Non-photosynthetic, brown loricate unicell *Fuscolorica viatorophila gen. et sp. nov.* (*Cercozoa,* ?*Nanofilidae*) on the marine red alga, *Viator vitreocola* (*Rhodophyta, Stylonemataceae*), and bacterial associates

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Following the 2011 earthquake and tsunami incident at Tohuku, Japan, massive quantities of debris were carried by the North Pacific Current to North America during 2012–2017. Two new red algae of the class *Stylonematophyceae*, *Tsunamia transpacifica* (West & al. 2016) on plastic drift and *Viator vitreocola* (Hansen & al. 2019) on glass drift. *Viator* cultures had numerous interesting bacteria and an unidentified non-photosynthetic eukaryotic loricate unicell that occurred in only one of ten *Viator* isolates from a fluorescent tube which will be formally described here.

The crustose red alga *Viator vitreocola* was isolated from a drift fluorescent tube collected at Otter Crest, Oregon (44.747333, -124.064833) on 25 March 2018 (Hansen & al. 2019). Of 10 subsamples (4881:1–10), individually scraped from the tube for culture, only isolate 4881-2 had a protist with a brown lorica as an associate. It grew well in culture intermixed with the alga *Viator* attached to glass and plastic substrates and was easily transferred with *Viator* to subcultures.

Unialgal cultures were prepared by placing single small (2–3 mm) strips into each 35-mm well of 6-well microculture plates, each containing 5 mL of quarter-strength Modified Provasoli's Medium (West & McBride 1999) with GeO_2 and Na Penicillin G added to supress diatom and cyanobacterial growth, respectively. A 13-mm circular glass coverslip was added to each well. The plates were sealed with tape and placed in 18–20°C in a 10:14 h light-dark cycle with 2-4 µmol photons m^{-2} s^{-1} LED cool-white lighting. These were monitored at 2-week intervals for recovery, growth, and reduction of contaminants by excising 1 mm pieces of material with microforceps and examining them under a stereomicroscope brightfield microscope. Clean segments were then transferred to similar 6-well plates or to 50 x 70-mm glass dishes containing 22-mm glass coverslips with the same medium and conditions.

To determine if *Fuscolorica* was dependent on a specific host, additional tests were carried out.

1. Glass coverslip fragments with only live *Fuscolorica* cells from 4881-2 culture were transferred to two different isolates 4881-9 and 4881-7 of live *Viator vitreocola* with clean coverslips on the dish bottom to see if the cells would reproduce and grow well in association with each different

4881 live isolate. This was indeed the case: *Fuscolorica* behaved in the same way as with the same original host 4881-2.

2. Glass coverslip fragments with aged thick-walled *Fuscolorica* cells (no *Viator*) were transferred to a culture of a genetically related crustose stylonematophycean isolate, *Tsunamia transpacifica* J.A.West, G.I.Hansen, Zuccarello & T.Hanyuda (isolate 4874, West & al. 2016), with clean coverslips on the dish bottom. These did not support reproduction or growth of *Fuscolorica* cells.

3. Two additional stylonematophycean hosts were tested: *Stylonema alsidii* (Zanardini) K.M.Drew JAW 4877-2 and *Goniotrichopsis reniformis* (Kajimura) N.Kikuchi JAW4563 (Kikuchi & al., 2006). These also did not support *Fuscolorica* cells.

4. Macerated dead *Viator* cells were tested. Glass coverslip fragments with *Fuscolorica* cells were transferred to a culture with clean coverslips on the dish bottom with *Viator* cells (Eppendorf tube containing a cell pellet macerated with pestle and heat sterilised with 800-mV microwave for 1 min) to determine if dead cells and soluble organic suspension would serve as a food source for bacteria/protists and direct nutrient uptake by *Fuscolorica*. In two months of culture with macerate no new *Fuscolorica* cells were found even though numerous bacteria developed on the coverslips. This suggests that some essential metabolite is released from live *Viator*.

The live cells adhering to plastic cover slips were stained with 1:10000 SYBR® Green (Thermofisher) for 5 min. Following staining, coverslips were washed in MiliQ water, mounted under a 22-mm coverslip on a slide in Antifade DAKO fluorescent mounting media (Agilent), and sealed with VALAP (1:1:1 mixture of paraffin, lanolin, and Vaseline® petroleum jelly) melted on a hotplate at 40°C). Imaging was achieved with a Nikon C2 confocal microscope (Biological Optical Microscopy Platform, University of Melbourne). Deconvolution and image reconstruction were done with NIS Elements software (Nikon).

Samples of *Viator* and *Fuscolorica* grown on glass coverslips were fixed for scanning electron microscopy (SEM) with 2.5% glutaraldehyde in culture medium for 1 h. The cells were then washed in polybutylene succinate three times and double distilled water once for 10 min each wash. The samples were subsequently dehydrated in a graded ethanol series for 1 h at each step before being critical-point-dried using a Leica CPD300 critical point dryer (Leica Microsystems, Australia). The samples were attached to aluminium stubs using carbon sticky tabs, carbon coated and viewed using an FEI Quanta SEM (Thermo Fisher) equipped with an Oxford INCA energy dispersive spectroscopy (EDS) detector (Nanospec).

Total genomic DNAs were extracted from five isolates using a modified 2X CTAB (ionic detergent cetyltrimethylammonium bromide) method. Briefly, samples were plunged directly into liquid nitrogen for 1 minute and thawed at 96°C for 1 min. This freeze-thaw cycle was conducted three times and then the samples went through a grinding process using a sterile micro pestle. After this homogenization step, CTAB DNA extraction method was followed with 2X CTAB lysis solution described in Stewart (1997).

For species identification, partial 18S rRNA was amplified from five isolates using Cercozoa specific 18S primers of the 18S-590F (5'CGGTAATTCCAGCTCCAATAGC3') and 18S-1256R (5'GCACCACCACCCAYAGAATCAAGAAAGAWCTTC3') (Bass & Cavalier-Smith 2004, Quintela-Alonso & al. 2011). PCR conditions consisted of an initial denaturing phase at 95°C for 2 min, and 30 cycles at 95°C for 20 seconds, annealing at the 53°C for 40 s, and 72°C for 1 min with

10-min extension. Amplified PCR products were purified using Labo Pass TM Gel Extraction Kit of Cosmo Genetech Inc. (Seoul, Korea), and sent to Macrogen Inc. (Seoul, Korea) for sequencing. 18S rRNA sequences of *Fuscolorica* were used as queries for BLASTn search (e- value ≤ 1e-5), to collect a homologous sequence. Among the 478 top-matched sequences, 81 of 18S rRNA sequences were not duplicated but represent Cercozoan classes that were chosen for phylogenetic analysis. These 18S rRNA sequences were aligned using MAFFT (V. 8.3.10) with default option. The Maximum likelihood phylogenetic tree was conducted using IQ-TREE software (V. 1.6.8.) with 1000 replications. The best evolutionary model (TIM3+F+I+G4) for this phylogeny was selected with the IQ-TREE basic option for model selection function.

Bacteria were initially considered possible prey of *Fuscolorica* and it was important to identify those associated with the red alga *Viator*.

Six replicate broken sections of glass coverslips with visible growth of *Viator vitreocola* and *Fuscolorica* in isolate 4881-2 (Hansen & al., 2019) were removed from fresh cultures: three were used to inoculate marine agar (MA; Difco™ Marine Agar 2216, Edwards) plates to culture associated bacteria, while the other three were placed in sterile 1.5 ml Eppendorf tubes for DNA extraction.

Each of three glass coverslip sections, along with 50 µL of seawater media, were spread-plate inoculated onto three replicate MA plates. All plates were incubated at 26°C for 7 d. Fifteen bacterial colonies were selected based on unique colony morphology and subcultured to purification. Colony PCR with the universal bacterial primers 27f (5' AGAGTTTGATCMTG-GCTCAG 3') and 1492r (5' TACGGYTACCTTGTTACGACTT 3'; Lane, 1991) was used to generate 16S rRNA gene amplicons from each isolate. Briefly, cells from each pure culture were suspended in 20 µL Milli-Q water and denatured at 95°C for 10 min. The suspension was then centrifuged at 2,000 rev min $^{-1}$ for 2 min and the supernatant was used as the DNA template for PCR amplification. The PCR was performed with 20 µL Mango Mix™ (Bioline), 0.25 µM of each primer and 2 µL of DNA template in a final volume of 40 µL with nuclease-free water (Ambion). The thermal cycling protocol was as follows: 95°C for 5 min; 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and a final extension of 10 min at 72°C. Amplicons were purified and Sanger sequenced on an ABI sequencing instrument by Macrogen Inc. (Seoul, South Korea) using the 1492r primer. Trimmed high quality read data from each isolate was used for presumptive identification by querying the 16S rRNA gene sequences via the Basic Local Alignment Search Tool (BLASTn).

Total DNA was extracted using a modified protocol described by Wilson & al.(2002) and Hartman & al. (2020). In preparation for DNA sequencing, the V5-V6 region of the bacterial 16S rRNA gene was amplified by PCR in triplicate using primers with overhang adapters to determine bacteria relative abundance: 784f [5′ GTGACCTATGAACTCAGGAGTCAGGATTAGATACCC-TGGTA 3′]; 1061r [5′ CTGAGACTTGCACATCGCAGCCRRCACGAGCTGACGAC 3′] & al., 2008). PCR for each sample was completed in independent triplicate reactions containing 7.5 µL MyTaq HS Mix polymerase (Bioline), 1 µL of DNA template, 0.4 µM of each primer and nuclease-free water up to 15 µL. PCR conditions consisted of an initial denaturation step at 95°C for 3 min, 18 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s followed by a final extension at 72°C for 7 min. Triplicate PCR products were pooled and cleaned using NucleoMag® NGS beads (Macherey-Nagel, Scientifix, Australia). A volume of 10 µL of cleaned DNA was added to 10 µL 2× Taq master mix (M0270S, New England Biolabs, Australia) and 0.25 µM of a forward and reverse indexing primer. Indexing PCR conditions consisted of an initial denaturation step at 95°C for 3 min, 24 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s followed by a

final extension at 72°C for 7 min. Each sample was checked for product size and quantity (2200 TapeStation, Agilent Technologies, Australia). Libraries were created by pooling 5 µL from each sample with other 16S samples and performing a final bead clean-up. Each library was checked for quality and quantity to guide pool normalization (2200 TapeStation). Normalized pools were sequenced at the Walter and Eliza Hall Institute (WEHI), Melbourne, Australia for sequencing on a single Illumina MiSeq V3 2 x 300 bp run.

Raw, demultiplexed MiSeq reads were joined in QIIME2 (Quantitative insights into microbial ecology 2) v2019.1.0 (Caporaso & al. 2010) after primers were removed using Cutadapt (Martin, 2011). Sequence denoising, chimera checking and trimming was performed in DADA2 (Callahan & al., 2016) to correct sequencing errors and remove low quality bases (mean Qscore < 30). Prokaryote taxonomy was assigned in QIIME2 against a SILVA database (version 132) trained with a naïve Bayes classifier against the same V5-V6 region targeted for sequencing & al., 2018). Amplicon sequencing variants (ASVs) identified as eukaryotes, mitochondria, or chloroplasts were filtered from the bacteria dataset. Given that these technical replicates all stem from one biological sample, the three triplicates were pooled for visualization and exploration of the bacterial communities. Metabarcoding raw reads are available in the Sequence Read Archive under BioProject PRJNA773122.

It was very clear that *Fuscolorica* was initially present in only one isolate of *Viator* (4881-2). The many testate cells commonly present on single coverslips varied greatly in size (8–40 µm), surface structure and shape as they enlarged (Fig. 1 A-C). The young and smallest oblong cells about 8–10 µm diam. had a thin colourless to light brown wall with a faint narrow orifice (cytostome) at one end and a small internal protoplast (Fig. 1 D, E) from which a faint filopodium extending a short distance outward from the cytostome was observed very infrequently. As the extracellular brown matrix deposition increased, internal cellular details were obscured when the original inner wall became slightly thicker. The flat, circular lorica usually seen around each cell had a variably shaped perimeter and variable surface texture firmly attached to the substrate (glass, plastic, or the host alga) (Fig. 1). Thin (1–2 µm diam.) channels, often branched, extending outward through the lorica matrix (Fig. 1 C, D & G) and may have been access for feeding. However, we observed many live loricate cells under 1000 x oil immersion light microscopy but rarely observed a cell with an extended filopodium (Fig. 1 E, arrow), and never observed 'feeding' anywhere. Numerous bacteria (unicells, colonial, mycelial) and other small colourless protists (non-motile and motile) were visible on the substrate (Fig. 1 B, F, G).

Very small (0.5–1 µm) brown scales, discoid with a pale dimple-like centre in face view, and variably open, flared, shallow cup-shaped (like a sorbet glass) with a short stem and base, side view (Fig. 1F, insert) were commonly scattered and aggregated around the expanding darker brown lorica, accumulating increasingly at the lorica perimeter (Fig. C, G, H). They did not appear to be like 'captured' uniform scales from other cercozoans that are seen in predatory cercozoans, but evidently, in *Fuscolorica*, scales developed via extracellular biomineralization processes around the cell exterior. In the lorica the homogeneous brown colour and texture of the matrix were variable, with tiny scales distributed around the surfaces (Fig. 1 C, D, G with insert).

SYBR® green stain showed a single spherical to slightly oblong nucleus (1.5-2.1 µm diam.; Fig. 2) in each cell but it was not possible to discern other DNA-containing organelles. SYBR® green stain also revealed numerous small colourless prokaryotes (unicells, colonial, mycelial) and tiny colourless eukaryotes of various sizes much smaller than *Fuscolorica* cells.

To investigate the morphology and possible elemental differences between *Viator* and the associated unicellular protist *Fuscolorica*, samples growing on glass coverslips were prepared for SEM and EDS. *Viator* showed the filamentous habit as described in Hansen & al. (2019), while *Fuscolorica* appeared as cells with variably shaped loricae (Fig. 3 A–C).

The elements present in the two species were compared using EDS (Fig. 3D). The results for *Fuscolorica* indicated the presence of manganese and oxygen with other elements: sulphur, calcium, potassium, and carbon. There appeared to also be elevated silicon in *Viator*; however, this may have been background from the silicon dioxide of the glass coverslip. Minimal differences were seen between oxygen, sodium, aluminium, and titanium. However, aluminium and titanium may have been background elements from the electron microscope and aluminium sample holders. Apart from zinc, copper and magnesium, heavy metals did not appear in *Viator*. Conversely, *Viator* showed the presence of higher amounts of potassium and carbon. Minimal differences were seen between oxygen, sodium, aluminium, and titanium. These latter two elements may also be background elements from electron microscope and aluminium sample holders.

We propose to describe a new genus and new species for this organism under the ICN (Turland & al. 2018):

Fuscolorica viatorophila J.A.West, G.I.Hansen, H.S.Yoon & Loiseaux-de Goër, *gen. et sp. nov.*

Description: Unicell, non-photosynthetic, adherent to substratum, ovate (slightly flat posterior) primary cell 7–9 µm diam., thin (<1 µm) pale-tan wall, that thickened and darkened somewhat with aging, a single narrow (< 1.5 µm) cytostome at apex, colourless protoplast, single nucleus (1.4-2 µm diam.) and probable light grey spherical nucleolus about 2 µm diam., no flagella seen; infrequently a small filipodium was seen emerging from the cytostome. As the lorica increased in size it became light brown to dark brown usually flat round disc about 10–40 µm diam. SEM/EDS analyses of *Fuscolorica* cell walls showed elemental content primarily of O, Mn. Si, C, Zn, Na & K, whereas *Viator* cell walls showed elemental content primarily of O, Si, C, Zn, Na, & K. Loricae of *Fuscolorica* contained much manganese and oxygen suggesting that brown/black colouration may result from +4 MnO_2. Older cells showed a variety of lorica shapes (uniform discs, irregular granular perimeter to dense oblong, cell bases with short and long necks) and surface texture (uniform, granular, warty). Minute (0.5–1 µm) brown, cup-shaped scales common on main lorica surface and around the outer perimeter, suggesting extracellular biochemical processes. Reproduction, cell division and flagella were not observed.

Holotype: Dried, metabolically inactive material of JAW4881-2 on a glass coverslip (**UC** 2085163).

Type locality: The original culture from which the type was isolated was obtained from a fluorescent tube collected by Gayle I. Hansen on the beach at Otter Crest, Lincoln County, Oregon, USA (44.747333, -124.064833) on March 25, 2018.)

Etymology: *Fusco* = prefix for brown, suffix *lorica* = shell, *viatorophila* = preference for the red alga genus *Viator*.

Representative culture: JAW4881-2 containing *Fuscolorica* and *Viator* was sent to the Culture Collection of Algae and Protozoa (CCAP)**,** Scotland.

DNA sequences of type specimens: OK316892 (18S rRNA; Fig. 4).

Partial 18S rRNA sequences (659 bp) using 590F-1256R primers from five isolates were all identical and one representative sequence uploaded to the NCBI (GenBank accession number: OK316892). Phylogenetic analysis indicated that *F. viatorophila* was grouped together with an uncultured Cercozoan obtained from a terrestrial ‘rhizobium’. The sequence (EU567234.1, Bass & al. 2009) with full bootstrap support (100%) (Fig. 4). The clade of *F. viatorophila* was positioned

within the *Granofilosea* with strong bootstrap support (96%) (Fig. 4). This class included several described species (e.g., *Nanofila marina* Cavalier-Smith & D.Bass, *Hedriocystis reticulata* Penard, *Clathrulina elegans* Cienowski, *Mesofila limnetica* Cavalier-Smith & D.Bass, and *Massisteria marina* J.Larsen & D.J.Patterson), but many uncultured environmental cercozoan sequences were also included.

Fuscolorica viatorophila and an unknown cercozoan were identical in the alignment. *Fuscolorica* was positioned within the *Granofilosea* with strong bootstrap support (98%). This group included mostly uncultured environmental *Cercozoa* sequences, except two *Massisteria marina* (AF174370.1 and AF411286.1) and two *Dimorpha*-like species ATCC-50522 (AF411283.1) and ATCC PRA-54 (EF455769.1).

Adl & al. (2007) assessed the relative abundance, methodologies, diversity, nomenclature, and taxonomy of the protistan organisms including the *Cercozoa*. Numerous publications on various protist amoebae groups deal with molecular phylogeny employing SSU rDNA sequences (e.g., Dumack & al. 2017, Shiratori & Ishida 2016,) and Partial 18S rRNA gene sequences (Bass & Cavalier-Smith 2004).

The cercozoan SAR class *Granofilosea* was proposed by Bass & al. (2009) based on environmental 18S rRNA sequences and phylogenetic analyses. The species in this class have branched or unbranched filopodia with granules in reticulated network, but test walls were not reported (Siemensma & Dumack, 2020). Although, it was phylogenetically positioned within the *Granofilosea* but unlike other genera of the class, *F. viatorophila* is covered by a lorica wall like that seen in testate amoebae. We did not observe amoeba-like pseudopodia during microscopic observation. *Fuscolorica* seems to have host-dependent lifestyle, co-occurring with the red alga *Viator vitreocola*. Perhaps, the lorica is somewhat similar to testate amoeba such as *Imbricatea* and host-dependant lifestyle is similar to the parasitic amoebae of the subphylum *Endomyxa* (Berney & al. 2013). A complete life history is still unknown in the *Granofilosea*, largely due to lack of available culture strains, and further observations are needed using this *F. viatorophila* strain.

Fifteen isolates were selected for Sanger sequencing based on morphology. Partial 16S rRNA gene sequences (~1000 bp) were used to identify the closest matches from GenBank using BLASTn. In total there were seven unique species cultured: *Alcanivorax dieselolei* C.L.Li & Z.Z.Shao (2x), *A. xenomutans* Rahul & al. (4x), *Cellulophaga algicola* Bowman, *Labrenzia aggregata* (Uchino & al.) Biebel & al. (2x), *Maribacter pelagius* Xie & al. (4x), *Micrococcus yunnanensis* Zhao & al., and *Tropicibacter phthalicius* Iwaki & al. Of these, *Alcanivorax* and *Labrenzia* ASVs were prominent members of the microbiome as identified with metabarcoding (Fig. 5). A total of 88,022 pairs of reads (ranging from 28,384 to 30,237) were obtained from the Illumina MiSeq. After filtering, denoising, merging and removal of chimeric sequences with DADA2, 59,693 reads remained in the data set (67.8% of the initial data set). The eight most abundant ASV's in the dataset accounted for 94.2% of all reads (Fig. 5).

Confocal microscopy on live *Fuscolorica* incubated with the DNA stain SYBR green showed a single nucleus and no evidence of non-nuclear DNA. This suggests a lack of concentrated organellar DNA and the absence of commensal or symbiotic partners. The lack of photosynthetic symbionts was confirmed by the lack of chlorophyll autofluorescence (not shown). It will require further work with DNA and organelle specific stains, as well as TEM with *Fuscolorica* in culture, to resolve internal organelle structure and DNA placement. The obligate firm adherence of *Fuscolorica* cells and their walls to the substrate requires use of plastic coverslips and special TEM embedding and sectioning methods not available in the present study.

Different testate 'amoebae' form their shells via biomineralisation or acquiring scales from their prey (Lahr & al., 2015; Kosakyan & al., 2016; Dumack & Siemensma 2020). The *Fuscolorica* loricae appear to have developed by biomineralisation of oxygen, carbon and manganese judging from the SEM/EDS evidence. Brown/black colouration of loricae may result from a +4 MnO_2 oxide state. However, the oxide state and colour were not mentioned for any other testate Cercozoa previously (e.g., Dumack & Siemensma 2020).

Adl & al. (2007) assessed the relative abundance, methodologies, diversity, nomenclature, and taxonomy of protists including the *Cercozoa*. Numerous publications on various protist amoeboid groups deal with molecular phylogeny employing SSU rDNA sequences (e.g., Dumack & al., 2017, Shiratori & Ishida 2016) and partial 18S rRNA gene sequences (Bass & Cavalier-Smith 2004). In our present investigation 18S rRNA and eukA/B gene sequences were used.

The class *Granofilosea* was proposed by Bass & al., (2009) based on environmental 18S rRNA sequences and its phylogenetic analysis. The species in this class are known to have branched or unbranched filopodia with granules in reticulated network, but test-wall details were not reported (Siemensma & Dumack 2020). Although *Fuscolorica viatorophila* was phylogenetically positioned within the *Granofilosea* it is covered by test wall similar to testate amoebae but dissimilar to the *Granofilosea*. We also were not able microscopically to observe amoeba-like pseudopodia (Fig. 1E). *Fuscolorica* seemed to have a host-dependent lifestyle, co-occurring with red alga *Viator vitreocola*. The *Fuscolorica* lorica wall resembled that of testate amoebae such as those referred to the class *Imbricatea* and the host-dependent lifestyle may be similar to that of parasitic amoebae in the subphylum *Endomxya* (Berney & al. 2013). These unique features of *Fuscolorica* differ from major *Granofilosea* species. The complete life history of most *Granofilosea* is still unknown, largely due to lack of available culture strains. Further observations are also needed for *F. viatorophila* strain.

The overwhelming majority of reads (72.3%) belonged to the genus *Granulosicoccus*, though no members of this genus were isolated from *V. vitreocola* samples. The Gram-negative non-pigmented bacterium *Granulosicoccus* was first described in 2007 as a member of the new family *Granulosicoccaceae* (Lee & al., 2007) of the order *Chromatiales* (Imhoff, 2005) and currently contains four described species: *G. antarcticus* K.Lee & al. (type), *G. coccoides* Kurilenko & al., *G. marinus* Baek & al., and *G. undariae* S.Park & al.. *Granulosicoccus* is frequently found in association with marine algae and plants, such as brown algae (Park & al.., 2014; Weigel & Pfister, 2019), red algae (Miranda & al. 2013), and the seagrass *Zostera marina* Linnaeus (Kurilenko & al., 2010). They have been characterized as aerobic, chemoheterotrophic bacteria that are capable of nitrate reduction to nitrite (Baek & al., 2014; Park & al., 2014). Functionally, the one full genome of *Granulosicoccus* (*G. antarcticus*) contains genes involved in sulphur metabolism (Kang & al., 2018). A gene for dimethyl sulfoniopropionate (DMSP) demethylase (*dmd*A) was found in the genome, rendering this species as one of the few marine gammaproteobacteria equipped with the potential for DMSP demethylation. In the demethylation pathway, *Dmd*A removes a methane group from DMSP producing methylmercaptopropionate (MMPA). MMPA can undergo an additional demethylation step to generate methanethiol (CH_3SH), which is assimilated by bacteria and eventually back to inorganic sulphur. In addition to its role as an osmoprotectant, DMSP and its other breakdown products [dimethyl sulphide (DMS), acrylate, dimethyl sulfoxide (DMSO)], and methanesulphonic acid (MSA) have been described as antioxidants (Sunda & al., 2002). DMSP lysis greatly increases antioxidant protection as the cleavage products (DMS and acrylate) together are 80 times more effective at scavenging hydroxyl radicals than DMSP (Sunda & al., 2002). As reactive oxygen species (ROS) are constantly being produced during photosynthesis, antioxidants play a critical role in maintaining cellular homeostasis for marine plants and algae. Other stressors

such as high salinity, elevated temperature, and increased UV radiation can cause photosystems to stimulate ROS production.

The second most abundant taxonomic group associated with *V. vitreocola* is the genus *Labrenzia*. Species of *Labrenzia* are routinely found associated with marine algae and two *Labrenzia aggregata* strains were isolated from these cultures. *Labrenzia aggregata* has been identified as a bacterial species that can not only breakdown DMSP to DMS but produce DMSP in the absence of any methylated sulphur compounds (Curson & al., 2017). The latter authors identified the first DMSP synthesis gene in any organism, *dsy*B, which can serve as a reporter for bacterial DMSP synthesis in marine alphaproteobacterial processes. Under low nitrogen environments, such as those of tropical marine systems, *L. aggregata* had a 9-fold increase in DMSP production & al., 2007). Nitrogen limitation increases oxidative stress within algal cells because of (1) decreased photosynthetic efficiency, as evidenced by lower variable to maximum photosystem II fluorescence (Berges and Falkowski, 1998); (2) decreased synthesis of antioxidant enzymes (e.g., ascorbate peroxidase) composed of nitrogen-rich protein (Logan & al.., 1999); and a restriction in enzyme systems that repair intracellular oxidative damage (Litchman & al., 2002).

Contaminating organisms in algal cultures are common, but too few have been studied properly. This investigation gave us the opportunity to provide information on a new, previously unknown eukaryotic unicell. In cultures of the red alga *Viator*, this rare contaminant was scattered over the red alga, glass and plastic substrates. The small non-photosynthetic loricate eukaryotic unicells stood out because they were often surrounded by a brown fringe of scales highlighting the cells. These scales were probably formed by extracellular biochemical processes. Culturing experiments revealed that the protistan unicell required living material of *Viator* to survive. Through further observations and sequencing, the protist was found to be new and a member of the phylum *Cercozoa*, class *Granofilosea*. Unlike other *Granofilosea*, *Fuscolorica* had lorica walls and very infrequent filopodia. Feeding was not observed. Many features of the *Granofilosea*, including life histories and some aspects of nutrition, are poorly known, so comparisons within the group were difficult. Numerous bacteria were identified in the *Fuscolorica-Viator* cultures. Many of these, including *Granulosicoccus* and *Labrenzia*, carry out critical chemical reactions in their field habitats. It seems likely that these bacteria along with the red alga provide nutrients that support the new protist. Our established *Fuscolorica-Viator* cultures can now be used with additional techniques (HPF, TEM, and chemical analyses) to further reveal the biological processes of this unique eukaryote protist.

Pending further studies can be carried out on such marine cercozoans, *Fuscolorica viatorophila* should be referred to the *Nanofilidae* Cavaliier-Smith & D.Bass, a family of the *Cryptofilida* (Bass & al. 2009; cf. Fig. 4).

Hwan Su Yoon is grateful for funding from the National Research Foundation of Korea (NRF-2017R1A2B3001923). We thank: Michael Guiry for assistance with the manuscript; Christopher Goodman who did nuclear stain observations using confocal microscopy at the Biological Optical Microscopy Platform, University of Melbourne; Allison van de Meene who employed SEM/EDS techniques at the BioSciences Microscopy Unit and the Bio21 Advanced Microscopy Platform, University of Melbourne; and Giulia Holland who assisted Ashley Dungan in culturing the bacteria isolated from a *Viator* culture.

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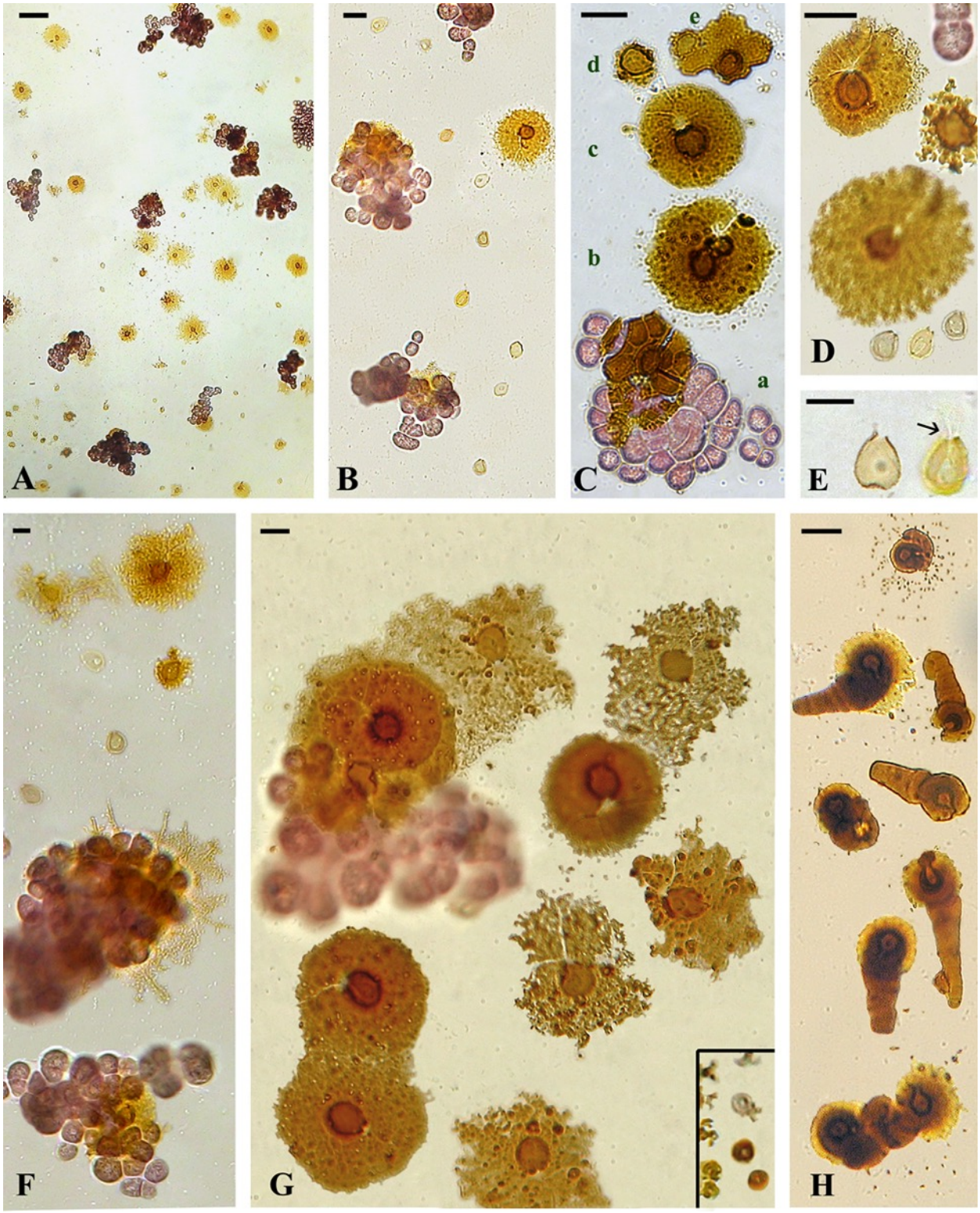


Fig. 1 A–H. *Fuscolorica viatorophila* (JAW4881-2). (A) Overview of many free *Fuscolorica*, small colourless walled and larger brown walled unicells, with red algal thalli of *Viator*. (B) Enlarged view of two *Fuscolorica* cells around *Viator* thalli, with scattered *Fuscolorica* free small cells and, at right, one free cell with large brown discoid lorica, faint channel below and outer halo of minute brown scales. (C) One *Fuscolorica* densely embedded in *Viator* thallus at bottom (a); one lorica in middle with two cells embedded in single disc matrix showing some small bumps, around disc perimeter is halo of minute brown scales; short and long channels visible from one

cell (b); large brown disc has large cytostome, single branched channel, faint surface bumps and two minute scales in external brown strands (c); small cell with cytotsome, thick brown wall and scales (d); irregularly shaped brown disc has two cells without visible channels (e). (D) Largest lorica at bottom, focussed on upper surface, showing regular pattern of brown scales, uppermost cell is focussed on inside of lorica showing irregular perimeter, both cells with conspicuous single and multiple channels extending from cytostome; in middle loricate cell with irregularly distributed brown scales at perimeter. (E) At left, young cell focussed in the middle, showing probable clear nucleus and probable inner grey nucleolus; young cell at right, with pale brown wall and filopodium extending from cytostome (arrow). (F) *Fuscolorica,* embedded in *Viator* thallus, showing lorica of outer, feathery-branched, brown threads. (G) Large uniform brown loricae, numerous clear to dark-centred bumps are probable scales, also with channels visible in three; other five loricae with very irregular perimeters, three with variable channels, scattered scales at perimeters. Insert: scales enlarged, in facial and lateral profiles shaped like sorbet cups. (H) Loricae with elongate necks and frequent transverse rings, some lorica with short bent necks appressed, some with light halos of faint brown scales around perimeters, channels faintly visible in several.

Scale bars: (A) 50 µm, (B) 20 µm, (C) 20 µm, (D) 20 µm, (E) 10 µm, (F) 30 µm, (G) 10 µm, (H) 20 µm.

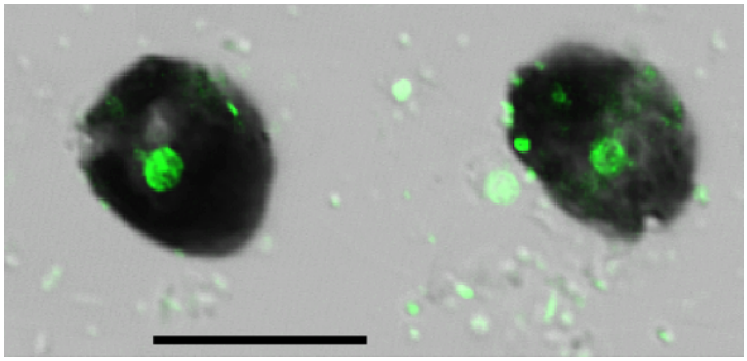


Fig. 2. SYBR® green stain of nucleus in *Fuscolorica* and nucleoids in other (non-photosynthetic prokaryote and eukaryote cells. Other intracellular DNA-containing organelles were not distinguishable in *Fuscolorica*. Scale bar: 10 µm.

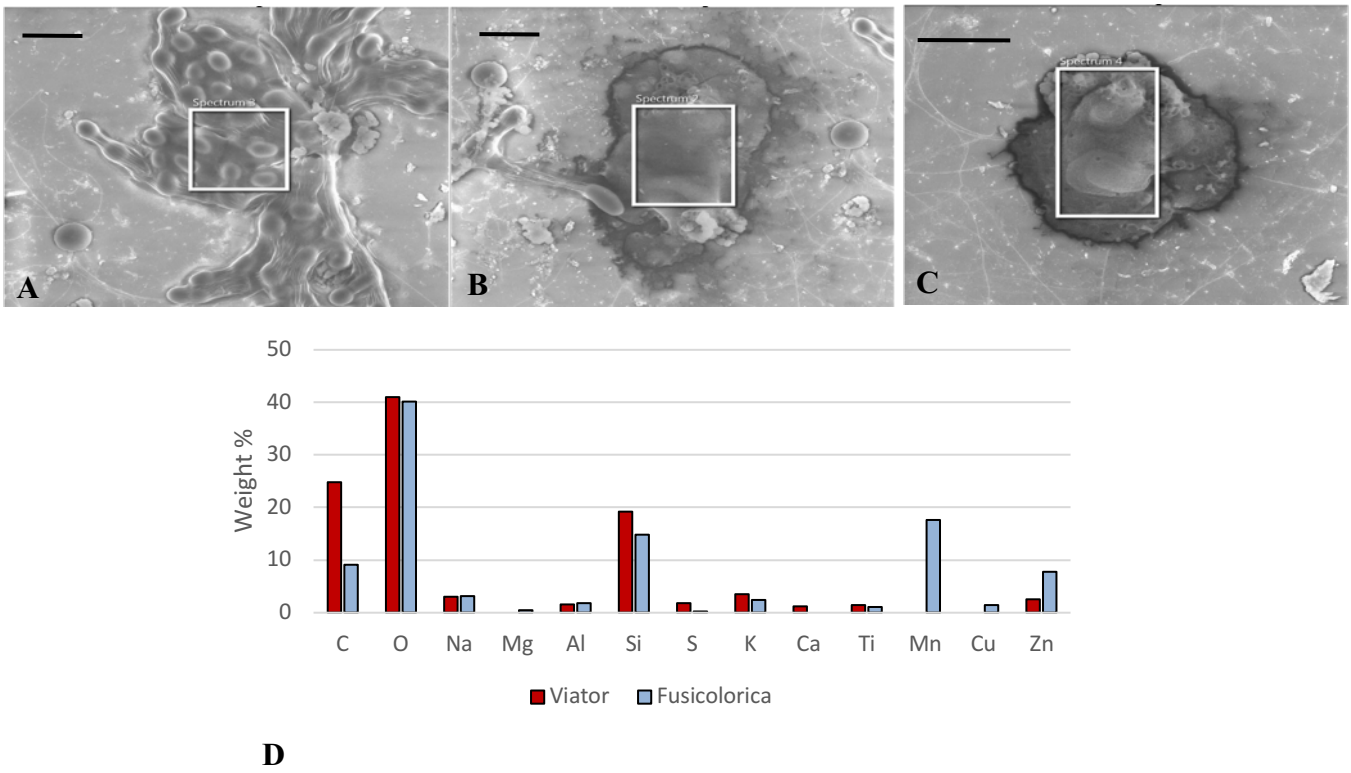


Fig. 3. SEM images of *Viator* (A) and *Fuscolorica* (B, C) showing cell morphology and areas of elemental analysis (white boxes). *Viator* (A) rounded cells in filaments while *Fuscolorica* cells (B-C) were more variable in shape. (D) Elemental analysis results using EDS indicated compositional differences between the two species. The heavy metals magnesium, manganese, copper, and zinc were present in higher amounts in *Fuscolorica*. Comparatively in *Viator*, the biological elements carbon, silicon, sulphur, potassium, and calcium were elevated. The elemental results are presented as percentage weight of the spectrum analysed. The average of three measurements for each sample are shown. Scale bars: A, B, 25 µm; C, 10 µm.

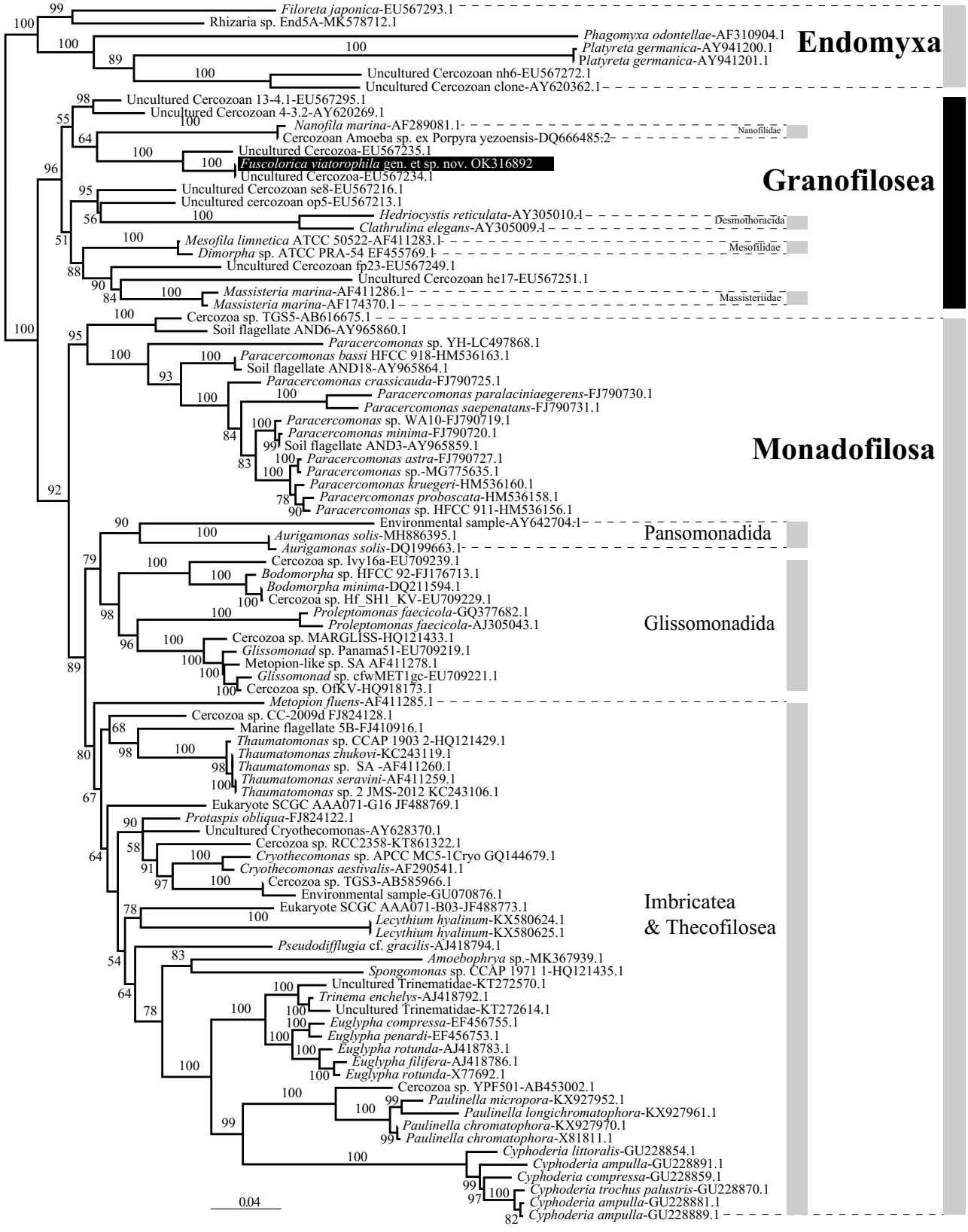
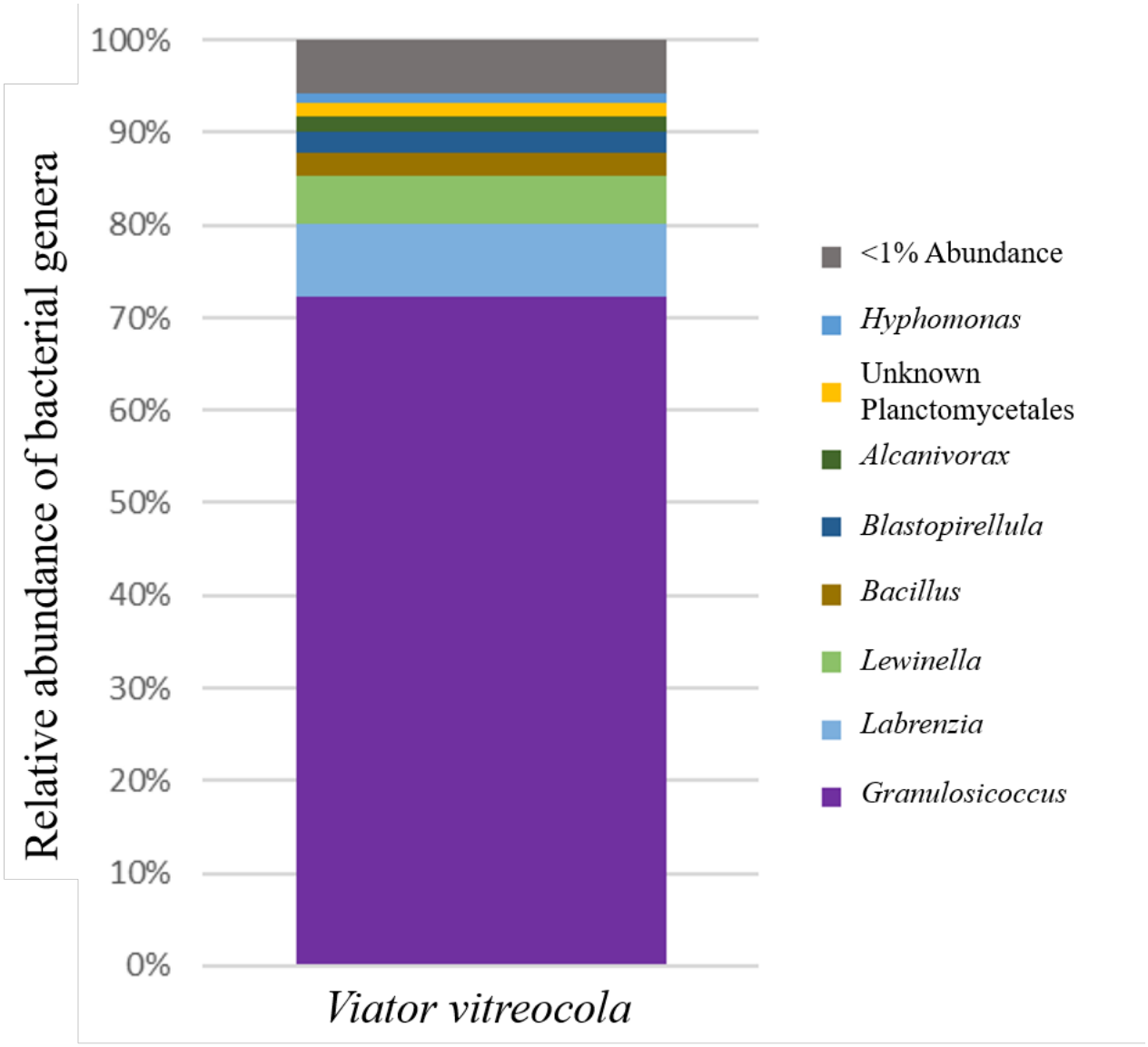


Fig. 4. Maximum likelihood (ML) phylogeny based on 18S rRNA sequences from 95 Cercozoan taxa. ML tree was inferred using IQ-TREE with TIM3+F+I+G4 evolutionary model. ML bootstrap values with 1000 replicates (≥50%) were shown at branches. GenBank accession numbers were added after species name.



Percentage	Genus
5.78%	Other
1.07%	*Hyphomonas*
1.39%	*Planctomycetales*
1.64%	*Alcanivorax*
2.26%	*Blastopirellula*
2.57%	*Bacillus*
5.11%	*Lewinella*
7.83%	*Labrenzia*
72.35%	*Granulosicoccus*

Fig. 5. Relative abundance of bacterial genera based on metabarcoding of the 16S rRNA gene, V5-6 regions. Each genus is represented by a single ASV. ASVs with abundances less than 1% were pooled in the category "1% Abundance". In total, 48 ASVs were identified in the triplicate samples with only 21 ASVs present in all technical replicates, including the 8 represented on the figure, which make up 94.2% of all reads.