGGATGC-3').

Description of “*Candidatus Finniella dimorpha*” sp. nov.

Finniella dimorpha (Fin.ni.el'la di.mor'pha; N.L. adj. *dimorphus*, with two forms, related to the dimorphic morphology of the bacterium)

Rod-shaped bacterium, endosymbiont living in the cytoplasm of the ciliates *Euplotes daidaleos*, *Euplotes eurystomus*, and *Euplotes octocarinatus* (Ciliophora, Spirotrichea), identified in host strains and populations collected in Italy. In *E. daidaleos*, observed in two distinct forms of approximately the same diameter but different lengths, 1.7 µm vs. 15-25 µm, respectively. Basis of assignment: 16S rRNA gene sequence (accession number: LR585336) and positive match with the oligonucleotide probe Fin_1025 (5'-GTGGACGCCAGCCAAACT-3').

Description of “*Candidatus Parafinniella ignota*” gen. nov. sp. nov.

Parafinniella ignota (Pa.ra.fin.ni.el'la ig.no'ta; N.L. fem. n. *Parafinniella*, similar to *Finniella*; N.L. adj. *ignotus*, unknown, mysterious)

Rod-shaped bacterium, about 1.3 µm in length. Endosymbiont living in the cytoplasm of the Italian population EMP belonging to the ciliate genus *Euplotes* (Ciliophora, Spirotrichea). Basis of assignment: 16S rRNA gene sequence (accession number: FR873728-30) and positive match with the oligonucleotide probe Paraf_838 (5'-AACCTCGGTCCCGACACC-3').

Supplementary Text S2

Synopsis of the known bacterial symbionts of *Euplotes*.

Betaproteobacteria Garrity et al 2006

Burkholderiales Garrity et al 2006

Burkholderiaceae Garrity et al 2006

Polynucleobacter Heckmann and Schmidt 1987

All *Euplotes* species in clade B live in freshwater or low-salinity aquatic environments and depend on essential intracellular bacteria. An ancient symbiotic event must hence have taken place in an ancestor of the clade. Since elongated, multiple-nucleoid *Polynucleobacter* occur in most clade B *Euplotes* species [12,15], they were long assumed to be ancestral mutualists co-evolving with their hosts in the fashion of aphids and *Buchnera* [65]. Recent environmental microbiology and genomic developments have however painted a more intriguing scenario. Most *Polynucleobacter* are in fact free-living, small, pleomorphic bacteria, common and abundant in freshwater bacterioplankton [16,66]. Conversely, extant symbiotic *Polynucleobacter* are products of multiple independent establishments of the symbiosis [17], coopted by *Euplotes* to perform some unknown function they may be pre-adapted for. During each acquisition, a previous symbiont (either another *Polynucleobacter* or a different bacterium) is replaced, arguably because it has lost too many genes to the process of genome erosion [7], and quickly dispatched (no multiple essential strains have ever been observed in the same host). This process must happen at a relatively high frequency, considering the low level of degeneration observed in symbiotic *Polynucleobacter* [17,67] compared to genuinely ancient symbionts [e.g. 68]. It certainly happens faster than the average speciation time of *Euplotes*, since certain species (such as *E. aediculatus*) are associated to multiple symbiotic lineages [17]. Strains sharing identical 16S rRNA gene sequence have never been found in different host species [14], also supporting the idea that *Polynucleobacter* cannot be horizontally transmitted (although they can be artificially transfected between hosts [12]). *Euplotes* is an evolutionary dead-end for its symbionts, so it is difficult to consider their relationship a mutualism, even if the partners depend on each other for survival. *Polynucleobacter* are recent symbionts in an old symbiotic system, the purpose of which remains a mystery.

“*Ca. Protistobacter*” Vannini et al 2013

“*Ca. Protistobacter heckmanni*”, the only species in the genus, was the second essential symbiont identified in *Euplotes* species of clade B [14,19]. It belongs to the same family as *Polynucleobacter* and it is functionally equivalent for the host, although transfection experiments have produced mixed results [69]. It differs from *Polynucleobacter* for the lack of multiple nucleoids and a slightly larger genome size (estimated by pulsed field electrophoresis [19]). “*Ca. Protistobacter*” is currently only known from *Euplotes*, and it is far less common than *Polynucleobacter*. It has been detected in strains of *E. eurytomus*, *E. octocarinatus*, *E. woodruffi* and, in this paper, *E. aediculatus*, but it can probably occur in most or all clade B species. In the absence of known close free-living

relatives, “*Ca. Protistobacter*” has been speculated to be the ancestral symbiont of clade B, replaced on multiple occasions by *Polynucleobacter* [14].

Alphaproteobacteria Garrity et al 2006

Rhizobiales Kuykendall 2006

Hyphomicrobiaceae Babudieri 1950

Devosia Nakagawa et al 1996

Devosia is a large genus of bacteria occurring mostly (but not exclusively) in soil environments [e.g. 70]. Almost all species are free-living or associated to plants, and can be easily cultivated under standard lab conditions [e.g. 71]. The exception is represented by *Devosia* harbored by *Euplotes*. “*Ca. Devosia symbiotica*” has been found in a single strain of *E. platystoma*, a clade B *Euplotes* species, where it is ecologically analogous to *Polynucleobacter* and “*Ca. Protistobacter*”: it is an obligate and essential symbiont that cannot survive outside its host and is required for host survival [18]. The closely related “*Ca. Devosia euplotis*” performs the same role in all investigated *E. magnicirratu*s, a species in clade A that represents the only known example of a marine *Euplotes* with essential symbiotic bacteria [25]. We have here recovered a third *Devosia* lineage in a strain of *E. enigma*, a clade A marine *Euplotes* that is not closely related to *E. magnicirratu*s [10]. Unfortunately, we were unable to confirm the symbiotic status of this uncultured *Devosia* with FISH experiments. Our finding does however corroborate the existence of a clade of *Devosia* associated with various *Euplotes* species. Genome analyses of symbiotic *Devosia* might tell us if these bacteria independently infected ciliates due to shared pre-adaptations, possibly replacing older symbionts like *Polynucleobacter* often does, or if they represent instead the remnants of an ancestral symbiotic event predating the split between clade A and clade B of *Euplotes*.

Rickettsiales Gieszczykiewicz 1939

Rickettsiaceae Pinkerton 1936

“*Ca. Megaira*” Schrällhammer et al 2013

The genus “*Ca. Megaira*” was first observed in ciliates [23], but related uncultured bacteria already suggested a wider host range, later confirmed by further surveys [42]. “*Ca. Megaira polyxenophila*”, associated with several ciliates and algae, was previously detected in *E. octocarinatus*, and we confirmed here its presence in *E. eurystomus* and another undescribed *Euplotes* species of clade B. “*Ca. Megaira venefica*”, a symbiont also found in *Paramecium* and possibly the hydrozoan *Hydra* [42], has been reported here for the first time in *Euplotes* (*E. woodruffi*). “*Ca. Megaira*” can coexist with both *Polynucleobacter* and “*Ca. Protistobacter*”, but it is never the only accessory symbiont in investigated *Euplotes*, being instead part of larger alphaproteobacterial communities. Host range proves that “*Ca. Megaira*” is capable of horizontal transmission, at least over evolutionary times, and it is possible that the *Euplotes* strains harboring “*Ca. Megaira*” are the ones that are more prone to be colonized by bacteria. It is also true that “*Ca. Megaira*” seems to be fairly stable within its hosts over years, and it is generally present in all inspected host cells. Hence, there is currently no evidence for any harmful effect on its host.

“Ca. Trichorickettsia” Vannini et al 2014

The flagellated rickettsiae “Ca. Trichorickettsia” and “Ca. Gigarickettsia”, characterized by a combination of flagella, flagellar motility, and/or capsid-like particles in their cytoplasm, were originally described in ciliates of the genera *Paramecium*, *Spirostomum*, and *Euplotes* [24]. “Ca. Trichorickettsia mobilis” was detected in a single *E. aediculatus* population harboring at least three accessory symbionts in addition to *Polynucleobacter*. Despite sharing a virtually identical 16S rRNA gene sequence with conspecific strains, the “Ca. Trichorickettsia” in *Euplotes* was atypical, lacking obvious flagella or capsid-like particles, suggesting that both features are either strain- or condition-specific (a condition possibly shared with “Ca. Megaira venefica” [42]).

“Ca. Midichloriaceae” Bandi et al 2013

“Ca. Anadelfobacter” Vannini et al 2010

“Ca. Anadelfobacter veles” was originally described as the only accessory symbiont of an *E. platystoma* (=“harpa”) strain harboring *Polynucleobacter* [20]. “Ca. Anadelfobacter sociabilis”, described here, was found in two strains of *E. octocarinatus*, one associated to *Polynucleobacter* and the other to “Ca. Protistobacter”, both harboring additional accessory alphaproteobacteria.

“Ca. Cyrtobacter” Vannini et al 2010

“Ca. Cyrtobacter comes” was originally described in an *E. platystoma* (=“harpa”) strain harboring *Polynucleobacter* [20]. The closely related “Ca. Cyrtobacter zanobii” was originally described in *E. aediculatus* [22] and it has been detected in two more strains of the same species here. Its distribution coincides with that of the gammaproteobacterium “Ca. Nebulobacter yamunensis” (see below). A third species was detected in a metagenomic screening of one *E. eurystomus* population, but it is not formally established in the absence of a confirmatory FISH result.

“Ca. Bandiella” Senra et al 2016

“Ca. Bandiella woodruffi” was originally described in *E. woodruffi* [44]. A related bacterial species, “Ca. Bandiella numerosa”, is reported here in two additional *E. woodruffi* strains (one harboring *Polynucleobacter* and one “Ca. Protistobacter”). This does not necessarily imply a high degree of host specificity for “Ca. Bandiella”, since closely related environmental sequences are associated to freshwater and marine cnidarians and other invertebrates. Like other representatives of *Rickettsiales*, “Ca. Bandiella” probably has some infectious capabilities. “Ca. Bandiella woodruffi” is the only *Euplotes* accessory symbiont where these abilities have been experimentally tested [44]: “Ca. Bandiella” could infect other strains of *Euplotes* in culture, but was quickly eradicated from them once the source host was removed, suggesting some mechanism of suppression from the ciliate. In natural *E. woodruffi* hosts, however, both species of “Ca. Bandiella” are found in high numbers and in every cell.

“Ca. Euplotella” Boscaro et al 2019, gen. nov.

“Ca. Euplotella sexta”, described here in an *E. octocarinatus* harboring *Polynucleobacter* and five accessory alphaproteobacteria, is a large, elongated and slightly curved rod-shaped bacterium. It is present in all inspected cells of its host, but

in relatively low numbers, probably due to its considerable size (and the presence of several other bacteria in the cytoplasm of the same host).

Holosporales Szokoli et al 2016

Holosporaceae Görtz & Schmidt 2006

“*Ca. Fujishimia*” Boscaro et al 2019, gen. nov.

The family *Holosporaceae* has been defined in many different ways [e.g. 72-74], but its core representatives are nuclear symbionts of peniculid ciliates (mainly *Paramecium*) characterized by a morphologically differentiated infectious stage. Beside the four genera that show this conspicuous synapomorphy (*Holospora* [75], “*Ca. Gortzia*” [72], “*Ca. Hafkinia*” [76] and “*Ca. Preeria*” [77]), several other taxa have been assigned to the family, some of which are also symbionts of ciliates (e.g. “*Ca. Paraholospora*” in *Paramecium sexaurelia*, which shuttles between the nucleus and the cytoplasm [78]) or other protists (e.g. “*Ca. Cytomitobacter*” in the diplonemid *Diplonema* [79]). Not much is known about the non-core *Holosporaceae*. The first representative in *Euplotes* is “*Ca. Fujishimia apicalis*” described here, detected in a single population of *E. octocarinatus* together with *Polynucleobacter* and four other alphaproteobacteria. Although we have no information yet on the biology of this bacterium, its coccoid shape and diminutive size (about 0.5 µm) stand out. “*Ca. Fujishimia*” is not distributed throughout most of the cytoplasm, but tends to be concentrated in localized areas at the margin of host cells, often in an anterior position. Additionally, “*Ca. Fujishimia*” is one of the few alphaproteobacterial symbionts of *Euplotes* that is not present in all cells of the host population, a characteristic shared with *Holospora* and allied genera that might suggest a parasitic, or at least infectious, lifestyle.

Paracaedibacteraceae Hess et al 2016

“*Ca. Finniella*” Hess et al 2016

“*Ca. Finniella*” was first described in the cytoplasm of amoeboflagellate viridiraptorids (Rhizaria, Cercozoa) [41]. Different viridiraptorid species harbored genotypically different symbionts, classified in two separate species (“*Ca. Finniella inopinata*” in *Viridiraptor invadens* and “*Ca. Finniella lucida*” in *Orciraptor agilis*). Not all strains of the host species harbored the bacteria, but all cells in infected strains did. “*Ca. Finniella*” did not disappear during starvation, suggesting some stability in the symbiosis. “*Ca. Finniella dimorpha*”, described in this paper, is the first species of the genus described in *Euplotes*, specifically *E. daidaleos*, *E. eury stomus*, and *E. octocarinatus*. “*Ca. Finniella dimorpha*” was found with both *Polynucleobacter* and “*Ca. Protistobacter*”, and either as the only accessory symbiont or as part of a larger community of alphaproteobacteria. The most striking feature of “*Ca. Finniella dimorpha*” in *E. daidaleos* Eda1 is the presence of two very different morphotypes in some of the host cells: long thread-like bacteria, up to a third of the host’s length, can be seen in addition to the smaller rod-like bacteria, that are the only form present in some other host cells. Elongated morphotypes are known for a few bacterial symbionts [e.g. 80] and often originate from incomplete cell division, either as a physiological aspect of the cell cycle or due to host-induced inhibition [81,82]. The presence of “*Ca. Finniella dimorpha*” in all inspected cells of Eda1 does not, however, suggest any kind of antagonistic interaction.

“Ca. Parafinniella” Boscaro et al 2019, gen. nov.

“Ca. Parafinniella ignota” is reported here for a population of an unclassified clade B *Euplotes* additionally harboring *Polynucleobacter* and three other accessory alphaproteobacteria. Not much is currently known about this bacterium, but it is present in fairly high numbers in the cytoplasm of all cells in the host population.

Caedimonadaceae Schrallhammer et al 2018

Caedimonas Schrallhammer et al 2018

One of the most unusual phenomena discovered by pioneer researchers working on ciliate endosymbionts was the “killer trait” conferred by some endosymbiotic bacteria to their *Paramecium* hosts [83,84]. Each “killer symbiont” produces one “R-body”, a tightly coiled proteinaceous ribbon that springs open when the bacterium is ingested by a *Paramecium* that is not its natural host, puncturing the digestive vacuole and releasing lethal toxins [85]. Host strains poison their surroundings by exocytosing their symbionts, killing potential congeneric competitors [86]. It was later realized that R-body-containing bacteria are not related to each other [87], and that the trait can be horizontally transmitted. *Caedimonas varicaedens*, reported in multiple *Paramecium* species and the dinoflagellate *Peridinium cinctum*, belongs to *Holosporales*, and it has been found here in *Euplotes* sp. EMP, a clade B population harboring *Polynucleobacter* as the essential symbiont. It is located in the cytoplasm, as is the case in *Peridinium* and some *Paramecium* (but not others, where it resides in the macronucleus) [29]. It is not known if the *Caedimonas* in *Euplotes* can confer the killer trait to its host. Since this is not the case for *Peridinium* [85], it is possible that either the presence of R-bodies or the killing phenotype is only present in strains harbored by *Paramecium*. More functional studies are needed, and a comparison between *C. varicaedens* in different hosts may provide valuable insights into the biology of the killers.

Gammaproteobacteria Garrity et al 2005

Thiotrichales Garrity et al 2005

Francisellaceae Sjöstedt 2005

Francisella Dorofe'ev 1947

The genus *Francisella* is comparatively well-known because some of its representatives are pathogenic in mammals (*Francisella tularensis* is the causative agent of tularemia) [88,89] and farmed sea animals [90]. *Francisella* is usually an intracellular opportunist (i.e., a facultative symbiont) [88]. In *Euplotes*, it was originally reported in the marine *Euplotes raikovi* (clade C) as the subspecies *Francisella noatunensis endociliophora* [26], later established as its own separate species. Another species, *Francisella adeliensis*, has recently been found associated with the Antarctic *Euplotes petzi* (clade E) [27]. Alongside *Polynucleobacter*, these two *Francisella* are the only *Euplotes* symbionts whose genome has been sequenced [27,91]. Another strain of *F. adeliensis* has been detected here in a marine *Euplotes magnicirratus* (clade A), suggesting that the genus *Francisella* is widespread in marine *Euplotes*. As is the case for *Rickettsiales* and *Holosporales*, however, the phylogeny of the bacteria rules out any ancient relationship or co-evolution with the host. It is even possible that *Francisella* are just “accidental” infections, and not long term symbionts, considering how they differ from most other

bacteria described here in having very low density within host cell, being usually distributed in small “clumps” in the *Euplotes* cytoplasm instead of evenly spread, and being able to grow outside the host.

Fastidiosibacteraceae Xiao et al 2018

“*Ca. Nebulobacter*” Boscaro et al 2012

“*Ca. Nebulobacter yamunensis*” was first described in *E. aediculatus* [21], and cultivation attempts showed that it either cannot grow outside *Euplotes* or it has very specific nutritional requirements. Two novel “*Ca. Nebulobacter*” strains with identical 16S rRNA gene sequence are reported here. All “*Ca. Nebulobacter*” strains were found in *E. aediculatus* harboring either *Polynucleobacter* or “*Ca. Protistobacter*” and the accessory alphaproteobacterium “*Ca. Cyrtobacter zanobii*”. If further confirmed, this might provide useful clues on the biology of both organisms as well as their relationship with the host. Recently, a bacterium differing from “*Ca. Nebulobacter yamunensis*” by a single-nucleotide in the 16S rRNA gene sequence has been described as *Fastidiosibacter lacustris* [92]. The paper makes no mention of “*Ca. Nebulobacter*”, despite the fact that sequence similarity would not warrant the establishment of two separate genera. We maintain here the older name for the symbiont, acknowledging that some taxonomic clarification might be needed in the future. Regardless, it is extremely interesting that *F. lacustris* can be maintained in pure culture, although with difficulty. The existence of very closely related free-living and obligate symbiont strains could mirror the case of *Polynucleobacter* and provide a second, possibly independent case-study to investigate recent symbioses in the same host.

Uncertain and unclassified.

“*Ca. Endonucleariobacter rarus*” was described in a population of the opisthokont amoeba *Nuclearia* that also harbored betaproteobacterial ectosymbionts [43]. “*Ca. Endonucleariobacter*” is not currently known from any other host, but a closely related 16S rRNA gene sequence has been detected here in a metagenomic screening of *E. eurytomus*. The result could not be validated by FISH, so it is not confirmed if a different species of “*Ca. Endonucleariobacter*” is an accessory symbiont in ciliates, instead of a free-living bacterium present in our cultures. Similarly, a betaproteobacterial sequence affiliated to family *Comamonadaceae* was previously reported from the *Polynucleobacter*-harboring unclassified *Euplotes* strain PUHP (clade B), but never confirmed by FISH [14]. It was considered a putative symbiont based on the fact that the signal from betaproteobacterial fluorescent probes did not completely overlap that of the *Polynucleobacter* probe, suggesting the presence of an additional betaproteobacterium in the ciliate cytoplasm. However, without a sequence-specific hybridization and considering that *Comamonadaceae* are fairly common in the environment, we consider the status of this bacterium dubious. Finally, it is worth mentioning that several reports of endosymbiotic bacteria in marine and freshwater *Euplotes* predate molecular techniques [reviewed in 93]. As it was customary at the time, many were classified using letters instead of binomial names (*Polynucleobacter* was originally “omicron”). The “epsilon” symbionts of *Euplotes minuta*, a marine species in clade A, were associated with a killer trait of the host reminiscent of that observed in *Paramecium* [94]. “Eta” [95] and “B1” [96] symbionts

apparently conferred a similar feature to another marine species, *Euplotes crassus*. A different strain of *E. crassus* is also the only reported case of *Euplotes* symbionts harbored in the macronucleus instead of the cytoplasm [50]. Other bacteria were only observed in electron microscopy sections, and no function was ever assigned to them. Many were observed in *Euplotes* clade B species in addition to the easily recognizable *Polynucleobacter* [12]. We know now that these accessory bacteria might have belonged to any number of various groups.

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