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1 NEW REPRESENTATIVES OF ORDER PIRSONIALES

2
3 **Refurbishing the marine parasitoid order Pirsoniales with newly (re)described marine**
4 **and freshwater free-living predators**

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20
21 **ABSTRACT** Pirsoniales is a stramenopile order composed of marine parasitoids of diatoms
22 with unique life cycle. Until recently, a single genus, *Pirsonia*, uniting six species, was known.
23 The recent identification of new free-living eukaryotrophic Pirsoniales *P. chemainus*, *Feodosia*
24 *pseudopoda* and *Koktebelia satura* changed our understanding of this group as exclusively
25 parasitic. However, their cell ultrastructure and feeding preferences were not fully studied due
26 to the death of the cultures. In this study, we re-isolated some of these Pirsoniales and
27 established six new strains exhibiting predatory behavior, including a first freshwater
28 representative. This allowed us to describe five new genera and species, as well as to emend
29 the diagnosis of the order Pirsoniales. 18S rRNA gene phylogenetic analysis revealed the
30 position of new strains within Pirsoniales and their relationships with parasitoid relatives and
31 environmental sequence lineages. Feeding experiments on novel Pirsoniales strains using
32 diverse algal prey showed that they were not able to form trophosomes and auxosomes. The
33 ability of cell aggregation in Pirsoniales was observed for the first time. One of the studied
34 strains contained intracellular gammaproteobacteria distantly related to *Coxiella*.
35 Ultrastructural analyses revealed a more complex cytoskeleton structure in Pirsoniales than
36 previously thought and supported the monophyly of Bigyromonadea and Pseudofungi.

37
38 **Keywords**

39 Bigyromonadea; eukaryotrophy; 18S rRNA gene phylogeny; protists; Pseudofungi;
40 ultrastructure.

41
42 Stramenopiles is a large and highly diverse group of eukaryotes, which includes free-living
43 heterotrophic flagellates, unicellular and multicellular photosynthetic algae (e.g., brown algae,
44 golden algae, xanthophytes, diatoms, etc.), and important parasites (oomycetes, *Blastocystis*)
45 (Derelle et al., 2016). They are part of the supergroup SAR (Stramenopiles-Alveolata-Rhizaria)
46 (Adl et al., 2019). However, the phylogenetic relationships of several major stramenopile
47 lineages remain poorly resolved due to the lack of massive sequence data, in particular for
48 heterotrophic representatives, including many MAST (MARine STRamenopile) lineages, whose
49 undescribed diversity was predicted by environmental DNA studies (Logares et al., 2012;
50 Massana et al., 2004, 2006, 2009, 2014, 2015).

51 The biology, morphology, and ecology of many stramenopiles also remain poorly
52 studied. In particular, we know almost nothing about most MAST lineages other than their
53 phylogenetic position in 18S rRNA phylogenetic trees and that they are common in diverse
54 aquatic ecosystems (Logares et al., 2012; Massana et al., 2004, 2006, 2009, 2014, 2015; Simon
55 et al., 2015). It remains to be determined what precise role they play in microbial communities
56 and what the ultrastructural organization of their cells is, although some efforts have been
57 carried out (Cavalier-Smith and Scoble, 2013; Cho et al., 2024; Shiratori et al., 2017). Even
58 long-known groups of heterotrophic stramenopiles, such as Bicosoecida, Labyrinthulea or
59 Hyphochytriomycota, remain poorly studied, and our understanding of entire large taxa is
60 based on scant data from the few described species available, which sometimes leads to
61 incorrect conclusions about the broader ecology and biology of these groups.

62 An illustrative example is that of the Bigyromonadea, a group of marine heterotrophs
63 comprising a few studied species belonging to the subgroups Pirsoniales and Delepliozoa (Cho
64 et al., 2022). Pirsoniales encompasses parasitoid protists initially described in the 1990s and,
65 until recently, included six species in the single genus *Pirsonia*: *P. eucampiae*, *P. diadema*, *P.*
66 *formosa*, *P. guinardiae*, *P. punctigera*, and *P. verrucosa* (Kühn et al., 1996; Schnepf et al.,
67 1990; Schweikert and Schnepf, 1997). One species, originally described as *Pirsonia mucosa*,
68 was transferred to *Pseudopirsonia* (Cercozoa) according to molecular phylogenetic data (Kühn
69 et al., 2004). Pirsoniales are characterized by a unique life cycle and are adapted to consume
70 large diatom cells. The zoospores are attracted to the diatom hosts by chemical signals and
71 form pseudopodia that penetrate the frustule through one or several frustule openings, e.g.,
72 through strutted or labiate processes, openings at the end of the setae, or gaps between girdle
73 bands or less silicified parts of the cingulum (Schweikert, 2015). Subsequently, the
74 pseudopodium forms a special structure inside the host called the trophosome. It digests the
75 host cytoplasm and transports organic matter to the main part of the cell that remains outside –
76 the auxosome. During feeding, auxosomes divide several times, producing secondary
77 auxosomes that attach to each other and to a common trophosome, forming a bouquet-like
78 group of up to 60 descendants (Schweikert and Schnepf, 1997). Several individual zoospores
79 can infect the same diatom cell and their trophosomes can merge. Usually, the Pirsoniales cells
80 retract their flagella during infection, with the exception of *P. eucampiae*, whose flagella are
81 only partially retracted (Kühn et al., 1996). Eventually, after the diatom protoplast is consumed,
82 secondary auxosomes detach and mature into individual zoospores, producing flagella and
83 changing the cell shape from spherical to oval. The trophosomes with residual bodies of
84 undigested chloroplasts remain inside the host (Kühn et al., 1996; Schnepf et al., 1990).

85 Although ultrastructural data obtained for *P. guinardiae*, *P. diadema*, and *P.*
86 *punctigerae* confirmed that they belong to stramenopiles, they also emphasized the uniqueness
87 of these organisms (Schnepf and Schweikert, 1997; Schweikert and Schnepf, 1997). Typical
88 stramenopile features included tripartite tubular mastigonemes on the anterior flagellum,
89 tubular mitochondrial cristae, and a helix in the transition zone of the flagella. Unique
90 structures in *Pirsonia* were the presence of cell body hairs and reduced flagellar apparatus (both
91 posterior flagellar roots are fibrous). The kinetosomes are oriented at a right angle to each other
92 and connected by two fibrous bands: one short and wide proximal striated connecting band and
93 one long and narrow electron-dense distal connecting band. The anterior kinetosome is
94 connected to the nucleus by two nuclear bands. Based on these ultrastructural features,
95 Cavalier-Smith created the new order Pirsoniales within stramenopiles, provisionally placing
96 it in the class Bicoecia, phylum Sagenista (Cavalier-Smith, 1998). The ability to form
97 trophosomes and auxosomes was indicated as a main taxonomic characteristic for this order
98 (Cavalier-Smith, 1998). The first 18S rRNA gene phylogenetic analysis confirmed the
99 placement of Pirsoniales in Stramenopiles, but as a sister clade to hyphochytriomycetes, both
100 related to ochrophytes (Kühn et al., 2004). Subsequent 18S rRNA gene phylogenetic analyses

101 confirmed the closer relationship of Pirsoniales with hyphochytrids, but also with oomycetes
102 and *Developayella elegans* (Cavalier-Smith and Chao, 2006), thus allowing to place them
103 within the phylum Pseudofungi. The authors also placed hyphochytrids, *Pirsonia* and
104 *Developayella* within the class Bigyromonadea.

105 Further phylogenetic and phylogenomic studies have shown contradictory results
106 regarding Pirsoniales (Aleoshin et al., 2016; Leonard et al., 2018; Noguchi et al., 2016; Thakur
107 et al., 2019; Weiler et al., 2021). The monophyly of Bigyromonadea has not been tested by
108 phylogenomic analysis until recently due to the lack of transcriptomic or genomic data, and
109 Bigyromonadea itself has shown different phylogenetic positions, branching with either
110 Ochrophytes or Oomycetes. Recently, transcriptomic data were obtained from three new
111 pirsoniales, *P. chemainus*, *Feodosia pseudopoda* and *Koktebelia satura*, as well as from four
112 new developean species (Cho et al., 2022). Subsequent phylogenomic analyses confirmed the
113 monophyly of Bigyromonadea, which includes two diverse subgroups, Developea and
114 Pirsoniales. Nevertheless, the position of Bigyromonadea within stramenopiles was not fully
115 resolved. Maximum likelihood multigene trees showed its sister relationship with oomycetes,
116 whereas Bayesian analyses and topology testing were inconclusive (Cho et al., 2022).

117 In contrast to all previously known Pirsoniales, the new strains studied by Cho et al.
118 (2022) were eukaryovorous predators feeding on bodonid flagellates in culture. However, they
119 did not actively pursue the prey and only partially consumed it (except for one species, *K.*
120 *satura*), and all the cultures died after a few months of cultivation. Thus, ultrastructural data
121 were not obtained, and the authors did not conduct feeding experiments to determine whether
122 these clones were able to parasitize diatoms and form trophosomes and auxosomes.
123 Nevertheless, Cho et al. (2022) proposed that these Pirsoniales represented motile zoospore
124 stages of parasites capable of facultative eukaryotrophy to survive between host bloom seasons.
125 The morphology of those strains was not studied in detail, and provisional names without
126 formulation of taxonomical diagnoses were provided. These authors also observed some
127 similarities between studied bigyromonadeans and oomycete zoospores in terms of cell
128 morphology, swimming patterns and, especially, the ability to self-aggregate, a feature that was
129 found in developeans for the first time. Taking into account the obtained phylogenomic and
130 morphological results, the authors speculated on the origin of oomycetes, assuming that the
131 ancestor of oomycetes was a phagoheterotrophic amoeboid protist (Cho et al., 2022).

132 Testing this hypothesis and better understanding the global evolution of stramenopiles
133 will require the characterization of other poorly known lineages. This is all true for Pirsoniales,
134 the diversity, morphology and biology of which have not been sufficiently studied despite the
135 fact that they are one of the major clades of the stramenopile tree. In this study, we reisolated
136 two of the three species studied by Cho et al. (2022) as well as three new pirsoniales from
137 different and geographically distant marine and freshwater habitats. All of them were predatory
138 flagellates that consume diverse prey. 18S rRNA gene phylogeny together with morphological
139 and ultrastructural observations allowed us to describe several new taxa and characterize the
140 morphology and biology of Pirsoniales.

141

142 **MATERIALS AND METHODS**

143

144 **Strain isolation and culture maintenance**

145 Nine clonal cultures of predatory pirsoniales (Colp-Nor4, Colp-Nor6, Colp-p1, Colp-S9, Kt49-
146 10a, NY0226, Pirs-sm2, Colp-RSA11, and Pirs-v1) were isolated from different marine and
147 freshwater environments (Table 1). Prey cultures were isolated from the same samples or
148 obtained from colleagues (Table 1). Water samples with the upper sediment layer were taken
149 with sterile 15 ml Falcon tubes. The samples were enriched with 1% YT medium (100 mg of
150 yeast extract and 200 mg of tryptone in 100 ml of distilled water as described in the protocol

151 from the National Institute for Environmental Studies, Japan) to stimulate bacterial growth,
 152 and incubated at 15°C. The samples were examined under a light microscope within 10 days
 153 in search of predatory flagellated cells. Strains were isolated by picking one cell using a glass
 154 micropipette and placed in marine or freshwater media (f/2 diatom media based on filtered and
 155 autoclaved marine water (Guillard and Ryther, 1962) and Volvic (France) mineral water,
 156 respectively) with cultures of eukaryotic prey at 15°C with a 10h light / 14h dark cycle. All the
 157 cultures are currently stored in a collection of live protist cultures in the laboratory Ecologie
 158 Systématique Evolution, CNRS, Université Paris-Saclay (except for Colp-RSA11 and Pirs-
 159 sm2, which were lost).

160 For the feeding experiments, diverse eukaryotic prey, including diatom algae, red algae, and
 161 heterotrophic flagellates (see Table 2), were used as food sources. Single predator cells were
 162 transferred into individual wells of 48-well plates containing 1 ml of enriched prey culture.
 163 Experiments with each predator/prey combination were repeated 6 times and observations were
 164 carried out for three weeks. If a predator multiplied and no eukaryotic contamination was
 165 observed in the prey culture, we assumed the ability of the predator to consume this prey (see
 166 Table 2).

167

168 **18S rRNA PCR amplification, sequence alignment, and phylogenetic analyses**

169 The 18S rRNA genes from all of our strains were amplified using the Platinum Taq polymerase
 170 reaction mix (Invitrogen, Thermo Fisher, France) in 25 µl reaction volumes and two
 171 combinations of universal eukaryotic primers: A) EK-1F (5'-CTGGTTGATCCTGCCAG-3')
 172 and 1520R (5'-CYGCAGGTTACCTAC-3'); B) EK-82F (5'-GAAACTGCGAATGGCTC-3')
 173 and 18S-1498R (5'-CACCTACGGAAACCTTGTTA-3'). PCR amplifications were carried out
 174 using a nested approach. For the first PCR, manually isolated single cells (with eight replicates
 175 of one cell per reaction for each strain) were added to PCR reaction mix with primer
 176 combination A. Then, we used 2 µl of the resulting non-purified PCR products as a template
 177 for subsequent PCR reactions using primer combination B. The touchdown amplification steps
 178 were as follows: initial denaturing period (95°C for 2 min) followed by 35 cycles of
 179 denaturation (95°C for 30 s), annealing (60°C for 30 s in the first cycle, decreasing at 1°C per
 180 cycle until reaching 55°C), extension (72°C for 1 min 30 s), and a final extension period (72°C
 181 for 5 min). In the case of strain Colp-S9, we needed a third nested PCR reaction (with primers
 182 612F (5'-GCAGTTAAAAGCTCGTAGT-3') and 1498R) to produce enough amplicon DNA
 183 for sequencing. Sanger sequencing of PCR amplicons using forward and reverse primers was
 184 performed by Azenta (Leipzig, Germany). For each strain, we used MAFFT (Katoh et al.,
 185 2002) with default settings to align forward and reverse sequences and generate a consensus
 186 sequence using the cloud-based platform Benchling (<https://www.benchling.com/>). The
 187 resulting 18S rRNA gene sequences were submitted to GenBank under accession numbers
 188 PP795565 (strain Colp-Nor4), PP795580 (strain Colp-Nor6), PP795920 (strain KT49-10a),
 189 PP795921 (strain Colp-S9), PP795933 (strain NY0226), PP808807 (strain Colp-RSA11),
 190 PP808838 (strain Pirs-v1), PP808806 (strain Colp-p1), PP808839 (strain Pirs-sm2), PP795975
 191 (strain str-V), PP795979 (strain diat-RSA1).

192 The obtained 18S rRNA gene sequences were added to a dataset containing 97
 193 sequences (collected from the GenBank database) representing main stramenopile clades, as
 194 well as seven outgroup sequences (alveolates and rhizarians). We also constructed a reduced
 195 dataset containing 75 sequences representing all Gyrista clades with and without environmental
 196 sequences. Multiple sequence alignments were constructed using the L-INS-i algorithm in
 197 MAFFT v.7.475 (Katoh and Standley, 2013). Alignments were trimmed using trimAl v.1.2
 198 (Capella-Gutiérrez et al., 2009) with the gappyout method. The numbers of analyzed sites after
 199 trimming were 1,437 bp for the full dataset, 1,514 bp for the reduced dataset, and 1,504 bp for
 200 the dataset with environmental sequences.

201 To infer Bayesian phylogenetic trees, the parallel MPI version of MrBayes v.3.2.7a
202 (Ronquist et al., 2012) was used with four categories of gamma-distributed among-site rate
203 variation under the GTR+I+GAMMA4 substitution model. To calculate posterior probabilities
204 of individual nodes, four independent Metropolis-coupled Markov chains were run for 20
205 million generations and summarized after the 50% burn-in. The convergence of log-likelihood
206 values and model parameters for chains was verified using a plot and convergence diagnostics
207 provided by the MrBayes sump utility. Maximum likelihood phylogenetic trees were inferred
208 using IQ-TREE v.2.2.2.6 (Nguyen et al., 2015) with 1,000 nonparametric bootstrap
209 pseudoreplicates under the GTR+GAMMA+I substitution model. Trees were visualized using
210 the cloud-based platform iTOL (<https://itol.embl.de/>).

211 **Microscopy**

212 Light microscopy observations were made using an inverted Zeiss Axiovert 40 CFL
213 microscope equipped with phase contrast objectives (20× and 40×) and an upright Zeiss
214 Axioplan 2 microscope equipped with an oil-immersion DIC objective (100×) and a water-
215 immersion phase contrast/DIC N-Achroplan objective (63×). Videos were recorded with a
216 Sony α9 digital camera. Images as still frames were captured using a Light Alloy video player
217 (<https://light-alloy.com/>).

218 For epifluorescent microscopy observations, live cells of *Feodosia pseudopoda* strain
219 NY0226 were incubated in the dark for 1 h with Hoechst 33342 (Molecular Probes, Thermo
220 Fisher, France) at a final concentration of 10 µg/ml. The cells were imaged directly in the
221 staining solution without washing with a Zeiss Axioplan 2 epifluorescence microscope
222 equipped with a 49 mm filter set.

223 For scanning electron microscopy (SEM), 1 ml aliquots of cell-rich cultures were mixed
224 with 1 ml of 4% glutaraldehyde (final concentration of 2%) diluted in sterile sea water (for
225 marine strains) or 0.2 M sodium cacodylate buffer, pH 7.2 (for the freshwater strain Pirs-vl).
226 After that, the mixture was deposited on a 9-mm circular cover slip coated with poly-L-lysine
227 and fixed for 30 min. After fixation, the cells were washed with 0.2 M sodium cacodylate buffer
228 (pH 7.2) and dehydrated in a graded series of ethanol baths (30%, 50%, 70%, 80%, 90%, 100%
229 ×3 times, 10 min each). Then, the coverslips with cells were washed with 100%
230 hexamethyldisiloxane three times for 15 min and subsequently dried at room temperature. Dry
231 coverslips were mounted on aluminium stubs, coated with platinum, and observed using a
232 GeminiSEM 500 (Carl Zeiss, Germany) scanning electron microscope.

233 For investigation of the cell ultrastructure in transmission electron microscopy (TEM),
234 aliquots of cell-rich cultures were mixed with glutaraldehyde (sample-to-fixative ratio 1:1)
235 diluted in cacodylate buffer (1.25-2% final concentration) and fixed for 2 h on ice or at room
236 temperature. After fixation, the cell pellets were collected by centrifugation for 10 min at 5,000
237 g, washed with 0.2 M cacodylate buffer and fixed with 2% osmium tetroxide in 0.2 M
238 cacodylate buffer for 1 h at room temperature. Alternatively, a mixture of both fixatives,
239 glutaraldehyde (2.5% final concentration) and osmium tetroxide (1% final concentration), in
240 0.2 M cacodylate buffer was added to the cell cultures and incubated for 30 min on ice. After
241 dehydration in an alcohol series (30, 50, 70, 80, 90, 96, 100% ×2 times, 10 min each) and
242 acetone (100% ×2 times, 10 min), the pellets were embedded in Low Viscosity resin (EM 0300
243 Sigma-Aldrich). Ultrathin sections (70 nm) were prepared with a Leica EM UC6
244 ultramicrotome (Leica Microsystems, Germany) and observed using a JEM 1400 transmission
245 electron microscope (JEOL, Japan).

246
247

248 **16S rRNA gene PCR amplification and phylogenetic analysis of the bacterial** 249 **endosymbiont of strain NY0226**

250 The 16S rRNA gene of endosymbiotic bacteria was amplified with Platinum Taq polymerase
251 reaction mix (Invitrogen, Thermo Fisher, France) in 25 µl reaction volumes and two
252 combinations of universal bacterial primers: A) 27F (5'- AGAGTTTGATCCTGGCTCAG-3')
253 and 1492R (5'- TACCTTGTTACGACTT-3'); B) 63F (5'- CAGGCCTAACACATGCAAGTC-
254 3') and B-1492R. PCR amplifications were carried out using a nested approach. For the first
255 PCR, we added manually isolated and three times washed single cells of strain NY0226 (eight
256 replicates) to a PCR reaction mixture containing primer combination A. Then, we used 1 µl of
257 a 50-fold dilution of the resulting non-purified PCR products as a template for subsequent
258 PCRs using primer combination B. The touchdown amplification steps were as follows: initial
259 denaturing period (95°C for 2 min) followed by 35 cycles of denaturation (95°C for 30 s),
260 annealing (60°C for 30 s in the first cycle, decreasing at 1°C per cycle until reaching 55°C),
261 extension (72°C for 1 min 30 s), and a final extension period (72°C for 5 min). Sanger
262 sequencing of eight amplification products was performed by Azenta (Leipzig, Germany). We
263 used MAFFT (Katoh et al., 2002) with default settings to align the sequences and obtain a
264 consensus sequence using the cloud-based platform Benchling (<https://www.benchling.com/>).
265 The resulting 16S rRNA gene sequence was submitted to GenBank under accession number
266 PP809757.

267 The new 16S rRNA gene sequence was added to a dataset containing 101
268 gammaproteobacterial sequences and seven sequences from Alphaproteobacteria as an
269 outgroup and aligned using MAFFT with default settings (Katoh et al., 2019). Ambiguously
270 aligned positions were trimmed off with trimAL v.1.2 (Capella-Gutiérrez et al., 2009) using a
271 gap threshold of 40% and a minimum percentage of the positions in the original alignment to
272 conserve of 20%. The number of analysed sites after trimming was 1,506 bp. Maximum
273 likelihood phylogenetic trees were inferred using IQ-TREE v.2.2.2.6 (Nguyen et al., 2015) with
274 1,000 nonparametric bootstrap pseudoreplicates under the best fit model (TPM3+R6 model)
275 determined by the in-built ModelFinder (Posada and Crandall, 1998). The tree was visualized
276 using the cloud-based platform iTOL (<https://itol.embl.de/>).

277

278 **RESULTS**

279

280 **18S rRNA gene phylogenetic analysis of new Pirsoniales strains**

281 Using single-cell PCR, we amplified and Sanger-sequenced 18S rRNA genes from nine
282 Pirsoniales strains. The lengths of amplicon sequences were as follow: 1,611 bp (KT49-10a),
283 1,664 bp (NY0226), 1,055 bp (Colp-S9), 1,644 bp (Colp-p1), 1,567 bp (Pirs-sm2), 1,593 bp
284 (Pirs-vl), 1,641 bp (Colp-RSA11), 1,629 bp (Colp-Nor6), and 1,642 bp (Colp-Nor4).

285 Preliminary BLAST (Altschul et al., 1990) searches against the nonredundant (nr)
286 GenBank database indicated that the new Pirsoniales were similar to *Pirsonia* spp., sharing
287 94.71-99.94% identity in the 18S rRNA sequences (Table S1). Therefore, we prepared a
288 multiple sequence alignment containing the newly obtained nine sequences and representative
289 sequences of Pirsoniales and Gyrista (Stramenopiles) for Maximum likelihood (ML) and
290 Bayesian (BI) phylogenetic analyses (Figure 1, S1, S2). Both analyses placed our strains within
291 Pirsoniales with full statistical support. The three studied strains (NY0226, Colp-S9, and KT49-
292 10a) grouped with *Feodosia pseudopoda* strain Chromo-2 with full support (Figures 1, S1, S2)
293 and shared 98.86-99.91% 18S rRNA nucleotide identity with this species (Table S1),
294 suggesting that they likely belong to the same species. A marine strain, Colp-p1, formed a sister
295 lineage to the *Feodosia* clade, separated from the known genera of Pirsoniales. It therefore
296 represented a new genus and species that we named *Bordeauxia parva* gen. et sp. nov. The 18S
297 rRNA gene sequences of two freshwater strains, Colp-RSA11 and Pirs-vl, were almost

298 identical (99.94% nucleotide identity) and formed an independent clade from other genera,
 299 sister to the *Feodosia* and *Bordeauxia* grouping. We propose that these strains represented a
 300 new genus and species, *Bullionia fluviatilis* gen. et sp. nov. Two of the studied strains (Colp-
 301 Nor4 and Colp-Nor6) grouped with the species *Koktebelia satura* strain Chromo-2 and shared
 302 98.98-99.66% 18S rRNA nucleotide identity with it (Table S1). Finally, the marine strain Pirs-
 303 sm2 formed a sister lineage to the clade uniting the representatives of the parasitoid family
 304 Pirsoniaceae, thus representing a new genus and species, *Noirmoutieria diatomophaga* gen. et
 305 sp. nov. The phylogenetic analyses that included environmental sequences (Figure S1)
 306 illustrated some undescribed diversity in the order Pirsoniales in natural environments, closely
 307 related to the genera *Noirmoutieria*, *Koktebelia* and *Bullionia*.

308

309 Overall morphology and feeding behavior

310 All studied strains exhibited a common morphological plan but possessed some characteristic
 311 differences from each other and from previously described Pirsoniales (Figures 2-4, Table S2).
 312 All strains were free-living colorless flagellates with elongated oval non-flattened cells but
 313 could undergo significant changes in cell shape depending on the shape of the swallowed prey
 314 (Video S1). The starved cells tended to be small (Figures 3B,R, 4D). All the strains had a
 315 longitudinal groove on the ventral side of the cell (Figures 2B, 3E,N,P,V, 4B). Two flagella
 316 were inserted subapically or almost laterally from a ventral bulge (protrusion) located in the
 317 middle region of the ventral groove and were directed oppositely (Figures 2B,
 318 3A,B,G,H,N,R,U, 4C-E), the anterior flagellum (AF) directed anteriorly and the posterior
 319 flagellum (PF) directed posteriorly. In this way, the anterior part of the ventral groove looked
 320 like the subapical anterior notch. Well-fed cells usually possessed large food vacuoles in their
 321 posterior part (Figures 3A,H,S, 4C,D) and their cytoplasm was filled with round refractive
 322 granules of reserve material, such as lipid droplets, which were also observed in thin sections
 323 (see below). Characteristic cell movements were a distinctive feature for all Pirsoniales. Cells
 324 swam in a straight line without rotation, with jerking movements and sudden sharp changes in
 325 direction, or attached to a substrate with the posterior flagellum while the anterior flagellum
 326 beat quickly (Video S1). Flattened and non-flattened resting cells varied in shape (Figures
 327 3F,L,M,O-T,V,Y-AB, 4B) and their flagella usually made slow sinusoidal movements.
 328 Swimming cells could be found in the water column; however, they were mostly concentrated
 329 at the bottom of the Petri dishes used for observations.

330 Unlike the previously described parasitoid *Pirsonia*, the new strains were able to
 331 swallow eukaryotic prey entirely (with some exceptions for the strain Colp-S9, see below)
 332 using a posterior pseudopodium. Our feeding experiments showed that the new strains
 333 consumed a wide range of eukaryotic prey (Table 2) depending on the strain, from a single
 334 acceptable prey to almost any proposed prey, and with varying growth success depending on
 335 the suitability of the proposed prey.

336 *Bordeauxia parva* strain Colp-p1 (Figures 2A,B, 3A-F)

337 Cells with a weakly pronounced longitudinal ventral groove (Figure 3E), but noticeable
 338 ventral bulge and anterior notch (Figure 3A,B). Cells are 6.5-14.0 μm in length and 4-8 μm
 339 in width. The starved cells are more elongated with a narrowed posterior end (Figure 3B). The
 340 AF is 2-2.5 times longer than the cell, and the PF is 1-2 times longer than the cell. The AF is
 341 acronematic, and both flagella are covered with hairs (Figures 2A). The food vacuoles and
 342 refractive granules of reserve material are positioned in the posterior part of the cell (Figure
 343 3A-C). Small pointed posterior pseudopodia are seen on some of the attached cells (Figure
 344 3C,D). Resting cells are oval, with slowly waving flagella directed sideways or backwards
 345 (Figure 3E,F). From the prey offered here, *Bordeauxia parva* feeds exclusively on the colorless
 346 chrysophyte alga *Paraphysomonas lucasi* Scoble et Cavalier-Smith, 2014 strain str-V (Table
 347 2). The cell population can reach high numbers in culture with abundant prey.

348 ***Bullionia fluviatilis* strains Pirs-vl and Colp-RSA11 (Figures 2C-E, 3G-M)**

349 Cells are oval but may take the shape of the diatom prey they swallow (Figure 3I-K,
 350 Video S1). Starved cells with narrowed anterior and posterior ends (Figure 3G). Cells are 8-15
 351 μm in length (up to 25 μm when a large diatom cell is consumed) and 6.5-12 μm in width. The
 352 single contractile vacuole is located close to the anterior end of the cell (Figure 3G-M). The
 353 ventral groove is poorly developed, and the ventral bulge is well pronounced (Figure 3G,H).
 354 AF is 1-1.5 times longer than the cell, and PF is very long, up to 4 times longer than the cell
 355 body (20-55 μm in length). The AF bears a short acroneme (Figure 2C,E). Well-fed cells
 356 contain many large round refractive granules of reserve material; some cells are fully filled
 357 with them (Figure 3L,M). Resting cells (Figure 3L,M) are usually oval, rarely round and
 358 flattened (Figure 3L). The flagella of resting cells are coiled near the cell body and remain
 359 motionless or very slowly wriggle in sinusoids.

360 Strain Colp-RSA11 feeds on the heterotrophic flagellate *Bodo saltans* Ehrenberg, 1832,
 361 but the culture died soon after isolation. Numerous attempts to re-isolate this strain from the
 362 same sample on diverse eukaryotic prey, including the green alga *Pseudomuriella engadinensis*
 363 (Kol et Chodat, 1934) Fuciková et al., 2011, the xanthophyte alga *Tribonema gayanum*
 364 Pascher, 1925, the naked amoeba *Paravanella minima* Kudryavtsev, 2014, and the chrysophyte
 365 heterotrophic flagellate *Spumella* sp., were unsuccessful. The strain Pirs-vl (Table 1) was
 366 isolated from another sample and fed on the freshwater diatom *Stephanocyclus meneghiniana*
 367 (Kützing, 1844) Kulikovskiy et al., 2022 (strain diat-RSA1) as a prey.

368 ***Koktebelia satura* strains Colp-Nor4 and Colp-Nor6 (Figure 3N-T)**

369 Cells are 10.5-16 μm in length and 8-13 μm in width. The AF is 1.5 times longer than
 370 the cell, and the PF is 1.5-2.5 times longer than the cell. Starved cells with a noticeable anterior
 371 notch and a ventral bulge from which flagella emerge (Figure 3R). Resting cells usually
 372 maintain an oval shape and possess a visible longitudinal ventral groove (Figure 3O,P, Video
 373 S2). Sometimes they produce several finger-like pseudopodia (Figure 3Q, Video S2). Flagella
 374 of the resting cells can extend into longitudinal planes and quickly undulate so that the flagella
 375 appear to double (Figure 3O, Video S2), or they can be pulled to the cell body and slowly
 376 wriggle sinusoidally (Figure 3R). Cells form clusters of resting cells on the substrate in starving
 377 cultures (Figure 3T, Videos S3). Despite 1% nucleotide dissimilarity in 18S rRNA gene
 378 sequence, both strains did not show any differences in morphology or pattern of cell movement
 379 and had identical food preferences (Table 2).

380 The strains consumed almost any prey offered to them, including all heterotrophic
 381 flagellates, almost all red algae (except for one species, *Galdieria* sp., which was partially
 382 consumed but predators reproduce slowly, and the culture eventually died), and most diatoms
 383 of small size suitable for swallowing.

384 ***Noimoutieria diatomophaga* strain Pirs-sm2 (Figure 3U-AB)**

385 Cells are elongated with a narrowed anterior end (Figure 3U,Y) and a well-pronounced
 386 longitudinal ventral groove (Figure 3V, Video S4). Cells can change shape after swallowing
 387 diatom prey (Figure 3W). Cells are 9-13 μm in length and 6-10 μm in width. AF is 2-2.5 times
 388 longer than the cell, and PF is 2.5-3.5 times longer than the cell. Cells rarely shed off flagella
 389 (Figure 3V, Video S4). Resting cells are oval, round and irregular in shape (Figure 3V,Y-AB),
 390 sometimes without flagella (Figure 3AB). Strain Pirs-sm2 consumed several species of diatoms
 391 and bodonids (Table 2) and reproduced slowly, never reached a high density in culture, and
 392 eventually died after a year of cultivation.

393 ***Feodosia pseudopoda* strains Colp-S9, KT49-10a, and NY0226 (Figures 2F-H, 4)**

394 Cells with well-developed longitudinal ventral groove (Figure 4B, Video S5), 12-22
 395 μm in length and 9-16 μm in width. Cells can change shape greatly after swallowing diatom
 396 prey (Figures 4E,F,G-J). Rarely produce finger-like pseudopodia at any part of the cell body
 397 (Figure 4A). Resting cells are oval, round or irregular. Both flagella are 1.5-2 times longer than

398 the cell (Figure 2F,G). Well-fed cells (Figure 4C) can be significantly larger than starved cells
399 (Figure 4D).

400 *Feodosia pseudopoda* consumes a variety of prey such as diatoms, red algae and
401 bodonids (Table 2). The grow success of the studied strains vary depending on the prey; they
402 grow best on the diatom *Attheya* sp. and *Chaetoceros neogracile*. In general, the prey range for
403 strain NY0226 slightly differs from other *Feodosia* strains (Table 2). When feeding on small
404 diatoms such as *Attheya* sp., the diatom frustules are compressed into compact lumps inside
405 the predator cell, sometimes accumulated from several prey cells, and subsequently excreted.
406 Such lumps can be found on the bottom of the culture Petri dish (Figure 4L,M).

407 Cells of the Colp-S9 strain are able to consume large diatoms (up to 100 µm in length),
408 such as *Licmophora* sp., *Coscinodiscus radiatus* and cf. *Pinnularia* sp. (Figure 4G-J,N-S) using
409 two different strategies. In the case of *Licmophora* sp. (Figure 4G-J), the predators are able to
410 engulf almost the whole diatom, which may reach up to 45 µm in length. This feeding strategy
411 was very successful, and all the diatoms in the culture were consumed. After feeding, the
412 unchanged empty frustules were released by the predator (Figure 4K), in contrast with the
413 deformed frustules of *Attheya* sp.

414 In the case of *Coscinodiscus radiatus* (Figure 4N-Q) and cf. *Pinnularia* sp. (Figure
415 4R,S), the diatoms were too large to be swallowed, and the predators used another strategy.
416 They attached to diatoms and penetrated with broad pseudopodia through some of the wide
417 openings of the diatom frustules. It is unclear which openings were used to reach the diatom
418 protoplast; however, openings should be large enough to allow parts of the protoplast and
419 individual chloroplasts to be transported outside the diatom frustule, as visible in diatom cells
420 after predator attack (Figure 4T). As confirmation of this, we observed large food vacuoles
421 with chloroplasts in the attached predator cells outside the diatom frustules (Figure 4O-Q), but
422 we never observed the formation of trophosomes. We hypothesize that *Feodosia* uses thin or
423 less silicified parts of the diatom cingulum for penetration, possibly during the diatom division
424 process when the cingulum becomes vulnerable. Several individual cells can attack one diatom.
425 Not all diatom cells are attacked in culture, and some diatom cells seem to be more suitable for
426 *Feodosia* than others. Flagella of the predator always remain full length and actively beating.

427 The strains Colp-S9 and KT49-10a can form giant irregularly shaped multinucleated
428 cells with several sets of flagella in the presence of a large amount of diatom prey (Figure 4V-
429 X, Videos S6, S7). These unusual forms are the result of several consecutive incomplete cell
430 divisions when daughter cells remain connected to each other by posterior ends or by long thin
431 pseudopodia. Often, such cells can share a common large food vacuole (Figure 4V-X, Video
432 S7) that is much larger than a single prey cell and represents a lump of accumulated deformed
433 diatom shells and undigested protoplasts. With a decrease in the number of prey cells, the
434 formation of multinucleated cells gradually fades and disappears.

435 We also observed self-aggregation of cells in strain KT49-10a (Figure 4U, Video S8)
436 when cells reached large numbers. Individual cells were actively attracted and merged into a
437 single mass as opposed to the above-described incomplete division.

438

439 **Ultrastructure of *Bordeauxia*, *Feodosia* and *Bullionia***

440 ***Bordeauxia parva* (Figure 5, 6, 7)**

441 Cells are covered with hair-like structures of about 0.2 µm in length (Figures 5A,C,F).
442 The nucleus is elongated or pear-shaped (Figure 5A), 2.5-3.5 µm long and 1.5-2 µm wide. Its
443 narrowed part bears two papillae (Figure 6B) connected with the posterior kinetosome by
444 fibrous bands (see below). Single nucleolus situated asymmetrically in the widest part of the
445 nucleus. Numerous elongated mitochondria are evenly distributed in the cell and contain
446 tubular cristae (Figures 5A,D,E) with a central filament in every crista (Figure 5E, insert).
447 Large flattened vesicles underlying the plasmalemma (Figure 5A-C, 6R) tend to swell during

448 fixation, as the nucleus envelope and endoplasmic reticulum (ER). Some flattened vesicles
449 contain flocculent material (Figure 5C) that may represent a fixation artifact. The cytoplasm
450 contains many lipid droplets 0.3-0.5 μm in diameter (Figure 5A,B,E) and microbodies (Figure
451 5I) possibly involved in lipid biosynthesis. The Golgi apparatus, with numerous small vesicular
452 bodies (Figures 5G, 6R), is located antero-ventrally in close connection with the nucleus and
453 kinetosomes.

454 The AF is covered with tubular mastigonemes, sometimes visible on thin sections (not
455 shown). The PF is covered with simple short hairs similar to the hair-like structures covering
456 the cell surface (Figure 5F). The flagella are frequently shed off during fixation. Tubular
457 mastigonemes are found in dilations of endoplasmic reticulum (Figure 5A,H).

458 Flagellar kinetosomes have cartwheel structures in the proximal part (Figure 6B). The
459 transition zone of the kinetosomes contains a transverse plate inside axoneme and a prominent
460 annular connection outside the axoneme, both at the same level (Figure 6A). The axosome is
461 located just above the transverse plate, sometimes with a central electron-dense plate (Figure
462 6A). A large and prominent central dense hub is located below the transverse plate (Figure 6A).
463 An indistinct single transitional helix with about 3-5 gyres is placed above the transverse plate
464 inside the doublets (Figure 6A). The distal part of the kinetosome is surrounded by transitional
465 fibers visible on both longitudinal (Figure 6A) and transverse sections (Figure 6E).

466 A schematic illustration of the flagellar apparatus is shown in Figure 7. The
467 kinetosomes diverge to each other in wide angle, of about 120° (Figure 6C,F-N, 7). The
468 proximal part of the posterior kinetosome (k1) is shifted to the side of the anterior kinetosome
469 (k2) (Figures 6F-N, 7). The kinetosomes are not in the same plane and k2 is slightly turned to
470 the left. The kinetosomes are connected to each other by two fibrous bands. A short and wide
471 proximal striated connecting band is adjacent to the proximal ends of kinetosomes (Figures
472 6L,N, 7) and a long and narrow distal connecting band is located closer to the plasmalemma
473 and connects the distal parts of the kinetosomes (Figures 6C, 7). Each kinetosome is supported
474 by some additional osmiophilic materials. K1 is connected to the nucleus by two nuclear bands
475 that run from both sides from the proximal part of the kinetosome to the nuclear papilla (Figure
476 6B). Several microtubules are originated in this region and directed to the narrow part of the
477 nucleus (Figure 5J-L).

478 There are four microtubular roots associated with the kinetosomes. Two microtubular
479 roots are associated with k1, corresponding to r1 and r2 in stramenopiles (Moestrup, 2000;
480 Yubuki and Leander, 2013). They originate from the left and right sides of k1 and are connected
481 proximally with each other by the same fiber, which passes near the kinetosome junction closer
482 to k1 (Figures 6B,E,K, 7). Both r1 and r2 are directed posteriorly along the left and right edges
483 of the ventral groove, respectively (Figures 6J-N). The left posterior root (r1) originates from
484 the left side of k1 (Figures 6D-N, 7) and consists of 3-5 microtubules. The right posterior root
485 (r2) originates from the right side of k1. It consists of 8-9 microtubules oriented in semicircle
486 in its proximal part (Figures 6B,D, 7) and of fibrous core material inside the semicircle, which
487 originates from k1 (Figure 7). The fibrous core material terminates in the middle part of r2,
488 while the r2 microtubules separate into two flattened parallel rows (Figure 7). Both posterior
489 roots meet with each other as soon as the ventral groove terminates and become parallel (Figure
490 6O).

491 Two anterior microtubular roots are associated with k2. A large and prominent right
492 anterior root (r3) originates near the right proximal end of k2 and is directed clockwise to the
493 anterior pole of the cell along the anterior part of the ventral groove (Figures 6J,P,Q, 7). It
494 consists of three microtubules, reinforced with fibrous material, and several secondary
495 microtubules (ribs) extending perpendicular to the three main microtubules towards the right-
496 dorsal side of the cell (Figures 6J,K,P,Q, 7). An inconspicuous right anterior root r4 consists

497 of 1-2 microtubules (Figure 7). It originates from the left side of k2 and is directed to the left
 498 under the plasmalemma, perpendicular to r3 (Figures 6R, 7).

499 ***Feodosia pseudopoda* strains NY0226 (Figure 8A-H) and KT49-10A (Figure 8I-P)**

500 As in *Bordeauxia parva*, cells are covered with hair-like structures of about 0.2-0.25
 501 μm in length (Figure 8B). Tubular mastigonemes are visible in dilated parts of the ER (Figure
 502 8E,I). The round nucleus is 2.5-3 μm in diameter (Figure 8A,I) with a slightly asymmetrical
 503 nucleolus. Two small nuclear papillae (protrusions) are located near the kinetosomes (Figure
 504 8G,H,K) and associated with two nuclear bands (see below). Sometimes large multinucleated
 505 cells are found on thin sections (not shown). Numerous elongated mitochondria with tubular
 506 cristae, each containing a central filament, were evenly distributed throughout the cell (Figure
 507 8L). In contrast with *B. parva*, this species does not contain flattened cortical vesicles. Cells of
 508 strain NY0226 contained numerous endosymbiotic bacteria located in the cytoplasm (more
 509 than 70 bacteria in each host cell). The size of these bacterial cells is about 0.7-1 \times 0.25 μm
 510 (Figure 8A,D,E). NY0226 cells also contain large spherical vesicles with flocculent material
 511 (Figure 8A,C), mostly on periphery of the cells, and flattened vesicles with similar flocculent
 512 material around the nucleus (not shown). Cells of strain KT49-10a are filled with numerous
 513 small vesicles (Figure 8I,J). Both strains have microbodies (Figure 8A,C), multivesicular
 514 bodies (Figure 8B), and lipid droplets of 0.7-2 μm in diameter (Figure 8J). Food vacuoles
 515 contain undigested crumpled diatom frustules (Figure 8J).

516 Although we did not study the flagellar apparatus of *F. pseudopoda* in detail, we found
 517 many similarities in its structure with *B. parva*. Kinetosomes are located in different planes and
 518 diverge in a wide angle of about 120° (Figure 8K,M). The proximal end of k1 is shifted to the
 519 side of k2 (Figure 8N-P). The transition zone contains a transverse plate with a sometimes
 520 visible electron-dense central thickening (Figure 8M). The prominent annular connection is
 521 slightly displaced distally to the transverse plate (Figure 8K,M-P). A single transitional helix
 522 with 3-5 gyres is located inside the axoneme above the transverse plate (Figure 8K,M-P). The
 523 axosome and central dense hub are adjacent to the transverse plate from distal and proximal
 524 sides, respectively (Figure 8K,M). Probable acorn-V filaments are sometimes visible at the
 525 level of the proximal end of the central dense hub (Figure 8K). The proximal part of the
 526 kinetosome has cartwheel structure (Figure 8K,M). Transitional fibers are visible outside the
 527 distal part of the kinetosomes, below the annular connection (Figure 8K). The kinetosomes are
 528 connected to each other by a proximal striated connecting band (Figure 8K) and a distal
 529 connecting band (Figure 8M). The k1 is connected to the nucleus by two long and thin nuclear
 530 bands (Figure 8G,H).

531 The posterior microtubular roots have similar structure with those in *B. parva*. Roots r1
 532 and r2 are connected proximally by fibrous material with k1 (Figure 8N) and both are directed
 533 backwards along the left and right edges of the ventral groove (Figure 8N-P). The right
 534 posterior root (r2) consists of about eight microtubules oriented in semicircle in its proximal
 535 part (Figure 8F) and a core fibrous material inside this semicircle (Figure 8F,N-P).

536 ***Bullionia fluviatilis* strain Pirs-v1 (Figure 9)**

537 Cells are covered with hair-like structures about 0.2 μm long (Figure 9F,H-J). The
 538 nucleus is roundish or irregular (Figure 9A,B), 2.5-3.5 μm in diameter and with a slightly
 539 asymmetric nucleolus. Two prominent nuclear papillae (Figure 9A,G-I) connect the nucleus
 540 with k1 by long and striated nuclear bands (Figure 9D,G-I). Elongated mitochondria contain a
 541 dense matrix and tubular cristae with central filaments (Figure 9A-C). The Golgi apparatus,
 542 with numerous vesicular bodies (Figures 9D,H-J), is located anterior-ventrally, in close
 543 connection with the nucleus and flagellar kinetosomes. Large lipid droplets (up to 2 μm) are
 544 found in the posterior part of the cell (Figure 9A).

545 Kinetosomes are oriented in a wide angle in different planes (Figure 9D,E). Their
 546 transition zones consist of a transverse plate (Figure 9D,E) with associated electron-dense

547 central thick region visible on some sections (Figure 9E). A dense annular connection is located
548 almost at the same level as the transverse plate outside of axoneme (Figure 9D,E). A single
549 transitional helix with six gyres (Figure 9D,E) is located inside the axoneme above the
550 transverse plate. The axosome and central dense hub are adjacent to the transverse plate from
551 distal and proximal sides, respectively (Figure 9D,E). A probable acorn-V system is visible at
552 the level of the proximal end of the central dense hub, right after the triplet microtubules are
553 replaced by doublets (Figure 8K). The proximal part of the kinetosome has cartwheel structure
554 (Figure 9B). The kinetosomes are connected to each other by a proximal striated connecting
555 band (not shown) and a distal connecting band (Figure 9D). Posterior microtubular roots are
556 connected by additional fibers (Figure 9H-J). The left posterior root r1 consist of about 4
557 microtubules arranged in a flat row (Figure 9F,H-J). The right posterior root r2 consists of
558 about 8-9 microtubules oriented in semicircle in its proximal part and a core fibrous material
559 inside the semicircle (Figure 9F,H-J).

560

561 **Bacterial endosymbiont of *Feodosia pseudopoda***

562 Using single cell picking and PCR, we isolated eight cells of strain NY0226 and amplified and
563 Sanger-sequenced the 16S rRNA gene of the intracellular bacterium observed in this species
564 (see above). The amplicon sequence was 1,388 bp long. Preliminary BLAST (Altschul et al.,
565 1990) searches against the nonredundant (nr) GenBank database retrieved the obligate
566 intracellular bacterial pathogen *Coxiella burnetii* as the first hit. Therefore, we included this
567 sequence in a multiple sequence alignment containing representatives of the main
568 Gammaproteobacteria lineages and reconstructed a ML phylogenetic tree. Phylogenetic
569 analysis placed the endosymbiont within a fully supported clade of marine environmental
570 sequences which was sister, albeit with moderate support, to *Coxiella* species (Figure 10A).

571 Using Hoechst fluorescent DNA staining, we visualized the bacterial endosymbionts
572 inside the four-nucleated host cells under a fluorescence microscope (Figure 10B-D). The
573 number and size of the intracellular bacteria matched the results obtained from the TEM thin
574 sections (Figure 8A,D,E).

575

576 **DISCUSSION**

577

578 **Feeding behavior of predatory and parasitoid Pirsoniales**

579 Pirsoniales is a group of marine parasitoids of diatoms with unusual life cycle adapted to
580 consume large diatom cells covered in hard siliceous frustules. A unique morphological
581 characteristic of this group is the separation of the cell into the main part (the auxosome), which
582 remains outside the host frustule, and the trophosome, whose function is only to digest diatom
583 protoplasts and transport disintegrated food to the auxosome through a very thin thread.

584 *Pirsonia* species have traditionally been considered parasitoids because they depend on
585 their host to complete their life cycle and the host almost always dies after an attack (Anderson
586 and May, 1978; Kühn et al., 1996; Schweikert, 2015; Skovgaard, 2014). The first observation
587 of predation (i.e., swallowing and digesting the whole prey cell) in the order Pirsoniales was
588 poorly documented (Cho et al., 2022). However, now we can assert with confidence that
589 predation of small diatoms and other protists is not only as common as parasitism in the order
590 Pirsoniales, but even prevails (given the larger number of identified predatory genera). The
591 assumption about the facultative nature of predation in the previously studied *Feodosia*
592 *pseudopoda* and *Koktebelia satura* (Cho et al., 2022) seems unlikely. These species survive
593 well on a variety of prey, and the success of reproduction and food consumption vary greatly
594 depending on the prey (Table 2). It is not surprising that previously studied predatory
595 Pirsoniales did not actively pursue the bodonid prey *Proccryptobia sorokini*, as also observed
596 in our feeding experiments (one strain, NY0226, did not consume this prey at all). On the basis

597 of our observations, it seems that algal prey is preferred by these species. All our cultures,
598 except for *Noirmoutieria diatomophaga*, were maintained for several years, which indicates
599 that they are stable and that their prey are suitable for prolonged growth. Facultative predation
600 still needs to be examined for *Pirsonia chemainus* and *Noirmoutieria diatomophaga* because
601 neither species survived in culture, and feeding experiments have been carried out partially for
602 *Noirmoutieria diatomophaga* (without experiments on large diatoms) or are completely absent
603 for *Pirsonia chemainus*. However, it is interesting to note that the parasitoid *Pirsonia* is closely
604 related to organisms capable of effective predation, which allows us to assume that parasitism
605 has only recently evolved in this group.

606 To further confirm that *Feodosia pseudopoda* and *Koktebelia satura* are not parasitoids,
607 we conducted feeding experiments with large diatom cells. Despite the ability of the *F.*
608 *pseudopoda* strain Colp-S9 to feed on large diatoms, we never observed the formation of
609 trophosomes inside the diatom frustules. At the same time, this strain demonstrated a variety
610 of feeding strategies for different types of diatom prey. These differences are probably due to
611 variations in the structure of diatom frustules and require further study. One of the largest
612 diatoms that Colp-S9 was able to consume by swallowing was the triangular diatom
613 *Licmophora* sp., which is up to 45 μm long. When consumed, *Licmophora* sp. frustules
614 maintain their shape and do not shrink into a shapeless lump, as occurs for less silicified
615 frustules of the diatom *Attheya* sp. Parts of the diatom protoplast are probably delivered into
616 the predator cell through some wide openings in the diatom frustule (e.g., openings at the end
617 of the setae or gaps between girdle bands). Strutted or labiate processes are too small to allow
618 large parts of the protoplast to be transferred outside of diatom frustules. We never observed
619 the formation of trophosomes inside the diatom. Thus, digestion should take place outside of
620 the diatom frustule. The ability of Colp-S9 cells to consume diatoms significantly larger than
621 themselves is not unique in stramenopiles. For example, *Paraphysomonas* sp. can swallow
622 diatom prey up to 10 times larger (Suttle et al., 1986).

623 The feeding strategy of Colp-S9 on two other large diatoms, *Coscinodiscus radiatus*
624 and cf. *Pinnularia* sp., was apparently different. Predators attach to diatom frustules and release
625 a wide pseudopodium, which is reminiscent of the attachment mechanism of the parasitoid
626 *Pirsonia*. However, this pseudopodium penetrates through some holes and transports parts of
627 diatom protoplast and individual chloroplasts to the main cell part of the predator without
628 formation of a trophosome inside the frustule. The food vacuole is formed in this main part of
629 the predator cell (Figure 4O-Q), which is a major difference from parasitoid behavior, where
630 digestion occurs in the trophosome, outside the main body of the parasite. Frustule holes, the
631 nature of which is unclear, should be wide enough to allow parts of diatom protoplasts and
632 even individual chloroplasts to pass through. They may be less silicified parts of the cingulum,
633 possibly during the diatom division process when the cingulum becomes vulnerable, or some
634 frustule damages. This is confirmed by the fact that less than half of the diatom cells were
635 consumed in culture. Interestingly, Colp-S9 was not able to consume many other large diatom
636 species.

637 Another marked difference in feeding on large diatoms between Colp-S9 and the
638 parasitoid *Pirsonia* is that predators maintain full-length and actively beating flagella, thus
639 continuing to swim. The flagella of the parasitoid *Pirsonia* always retract during infection,
640 except *P. eucampiae*, whose flagella are only partially retracted (Kühn et al., 1996). Likewise,
641 when Colp-S9 forms multiflagellated cells through multiple sequential incomplete cell
642 divisions, daughter flagellates remain active and determine the movement of the entire
643 organism. On the other hand, some resting cells of *Noirmoutieria diatomophaga* shed their
644 flagella, resulting in non-flagellated round or irregular amoeboid cells resembling auxosomes,
645 as was noted for eukaryotrophic Pirsoniales (Cho et al., 2022).

646 It is important to note that parasitoid *Pirsonia* species also demonstrate variability in
 647 feeding mechanisms. For example, *P. diadema* and *P. punctigerae* use only strutted processes
 648 to enter diatom frustules, while *P. guinardiae* can also use the overlap between the two valvae
 649 in the girdle region, and *P. punctigerae* sometimes uses large areolae in the center of the valvae
 650 (Schweikert and Schnepf, 1997). When most *Pirsonia* species feed, the host cytoplasm
 651 migrates to the site of infection (Kühn et al., 1996; Schnepf et al., 1990), whereas when infected
 652 by *P. punctigerae*, the host cytoplasm moves in the opposite direction (Schweikert and
 653 Schnepf, 1997). On the basis of the limited studies available, most *Pirsonia* species appear to
 654 be host-specific (Table S2), with the exception of *P. formosa*, which is able to infect up to nine
 655 diatom species (Kühn et al., 1996, 2004). Little is known about the feeding strategies of *P.*
 656 *formosa* in relation to its diverse hosts. One possible example of adaptation to parasites on
 657 different diatom species is the ability to infect different life stages of the hosts (vegetative cells,
 658 dormant spores or naked protoplasts only) (Kühn et al., 1996, 2004). It is also known that the
 659 size of the infected diatom determines the growth of the parasite (the larger the host cell is, the
 660 greater the number of parasite cell divisions).

661

662 **Morphological features of Pirsoniales and comparison with related groups**

663 The presence of a ventral groove interspersed with a protrusion of the flagella emergence site
 664 is a common morphological feature of all Pirsoniales. This feature differentiates them from the
 665 sister group Developea. Although developeans have a ventral depression that is homologous to
 666 a groove, they lack a protrusion dividing it into anterior and posterior parts. Unlike in
 667 Pirsoniales, where flagella emerge from the ventral protrusion, flagella of developeans emerge
 668 from the bottom of the anterior part of the ventral depression (Aleoshin et al., 2016; Cho et al.,
 669 2022; Tong, 1995; Weiler et al., 2021). The ventral groove of developeans is not divided into
 670 anterior and posterior parts. Thus, the body plane of Pirsoniales is more similar to that of
 671 oomycete zoospores, which have well-defined ventral grooves and prominent protrusions (Ho
 672 et al., 1968; Lunney and Bland, 1976).

673 The ventral groove is widespread in stramenopiles. In addition to Bigyromonadea and
 674 oomycetes, it is found in labyrinthulomycetes (Barr and Allan, 1985), *Kaonashia insperata*
 675 (Weston et al., 2023), *Platysulcus tardus* (Shiratori et al., 2015; Thakur et al., 2019), and a
 676 likely homologous structure is present also in bicosoecids and placidideans (Moriya et al.,
 677 2000, 2002; O'Kelly and Patterson, 1996; Teal, 1998). Outside the stramenopiles, the ventral
 678 groove can be found in many diverse eukaryotic groups: jakobids, most metamonads and
 679 malawimonadids (Simpson, 2003), alveolates (Tikhonenkov et al., 2014), rhizarians (Howe et
 680 al., 2011), provorans (Tikhonenkov et al., 2022), and collodictyonids (Brugerolle, 2006;
 681 Brugerolle et al., 2002). This structure is always supported by elements of an extremely
 682 conserved flagellar apparatus and is thus considered an ancestral structure for all eukaryotes
 683 (Cavalier-Smith, 1978; Yubuki and Leander, 2013). Identifying and studying this structure in
 684 new taxonomic groups further supports the adaptive importance of this trait and its likely
 685 occurrence in the last eukaryotic common ancestor.

686 We observed pseudopodia in all our strains. In parasitoid *Pirsonia*, pseudopodia play
 687 an important role in feeding due to their involvement in frustule invasion and trophosome
 688 formation (Schnepf and Schweikert, 1997; Schweikert and Schnepf, 1997). In predatory
 689 Pirsoniales, which do not possess any specialized cellular structure for nutrition, such as a
 690 cytostome, pseudopodia also play a role in feeding. Sometimes, they are also used for
 691 temporary substrate attachment. In the resting cells of *Koktebelia satura*, we observed the
 692 release of pseudopodia of unknown function. An interesting observation was made on starved
 693 *Pirsonia punctigera* cells, which became motionless, settled on the substrate and formed long
 694 filopodia-like processes in contact with each other (Schweikert and Schnepf, 1997). This is
 695 reminiscent of the formation of an ectoplasmic network in labyrinthulomycetes (Anderson and

696 Cavalier-Smith, 2012; Gomaa et al., 2013; Iwata et al., 2016), which possess amoeboid stages
697 in the life cycles and rhizopoda-like structures (Anderson and Cavalier-Smith, 2012; Benneth
698 et al., 2017; Gomaa et al., 2013; Takahashi et al., 2014). It is known that the trophosomes of
699 different *Pirsonia* cells may fuse inside the cell of the same diatom during multiple infections
700 (Kühn et al., 1996). Apparently, similar amoeboid features unite labyrinthulomycetes and
701 Pseudofungi and were present ancestrally before the divergence of these groups.

702 The species *Feodosia pseudopoda* is capable of forming large multinucleated and
703 multiflagellated cells. This could be a single large oval cell with two, four, etc., nuclei and
704 associated sets of flagella, as illustrated by Hoechst staining (Figure 10). Such structures
705 represent compact multinucleated forms that can be considered as predecessors of
706 multicellularity (Lamza, 2023). Sometimes cells undergo several consecutive incomplete cell
707 divisions without complete separation, thus forming temporary colonies of independent cells
708 that are attached to each other and usually to a large common food vacuole by long posterior
709 protrusions. The formation of these facultative colonies occurs in the presence of a large
710 amount of food (diatoms) and disappear gradually as the amount of diatom prey decreases. It
711 remains unclear what causes the formation of compact multinucleated cells and what they
712 subsequently transform into; whether into individual cells or into temporary colonies. The
713 question is also whether this process is related to sexual reproduction. A detailed study of the
714 life cycle of *F. pseudopoda* is needed to address these questions.

715 The formation of facultative colonies in *F. pseudopoda* is likely related to the formation
716 of auxosome-type colonies in parasitic Pirsoniales. Both phenomena are limited by the amount
717 of food available. The formation of a large common food vacuole filled with brownish material
718 of undigested diatom shells and chloroplasts in *F. pseudopoda* is reminiscent of trophosome
719 formation in parasitoid *Pirsonia* species (Schnepf and Schweikert, 1997; Schweikert and
720 Schnepf, 1997). Such food vacuoles may be up to 20 times larger than a single diatom cell. The
721 presence of both facultative colonies and large shared accumulative food vacuoles observed in
722 *F. pseudopoda* suggests that they may represent ancestral characteristics of all Pirsoniales that
723 contributed to the development of parasitism in the *Pirsonia* clade.

724 Another remarkable feature of the novel *F. pseudopoda* strain KT49-10a is its ability
725 to self-aggregate with possible cell fusion, which differs from the colonial formation resulting
726 from cell division. This is the first observation of self-aggregation in Pirsoniales. Individual
727 cells, probably attracted by chemical signals, join to form a common pellet of cells that are
728 pressed tightly to each other (Figure 4U). This behavior is likely initiated by starvation-induced
729 stress. Self-aggregation is a very rare phenomenon well known to occur in oomycete zoospores
730 (Bassani et al., 2020; Ko and Chase, 1973; Reid et al., 1995; West et al., 2003) and recently
731 detected in developoans (Cho et al., 2022). The discovery of this feature in Pirsoniales suggests
732 that it is a very likely ancestral character of Pseudofungi. We also frequently observed loose
733 clusters of aggregated cells in starved cultures of *Koktebelia satura* without cell fusion. Similar
734 behavior was noted for the parasitoid *Pirsonia guinardiae*, which assembles on the inner
735 surface of empty diatom frustules in starved cultures (Kühn et al., 1996). Interestingly, all three
736 documented self-aggregation behaviors in Pirsoniales are related to starvation.

737 Chemotaxis is likely involved in self-aggregation of oomycete zoospores (Bassani et
738 al., 2020; Reid et al., 1995), as it is involved in their aggregation on plants. It is assumed that
739 this may occur due to the release of certain chemicals by the zoospores themselves, which
740 increases the likelihood of infection due to the lack of stimulation from the host (Savoty et al.,
741 2014). Genomic and transcriptomic studies of oomycetes have revealed the presence of
742 proteins associated with the adhesion and signaling homologous to those required for the
743 multicellular development of metazoans and their closest protist relatives (Bassani et al., 2020;
744 King et al., 2003, 2008; Sebé-Pedrós et al., 2013). For example, a whole family of oomycete-
745 specific cadherins has been discovered (Fletcher et al., 2016). Cadherins are also involved in

746 cell adhesion and aggregation in *Dictyostelium* (Hulpiau et al., 2013; Niklas and Newman,
 747 2013). The developing auxosomes and newly-formed flagellated cells of *Pirsonia* are held
 748 together by a layer of mucilage, which is noticeable only via SEM (Schweikert and Schnepf,
 749 1997). Our SEM observation revealed similar coverage of individual cells of pirsoniales
 750 (Figure 2). Further genomic and transcriptomic studies of these organisms, together with
 751 ultrastructural observations of amoeboid and aggregative stages, will help to elucidate the
 752 nature and mechanisms of self-aggregation in this group, as well as the relatedness of this
 753 phenomenon to self-aggregation in oomycetes.

754 We did not find cysts in our cultures, although all studied strains had resting cells that
 755 retained flagella (except for the species *Noirmoutieria diatomophaga*) and that varied in shape,
 756 from non-flattened ovals with preserved ventral groove to rounded or irregularly amoeboid and
 757 flattened ones. Among *Pirsonia* species, round cysts with red-brown or dark brown walls filled
 758 with refractive granules, are found only in *P. guinardiae* (Schnepf et al., 1990). These cysts
 759 appear to be true resting cysts, in contrast with the resting cells we observed.

760

761 **Ultrastructure of the novel Pirsoniales**

762 The ultrastructure of the new strains is very similar to that of known *Pirsonia* species (Schnepf
 763 and Schweikert, 1997; Schweikert and Schnepf, 1997). All cells we studied were covered with
 764 hair-like structures, similar to the hairs covering the posterior flagellum. Similar structures,
 765 named as knotted body hairs, have been found in *Pirsonia* (Schweikert and Schnepf, 1997).
 766 However, the length of the hair-like structures we observed was twice shorter, 0.2-0.25 μm vs.
 767 0.4-0.5 μm in *Pirsonia* (Schnepf and Schweikert, 1997; Schweikert and Schnepf, 1997),
 768 although previous observations were based on whole mount TEM. In contrast to our species,
 769 body hairs in *Pirsonia* appear only at certain life cycle stages. In particular, life cycle stages
 770 associated with parasitism, such as auxosomes, developing flagellated cells, and flagellated
 771 cells before attachment to diatoms, lack body hairs (Schnepf and Schweikert, 1997; Schweikert
 772 and Schnepf, 1997). It is expected that we would observe hair-like structures on all studied
 773 cells since the novel predatory Pirsoniales do not have life cycle stages associated with
 774 parasitism (see above). These hair-like structures are specific to the order Pirsoniales since they
 775 have not been observed in other stramenopiles (Aleoshin et al., 2016; Beakes and Thines, 2017;
 776 Bennett et al., 2017; Kristiansen and Škaloud, 2017; Shiratori et al., 2015, 2017; Thakur et al.,
 777 2019; Tong, 1995; Weston et al., 2023). The structure and role of these hair-like structures in
 778 Pirsoniales need to be studied.

779 Different types of cell body hairs, somatonemes, cover the posterior half of the cell of
 780 the flagellate *Proteromonas lacerate* (Opalozoa, Stramenopiles), a tetrapod gut commensal
 781 (Brugerolle and Bardele, 1988). Somatonemes are very similar to mastigonemes: they are also
 782 tubular and tripartite and are synthesized in the ER cisternae. Additionally, somatonemes are
 783 anchored to cortical microtubules in the same way that mastigonemes are anchored to axonemal
 784 microtubules (Brugerolle and Bardele, 1988). Somatonemes probably evolved to adapt to an
 785 endobiotic habitat and transition from phagocytosis to pinocytosis by protecting cells from
 786 large and sharp food particles that can stick to the cell and interfere with pinocytosis, as well
 787 as damage the cell surface (Cavalier-Smith, 1998). Further studies will help to investigate the
 788 evolutionary relationship between both types of hair-like structures and whether these
 789 structures have a common evolutionary origin or have arisen independently.

790 The known parasitoid *Pirsonia* has a spherical nucleus, as we observed in two of three
 791 of our species (*Bullionia fluviatilis* and *Feodosia pseudopoda*). By contrast, the nucleus of
 792 *Bordeauxia parva* is elongated pear-shaped, similar to the nuclei of most stramenopiles,
 793 including developans, oomycetes, hyphochytriomycetes, labyrinthulomycetes, many
 794 opalozoans and some ochrophytes (Aleoshin et al., 2016; Barr, 1981; Barr and Allan, 1985;
 795 Gotelli and Hanson, 1987; Hardham, 1987; Lunney and Bland, 1976; Maistro et al., 2017;

796 Moriya et al., 2000; 2002; Tong, 1995). A spherical nucleus with eccentric nucleolus has
797 been observed in young oomycete zoospores released from sporangia (Lunney and Bland,
798 1976). Like the parasitoid *Pirsonia*, all our species have two nuclear papillae (protrusions) from
799 the side of the nucleus located closer to the kinetosomes. Such nuclear papillae are not known
800 even for the closely related developans and are likely a characteristic feature of the order
801 Pirsoniales. These papillae serve to attach the nucleus to the posterior kinetosome.

802 The cytoplasm of our new species contains diverse round and flattened vesicles,
803 microbodies, multivesicular bodies, and lipid droplets. We found large and roundish vesicles,
804 not necessarily associated with the cell periphery, in *Feodosia pseudopoda*, while flattened
805 vesicles in *Bordeauxia parva* always underlay the plasmalemma. Different types of vesicles
806 are found in many stramenopile groups. Flattened cortical vesicles in *B. parva* resemble those
807 found in developans (Aleoshin et al., 2016; Tong, 1995) and *Kaonashia insperata* (Weston et
808 al., 2023). A single large flat vesicle surrounds the cytoplasm with the nucleus, mitochondria,
809 and microbodies in the cells of *Platysulcus tardus* (Shiratori et al., 2015). Large round
810 peripheral vesicles are also found in *Pseudophyllomitus vesiculosus* (Shiratori et al., 2017). In
811 *K. insperata*, flattened vesicles are intermitted by extrusomes; in developans, they are
812 intermitted by densely stained bodies. Interestingly, both hyphochytrid and oomycete
813 zoospores have several types of peripheral vesicles, including very thin peripheral cysternae
814 and densely stained vesicles involved in adhesion and incorporation into the cyst coat (Beakes
815 et al., 2014). It remains unclear whether such structures are homologous to cortical vesicles
816 and densely stained bodies in developans and Pirsoniales.

817 The kinetosomal structure, and especially the transitional zone of kinetosomes, is
818 considered an extremely important morphological structure to study phylogenetic relationships
819 (Cavalier-Smith, 2022; Manton, 1964). The kinetosome structure is similar in all our studied
820 strains and corresponds to that of known *Pirsonia* species (Schnepf and Schweikert, 1997;
821 Schweikert and Schnepf, 1997). However, the transitional zone of *Pirsonia* was not described
822 in detail. It is very difficult to obtain good images of pirsonial kinetosomes with attached
823 flagella, as they tend to detach during fixation. The transitional zone of flagella of our studied
824 species possesses a single transitional helix with 3-6 gyres, as described for *Pirsonia* (Schnepf
825 and Schweikert, 1997; Schweikert and Schnepf, 1997). In general, transitional helices are
826 widely present in stramenopiles and are extremely rare outside this group, being their
827 characteristic feature (Thakur et al., 2019). In addition to Pirsoniales, single transitional helices
828 are present in most ochrophytes (Andersen, 2004) and *Pseudophyllomitus vesiculosus*
829 (Shiratori et al., 2017). A double helix is known in developans (Tong, 1995),
830 hyphochytriomycetes and most oomycetes (Barr, 1981; Barr and Allan, 1985; Barr et al., 1985;
831 Beakes and Thines, 2017; Beakes et al., 2012, 2014), Placididea (Moriya et al., 2000, 2002),
832 and ochrophyte Xanthophyceae (Andersen, 2004). Most bicosoecids, some ochrophytes
833 (Bolidophyceae, diatoms, Phaeophyceae, and Raphidophyceae) and *Platysulcus tardus* have
834 lost this structure (Andersen, 2004; Cavalier-Smith and Chao, 2006; Karpov et al., 2001;
835 O'Kelly and Patterson, 1996; Preisig, 1989; Shiratori et al., 2015; Teal et al., 1998; Yubuki et
836 al., 2010), while labyrinthulomycetes have similarly placed cone-like structures (Bennett et al.,
837 2017). The double helix in the transition zone of the flagellum, as the most common type, is
838 considered the ancestral feature for stramenopiles (Thakur et al., 2019), however, such a
839 structure may be modified in closely related groups. The transitional zone of our new strains
840 also contained a transverse plate and annular connections, both located almost at the same level.
841 These structures are well-known for stramenopiles and other eukaryotes (Cavalier-Smith,
842 2022). Electron-dense central thickening of the transverse plate is sometimes visible on some
843 sections. Acorn-V system, recognized by Cavalier-Smith (2022) in stramenopiles for the first
844 time, is probably present in all studied strains, but it is difficult to notice. It is visible as thin
845 transverse filaments on longitudinal sections of *Feodosia*, just above central dense hub (Figure

846 8), and as lattice structure on transverse sections of *Bullionia*, immediately after the transition
847 from triplets to doublets (Figure 9G).

848 We have observed a difference between parasitoid and predatory Pirsoniales in the
849 orientation of the kinetosomes relative to each other. In parasitoid *Pirsonia* species, they
850 diverge at a right angle (Schnepf and Schweikert, 1997; Schweikert and Schnepf, 1997)
851 whereas in our new predatory strains they are oriented at a wide angle. In the closely related
852 developans, this angle ranges between 60° and 120° (Aleoshin et al., 2016; Tong, 1995) and
853 depends on the type of cell, swimming or feeding ones (Aleoshin et al., 2016). A wide angle
854 between kinetosomes is common in zoospores of oomycetes (Barr and Allan, 1985; Gotelli and
855 Hanson, 1987; Lunney and Bland, 1976; Randolph and Powell, 1992) and
856 hyphochytriomycetes (Barr, 1981; Barr and Allan, 1985). Deep branching stramenopiles and
857 ochrophytes mostly have right or narrow angles between kinetosomes (Andersen, 2004; Honda
858 and Inouye, 1995; Iwata et al., 2017; Kristiansen and Walne, 1977; Moriya et al., 2000, 2002;
859 Mylnikov et al., 2008; O’Kelly and Patterson, 1996; Owen et al., 1990; Shiratori et al., 2015,
860 2017; Teal et al., 1998; Yubuki et al., 2010).

861 Here we need to note that anterior and posterior kinetosomes were probably mixed up
862 in previous studies of Pirsoniales (Schnepf and Schweikert, 1997; Schweikert and Schnepf,
863 1997). They are difficult to identify on thin sections since obtaining well-fixed preparations for
864 TEM is problematic and the flagella almost always detach during fixation. During fixation,
865 Pirsoniales do not retain clear outlines of cell body shape and do not have a clear localization
866 of most cell compartments. The ventral groove is not always pronounced, and kinetosomes are
867 located halfway along the ventral groove, making it difficult to identify the anterior and
868 posterior parts of the cell. According to the TEM images on Figures 20 and 21 from Schnepf
869 and Schweikert (1997), the proximal end of the anterior kinetosome (with a distinctive
870 cartwheel structure) is directed toward the side of the posterior kinetosome. By contrast, new
871 Pirsoniales strains, as well as other studied stramenopile groups, having the opposite
872 configuration, i.e. the proximal end of the posterior kinetosome is directed toward the side of
873 the anterior kinetosome (Barr and Allan, 1985; Beakes et al., 2014; Iwata et al., 2017; Owen et
874 al., 1990; Shiratori et al., 2015, 2017; Tong, 1995). In addition, the schematic illustration of
875 the flagellar apparatus in Figure 27 from Schnepf and Schweikert (1997) contradicts to their
876 own TEM illustrations and goes in accordance with ‘canonical’ stramenopile kinetosome
877 connection. Also, according to our observations, the posterior kinetosome is connected to the
878 nucleus by nuclear bands (Figures 6B, 8G,H, 9H-J), while previous descriptions of parasitic
879 *Pirsonia* referred to the anterior kinetosome (Schnepf and Schweikert, 1997). In this case, we
880 are confident that the nuclear bands extend from the posterior kinetosome on the basis of the
881 associated posterior root R2 with the characteristic semicircular orientation of its microtubules
882 (Figures 6B, 8G,H, 9H-J).

883 In all known Pirsoniales, the kinetosomes are connected to each other by two fibrillar
884 bands: a short and wide proximal striated connecting band and a long and thin distal connecting
885 band (Schnepf and Schweikert, 1997; Schweikert and Schnepf, 1997). These bands may be
886 present in developans but have not been studied in detail (Aleoshin et al., 2016; Tong, 1995).
887 The dorsal connecting band is probably visible in Figure 15 (Tong, 1995) and in Figures
888 3C,D,F-H (Aleoshin et al., 2016). The proximal striated band and its modifications have been
889 found in diverse stramenopile groups, such as a striated fan-shaped band in oomycetes (Barr
890 and Allan, 1985; Gotelli and Hanson, 1987; Holloway and Heath, 1977; Lunney and Bland,
891 1976; Randolph and Powell, 1992) and two striated bands in bicosoecids and placidideans
892 (Moriya et al., 2000, 2002; O’Kelly and Patterson, 1996; Teal et al., 1998). By contrast, the
893 distal connecting band seems to be specific of Bigyromonada.

894 As mentioned before, the posterior kinetosome is connected to the nucleus by two
895 electron-dense fibrous nuclear bands. These nuclear bands appear to be unique of Pirsoniales,

896 although homologous structures are likely present in oomycetes as a single striated strap
897 extending from the posterior kinetosome along the surface of the nuclear envelope (Barr and
898 Allan, 1985; Holloway and Heath, 1977; Randolph and Powell, 1992; Schnepf et al., 1978).
899 Most ochrophytes are known to have a wide and prominent striated structure, the rhizoplast,
900 connecting the anterior kinetosome with the nucleus, which is positioned at some distance from
901 the kinetosomes (Andersen, 2004; Bouck and Brown, 1973; Kristiansen and Škaloud, 2017;
902 Kristiansen and Walne, 1977; Owen et al., 1990).

903 The flagellar root system in our studied species is much more complex than that in
904 known *Pirsonia* species and more similar to those of other stramenopiles. All four flagellar
905 roots are microtubular in our strains, whereas only the roots associated with the posterior
906 (indicated as “anterior” in Schnepf and Schweikert (1997)) kinetosome are microtubular in
907 *Pirsonia* (Schnepf and Schweikert, 1997). Anterior (mentioned as “posterior” in Schnepf and
908 Schweikert (1997)) roots in *Pirsonia* are fibrous, and posterior roots (“anterior” according to
909 Schnepf and Schweikert (1997)) are highly reduced: four microtubules and a fibrillar band in
910 root r1 and a single microtubule and several fibrillar bands in r2. However, Schnepf and
911 Schweikert (1997) did not explain in detail the orientation of the roots in the cell, which makes
912 it difficult to compare the roots of *Pirsonia* with the homologous roots in other eukaryotes
913 (Moestrup, 2000; Yubuki and Leander, 2013). In most eukaryotes, the posterior root is more
914 developed and is always associated with the ventral groove if such a structure is present
915 (Yubuki and Leander, 2013). Therefore, it is more likely that the anterior root, not the posterior
916 root, is reduced in *Pirsonia*. In any case, the flagellar root system of the parasitoid *Pirsonia* is
917 reduced compared to that of other stramenopiles, which might be due to their parasitoid
918 lifestyle. For example, some roots might be absent or poorly developed at some non-flagellated
919 stages of the life cycle. This is consistent with the general trend of independent loss of
920 complexity of the flagellar apparatus in some eukaryotic lineages during the transition from a
921 free-living lifestyle to a symbiotic or parasitic lifestyle (Yubuki et al., 2016). These transitions
922 are most likely related to changes in cell motility, attachment and nutrition. Well-known
923 examples of microtubular reduction in parasitic lineages in comparison with their free-living
924 relatives are slopalinids and *Blastocystis* (Stramenopiles), apicomplexans (Alveolates),
925 diplomonads (Fornicata), and trypanosomatids (Euglenozoa) (Yubuki et al., 2016).

926 The most conspicuous right posterior root, r2, has 8-9 microtubules that are oriented in
927 semicircle in its proximal part and reinforced with fibrous core material inside the semicircle.
928 The same structure of the proximal part of r2 (U-shaped or L-shaped, which can be considered
929 a modification of a semicircle) is present in most stramenopiles, e.g., developans (Aleoshin et
930 al., 2016; Tong, 1995), labyrinthulomycetes (Kazama, 1972, 1980; Perkins, 1974),
931 *Pseudophyllomitus vesiculosus* (Shiratori et al., 2017), opalozoans (Moriya et al., 2000, 2002;
932 O’Kelly and Patterson, 1996; Teal et al., 1998), *Platysulcus tardus* (Shiratori et al., 2015), and
933 ochrophytes (Andersen, 2004; Honda and Inouye, 1995; Mylnikov et al., 2008; Owen et al.,
934 1990). Oomycetes differ by having a flat arrangement of microtubules in r2 (Barr, 1981; Barr
935 and Allan, 1985; Gotelli and Hanson, 1987; Randolph and Powell, 1992), and
936 hyphochytriomycetes have greatly reduced r2 due to the loss of the second flagellum (Barr,
937 1981; Barr and Allan, 1985; Beakes and Thines, 2017). The fibrous material supporting this
938 root from inside the semicircle and connecting it to the posterior or to both kinetosomes is also
939 widely present in stramenopiles, e.g., fibrous core material inside the semicircle in developans
940 and placidideans (Aleoshin et al., 2016; Moriya et al., 2000; Tong, 1995), electron-dense
941 backing material in labyrinthulomycetes (Kazama, 1972, 1980; Barr, 1981; Perkins, 1974), a
942 striated band in bicosoecids (O’Kelly and Patterson, 1996; Teal et al., 1998; Yubuki et al.,
943 2010), multilayered buttress in oomycetes (Barr, 1981; Barr and Allan, 1985; Gotelli and
944 Hanson, 1987; Randolph and Powell, 1992; Holloway and Heath, 1977), and fibrous material
945 in ochrophytes (Andersen, 2004; Owen et al., 1990; Mylnikov et al., 2008).

946 The left posterior root r1 in the new Pirsoniales species possesses a larger number of
 947 microtubules (3-5) compared with those (1-2) in other stramenopile groups (Aleoshin et al.,
 948 2016; Andersen, 2004; Barr, 1981; Barr and Allan, 1985; Gotelli and Hanson, 1987; Moriya et
 949 al., 2000, 2002; Owen et al., 1990; Randolph and Powell, 1992; Shiratori et al., 2015). A similar
 950 number of microtubules (3-4) was found only in *Platysulcea*, (Shiratori et al., 2015). The root
 951 r1 is absent in hyphochytriomycetes (Beakes and Thines, 2017) and in some ochrophytes
 952 (Andersen, 2004).

953 The right anterior root r3 of the novel Pirsoniales was also more similar to that of most
 954 stramenopiles than to *Pirsonia* in terms of its ribbed structure (the presence of secondary
 955 microtubules emerging at the right angle from the main microtubules) and in its clockwise
 956 direction. Secondary microtubules run anterior-dorsally and support the anterior part of the
 957 cell. The same structure (ribbed r3 with 2-3 main microtubules) is present in most
 958 stramenopiles (Aleoshin et al., 2016; Andersen, 2004; Barr, 1981; Barr and Allan, 1985; Gotelli
 959 and Hanson, 1987; Holloway and Heath, 1977; Iwata et al., 2017; Moriya et al., 2002; O'Kelly
 960 and Patterson, 1996; Randolph and Powell, 1992). Apparently, secondary microtubules are
 961 absent in Eustigmatophyceae, *Platysulcus*, *Pseudophyllomitus* and *Wobblia lunata* (Andersen,
 962 2004; Moriya et al., 2000; Shiratori et al., 2015, 2017). In oomycetes, r3 is also strengthened
 963 with osmiophilic material along its way (Barr, 1981; Barr and Allan, 1985; Gotelli and Hanson,
 964 1987; Holloway and Heath, 1977; Randolph and Powell, 1992).

965 The most inconspicuous left anterior root, r4, contains 1-2 microtubules, as in many
 966 stramenopiles (Andersen, 2004; Barr, 1981; Barr and Allan, 1985; Gotelli and Hanson, 1987;
 967 Iwata et al., 2017; Moriya et al., 2000, 2002; O'Kelly and Patterson, 1996; Shiratori et al.,
 968 2015, 2017; Teal et al., 1998).

969 The cytoplasmic and nuclear microtubules that we observed are common for many
 970 stramenopile groups (Barr, 1981; Barr and Allan, 1985; Gotelli and Hanson, 1987; Randolph
 971 and Powell, 1992; Holloway and Heath, 1977; Moriya et al., 2002; O'Kelly and Patterson,
 972 1996; Teal et al., 1998; Andersen, 2004). Notably, such cytoplasmic microtubules are difficult
 973 to detect in thin sections (Barr, 1981; Barr and Allan, 1985).

974

975 **Taxonomy, phylogeny and evolution**

976 We identified five new genera in the order Pirsoniales, which indicates that this group of
 977 eukaryotes remains poorly studied in terms of its taxonomic diversity. Two of the studied
 978 Pirsoniales (*Feodosia pseudopoda* and *Koktebelia satura*) were described previously under
 979 provisional names because the obtained data were insufficient to describe new genera and
 980 species (Cho et al., 2022). Here, we studied their biology and morphology, which allowed us
 981 to formally describe them. We keep the provisional names proposed for these taxa in their
 982 taxonomic descriptions to avoid further confusion (see the taxonomic summary).

983 The 18S rRNA phylogenetic trees clarified the position of the novel strains within
 984 Pirsoniales and their relationships with already known parasitoid and predatory protists and
 985 environmental sequences. According to these results, close environmental lineages exist for
 986 almost all new Pirsoniales, except for *Feodosia* (Figure S1). Further research efforts should be
 987 directed to find and investigate these lineages, as well as to a more extensive survey of current
 988 metabarcoding datasets to explore the distribution of Pirsoniales. The largest number of
 989 lineages identified by environmental sequencing corresponds to the *Noirmoutieria* clade.
 990 Interestingly, they are all associated with anoxic or oxygen-limited habitats. A study of the
 991 tolerance of *Noirmoutieria* to low-oxygen conditions would be useful when the culture is
 992 reisolated.

993 We also identified the first freshwater representative of the Pirsoniales clade, which
 994 likely had an ancestor of marine origin. The transition of *Bullionia fluviatilis* to freshwater
 995 ecosystems probably occurred early in the evolution of Pirsoniales, but poor phylogenetic

996 support and more thorough sampling hinder the interpretation of this eco-evolutionary
997 scenario.

998 The genera *Pirsonia* and *Noirmoutieria* constitute the family Pirsoniaceae. Concerning
999 the other genera within the order Pirsoniales, the topology of our 18S rRNA phylogenetic tree,
1000 highly or moderately supported depending on the branch, is not necessarily consistent with the
1001 results of previous phylogenomic analyses (Cho et al., 2022, 2024). This is likely due to the
1002 limited information from this single marker, although differential taxon sampling can also have
1003 an effect. Therefore, we refrain from describing new families at this stage; they should be
1004 defined in subsequent studies using more in-depth phylogenomic data. This will help to clarify
1005 the relationships between the main clades of Pirsoniales and also to verify the monophyly of
1006 Bigyromonadea and their position within Stramenopiles. Molecular phylogenetic studies of
1007 small heterotrophic groups, such as Pirsoniales and Delepliozoa, can impact our interpretation
1008 of the evolution of large and important groups, such as oomycetes and ochrophytes. If
1009 Bigyromonadea are sister to oomycetes, as shown in previous studies (Cavalier-Smith and
1010 Chao, 2006; Cho et al., 2022, 2024; Noguchi et al., 2016; Thakur et al., 2019), this will help to
1011 reconstruct the ancestral state of oomycetes and advance our understanding of the origins of
1012 parasitism in this economically important group.

1013 Alternatively, the possible relationship of Bigyromonadea with ochrophytes, as shown
1014 in a number of earlier studies (Aleoshin et al., 2016; Cho et al., 2022, 2024; Kühn et al., 1996;
1015 Leonard et al., 2018) also looks intriguing and, if confirmed, could help to understand the
1016 processes underlying the acquisition of plastids in one of the most important group of
1017 photosynthetic eukaryotes of planetary-scale ecological significance. Previously, mostly
1018 bacteriophilic species were known for being closely related to ochrophytes (Aleoshin et al.,
1019 2016). However, further research demonstrated that eukaryotrophic protists are commonly
1020 found at the base of the most important stramenopile lineages (Aleoshin et al., 2016; Cho et
1021 al., 2022, 2024; Weston et al., 2023). Our finding of the ability of new Pirsoniales to
1022 successfully consume red algae is particularly intriguing given the rhodophyte ancestry of the
1023 ochrophyte plastids (Yoon et al., 2002). Red algae are considered unsuitable prey for most
1024 predators due to their thick polysaccharides and glycoprotein cell wall that serves to protect
1025 against recognition as prey and digestion (Martel, 2009; Ucko et al., 1997, 1999;). Therefore,
1026 the discovery of protists in natural ecosystems that can consume red algae and that branch close
1027 to ochrophytes is very relevant from an evolutionary point of view.

1028

1029 CONCLUSIONS

1030

1031 The identification and morphological and phylogenetic study of new members of the order
1032 Pirsoniales, including the first freshwater species, resulted in the description of five new genera
1033 and the emendation of the diagnosis of the order, previously known to be parasitic, as it now
1034 contains also predatory flagellates.

1035 The formation of multiflagellated cells with a common large food vacuole, which is
1036 found in *Feodosia pseudopoda*, is reminiscent of trophosome and auxosome formation in
1037 *Pirsonia* and suggests its preparasitic origin. We observed self-aggregation in Pirsoniales for
1038 the first time and propose that this trait was present in the common ancestor of Pseudofungi.
1039 Our new species illustrate the astonishing diversity of strategies evolved in the order Pirsoniales
1040 for the effective consumption of diatoms, including parasitism with the formation of
1041 trophosomes and auxosomes, predation via the capture of whole cells, or via the use of
1042 pseudopodia to absorb/scavenge parts of protoplasts and chloroplasts through holes in the
1043 frustules.

1044 The ultrastructure of the new strains shows that they share more features in the flagellar
1045 apparatus with other stramenopiles than with the known *Pirsonia* species. These features

1046 include four microtubular roots, an arrangement of r2 microtubules in a semicircle and its
1047 division into two parallel rows, a ribbed r3 backed with fibrous material, and the orientation of
1048 kinetosomes. Hair-like structures covering the cell body and two nuclear bands connecting the
1049 nucleus with the posterior kinetosome are unique structures for the order Pirsoniales that define
1050 this taxon. Our results provide further support for the similarity of Bigyromonadea cells and
1051 oomycete zoospores, including new ultrastructural traits, such as general cell structure,
1052 orientation of kinetosomes relative to each other and the nucleus, direction of flagellar roots
1053 and the number of microtubules they contain.

1054

1055 **Taxonomic summary**

1056 Zoobank Registration for this publication: urn:lsid:zoobank.org:pub:D320D0CD-FBC0-493C-
1057 8F6C-CBC4C5328BEF.

1058 Taxonomy: Eukaryota; Sar; Stramenopiles; Gyrista; Bigyromonadea.

1059

1060

1060 **Order Pirsoniales Cavalier-Smith, 1998 emend.**

1061 Emended diagnosis: Biflagellated parasitoids of diatoms and eukaryotrophic predators.
1062 Parasitoid species differentiate into an intracellular feeding part (trophosome) and an external
1063 generative part (auxosome). With non-tubular hair-like structures on the cell body.
1064 Longitudinal ventral groove divided in anterior and posterior parts intermitted by a protrusion
1065 from which flagella emerge. Tubular mitochondrial cristae with a filament inside each crista.
1066 Kinetosomes connected to each other by a proximal striated connecting band and a distal
1067 connecting band. Posterior kinetosome connected with the nucleus by two nuclear bands.
1068 Transition zone of the flagellum contains a single helix above the transverse plate.

1069

1070

1070 **Family Pirsoniaceae Cavalier-Smith, 1998 emend.**

1071 Emended diagnosis: Biflagellated parasitoids of diatoms and eukaryotrophic predators.
1072 Parasitic species differentiate into an intracellular feeding part (trophosome) and an external
1073 generative part (auxosome). Round nucleus. Kinetosomes oriented at right angles to each other.
1074 Posterior kinetosome connected to the nucleus by a nuclear connecting band. Roots of posterior
1075 kinetosome are fibrillar (non-microtubular). Cells are covered with hair-like structures only at
1076 some life cycle stages. Cysts present in some species.

1077 Type genus: *Pirsonia* Schnepf et al., 1990.

1078

1079

1079 ***Noirmoutieria* gen. nov.**

1080 Diagnosis: Predators consuming heterotrophic flagellates and diatom algae. Elongated
1081 cells with well-developed longitudinal ventral groove. Oval, round, or irregular resting cells
1082 rarely shed flagella.

1083 Etymology: Genus name reflects the source of the isolate, Noirmoutier-en-l'Île, France.

1084 Zoobank Registration: urn:lsid:zoobank.org:act:34AD0014-5CA4-47E8-998E-
1085 DC7489442F4D.

1086 Type species: *Noirmoutieria diatomophaga*

1087

1088

1088 ***Noirmoutieria diatomophaga* sp. nov.**

1089 Diagnosis: Same as for the genus.

1090 Type figure: Figure 3U illustrates a live cell of strain Pirs-sm2.

1091 Type locality: Marine sediments of Noirmoutier-en-l'Île, France.

1092 Etymology: Species named after its feeding mechanism, ability to swallow diatom cells.

1093 Gene sequence: The 18S rRNA gene sequence has the GenBank Accession Number
1094 PP808839.

1095 Zoobank Registration: urn:lsid:zoobank.org:act:4924A885-A9E4-4D6E-BD54-
1096 C582F61D5C09.

1097

1098 ***Feodosia* gen. nov.**

1099 Diagnosis: Eukaryotrophic predators. Round nucleus with small nuclear papillae.
1100 Slightly acentric nucleolus. Transitional helix in flagellum transition zone with 3-5 gyres.
1101 Large and round peripheral vesicles and flattened vesicles around the nucleus. Long and narrow
1102 nuclear bands. Presence of facultative multiflagellated colonial life cycle stages. Able to self-
1103 aggregate.

1104 Etymology: Genus name kept as provisional from Cho et al. (2022) and reflects the
1105 source of isolate, the settlement Beregovoye, Feodosiya, Crimea.

1106 Zoobank Registration: urn:lsid:zoobank.org:act:20F4AC14-1E06-4F44-A9A6-
1107 5B4208248795.

1108 Type species: *Feodosia pseudopoda*

1109

1110 ***Feodosia pseudopoda* sp. nov.**

1111 Diagnosis: Same as for the genus.

1112 Type figure: Figure 4A illustrates a live cell of strain NY0226.

1113 Type culture: Strain NY0226 is deposited in the DEEM live culture collection, CNRS
1114 and Université Paris-Saclay (France).

1115 Type material: A block of chemically fixed resin-embedded cells of the type strain,
1116 NY0226, is deposited in the DEEM culture collection, CNRS and Université Paris-Saclay
1117 (France). This constitutes the name-bearing type of the species (a hapantotype).

1118 Type locality: Marine sediments of plage de Jean Blanc near Saint-Tropez, France.

1119 Etymology: Species name kept as provisional from Cho et al. (2022) and reflects the
1120 ability to produce pseudopodia.

1121 Gene sequence: The 18S rRNA gene sequence of the strain NY0226 has the GenBank
1122 Accession Number PP795933.

1123 Zoobank Registration: urn:lsid:zoobank.org:act:DBB3C346-E4F1-404A-A47B-
1124 11C0BBA54778.

1125

1126 ***Koktebelia* gen. nov.**

1127 Diagnosis: Eukaryotrophic predators. Longitudinal ventral groove well developed.
1128 Resting cells are oval, with finger-like pseudopodia. Form clusters of resting cells in starved
1129 cultures.

1130 Etymology: Genus name kept as provisional from Cho et al. (2022) and reflects the
1131 source of the isolate, Koktebel bay, Crimea.

1132 Zoobank Registration: urn:lsid:zoobank.org:act:3D31C141-F8A9-4FA6-8228-
1133 30E7F7D7F4F0.

1134 Type species: *Koktebelia satura*

1135

1136 ***Koktebelia satura* sp. nov.**

1137 Diagnosis: Same as for the genus.

1138 Type figure: Figure 3N illustrates a live cell of strain Colp-Nor4.

1139 Type culture: Strain Colp-Nor4 is deposited in the DEEM live culture collection, CNRS
1140 and Université Paris-Saclay (France).

1141 Type locality: Marine sediments of Yport, France.

1142 Etymology: Species name kept as provisional from Cho et al. (2022) and coming from
1143 satur (lat.), well-fed.

1144 Gene sequence: The 18S rRNA gene sequence of the strain Colp-Nor4 has the GenBank
1145 Accession Number PP795565.

1146 Zoobank Registration: urn:lsid:zoobank.org:act:8DD522CF-B652-43A7-A5D4-
1147 D3C640E8146D.

1148

1149 ***Bordeauxia* gen. nov.**

1150 Diagnosis: Eukaryotrophic predators. Longitudinal ventral groove poorly developed.
1151 Able to form thin pseudopodia. Pear-shaped nucleus with a narrowed part connected to
1152 kinetosomes by two nuclear bands. Flattened vesicles underlying plasmalemma. Transitional
1153 helix in flagellum transition zone with 3-5 gyres. Cytoplasmic microtubules originating from
1154 kinetosomes and conically radiating along the nucleus. Right posterior root r2 divided in two
1155 parallel rows in its middle part. Right anterior root r3 consists of three microtubules backed
1156 with fibrous material and perpendicularly oriented secondary microtubules. Left anterior root
1157 r4 consists of two microtubules and is oriented laterally.

1158 Etymology: Genus name reflects the source of the isolate, Gironde estuary near
1159 Bordeaux, France.

1160 Zoobank Registration: urn:lsid:zoobank.org:act:B85AC3F7-C518-4F59-8D16-
1161 B900B5CE10A3.

1162 Type species: *Bordeauxia parva*

1163

1164 ***Bordeauxia parva* sp. nov.**

1165 Diagnosis: Same as for the genus.

1166 Type figure: Fig. 3A illustrates a live cell of strain Colp-p1.

1167 Type culture: Strain Colp-p1 is deposited in the DEEM live culture collection, CNRS
1168 and Université Paris-Saclay (France).

1169 Type material: A block of chemically fixed resin-embedded cells of the type strain,
1170 Colp-p1, is deposited in the DEEM culture collection, CNRS and Université Paris-Saclay
1171 (France). This constitutes the name-bearing type of the new species (a hapantotype).

1172 Type locality: Gironde estuary near Talmont-sur-Gironde, Nouvelle-Aquitaine, France.

1173 Etymology: Species name means small in Latin.

1174 Gene sequence: The 18S rRNA gene sequence of the strain Colp-p1 has the GenBank
1175 Accession Number PP808806.

1176 Zoobank Registration: urn:lsid:zoobank.org:act:8DD70CA4-51F5-4ABE-A86A-
1177 B21DF7BD5743.

1178

1179 ***Bullionia* gen. nov.**

1180 Diagnosis: Eukaryotrophic predators. Longitudinal ventral groove poorly developed.
1181 Round or irregular nucleus with prominent nuclear papillae. Long striated nuclear bands.
1182 Slightly acentric nucleolus. Large reserve lipid droplets. Transition helix in flagellum transition
1183 zone with six gyres. Round flattened resting cells.

1184 Etymology: Genus name reflects the source of the first isolate of this organism, near
1185 the town of Bullion, Ile-de-France, France.

1186 Zoobank Registration: urn:lsid:zoobank.org:act:66362B67-A30D-4D0D-BCD2-
1187 AE7985F88A55.

1188 Type species: *Bullionia fluviatilis*

1189

1190 ***Bullionia fluviatilis* sp. nov.**

1191 Diagnosis: Same as for the genus.

1192 Type figure: Figure 3H illustrates a live cell of strain Pirs-vl.

1193 Type culture: Strain Pirs-vl is deposited in the DEEM live culture collection, CNRS
1194 and Université Paris-Saclay (France).

1195 Type material: A block of chemically fixed resin-embedded cells of the type strain,
1196 Pirs-vl, is deposited in the DEEM culture collection, CNRS and Université Paris-Saclay
1197 (France). This constitutes the name-bearing type of the new species (a hapantotype).

1198 Type locality: Valmont river, Valmont, Normandie, France.

1199 Etymology: Species name refers to freshwater habitat in a river.

1200 Gene sequence: The 18S rRNA gene sequence of the strain Pirs-vl has the GenBank
1201 Accession Number PP808838.

1202 Zoobank Registration: urn:lsid:zoobank.org:act:91FB4FE3-0833-48EF-BF5D-
1203 7E193B13EFC5.

1204

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1218

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1537
1538 **FIGURE LEGEND**

1539 **FIGURE 1.**

1540 Phylogenetic tree generated from Bayesian analysis of 18S rRNA gene sequences. The tree
 1541 contains 75 stramenopile sequences, including sequences of 7 new pirsoniales strains and 2
 1542 prey strains (ochrophytes), and 5 outgroup sequences. Bayesian posterior probabilities (BPP,
 1543 GTR+I+GAMMA4) and Maximum Likelihood (ML, **GTR+GAMMA+I** model) bootstrap
 1544 values are indicated at branches (only values >0.8/>50 are shown); black dots indicate values
 1545 of BPP = 1.00 and ML bootstrap = 100%; dt – different topology. Sequences generated in this
 1546 study are shown in bold.

1547 **FIGURE 2.** Scanning electron microscopy of fixed cells of new pirsoniales strains: (A,B)
 1548 *Bordeauxia parva* gen. et sp. nov. strain Colp-p1; (C-E) *Bullionia fluviatilis* gen. et sp. nov.
 1549 strain Pirs-vl, showing a predatory cell swallowing a prey cell of the diatom *Cyclotella*
 1550 *meneghiniana* (C,D) and a starved cell (E); (F-I) *Feodosia pseudopoda* strain NY0226 (F,G)
 1551 and strain Colp-S9 (H,I). Abbreviations: **an** – acronema, **af** – anterior flagellum, **avg** – anterior
 1552 ventral groove, **f** – flagellum, **pf** – posterior flagellum; **vg** – ventral groove. Scale bars: A-H –
 1553 5 µm, I – 1 µm.

1554 **FIGURE 3.** Phase contrast (A,B,H-K,M,S,T,W,X) and differential interference contrast (C-
 1555 G,L,N-R,U,V,Y-AB) light micrographs of new pirsoniales strains. (A-F) *Bordeauxia parva*
 1556 gen. et sp. nov. strain Colp-p1: swimming cells (A,B), attached cells with emerged pseudopodia
 1557 (C,D), and resting cells (E,F). (G-M) *Bullionia fluviatilis* gen. et sp. nov. strain Pirs-vl:
 1558 swimming cells (G-K), cell with a well-developed food vacuole (H), cells swallowing cells of
 1559 the prey diatom *Stephanocyclus meneghiniana* (I-K), resting cell (L), dividing cell (M). (N-T)
 1560 *Koktebelia satura* gen. et sp. nov. strains Colp-Nor6 (N-Q, T) and Colp-Nor4 (R,S): swimming
 1561 cell (N), resting cells (O-T), including a cell with emerged pseudopodia (Q), cell with a
 1562 swallowed cell of the prey red alga *Erythrolobus coxiae* (S), and cluster of cells (T). (U-AB)
 1563 *Noirmoutieria diatomophaga* gen. et sp. nov. strain Pirs-sm2: swimming cells (U,W,X), cell
 1564 with a swallowed cell of the prey diatom *Attheya* sp. (W), resting cells of regular (V,Y), round
 1565 (Z), and irregular (AA,AB) shape, including resting cells with detached flagella (V,AA,AB).
 1566 Abbreviations: **af** – anterior flagellum, **an** – anterior notch, **bg** – bulge from which flagella
 1567 emerge, **cv** – contractile vacuole, **dp** – diatom prey, **fv** – food vacuole, **ld** – lipid droplets, **pf** –
 1568 posterior flagellum, **ps** – pseudopodia, **ra** – red algae prey, **vg** – ventral groove. Scale bars: A-
 1569 C, U-AB – 5 µm, T – 15 µm.

1570 **FIGURE 4.** Differential interference contrast (A,B,U-X) and phase contrast (C-T) light
 1571 micrographs of *Feodosia pseudopoda* gen. et sp. nov. strains NY0226 (A,B), KT49-10a
 1572 (C,D,U-X), and Colp-S9 (E-J,N-S): swimming cells (A,C-F); resting cell with noticeable
 1573 ventral groove (B); cells with swallowed cells of the prey diatom *Attheya* sp. (E, F) and food
 1574 vacuoles with remains of diatom prey (C, D); cells with swallowed cells of the prey diatom
 1575 *Licmophora* sp. (G-J); undigested *Licmophora* sp. frustules after predator attack (K); defecated
 1576 lumps of deformed compressed frustules of diatom *Attheya* sp. (L, M); cells feeding on the
 1577 diatom *Coscinodiscus radiatus* (N-Q); cells feeding on the diatom cf. *Pinnularia* sp. (R, S); cf.
 1578 *Pinnularia* sp. frustule after predator attack, chloroplasts are released out through the damaged
 1579 frustule (T); cell aggregation of starved cells (U); large irregular multiflagellated cells (V-X).
 1580 Abbreviations: **af** – anterior flagellum, **bg** – bulge from which flagella emerge, **ch** –
 1581 chloroplast, **dp** – diatom prey cell, **fv** – food vacuole, **pf** – posterior flagellum, **ps** –
 1582 pseudopodia, **vg** – ventral groove. Scale bars: A-T, V-X – 10 µm, U – 20 µm.

1583 **FIGURE 5.** Transmission electron micrographs of *Bordeauxia parva* gen. et sp. nov. strain
 1584 Colp-p1: (A) oblique section showing the general cell structure; (B) transverse section at the
 1585 middle part of the cell showing the ventral groove; (C) flattened vesicle with flocculent
 1586 material; (D,E) mitochondria with tubular cristae and central filament in each crista (E, insert);
 1587 (F) posterior flagellum; (G) Golgi apparatus adjacent to the nucleus; (H) dilations of
 1588 endoplasmic reticulum with tubular mastigonemes; (I) microbody; (J-L) serial sections

1589 showing microtubules (indicated by arrows) conically radiating from kinetosomes and
 1590 surrounding the nucleus. Abbreviations: **fv** – food vacuole; **ga** – Golgi apparatus; **k1** – posterior
 1591 kinetosome; **k2** – anterior kinetosome; **ld** – lipid droplets; **mt** – mitochondrium; **n** – nucleus;
 1592 **nu** – nucleolus; **pf** – posterior flagellum; **ve** – flattened vesicles; **vg** – ventral groove. Scale
 1593 bars: A – 1 μm , B-L – 500 nm.

1594 **FIGURE 6.** Transmission electron micrographs of the flagellar apparatus of *Bordeauxia parva*
 1595 gen. et sp. nov. strain Colp-p1: (A) longitudinal section of the anterior kinetosome; (B)
 1596 proximal part of the posterior kinetosome connected with the nucleus by two nuclear bands;
 1597 (C) distal connecting band between the kinetosomes; (D) proximal part of the posterior
 1598 kinetosome with associated roots; (E) distal part of the kinetosome with transitional fibers; (F-
 1599 I) serial sections of the kinetosomes connected with each other by a striated proximal band; (J-
 1600 N) serial sections of the kinetosomes and associated roots and fibers; (O) posterior part of the
 1601 cell showing joined r1 and two-rowed r2; (P,Q) serial sections of k1 with associated r3; (R)
 1602 lateral side of the cell with left anterior root r4. Abbreviations: **ac** – annular connection; **ax** –
 1603 axosome; **cf** – core fibrous material; **cw** – cartwheel structure; **db** – distal connecting band; **dh**
 1604 – central dense hub; **fb** – additional fibers; **ga** – Golgi apparatus; **k1** – posterior kinetosome;
 1605 **k2** – anterior kinetosome; **n** – nucleus; **nb** – nuclear bands; **r1** – left posterior root; **r2** – right
 1606 posterior root; **r3** – ribbed right anterior root; **r4** – left anterior root; **sb** – striated proximal
 1607 band; **tf** – transitional fibers; **th** – transitional helix; **tp** – transverse plate; **ve** – flattened vesicle.
 1608 Scale bars: 250 nm.

1609 **FIGURE 7.** Diagram illustrating the proximal part of the flagellar apparatus of *Bordeauxia*
 1610 *parva* gen. et sp. nov. viewed from the ventral face (A) and general cell view and orientation
 1611 of flagellar roots from the ventral side (B) and the lateral side (C). The diagram does not
 1612 illustrate the nuclear bands connecting the posterior kinetosomes with the nucleus.
 1613 Abbreviations: **af** – anterior flagellum; **cf** – fibrous core material of r2; **db** – distal connecting
 1614 band; **fb** – additional fiber connecting r1 and r2 together and with k1; **k1** – posterior
 1615 kinetosome; **k2** – anterior kinetosome; **n** – nucleus; **pf** – posterior flagellum; **r1** – left posterior
 1616 root; **r2** – right posterior root; **r3** – ribbed right anterior root; **r4** – left anterior root; **sb** –
 1617 proximal striated connecting band; **arrows** indicate nuclear papillae.

1618 **FIGURE 8.** Transmission electron micrographs of *Feodosia pseudopoda*. (A-H) strain
 1619 NY0226: (A) whole cell; (B,C) peripheral part of the cell with vesicles, microbody and
 1620 multivesicular body; (D,E) intracellular bacteria and dilations of endoplasmic reticulum with
 1621 tubular mastigonemes (see insert); (F) transverse section of the proximal part of the posterior
 1622 kinetosome with associated r2; (G,H) serial sections of the nucleus and proximal parts of the
 1623 kinetosomes. (I-P) strain KT49-10a: (I) longitudinal section of the whole cell; (J) transverse
 1624 section of the whole cell through a large food vacuole; (K) part of cell showing the nucleus,
 1625 mitochondrium, and longitudinal section of the posterior kinetosome; (L) mitochondria with
 1626 tubular cristae; (M) longitudinal section of both kinetosomes; (N-P) serial sections of the
 1627 posterior kinetosome and associated roots and fibers.

1628 Abbreviations: **ac** – annular connection; **av** – probable acorn-V filaments; **ax** – axosome; **b** –
 1629 symbiotic intracellular bacteria; **cf** – core fibrous material; **ct** – central thickening of transverse
 1630 plate; **cw** – cartwheel structure; **db** – distal connecting band; **df** – diatom frustule; **dh** – central
 1631 dense hub; **er** – endoplasmic reticulum; **fb** – additional fibers; **k1** – posterior kinetosome; **k2**
 1632 – anterior kinetosome; **ld** – lipid droplets; **mb** – microbody; **mt** – mitochondrium; **m vb** –
 1633 multivesicular body; **n** – nucleus; **nb** – nuclear bands; **np** – nuclear papilla; **nu** – nucleolus; **pf**
 1634 – posterior flagellum; **r1** – left posterior root; **r2** – right posterior root; **sb** – striated proximal
 1635 band; **tf** – transitional fibers; **th** – transitional helix; **tp** – transverse plate; **ve** – vesicle; **vg** –
 1636 ventral groove. Scale bars: A, I, J – 1 μm , B-H, K-P – 250 nm.

1637 **FIGURE 9.** Transmission electron micrographs of *Bullionia fluviatilis* gen. et sp. nov. strain
 1638 Pirs-vl: (A) longitudinal section of whole cell; (B) transverse section of whole cell at the level

1639 of the proximal part of the posterior kinetosome; (C) mitochondria with tubular cristae; (D)
 1640 part of cell with Golgi apparatus, longitudinal section of the posterior kinetosome connected to
 1641 the anterior kinetosome by the distal connecting band; (E) longitudinal section of the posterior
 1642 kinetosome showing detailed transitional zone; (F,G) serial sections through the proximal part
 1643 of the posterior kinetosome with associated posterior roots, and probable acorn-V filaments
 1644 (G) at the point of transition from triplets (F) to doublets (G); (H-J) serial sections of the
 1645 posterior kinetosome and associated roots and fibers, and striated nuclear bands connecting the
 1646 kinetosome with the nucleus. Abbreviations: **ac** – annular connection; **ax** – axosome; **cf** – core
 1647 fibrous material; **ct** – central thickening of transverse plate; **db** – distal connecting band; **dh** –
 1648 central dense hub; **fb** – additional fibers; **ga** – Golgi apparatus; **k1** – posterior kinetosome; **k2**
 1649 – anterior kinetosome; **ld** – lipid droplets; **mt** – mitochondrium; **n** – nucleus; **nb** – nuclear
 1650 bands; **nu** – nucleolus; **r1** – left posterior root; **r2** – right posterior root; **th** – transitional helix;
 1651 **tp** – transverse plate. Scale bars: A, B – 1 μ m, C-I – 250 nm.

1652 **FIGURE 10.** (A) Maximum likelihood (ML) 16S rRNA phylogenetic tree showing the
 1653 position of the bacterial endosymbiont of *Feodosia pseudopoda* strain NY0226 within
 1654 Gammaproteobacteria. The tree contains 102 gammaproteobacterial sequences and 7 outgroup
 1655 sequences of Alphaproteobacteria. The tree was constructed using the TPM3+R6 sequence
 1656 evolution model. Branches with 100% ML bootstrap values are indicated by black dots, values
 1657 lower than 50% are not shown. The branch lengths denoted by double slash (//) was shortened
 1658 (2 \times) to improve visualization. For the full tree, see Figure S3. (B-D) Different views of a single
 1659 *F. pseudopoda* cell under Hoechst fluorescent staining showing the presence of four host nuclei
 1660 (indicated by white arrows) and numerous endosymbiont bacteria. Scale bars: 5 μ m.

1661

1662 SUPPORTING INFORMATION

1663 **FIGURE S1.** Bayesian phylogenetic tree of 18S rRNA gene sequences. The tree contains 86
 1664 stramenopile sequences, including sequences of 7 new pirsoniales strains, 2 new ochrophyte
 1665 strains (prey), 11 environmental sequences, and 5 outgroup sequences. Bayesian posterior
 1666 probabilities (BPP, GTR+I+GAMMA4) and Maximum Likelihood (ML, TN+F+I+G4 model)
 1667 bootstrap values are indicated at branches (only values >0.8/>50 are shown); black dots
 1668 indicate values of BPP = 1.00 and ML bootstrap = 100%; dt – different topology. Sequences
 1669 generated in this study are shown in bold.

1670 **FIGURE S2.** Bayesian phylogenetic tree of 18S rRNA gene sequences. The tree contains 97
 1671 stramenopile sequences, including sequences of 7 new pirsoniales strains and 2 new ochrophyte
 1672 strains (prey), and 7 outgroup sequences. Bayesian posterior probabilities (BPP,
 1673 GTR+I+GAMMA4) and Maximum Likelihood (ML, TN+F+I+G4 model) bootstrap values are
 1674 indicated at branches (values >0.8/>50 are shown); black dots indicate values of BPP = 1.00
 1675 and ML bootstrap = 100%; dt – different topology. Sequences generated in this study are shown
 1676 in bold.

1677 **FIGURE S3.** Maximum likelihood (ML) 16S rRNA phylogenetic tree showing the position of
 1678 the bacterial endosymbiont of *Feodosia pseudopoda* strain NY0226 within
 1679 Gammaproteobacteria. The tree contains 102 gammaproteobacterial sequences, and 7 outgroup
 1680 sequences of Alphaproteobacteria. The tree was constructed using the TPM3+R6 sequence
 1681 evolution model. Branches with 100% ML bootstrap values are indicated by black dots, values
 1682 lower than 50% are not shown.

1683 **TABLE S1.** Comparison of the identity in the 18S rRNA sequences of new pirsoniales strains.

1684 **TABLE S2.** Morphological characteristics of all known Pirsoniales species.

1685 **Video S1.** *Bullionia fluviatilis* strain Pirs-v1 swimming and attached (resting) cells that
 1686 swallowed diatoms, PhC \times 63, water immersion.

1687 **Video S2.** *Koktebelia satura* strain Colp-Nor6 attached (resting) cell with pseudopodia, DIC
 1688 \times 63, oil immersion.

- 1689 **Video S3.** *Koktebelia satura* strain Colp-Nor6 cluster of cells, PhC ×63, water immersion.
1690 **Video S4.** A *Noirmoutieria diatomophaga* strain Pirs-sm2 attached (resting) cell sheds off its
1691 flagellum, DIC ×100, oil immersion.
1692 **Video S5.** *Feodosia pseudopoda* strain Colp-S9 attached (resting) cells with slowly undulating
1693 flagella, DIC ×100, oil immersion.
1694 **Video S6.** *Feodosia pseudopoda* strain Colp-S9 multiflagellated swimming cell, DIC ×63,
1695 water immersion.
1696 **Video S7.** *Feodosia pseudopoda* strain Colp-S9 multiflagellated resting cell with large food
1697 vacuole, DIC ×63, water immersion.
1698 **Video S8.** *Feodosia pseudopoda* strain KT49-10a cell aggregation, DIC ×63, water immersion.

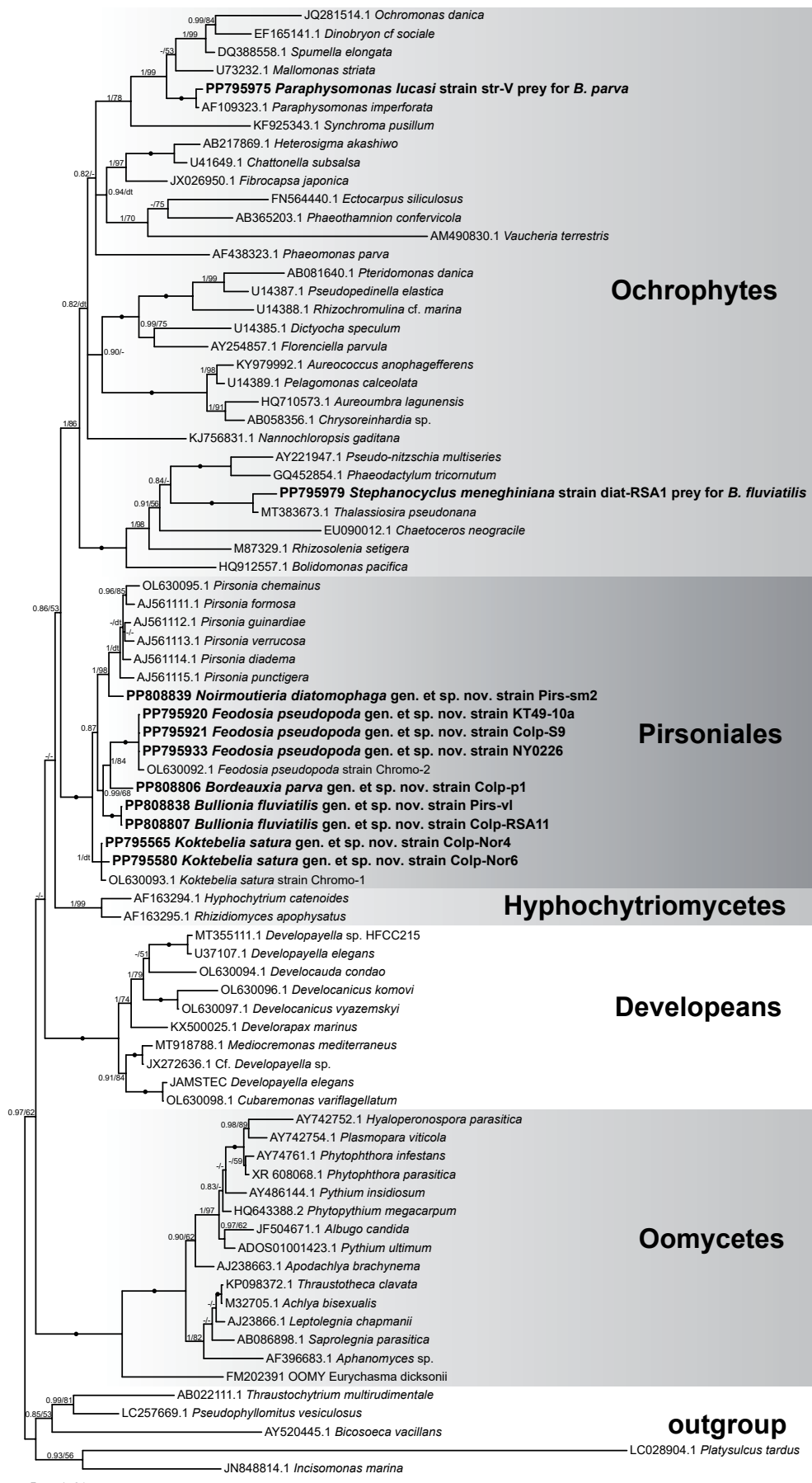
Table 1. Information about the studied strains and samples from which they were isolated

Species	Strain	Sample collection/culture source	Coordinates	Date
<i>Bordeauxia parva</i> gen. et sp. nov.	Colp-p1	France, Talmont-sur-Gironde, Gironde estuary, Atlantic ocean	45°32'03.5"N 0°54'24.0"W	01.07.2021
<i>Paraphysomonas lucasi</i> Scoble et Cavalier-Smith, 2014	str-V			
<i>Bullionia fluviatilis</i> gen. et sp. nov.	Pirs-v1	France, Normandie, Valmont, river Valmont	49°44'50.7"N 0°30'26.3"E	1.08.2022
	Colp-RSA11	France, Ile-de-France, Bullion, river Saint Anne	48°36'43.7"N 1°58'40.6"E	14.03.2021
<i>Cyclotella meneghiniana</i> Kütz., 1844	Diat-RSA1			
<i>Feodosia pseudopoda</i> Tikhonenkov et al., 2022	NY0226	France, Plage de Jean Blanc near Saint-Tropez, Mediterranean sea	43°08'57.9"N 6°24'44.6"E	20.09.2019
	Colp-S9	Italy, Sicily, near the salt lake reserve, Mediterranean sea	36°48'02.6"N 15°05'41.1"E	05.08.2021
	KT49-10a	Russia, Krasnodar krai, Sennoy village, Taman Bay of Azov sea	45°19'29.8"N 36°59'58.5"E	15.08.2021
<i>Noirmoutieria diatomophaga</i> gen. et sp. nov.	Pirs-sm2	France, Noirmoutier-en-l'Île, Atlantic ocean	47°01'12.3"N 2°14'43.0"W	2.08.2022
<i>Koktebelia satura</i> Tikhonenkov et al., 2022	Colp-Nor4	France, Yport, La Manche	49°44'23.8"N 0°18'33.7"E	27.07.2023
	Colp-Nor6	France, Vaucottes, La Manche	49°44'17.7"N 0°17'26.9"E	27.07.2023
Cf. <i>Developayella</i> sp.	str-opiA	Russia, Sochi, Black sea	43°35'00.7"N 39°42'49.8"E	21.10.2021
<i>Attheya</i> sp.	NY0228	France, Saint-Tropez, Plage de Jean Blanc, Mediterranean sea	43°08'57.9"N 6°24'44.6"E	20.09.2019
<i>Odontella granulata</i> (Roper) Ross, 1986	D1-val	Spain, Valencia, Mediterranean sea	39°28'28.3"N 0°19'21.9"W	2.08.2023
<i>Lithodesmium undulatum</i> Ehrenberg, 1839	D10-val			
<i>Cylindrotheca closterium</i> (Ehrenberg) Reimann et Lewin, 1964	D11-val			
<i>Amphiprora alata</i> (Ehrenberg) Kützling, 1844	D4-Nor2	France, Fecamp, La Manche	49°45'47.1"N 0°21'47.2"E	27.07.2023
<i>Parlibellus</i> sp.	D5-Nor2			
cf. <i>Pinnularia</i> sp.	D6-1-Nor2			
<i>Gyrosigma</i> sp.	D6-2-Nor2			
<i>Navicula</i> sp.	D8-Nor2			
<i>Tabellaria</i> sp.	D12-Nor2			
<i>Amphora</i> sp.	D13-Nor2			
<i>Coscinodiscus</i> sp.	D14-Nor2			
<i>Licmophora</i> sp.	D2-Nor9	France, Etretat, La Manche	49°42'29.5"N 0°12'06.2"E	27.07.2023
<i>Ditylum brightwellii</i> (West) Grunow, 1885	CCMP359	Benjamin BAILLEUL	-	-

<i>Phaeodactylum tricornutum</i> Bohlin, 1897	CCMP2561	Benjamin BAILLEUL	-	-
<i>Skeletonema costatum</i> (Greville) Cleve, 1873	RCC1617	Benjamin BAILLEUL	-	-
<i>Minidiscus spinulatus</i> (Takano) Park et Lee in Park et al., 2017;	RCC4659	Benjamin BAILLEUL	-	-
<i>Leptocylindrus danicus</i> Cleve, 1889	CCMP1856	Benjamin BAILLEUL	-	-
<i>Thalassiosira pseudonana</i> Hasle et Heimdal, 1970	CCMP1335	Benjamin Bailleul	-	-
<i>Opephora guenter-grassii</i>	DCG 044	Benjamin Bailleul	-	-
<i>Chaetoceros muelleri</i> Lemmermann, 1898	CCAP 1010-3	Benjamin Bailleul	-	-
<i>Porphyridium purpureum</i> (Bory) Drew et Ross, 1965	CCAP 1388/4	Fabian van Beveren	-	-
<i>Erythrolobus coxiae</i> Scott et al., 2006	CCAP 1393/6	Fabian van Beveren	-	-
<i>Galdieria sp.</i>	ACUF613	Fabian van Beveren	-	-
<i>Timspurckia oligopyrenoides</i> Yang et al., 2010	CCAP 1393/1	Fabian van Beveren	-	-
<i>Rhodella violacea</i> (Kornmann) Wehrmeyer, 1971	SAG 115.79	Fabian van Beveren	-	-
<i>Rhodaphanes brevistipitata</i> West et al., 2007	CCAP 1387/1	Fabian van Beveren	-	-
<i>Procryptobia sorokini</i> (Zhukov, 1975) Frolov et al., 2001	B-69	Denis Tikhonenkov	-	-
<i>Coscinodiscus radiatus</i> Ehrenberg, 1840	PLY1	Varsha Mathur	-	-

Table 2. Prey range of new Pirsoniales species. Data from feeding experiments: + well suitable prey, predators consumed prey actively and multiplied fast; +/- predators did not actively pursue prey and only partially consumed it, multiplied slowly and eventually culture died; - predators did not consume this prey; n/d – no data

Species name	<i>Feodosia pseudopoda</i>			<i>Bordeauxia parva</i>	<i>Noirmoutieria diatomophaga</i>	<i>Koktebelia satura</i>	
Strain name	NY0226	Colp-S9	KT49-10a	Colp-P1	Pirs-sm2	Colp-Nor4	Colp-Nor6
Heterotrophic flagellates							
<i>Procryptobia sorokini</i>	-	+	+	-	+	+	+
Cf. <i>Developayella</i> sp.	-	-	-	-	-	+	+
<i>Paraphysomonas lucasi</i>	-	-	-	+	-	+	+
Diatom algae							
<i>Attheya</i> sp.	+	+	+	-	+	+	+
<i>Chaetoceros muelleri</i>	+	+	+	-	+/-	+	+
<i>Coscinodiscus radiatus</i>	-	+	-	-	n/d	-	-
<i>Gyrosigma</i> sp.	-	+/-	-	-	n/d	-	-
<i>Licmophora</i> sp.	-	+	+	-	n/d	-	-
<i>Navicula</i> sp.	+	+	+	-	n/d	-	-
<i>Opephora guenter-grassii</i>	+/-	+/-	+/-	-	+/-	+	+
cf. <i>Pinnularia</i> sp.	-	+	-	-	n/d	-	-
<i>Tabellaria</i> sp.	+	+	+	-	n/d	+/-	+/-
<i>Thalassiosira pseudonana</i>	+/-	+/-	+/-	-	-	+	+
<i>Amphiprora alata</i> , <i>Amphora</i> sp., <i>Coscinodiscus</i> sp., <i>Cylindrotheca</i> <i>Closterium</i> , <i>Lithodesmium undulatum</i> , <i>Odontella granulata</i> , <i>Parlibellus</i> sp.	-	-	-	-	n/d	-	-
<i>Leptocylindrus danicus</i>	-	-	-	-	-	-	-
<i>Ditylum brightwellii</i> , <i>Minidiscus spinulatus</i> , <i>Phaeodactylum tricornutum</i> , <i>Skeletonema costatum</i>	-	-	-	-	-	n/d	n/d
Red algae							
<i>Erythrolobus coxiae</i>	-	+	+	-	n/d	+	+
<i>Galdieria</i> sp.	-	-	-	-	n/d	+/-	+/-
<i>Porphyridium purpureum</i>	+/-	+	+	-	n/d	+	+
<i>Rhodaphanes brevistipitata</i>	-	-	-	-	n/d	+	+
<i>Rhodella violacea</i>	+/-	-	-	-	n/d	+	+
<i>Timspurckia oligopyrenoides</i>	-	-	-	-	n/d	+	+



Bigyromonadea
Bigyromonadea
Pseudofungi

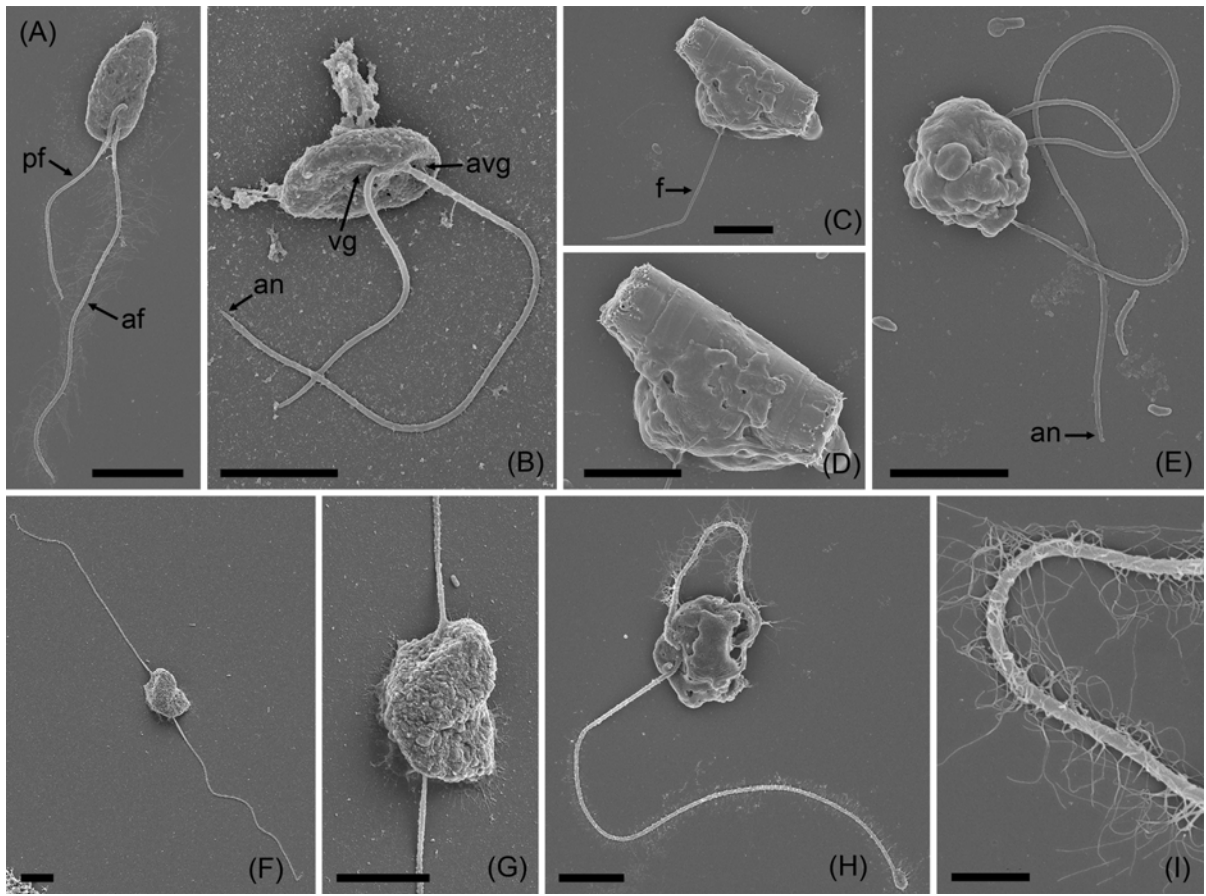


Figure 2

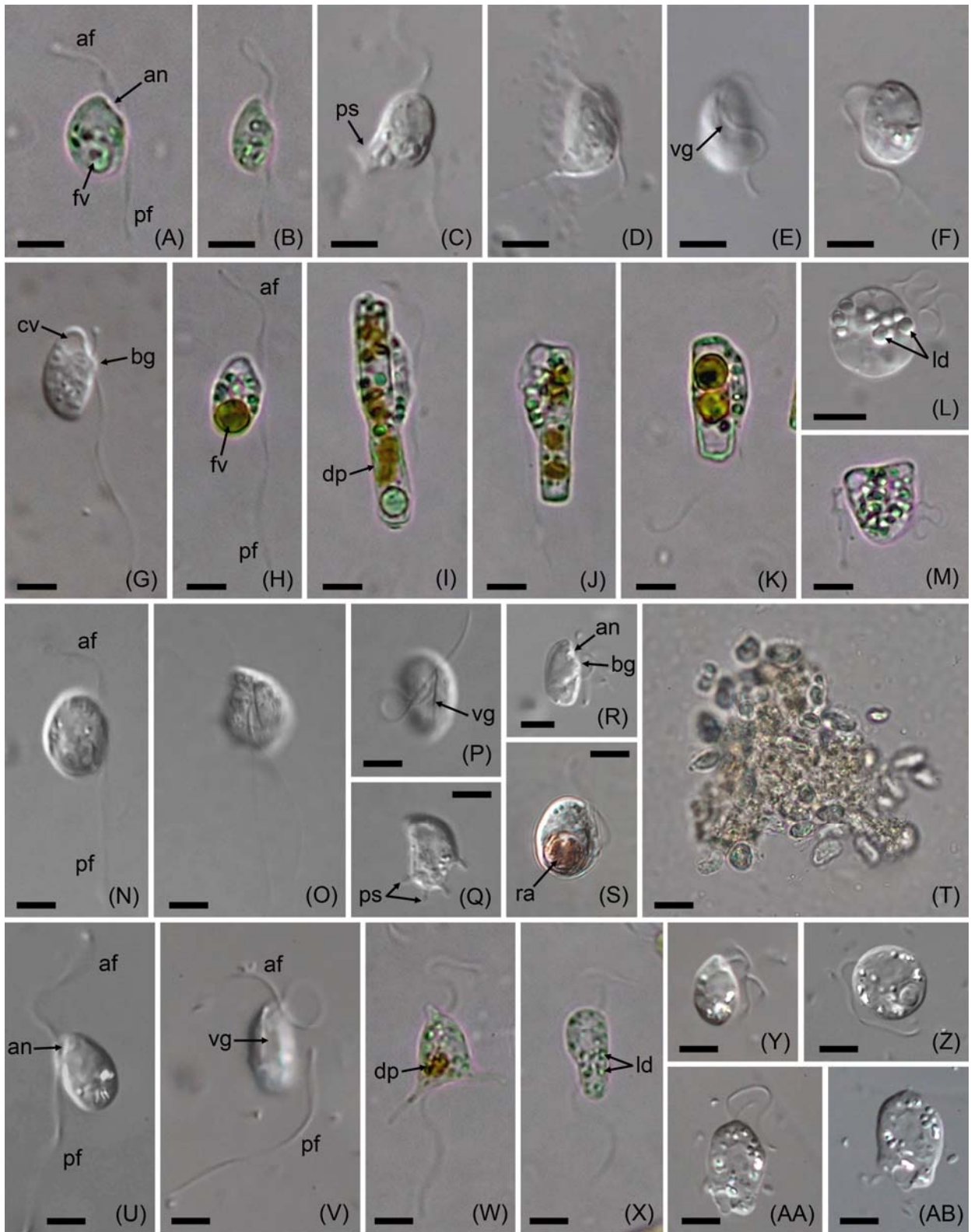


Figure 3

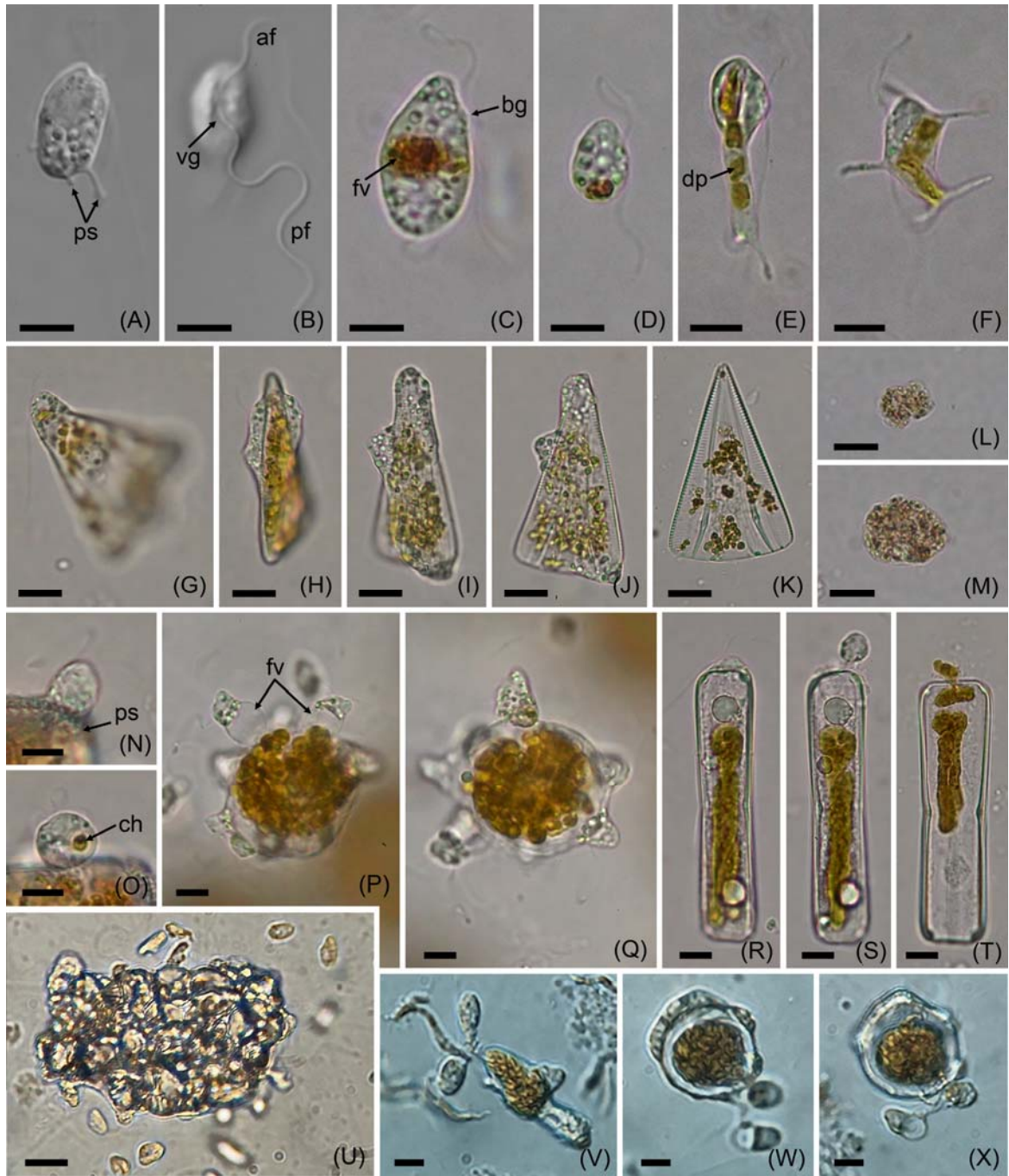


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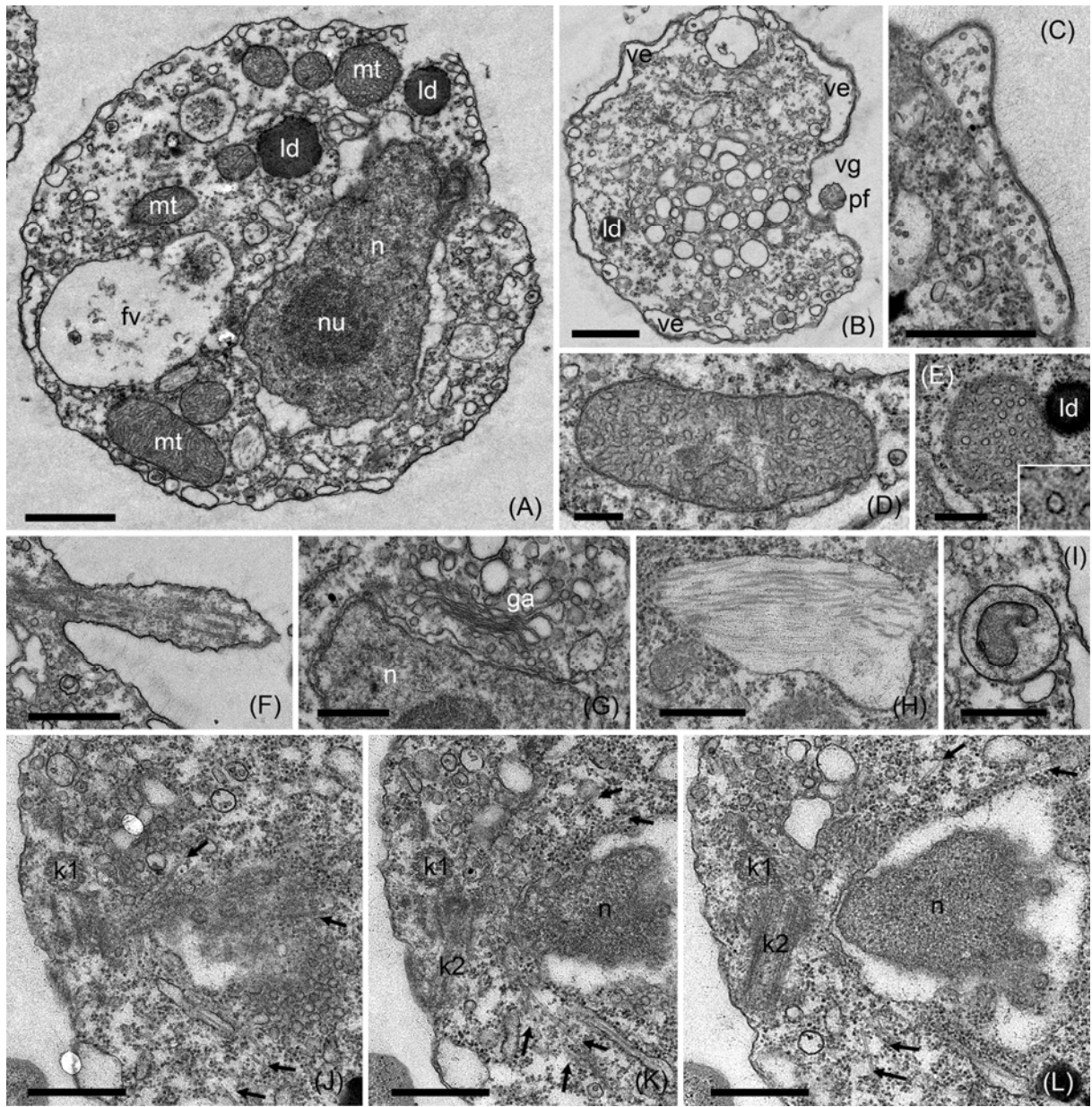


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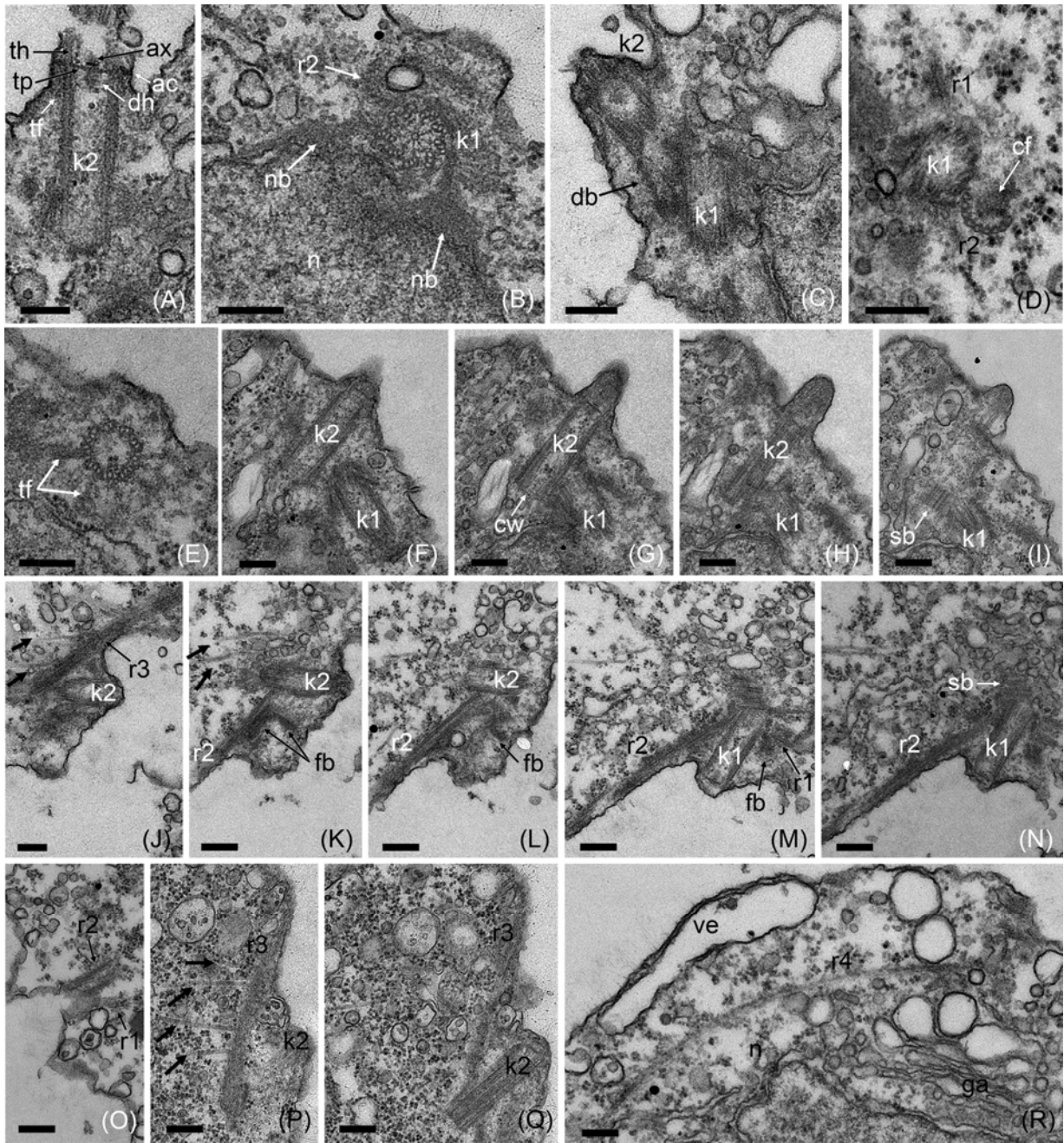


Figure 6

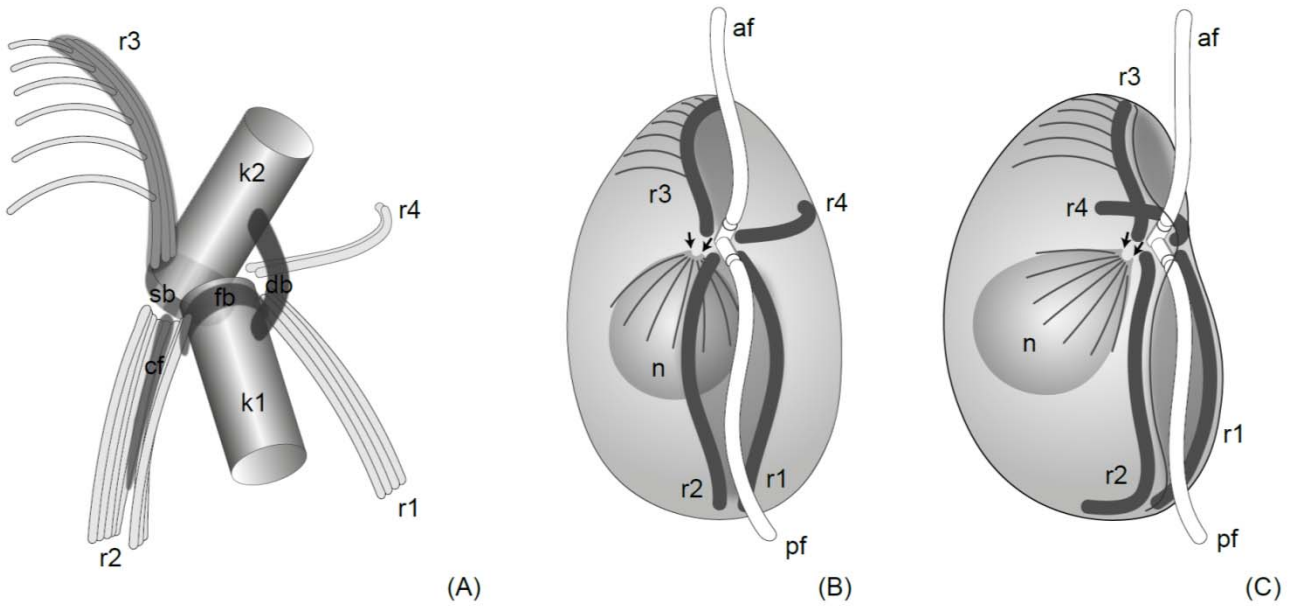


Figure 7

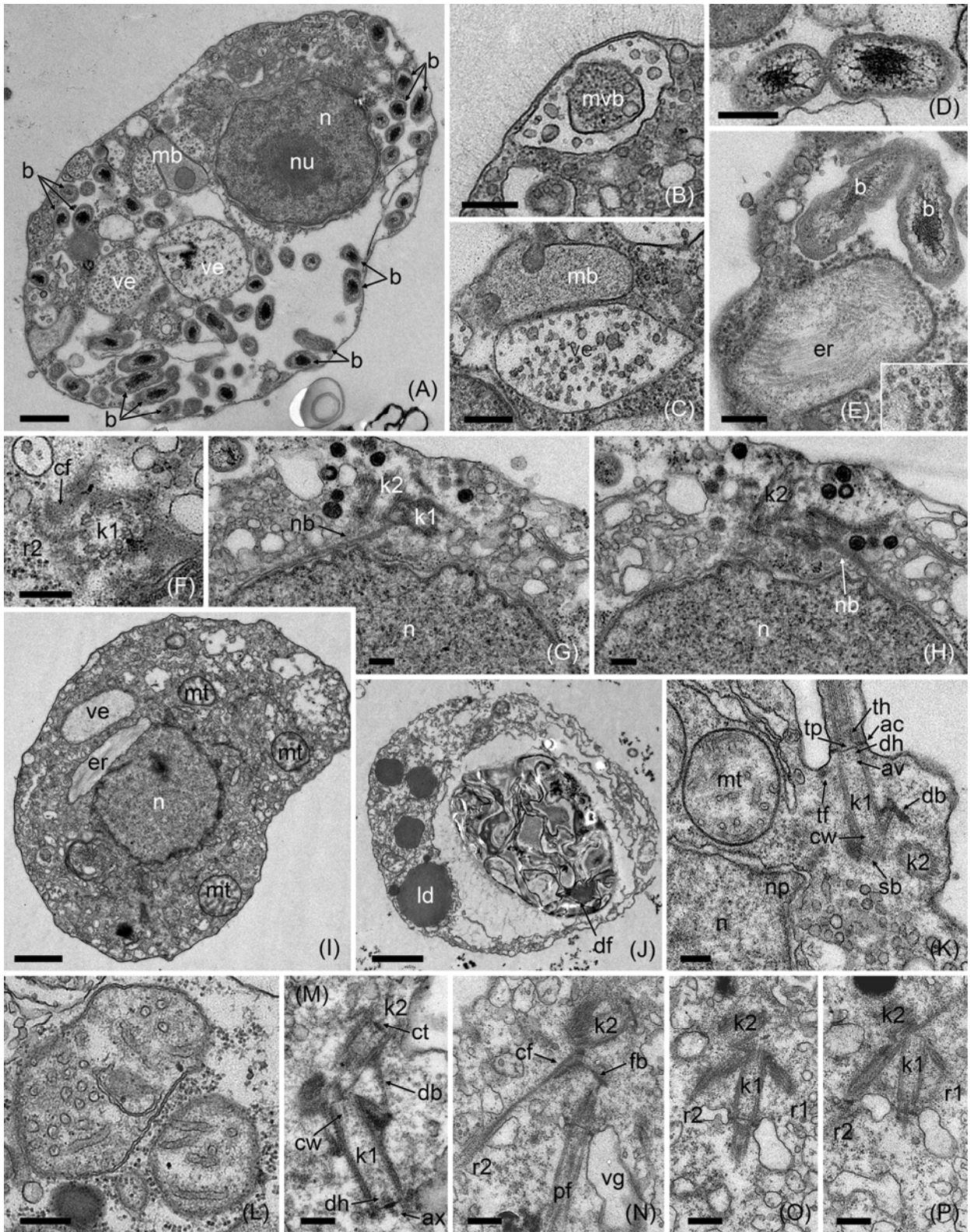


Figure 8

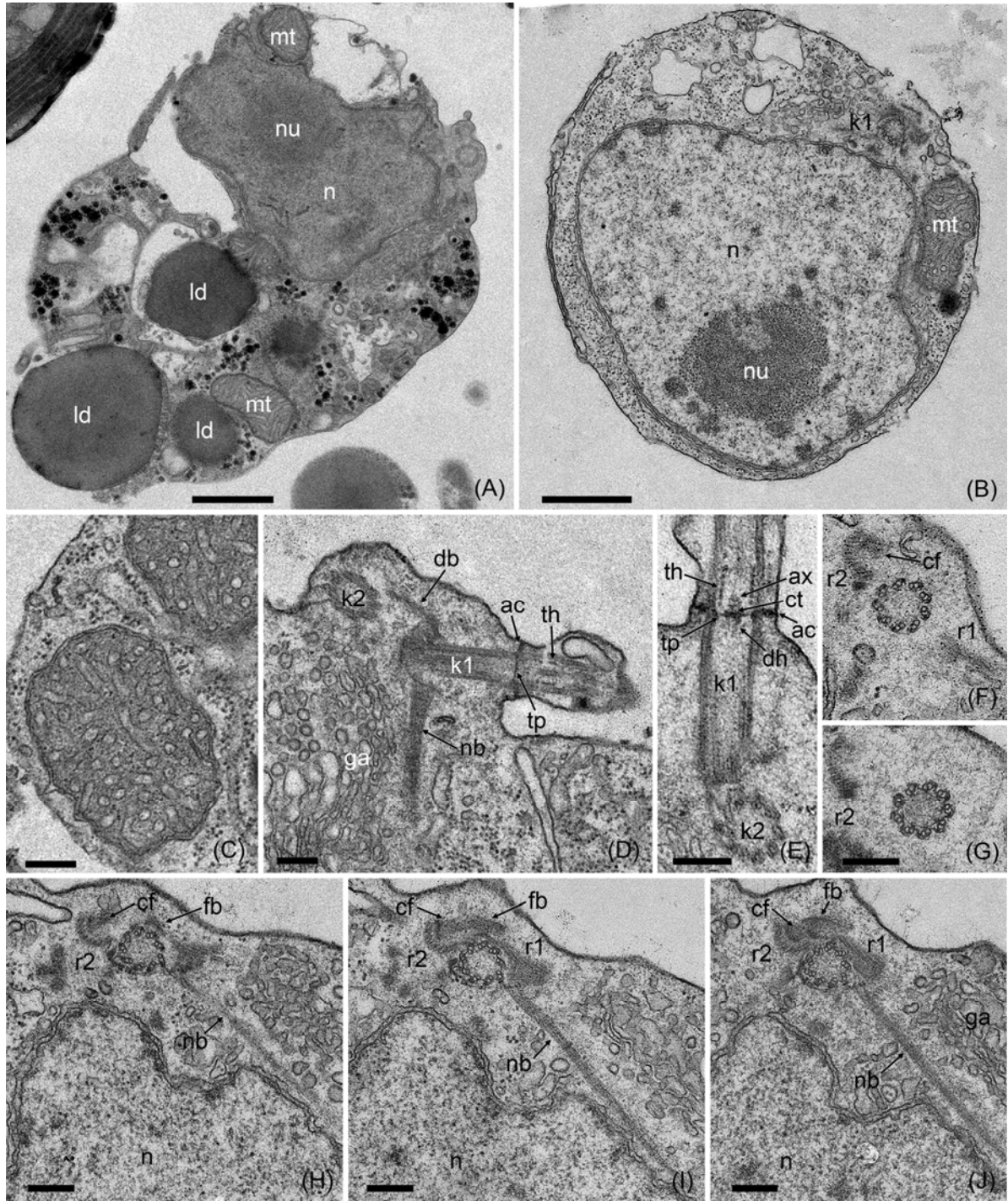


Figure 9

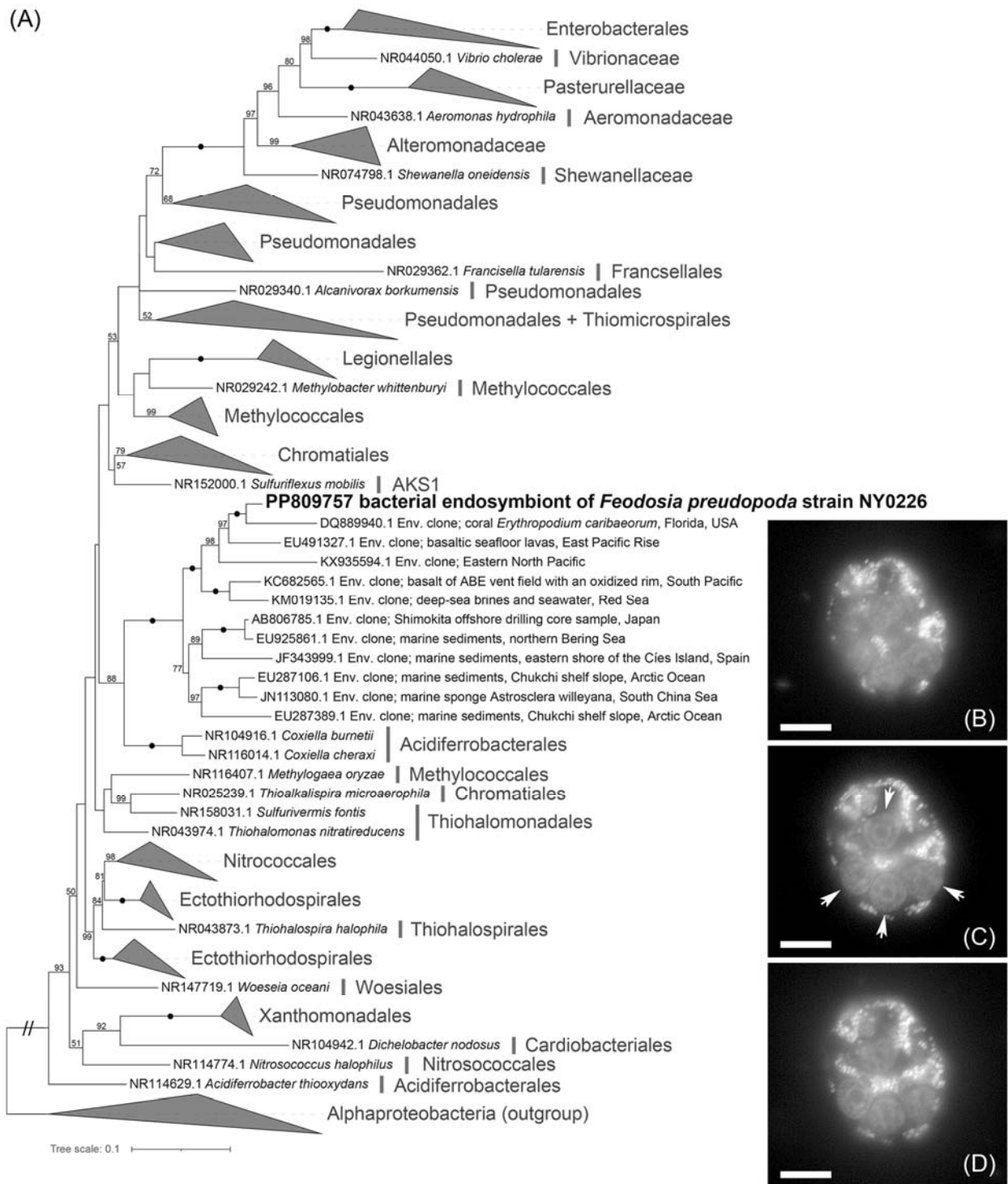


Figure 10