**Gene identification and functional characterization of a $\Delta 12$ fatty acid desaturase in
Tetrahymena thermophila and its influence in homeoviscous adaptation to low
temperature**

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Abstract

Homeoviscous adaptation in poikilotherms is based in the regulation of the level of desaturation of fatty acids, variation in phospholipids head groups and sterol content in the membrane lipids, in order to maintain the membrane fluidity in response to changes in environmental temperature. Increased proportion of unsaturated fatty acids is thought to be the main response to low-temperature acclimation, which is mostly achieved by fatty acid desaturases. Genome analysis of the ciliate *Tetrahymena thermophila* and a gene knockout approach has allowed us to identify one $\Delta 12$ FAD and to study its activity in the original host and in a yeast heterologous expression system. The “PUFA index” -relative content of polyunsaturated fatty acids compared to the sum of saturated and monounsaturated fatty acid content- was ~57% lower at 15°C and 35°C in the $\Delta 12$ FAD gene knockout strain (KO $\Delta 12$) compared to WT strain. We characterized the role of *T. thermophila* $\Delta 12$ FAD on homeoviscous adaptation and analyzed its involvement in cellular growth, cold stress response, and membrane fluidity, as well as its expression pattern during temperature shifts. Although these alterations allowed normal growth in the KO $\Delta 12$ strain at 30°C or higher temperatures, growth was impaired at temperatures of 20°C or lower, where homeoviscous adaptation is impaired. These results stress the importance of $\Delta 12$ FAD in the regulation of cold adaptation processes, as well as the suitability of *T. thermophila* as a valuable model to investigate the regulation of membrane lipids and evolutionary conservation and divergence of the underlying mechanisms.

Keywords

Fatty acid desaturases; $\Delta 12$ fatty acid desaturase; *Tetrahymena thermophila*; Membranes; Homeoviscous adaptation; cold stress.

Highlights

- A $\Delta 12$ FAD was identified and characterized from *T. thermophila*.
- Gene knockout of $\Delta 12$ FAD decreased PUFA index by 60%.
- Cell growth at low temperatures was impaired in the $\Delta 12$ FAD knockout strain.
- Phospholipids and tetrahymanol content were altered in the $\Delta 12$ FAD knockout strain.
- $\Delta 12$ FAD plays a critical role in the regulation of homeoviscous adaptation.

Abbreviations

- 6-AS: 6-(9-anthroyloxy) stearic acid
12-AS: 12-(9-anthroyloxy) stearic acid
AEPL: 2-aminoethyl-phosphonolipid
DES12: $\Delta 12$ -desaturase gene
DES6A: $\Delta 6$ -desaturase 1 gene
DES6B: $\Delta 6$ -desaturase 2 gene
FAD: fatty acid desaturase
FAME: fatty acid methyl ester.
HGT: horizontal gene transfer
KO $\Delta 12$: $\Delta 12$ -desaturase knockout mutant strain
MSTFA: N-methyl-N-(trimethylsilyl) trifluoroacetamide
MTT: metallothionein
MUFA: monounsaturated fatty acid
OLE1: stearoyl-CoA 9-desaturase 1 gene
OLE2: stearoyl-CoA 9-desaturase 2 gene
ORF: open reading frame
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PUFA: polyunsaturated fatty acid
SFA: saturated fatty acid
TL: total lipids
WT: wild type strain

1. Introduction

Poikilothermic organisms do not control their temperature and must adapt their membrane lipid composition in order to maintain membrane fluidity in response to changes in environmental temperature. This adaptive response, known as “homeoviscous adaptation”, can be achieved by regulating the level of desaturation of fatty acids, type of phospholipids/head-groups and sterol content in cell membranes [1]. Homeoviscous adaptation has been studied in prokaryotes and eukaryotes, including the bacteria *Escherichia coli* [2] and *Bacillus subtilis* [3], the phototrophic cyanobacteria *Synechocystis* and *Synechococcus*, higher plants like *Arabidopsis thaliana* and *Glycine max* [4], the algae *Chlamydomonas reinhardtii* [5], metazoans such as *Oncorhynchus mykiss* (rainbow trout) and *Carassius auratus* (goldfish) [6,7], the fungi *Saccharomyces cerevisiae* and *Neurospora crassa* [8], and protozoans such as the ciliate *Tetrahymena thermophila* [9].

Ciliates belong to the monophyletic lineage Alveolate, which also includes dinoflagellates and the parasitic Apicomplexa [10] (Fig. 1A). In addition to their structural and evolutionary complexity, they share three main features: the presence of cilia, nuclear dimorphism, and conjugation as a sexual process [11]. Its convenient cellular features, the availability of a varied set of tools for molecular-genetic manipulation, as well as its easy culture and rapid growth (comparable to *Saccharomyces cerevisiae*), have made *T. thermophila* a model organism widely used for studying a number of fundamental processes such as the nature of telomeres, telomerase and self-splicing RNA [12] and many biotechnological applications [13].

As any eukaryotic cell, *Tetrahymena* is organized at the subcellular level in organelles, but it also displays some peculiarities, including a slightly different Golgi apparatus [14], an extensive anastomosing system of alveolar sacs, an oral apparatus consisting of four ciliary elements and about 1000 cilia surrounding the surface of the cell. These features reveal a complex network of interconnected membranes, and their structure and function, as well as their role in the adaptive regulation to environmental stresses, have been previously studied [9].

Major phospholipids forming *Tetrahymena* membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), and the unusual 2-aminoethyl-phosphonolipid (AEPL) that contains an ether-linked hydrocarbon side chain instead of the typical fatty acyl group [15]. Regarding neutral lipids, the principal compound found in the ciliate is the pentacyclic triterpenoid tetrahymanol, which serves as a fluidity modulator similar to hopanoids in bacteria or sterols such as cholesterol, ergosterol, and phytosterol in mammals, yeast and plants, respectively [16]. The main fatty acid

species reported in *Tetrahymena* include palmitic acid (16:0), palmitoleic acid (16:1^{Δ9}), stearic acid (18:0), oleic acid (18:1^{Δ9}), linoleic acid (18:2^{Δ9,12}) and gamma-linolenic acid (18:3^{Δ6,9,12}) [17].

The biophysical properties of *Tetrahymena* membranes have been studied with a varied range of techniques including electron spin resonance (ESR), X-ray diffraction, fluorescence depolarization and [³¹P]NMR. Additionally, the lateral movement of proteins within different membrane fractions was characterized by freeze-fracture electron microscopy [9]. These studies revealed significant differences in fluidity among membranes of organelles, particularly between the more fluid endoplasmic reticulum and pellicles (outer alveolar membrane joined to the plasma membrane) to the less fluid cilia. These effects were attributed mainly to the higher level of tetrahymanol and AEPL, as well as to lower degree of unsaturation in fatty acyl chains in ciliary membranes than in microsomes and pellicles membranes [15,16].

Related to the adaptive response to environmental stress in *Tetrahymena* cells, e.g. the shift to low temperatures, it was shown that cell division is arrested and cells remain motile but relatively inactive for a period of several hours [9]. Alveolar membrane displays a lateral movement of protein particles into dense aggregates, leaving large particle-free areas of lipids in the crystalline phase. Membrane lipid composition has drastic alterations such as an increase in AEPL and decrease of PE, as well as a rapid rise of palmitoleic acid (16:1^{Δ9}) concurrent with a decrease of palmitic acid (16:0) and followed by a decline of other saturated fatty acids [17]. All these changes increase the fluidity of microsomal and pellicular membranes, whereas the fluidity in mitochondrial membranes remains unaltered.

The increased proportion of unsaturated fatty acids in phospholipids is considered the main response to environmental temperature decrease. The sensing mechanism involved in the regulation of the fatty acyl chain profile is still unknown for most eukaryotes including the ciliate, although the role of Δ9 fatty acid desaturase (FAD) in homeoviscous adaptation has been extensively demonstrated [18]. Upon exposure of the ciliate to low temperatures, the activity of the Δ9 FAD is increased [19], mainly due to enhanced expression of the gene encoding this enzyme [20]. Whereas the introduction of the first unsaturation at Δ9 position has critical effects on membrane fluidity, successive unsaturations have been demonstrated to be also very important [21]. It has been shown that *Tetrahymena* spp. showed that this ciliate has enzymes differing in their desaturase activities and introducing double bonds at Δ9, Δ6 and Δ12 positions of fatty acids [22]. Genome analysis of the ciliate *T. thermophila* has allowed us to identify 16 putative sequences belonging to the FAD superfamily including two paralogs of stearoyl-CoA desaturase (Δ9 desaturase), two of Δ6 FAD and one Δ12 FAD [23]. In this work, we identified the unique Δ12 FAD from *T. thermophila* and investigated its functions by a gene knockout approach and heterologous expression in yeast.

1 Furthermore, we studied its role in the homeoviscous adaptation in *T. thermophila* by characterizing
2 the expression patterns of the newly identified $\Delta 12$ FAD and other desaturases during temperature
3 shifts.

2. Materials and methods

2.1. Strains, plasmids, and *T. thermophila* growth conditions

The *T. thermophila* strain CU428 (mpr1-1/mpr1-1; mp-s, VII), designated as “wild type” (WT) in this study, and the plasmid pBS-MnB-3 were a gift from M. A. Gorovsky (University of Rochester, NY, USA) [24]. *T. thermophila* cells were grown in 250-ml Erlenmeyer flasks containing 25 ml SPP (superproteose peptone) medium with the following composition (w/v): 1% proteose-peptone (Oxoid, UK), 0.1% yeast extract (Merck, Germany), 0.2% glucose (Merck), and 0.003% iron citrate (Sigma-Aldrich, USA). When indicated, paromomycin (Sigma-Aldrich) was added, together with 1 µg/ml of CdCl₂. For fatty acid supplementation, oleic acid (18:1Δ⁹) and linoleic acid (18:2Δ^{9,12}) were prepared in ethanol and added at the beginning of the culture at a concentration of 1.0 µM of fatty acid and 0.5 % (v/v) of ethanol [25]. Cultures were inoculated daily with a 1:10 dilution of a 24-h culture. Cultivation was carried out in a rotary shaker (80 rpm) at different temperatures ranging from 15°C to 35°C depending on the experiment: (1) for PCR and Reverse transcription-PCR (RT-PCR), microscopy cultures were grown at 30°C; (2) for growth curves, cells were grown at 15°C, 20°C, 30°C and 35°C; (3) for q-PCR analyses, cells were first grown at 35°C, gradually chilled to 15°C (during a period of 20 min) and subsequently maintained at this temperature for 2 h. Cells were counted in a Neubauer chamber. Plasmid pBS-MnB-3, containing the neomycin resistance gene under a cadmium-inducible metallothionein (MTT) promoter (the neo 3 cassette) expressing paromomycin resistance, was also used in this study [24]. The recovery of viable cells was determined as freezing resistance in liquid nitrogen by serially diluting the 0.3 ml of thawed cells in Neff medium, adding 100 µl per well of the 10⁻⁴, 10⁻⁵, and 10⁻⁶ dilutions to duplicate 96 well microtiter plates, counting the total number of wells yielding viable cells, and applying the Poisson distribution [26].

2.2. Standard DNA and RNA manipulation procedures for PCR, RT-PCR and q-PCR analyses

Genomic DNA from *T. thermophila* CU428 (WT strain) was prepared as previously described [27]. The isolation of plasmid DNA from *E. coli* was performed with a QIAprep Spin Miniprep DNA purification system kit (Qiagen, Germany). Total RNA was prepared from *T. thermophila* cultures using TRIzol reagent (Invitrogen, CA, USA). Nucleic acid fragments were amplified by PCR using FIREPol DNA polymerase (Solis Biodyn, Estonia). Reverse transcription (RT) reactions were carried out using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, USA) according to the manufacturer’s instructions. Reactions were conducted in a StepOne™ PCR Real-Time PCR System, using MicroAmp™ Fast 96-well reaction plates and the StepOne™ version 2.3

software (Life Technologies GmbH). Thermal cycling conditions were as follows: holding stage at 50°C for 2 min, followed by a cycling stage of 40 cycles at 95°C for 15 s and 60°C for 1 min, during which fluorescent data were collected. Melting curves were performed after the end of the amplification cycles to validate the specificity of the amplified products. Standard curves were generated using 10-fold serial dilutions of DNA from WT cells (10^5 - 10^1) and normalized to three copies of the 17S rRNA sequence per WT cell. All standard dilutions, controls, and individual samples were run in biological triplicate and technical duplicates. Standard curves were accepted when slopes were between -3.10 and -3.74 and the R^2 was >0.99 [28]. Relative quantification of mRNA level was based on Ct (threshold cycle) values. The change in the target mRNA was calculated using the method described by Pfaffl [29] and expressed as fold increase relative to control values. The primers used for all amplification reactions are listed in the Supplementary Table S1.

2.3. Functional characterization of the *T. thermophila* desaturase in yeast

The open reading frame (ORF) of the *T. thermophila* $\Delta 12$ FAD was synthesized by GenScript with codon usage optimized according to Optimum Gene algorithm [30] and cloned into pUC57 vector to produce the construct pUC57- $\Delta 12$ FAD. The ORF contained restriction sites for *Bam*HI and *Xho*I (New England Biolabs, USA) at the 5' and 3' ends, respectively, and thus allowing subsequent cloning into similarly restricted pYES2 vector (Invitrogen, UK) to produce pYES2- $\Delta 12$ FAD for heterologous expression in yeast *S. cerevisiae*. Transformation and culture of yeast were conducted as previously described [31].

To test the desaturation activity of the *T. thermophila* $\Delta 12$ FAD, two clones (rDES12.1 and rDES12.2) of yeast transformed with pYES2- $\Delta 12$ FAD were grown in *Saccharomyces cerevisiae* minimal medium lacking uracil (SCMM^{ura}) in the absence of exogenously added fatty acid substrates. Expression of the *T. thermophila* $\Delta 12$ FAD was induced by supplementation of galactose 0.02 % (w/v). A control treatment consisting of yeast transformed with empty pYES2 was also run under the same conditions. Yeast cultures were run in triplicate. Function of the *T. thermophila* $\Delta 12$ FAD was estimated by calculating the $\Delta 12$ desaturation capacity as $[\text{absolute area of } 18:2^{\Delta 9,12} / (\text{absolute area } 18:2^{\Delta 9,12} + 18:1^{\Delta 9}) \times 100]$ in both control yeast and yeast transformed with pYES2- $\Delta 12$ FAD. Further desaturase activities of the *T. thermophila* $\Delta 12$ FAD were tested by growing yeast transformed with pYES2- $\Delta 12$ FAD in the presence of exogenously added 18:2 ^{$\Delta 9,12$} (linoleic acid) (Nu-Chek Prep., MN, USA) at 0.5 mM final concentration. After 48 h culture at 30°C, yeasts were harvested, washed twice in distilled water and homogenized in chloroform/methanol (2:1, v/v) containing 0.01 % (w/v) butylated hydroxyl toluene (BHT, Sigma-Aldrich) as antioxidant before

total lipids (TL) were extracted. Chemicals used to prepare yeast media were from Sigma-Aldrich, except for the bacteriological agar obtained from Oxoid Ltd. (UK).

2.4. Construction of $\Delta 12$ FAD KO strain and $\Delta 12$ FAD-GFP fusion in *T. Tetrahymena*

For $\Delta 12$ FAD gene disruption in *T. thermophila*, the transformation sequence C5680 was constructed. The 0.74 kb upstream (UP) and 0.87 kb downstream (DW) flanking regions of the putative sequence TTHERM_00535680 (I7MA19) were synthesized separately (Genscript, USA). Both sequences were cloned into plasmid pBS-MnB-3 and transformed into competent *E. coli* DH5 α cells. The C5680 construct was then released with *Xho*I restriction enzyme (New England Biolabs) from the vector. For localization of $\Delta 12$ FAD in *T. thermophila*, the transformation sequence $\Delta 12$ FAD-GFP was constructed. A 1041 base pair (bp) C-terminal fragment of the $\Delta 12$ FAD gene (Up), lacking the stop codon, was amplified by PCR with specific primers adding *Bam*HI and *Sph*I restriction sites (New England Biolabs) at each fragment's ends. The sequence was cloned into pmEGFP. A downstream flanking region of 1130 bp (Dw) was also amplified adding *Xho*I and *Kpn*I restriction sites at each fragment's ends and cloned into pmEGFP+Up between *Xho*I and *Kpn*I sites. The $\Delta 12$ FAD-GFP construct was then released with *Sph*I and *Kpn*I restriction enzymes from the vector. The constructs were introduced into the macronucleus of *T. thermophila* CU428 cells by biolistic transformation [27]. Transformants were recovered in 50 ml SPP medium containing 1.0 μ g/ml CdCl₂, selected upon growing in 120 μ g/mL paromomycin, and subsequently transferred to fresh SPP medium with increasing concentrations of paromomycin until no further growth could be obtained (100 mg/ml).

2.5. Lipid headgroup composition and fatty acid analysis

T. thermophila cells from cultures grown at 15°C and 35°C were collected by centrifugation at 3000 g for 2 min at 4°C, washed twice with 20 ml of distilled water, and TL were extracted [32]. For lipid headgroup composition, phospholipids were separated on silica gel G thin-layer chromatography (TLC) eluted with chloroform: acetic acid: methanol: water (75: 25: 5: 2.2, v/v) and detected with Dittmer reagent [15]. For fatty acid identification, the organic phase was evaporated under N₂ stream and methylated with two volumes of methanol: hydrochloric acid (5:1, v/v) overnight at 80°C. Samples were extracted twice with 4 ml n-hexane, the organic solvent was evaporated under N₂ stream and the residue was resuspended in 1 ml of n-hexane. Each sample was sowed in a pre-conditioned silica cartridge (Sep-Pak Vac 3cc) and eluted with 5 ml of ethyl acetate. After evaporating under N₂ stream, the samples were resuspended in 50 ml hexane. The composition in fatty acid methyl ester (FAME) derivatives was analyzed in a Hewlett Packard HP 6890 gas

1 chromatograph equipped with a Zebron ZB-5 column (30 m long, 0.53 mm inner diameter;
2 Phenomenex, California, USA). The increase of column temperature was programmed at 10°C/min
3 from 100 to 310°C and subsequently held for 10 min at 310°C. Mass spectrometry (MS) detection
4 was carried out using an HP mass selective detector (Mass Spec Model 5975VL) operated at an
5 ionization voltage of 70 eV with a scan range of 50–600 atomic mass units (amu). Fatty acid
6 identification was performed by comparison with standards present in NIST database (National
7 Institute of Standards and Technologies, MD, USA). The relative PUFA content towards SFA and
8 MUFA content was calculated as “PUFA index” (Eq. 1). For yeast samples from the functional
9 assay, FAMES were prepared from the entire TL fraction obtained from 5 mL yeast subcultures.
10 FAME were subsequently identified and quantified using a Thermo (Thermo Trace GC Ultra,
11 Thermo Electron Corporation, MA, USA) gas chromatograph equipped with a fused silica 30 m x
12 0.25 mm open tubular column (Tracer, TR-WAX, film thickness: 0.25 µm; Teknokroma, Spain)
13 using helium as carrier, and a 50–220°C thermal gradient during the running of samples. A personal
14 computer system equipped with Azur Datalys version 4.0.2.0 (France) software was used in the
15 recording and processing of the data from the flame-ionization detector. Individual FAMES were
16 identified by comparison with known standards confirmed by mass spectrometry as described in
17 [31].

18 **2.6. Tetrahymanol and total phosphorus content analysis**

19 To analyze tetrahymanol content in 15°C- and 35°C-grown cells, the sample remaining after the
20 procedures mentioned above was evaporated under N₂ stream and the residue was resuspended in 1.5
21 ml distilled water and then saponified in 1.5 ml 2 M NaOH in methanol:water (1:1, v/v) at 80 °C for
22 1 h. After cooling, sterols were extracted twice with 5 ml of n-hexane and dried under N₂ stream.
23 Stigmasterol (5a-cholest-5,22-dien-24bethyl-3b-ol) was used as the internal standard and was added
24 prior to extraction. To identify specific sterols in the samples, extracts were re-suspended in 100 µl
25 of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (Sigma-Aldrich), followed by 10 min
26 incubation at 60°C. Steryltrimethylsilyl ester derivatives were analyzed by running samples through
27 an HP-5MS (30 m x 0.25 mm x 0.25 µm, Agilent Technologies) in a Hewlett Packard HP 6890 gas
28 chromatograph. The column was temperature programmed at 10°C/min from 100 to 310°C and
29 subsequently held for 10 min at 310°C. MS was carried out using an HP mass selective detector
30 (model MSD 5973), operated at an ionization voltage of 70 eV, with a scan range of 50-600 amu.
31 The retention time and mass spectra of all newly identified peaks obtained were compared to those of
32 standards (Steraloids, USA) and/or available in the NIST library. Total phosphorus analysis was
33 carried out following the method of Taussky and Shorr [33].

2.7. Microscopic Analysis

Tetrahymena expressing $\Delta 12$ FAD-GFP were fixed with 4% (w/v) paraformaldehyde in 50 mM HEPES pH 7.0 for 10 min at room temperature, washed with ice-cold HEPES two times and permeabilized with ice-cold 0.2% Triton X-100 in 50 mM HEPES pH 7.0 for 8 min. After being washed with ice-cold HEPES three times, the fixed cells were treated with blocking solution (1% [w/v] bovine serum albumin [BSA] in TBS buffer) for 1 h at room temperature, and incubated for 1 h in blocking solution with anti-GFP primary antibody (A-11122; Invitrogen) at a 1:200 dilution. Cells were washed three times for 5 min each with TBS buffer containing 0.1% (w/v) BSA and subsequently incubated for 1 h at room temperature with goat anti-mouse-Alexa Fluor 594 secondary antibody (A-11020; Invitrogen) at a 1:400 dilution in blocking solution. After one wash in TBS buffer containing 0.1% (w/v) BSA the cells were incubated with 1 mM DAPI (4',6-diamidino-2-phenylindole in EtOH) for 15 min at room temperature. Finally, cells were washed twice with 50 mM HEPES pH 7.0 and mounted with Trolox antibleaching solution. Digital images were collected using a Carl Zeiss Axio imager M2 fluorescence microscope. Images were analyzed with Image J 1.50i (Wayne Rasband, National Institute of Health, USA).

2.8. Bioinformatics and phylogenetic analyses

A dataset comprising the methyl-end FADs was generated by extensive homologous sequence searches using as query sequences from representative prokaryotic and eukaryotic methyl-end FADs [34]. Sequences were retrieved from the National Center of Biotechnology Information (NCBI) and from the genomes of the ciliates *E. octocarinatus* (<http://ciliates.ihb.ac.cn/database/species/eo>), *I. multifiliis* (<http://ich.ciliate.org/index.php/home>), *O. trifallax* (<http://oxy.ciliate.org/index.php/home>), *S. lemnae* (<http://stylo.ciliate.org/index.php/home>), *T. thermophila* (<http://ciliate.org/index.php/home>), *P. tetraurelia* (<http://paramecium.cgm.cnrs-gif.fr/>) and *P. persalinus* (<http://ciliates.ihb.ac.cn/database/species/pp>). OrthoMCL database v5 was also used to retrieve the sequences from $\Delta 12$ and $\Delta 15$ FADs (OG5_129048 and OG5_143101). Redundant sequences were excluded from the dataset sequences using BLASTp tool at NCBI's homepage with the default setting parameters (Substitution matrix: BLOSUM62; word size: 6; gap opening cost: 11; gap extension cost: 1). Pairwise alignments of each individual sequence from the dataset against the entire dataset were retrieved. Those sequences with an identity of 100 % with another sequence from the dataset were considered as repeated sequences and therefore were excluded from the final dataset. CD-HIT with a sequence identity cut-off of 0.99 was also used to exclude redundant sequences. For phylogenetic analyses, PROMALS3D with default parameters was used to obtain datasets multiple alignments (MSA). Poorly aligned sequences were removed using trimAl

(<http://trimal.cgenomics.org/>) with the gappypout-tool. This MSA was used to construct the phylogeny by Maximum Likelihood using PhyML 3.0 optimized by SPR and NNI and a support of 100 bootstraps. The model LG+I+G for the MSA was selected using Prottest 3.0 server [35,36]. The sequences used for phylogenetic analyses are listed in the Supplementary Table S2.

2.9. Evaluation of membrane fluidity

The fluorescent probes 6-(9-anthroyloxy)stearic acid (6-AS), 12-(9-anthroyloxy) stearic acid (12-AS) were purchased from Invitrogen/Molecular Probes Inc. (OR, USA). These probes alter their ability to polarize their fluorescence depending on the packing and motion of lipids in their immediate surroundings, being the fluorescence anisotropy inversely related to membrane fluidity [37].

WT and KO *Tetrahymena* cells were grown at 35°C. After 24 h, cells of each strain were kept for further 24 h at 35°C and another part of the cell suspension was incubated at 15°C for further 24 h. After incubation, cells were washed, resuspended in sterile water and labeled with either the fluorescent probe 6-AS or 12-AS (probe:lipid ratio~1:500) and incubated for 30 min at their respective growth temperature (35°C and 15°C) to allow the incorporation of the probe to the plasma membrane. Aliquots containing 80 µl of cell suspensions were separated before and after treatment to measure cell viability. Membrane fluidity was evaluated between 15 and 35°C from the fluorescence anisotropy of the probes measured at 435 nm ($\lambda_{\text{excitation}} = 384 \text{ nm}$) in a PerkinElmer LS-55 spectrofluorometer (PerkinElmer Ltd., United Kingdom) equipped with temperature control.

2.10. Statistical analysis

For expression analysis of FADs in *T. thermophila* cells statistical analysis was performed using one-way ANOVA. For functional analysis in yeast, *t*-test was used to assess differences in fatty acid profiles of yeast from functional assays of ω x desaturases. Two-way ANOVA followed by Bonferroni's multiple comparison test was used to assess differences in fatty acid profiles of *T. thermophila* WT and KO strains. Tetrahymanol and total phosphorus content analysis were analyzed using the *t*-test. For cell growth and membrane fluidity assays, statistical analysis was performed by applying two-way ANOVA followed by Sidak's multiple comparison test.

3. Results

3.1. Identification and functional analyses of $\Delta 12$ FAD from *T. thermophila*

Blast and orthology analysis using as query reviewed (Swiss-Prot) $\Delta 12$ -desaturases from plants, fungi, metazoan and cyanobacteria identified only one putative sequence (UniProt ID: I7MA19) in the genome of *T. thermophila*. As expected, no putative sequences were retrieved when the query was made with known $\Delta 15$ -desaturases. Phylogenetics analysis of methyl-end FADs grouped I7MA19 sequence into a well-defined monophyletic branch together with other putative $\Delta 12$ -desaturases from oligohymenophorea ciliates. The ciliate branch shows a close relationship with a branch composed of $\Delta 12$ -desaturases sequences from cyanobacteria and a variety of eukaryotic organisms (*Chlorophyta*, *Rhodophyta*, *Cryptophyta*, *Stramenopiles*, and *Rhizaria*) (Fig. 1A, B).

To evaluate whether I7MA19 is a $\Delta 12$ -desaturase, we investigated their enzymatic activity using a yeast heterologous expression system. As shown in Fig. 2A, the transgenic yeast expressing the *Tetrahymena* gene, exhibited $\Delta 12$ desaturase activity, enabling the biosynthesis of linoleic acid ($18:2\Delta^{9,12}$) from oleic acid ($18:1\Delta^9$). We further tested the $\Delta 15$ desaturase activity of the *T. thermophila* FAD using $18:2\Delta^{9,12}$ as a potential substrate. Our results showed that no additional fatty acids were detected and so confirmed that the *T. thermophila* FAD lacks $\Delta 15$ activity (data not shown).

To further confirm that I7MA19 is a $\Delta 12$ desaturase, a KO mutant strain (KO $\Delta 12$) was generated by eliminating the coding gene through a somatic knockout procedure (Fig. 2B and S2). Fatty acid composition of total lipids from WT and KO $\Delta 12$ strains grown at 35°C are shown in Table 1. The relative amount of main fatty acid species from the WT strain followed the order $18:3\Delta^{6,9,12} > 16:0 > 18:2\Delta^{9,12} > 16:1\Delta^9$, $18:1\Delta^9$. In contrast, main fatty acids from the KO $\Delta 12$ strain grown at the same growth temperature were $18:1\Delta^9$, $18:2\Delta^{6,9} > 16:0 > 16:1\Delta^9$, and no fatty acids with a double bond at $\Delta 12$ position were detected (Table 1).

To localize the enzyme in *T. thermophila*, we tagged $\Delta 12$ FAD at the C terminus with EGFP (Fig. 3A). To reduce potential misexpression artifacts in the ciliate, we targeted the tagged gene to its endogenous locus. Due to high autofluorescence background even in WY cells, we increased the selectivity of the signal using anti-GFP antibody and indirect immunofluorescence microscopy. Microscopic analysis of $\Delta 12$ FAD-GFP showed a strong signal around the nucleus and a more diffuse one in the cytoplasm, a pattern that is consistent with the endoplasmic reticulum localization (Fig. 3B).

3.2. $\Delta 12$ -desaturase KO strain has impaired growth at low temperature, modifications in fatty acid profile and a decrease of cell viability

We examined the growth of WT and KO $\Delta 12$ strains at various temperatures, ranging from 15°C to 35°C. As shown in Fig. 4A, WT, and KO strains at 30°C and 35°C reached similar cell densities (1.0×10^6 and 1.2×10^6 cells/ml, respectively) after 24 h. However, at 15°C and 20°C, both strains differed largely in their responses. The WT strain achieved identical biomass yields than those at higher temperatures, albeit with expanded log phase (Fig. 4A). Importantly, the growth of the KO $\Delta 12$ strain was impaired, reaching a maximal cell density of 1.0×10^5 cells/ml after 6 d in culture. Fig. 4B shows that the impaired growth of the KO strains at 15°C can be restored by the supplementation of linoleic acid (18:2 $\Delta^{9,12}$) but not oleic acid (18:1 Δ^9). These results highlight the importance of *DES12* on cellular growth at low temperatures.

To understand the mechanism underlying *T. thermophila* adaptation to low temperatures, we analyzed fatty acid profiles from both strains grown at 15°C and 35°C (Table 1). For both temperatures, increased MUFAs and concomitant decreased PUFAs were found in KO $\Delta 12$ strain in comparison to WT strain, with SFA remaining unchanged. These changes in the unsaturation pattern were mostly due to increased 16:1 Δ^9 , 18:1 Δ^9 , 18:2 $\Delta^{9,6}$ and 20:1 Δ^{11} , and decreased 18:2 $\Delta^{9,12}$ and 18:3 $\Delta^{6,9,12}$. Together, these changes in the fatty acid profile of the KO $\Delta 12$ strain lead to a lower PUFA index (61.6% lower at 15°C and 49.8% lower at 35°C) compared to that of the WT strain (Table 1).

Another phenotype tested was the cell viability, measured as freezing resistance in liquid nitrogen. As shown in Fig. 4C, the recovery of viable KO $\Delta 12$ cells was significantly decreased when cells were grown at 15°C prior to freezing, while no change was detectable when WT and KO strains were grown at 35°C prior to freezing. The effect of the temperature also showed to play an important role in cell survival. On both strains, the recovery of viable cells was significantly lower at 15°C than 35°C.

3.3. Regulation of membrane fluidity by *DES12*

We examined if membrane fluidity could be altered in the KO $\Delta 12$ strain and if this could be a reason for the decreased growth rate observed when the cells were grown under low-temperature conditions. Membrane fluidity was evaluated as the fluorescence anisotropy of probes. A high degree of fluorescence anisotropy represents a high structural order or low cell membrane fluidity [38]. Two fluorescent probes were selected to monitor the fluidity of whole-cell plasma membrane, 6-AS, and 12-AS, which sense the fluidity at the membrane surface and the hydrophobic core, respectively [39]. Table 2 shows the anisotropy values for both probes measured at the optimal growth

temperature. No significant differences were observed between WT and KO Δ 12 strain, regardless of the probe or the temperature assessed. Next, the variations in the anisotropy values were recorded in the temperature range from 15 to 35 °C and normalized to the value recorded at the corresponding growth temperature (Fig. 5A). As expected, anisotropy values decreased with the increase in recording temperature, indicating the fluidification of the membrane. When grown at 35°C, no significant differences were observed between WT and KO Δ 12 cells in the temperature-dependence of anisotropy values, regardless of the probe assessed. In contrast, for KO Δ 12 strain grown at 15°C, lower 6-AS anisotropy values were recorded throughout the range of temperatures assessed, indicating higher membrane fluidity in comparison to that of WT cells. These differences were not observed when cells were labeled with the probe 12-AS.

We also measured the composition of phospholipids and the tetrahymanol to phospholipid ratio in membranes from WT and KO Δ 12 strain grown either at 15°C or 35°C to evaluate the participation of other mechanisms that could modulate membrane fluidity. At both temperatures, the relative content of phosphatidylethanolamine (PE) decreased in the KO Δ 12 strain, while phosphatidylcholine (PC) was increased, thus decreasing the PE/PC ratio in this strain (Fig. 5B). Regarding the tetrahymanol to phospholipid ratio, the ratio was lower in both strains when cells were grown at 15°C respect to the value measured at 37°C (Fig. 5C). For both temperatures, this ratio was markedly lower in KO Δ 12 mutants respect to the WT.

The results obtained that, in order to maintain the fluidity of the membrane close to control values, KO Δ 12 mutants, which have altered composition in acyl chains, modify the relative amount of PE in their membranes. As PE headgroup establishes hydrogen bonds with its neighbors, this phospholipid enhances lipid packing and decreases the fluidity at the membrane surface [40]. Hence, a decrease in PE content alleviates the higher rigidity in KO Δ 12 mutants imposed by the lack of PUFAs.

3.4. Regulation of FADs gene expression at low temperatures

In order to gain further insight about the regulation at low temperatures, we analyzed the transcription of the genes encoding a range of FAD enzymes after 20 min and 2 h of a temperature shift from 35°C to 15°C. As shown in Fig. 6, *OLE1*, *OLE2*, and *DES12* genes increased their expression between 1.5- and 3.0-fold. This effect was absent for *DES6* genes, showing decreased expression of *DES6A* gene but no variations in *DES6B* gene expression. After 2 h at 15°C, all genes except *OLE2* had marked decreased expression, even lower than the observed at 35°C. However, *OLE2* expression showed a 7-fold increase at 2 h, becoming the highest variation for gene expression observed in the current study.

1 The transcription analysis of the same genes in the KOΔ12 strain grown at 35°C showed a different
2 pattern with increased transcription of *OLE1* and *DES6A* genes, decreased transcription of *OLE2*
3 gene and no change in expression of *DES6B* gene. Upon temperature decrease to 15°C, no variations
4 in the expression of FADs were observed, neither at 20 min nor 2 h of the temperature shift. In fact,
5 and accompanying the arrest in their growth, decreased transcription of *OLE1*, *DES6A*, and *DES6B*
6 genes (at both sampling times) and no significant change of *OLE2* at 2 h were observed (Fig. 6).

4. Discussion

Using a bioinformatics approach and confirmed by yeast heterologous expression and KO strategy, the present study demonstrates that *DES12* gene encodes a $\Delta 12$ FAD in the ciliate *T. thermophila*. This is the third FAD identified and characterized in the ciliate after $\Delta 9$ and $\Delta 6$ FADs (*OLE1* and *DES6A*, respectively) [20,41]. Phylogenetic analysis grouped the *T. thermophila* *DES12* gene in a cluster with other putative $\Delta 12$ FADs from oligohymenophorean ciliates. Noteworthy, no methyl-end desaturases have been retrieved from the genome of other ciliates (*Heterotrichea* and *Spirotrichea*). This pattern, i.e. presence in oligohymenophorea and absence in other classes of ciliates, has been also reported for other enzymes belonging to the FAD superfamily [23]. The most parsimonious explanation suggests the gene loss in different periods of *Heterotrichea* and *Spirotrichea* evolution rather than horizontal gene transfer (HGT) events in oligohymenophorea lineage [23]. Phylogenetic analysis also positioned the ciliated $\Delta 12$ FAD distant to a deep node of sequences of cyanobacterial and plant methyl-end FADs. This finding is in agreement with Scott et. al. [42] who suggested that $\Delta 12$ FAD, together with $\Delta 9$ and $\Delta 6$ FADs, have been originated in a cyanobacteria that already contained three enzymes. The presence of $\Delta 12$ FADs in the alveolate lineage is not a widespread phenomenon. The search for orthologs in alveolate sequences including the genomes of 57 apicomplexans, 23 ciliates of different classes, 4 dinoflagellates, 2 chomerid, and 1 perkinssozoas only retrieved putative genes in the oligohymenophorean ciliates, the perkinsozoa *Perkinsus marinus* and the chromerid *Chromera velia*. A possible explanation to this pattern could be attributed to gene loss in the different lineages along evolution, as it was reported for vertebrates and ciliates [43,44], or to HGT events as found for some invertebrates [31].

$\Delta 12$ FADs belong to the fatty acid desaturase superfamily (pfam: PF00487). These enzymes are integral membrane proteins that localize in the endoplasmic reticulum. This is in accordance with the ER localization of the $\Delta 12$ FAD-GFP fusion in the ciliate and is consistent with the localization of other FADs from other organisms such as tobacco plant [45] as well other desaturases of ER localization in the ciliate [46,47].

$\Delta 12$ FAD has been identified in cyanobacteria [48] and many eukaryotic organisms such as plants and algae [49,50], trypanosomatids [51], stramenopiles [52], many fungi [53,54], multiple invertebrates [31,55,56], and now in the ciliate *T. thermophila*. Using yeast heterologous expression system, we confirmed the presence of $\Delta 12$ FAD –but not $\Delta 15$ FAD – activity. These results indicate that the *T. thermophila* *DES12* studied herein is not a bifunctional $\Delta 12/\Delta 15$ enzyme as reported in other organisms such as fungi and some invertebrates [57,58].

1 In poikilothermic organisms, the regulation of fatty acid content, the type of phospholipids/head-
2 groups and sterol content in cell membranes play an essential role in maintaining the fluidity of the
3 membrane upon changes in the environmental temperature. In response to its inability to synthesize
4 18:3^{Δ6,9,12} and 18:2^{Δ9,12} fatty acids, the KOΔ12 mutant strain shows marked lipid alterations. It
5 accumulates 18:2^{Δ6,9}, which is normally absent in the WT, and has an altered fatty acid profile
6 evidenced by increased MUFAs and decreased SFAs and PUFAs. This strain also shows decreased
7 PE/PC and tetrahymanol/phospholipid ratios. These two mechanisms allow cells regulating and
8 maintaining the fluidity of the membrane. As mentioned before, by decreasing the relative content of
9 PE, fewer hydrogen bridges are established between the polar headgroup of phospholipids and
10 therefore the membrane becomes more fluid. The lower relative content of tetrahymanol decreases
11 membrane packing, thus increasing the overall fluidity of the hydrophobic region of the membrane
12 [17]. Hence, the greater rigidity of the membranes imposed by the lack of PUFAs in the mutants is,
13 at least, partially counteracted by increased contents of tetrahymanol.

14 Despite these alterations allowed normal growth in the mutant strain at 30°C and higher
15 temperatures, they impaired its growth at temperatures such as 20°C and below. The
16 supplementation of the culture medium with linoleic acid, but not with oleic acid, restored only
17 partially the capacity of mutants to proliferate, demonstrating the importance of Δ12 fatty acyl chains
18 in this process. However, it did not compensate fully for growth, suggesting other secondary causes
19 derived to the loss of Δ12 fatty acyl chains. In addition, and despite having similar basal fluidity –
20 measured at their respective growth temperature–, KOΔ12 strain differed in its response to short-
21 term (5 min) alterations in environmental temperature. Under these experimental conditions, no
22 compensatory mechanisms could be triggered. Thus, the differences in the fluidity measured with the
23 probe 6-AS in the 15-35°C range relate the altered membrane composition with their capacity for
24 immediate adaptation towards changing environments. These differences were not observed when
25 cells were labeled with the probe 12-AS, giving further evidence that the alteration in membrane
26 composition in KOΔ12 mutant is affected mostly in the outer regions of the lipid bilayer.

27 Another phenotype observed in the KO strains was the significantly decreased cell viability when
28 cells were grown at 15°C prior to freezing. Although the decreased cell viability due effect of
29 lowering the temperature at the time of freezing has been previously demonstrated in the ciliate [26],
30 the KO strain showed a larger significant effect, suggesting a possible altered physiological state.
31 During the cryopreservation the cells are exposed to temperature changes that affect the viable cell
32 recovery, therefore the alterations in the capacity for immediate adaptation of membrane fluidity
33 observed in the KO strain at low temperature could be one reason for the low cell viability observed.

Another reason could be the different permeability in the KO cells grown at low temperature to the cryoprotectant DMSO due to the alteration in membrane composition. Nevertheless, this parameter was not examined.

The physiological impact of knocking out $\Delta 12$ FAD has been studied in several organisms. For example, in cyanobacteria *Synechocystis* sp., the impairment of this activity led to diminished photosynthetic activity and decreased growth in comparison to the WT strain incubated at the same temperature. In addition, studies performed using a strain of *Synechococcus* with no measurable $\Delta 12$ FAD activity and unable to acclimate to low temperatures, demonstrated that its complementation with a $\Delta 12$ FAD (*desA* gene) from a different strain of *Synechocystis*, not only restored the missing activity but also reverted the acclimation deficiency of this strain [59]. In *Arabidopsis*, the elimination of $\Delta 12$ FAD genes from ER (*fad2*) and chloroplastids (*fadC*) generated cold-sensitive mutant strains [60]. In *C. elegans*, *fat-2* mutants with altered $\Delta 12$ FAD activity, showed low growth rates, impaired development, and abnormal body shape, among other defects [56]. Furthermore, $\Delta 12$ FAD participates in salt tolerance in yeast and plants [61]. Therefore, the physiological effects of $\Delta 12$ FAD observed in evolutionary distant organisms like ciliate (alveolata) from other studied organisms such as fungi and animal (opisthokonts), land plant and algae (archaeplastids), and cyanobacterias (prokaryotes), highlight the importance and conservation of this enzyme in the process to low-temperature acclimation in poikilothermic organisms across the tree of life.

In *T. thermophila*, all FADs are expressed following a similar pattern, with high transcription levels during cell growth and very low ones during starvation and conjugation stages (Fig. S1). This is a crucial feature for membrane generation during cell growth (when all membranes are duplicated), starvation (when cells elongate and develop a long caudal cilium), and conjugation (when nuclear rearrangement processes occur) [62]. Importantly, in the WT strain *OLE1*, transcription level is 4-fold higher than the rest of FADs genes and 250-fold higher than basal transcription levels during cell growth [63]. The fatty acid content of the wild type strain observed in our experiments at 35°C correlates this pattern, showing a 61.04% of $\Delta 9$ fatty acyl chains, 39.79% of $\Delta 12$ fatty acyl chains and 28.96% of $\Delta 6$ fatty acyl chains. During the cold acclimation, all desaturases except *DES6B* are regulated, albeit in different ways. *DES12*, *OLE1*, and *OLE2* are up-regulated after only 20 min of temperature shift between 1.5- and 3.0-folds. There is a general consensus that the adjustment in gene transcription for all desaturases takes place in a minutes scale [64]. The 20 min period used in our experiments allowed us the detection of these changes, in agreement with the reported 2.6-fold up-regulation of the *T. thermophila* *OLE1* observed at 25 min upon the cooling process [20] and other reports that include $\Delta 9$ and $\Delta 12$ FAD expression in organisms of distant phyla [65–67].

However, the down-regulation of the *DES6A* gene observed in this work was unexpected. The expression of genes with similar function during temperature adaptation ranged from up-regulation in cyanobacteria [68] to no-regulation in teleost fish [69]. However, to the best of our knowledge, no down-regulation of $\Delta 6$ FAD has been reported yet, suggesting that the expression of $\Delta 6$ FAD has not a defined pattern during the cold acclimation. The different fatty acyl chains, of the wild type strain growth at 15°C observed in our experiments showed no significant changes from the growth at 35°C (64.61% vs 61.04% of $\Delta 9$ fatty acyl chains, 39.74% vs 39.79% of $\Delta 12$ fatty acyl chains and 24.9% vs 28.96% of $\Delta 6$ fatty acyl chains) suggesting that FADs regulation is more important during the cold acclimation process. At the time of sampling (when the cells are acclimatized), the level of expression could probably be different and the regulation of membrane fluidity was achieved not only with different species of fatty acids but also with a different composition of the phospholipid head group and tetrahymanol content, as discussed above. The expression of FADs in the KO $\Delta 12$ strain at 35°C showed to be different from wild type strain in *OLE1*, *OLE2*, *DES6A* but not in *DES6B*. During the acclimation process, the pattern of expression was similar for all the desaturases but not for the *OLE2* showing a marked decrease. Although the factors involved in the fatty acid regulation in ciliates (no ortholog genes of PPAR α and SREBP-1c which are main regulators in mammals [70] are not known, these results suggest that key transcription factors and a complex regulatory network must be present.

Additionally, the impaired expression of all FADs observed in the KO $\Delta 12$ strain is another evidence that these enzymes are tightly regulated by the decrease of temperature, although the mechanisms underlying such regulation still need to be elucidated. As a matter of fact, an ortholog search for the few membrane sensors described in bacillaceae (*DesK*), cyanobacteria (*Hik33*), and fungi (*Mga2*) [71] did not retrieve any putative gene in *T. thermophila*, suggesting a different mechanism for the regulation of homeoviscous response than those described for fungi (rotation-based mechanism) or for bacillaceae and cyanobacteria (hydrophobic thickness) [72].

In summary, current results stress the importance of the $\Delta 12$ FAD in the lipid membrane composition, the regulation of homeoviscous response, as well as the suitability for the use of *T. thermophila* as a valuable model to investigate the regulation of membrane lipids as well as evolutionary conservation and divergence of the mechanisms that control those processes.

1 **Acknowledgments**

2 A.D. Nusblat, C.B. Nudel and S. V. Verstraeten are members of the Consejo Nacional de
3 Investigaciones Científicas y Técnicas.

4

5 **Disclosure statement**

6 No potential conflict of interest was reported by the authors.

7

8 **Funding**

9 This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas, PIP
10 11220120100213; Ministerio de Ciencia, Tecnología e Innovación Productiva, PICT 2013–0701, and
11 Universidad de Buenos Aires, UBACYT 20020130100680BA. Further funding was obtained
12 through a Proyecto Intramural Especial of CSIC (201840I016) awarded to OM.

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Figures and tables

Figure 1: **A.** Schematic representation of the phylogenetic tree, showing major eukaryotic supergroups and the position of phyla based on phylogenetic analysis by He et. al. [73]. Circles indicate the presence of methyl-end desaturases of phylogenetic groups shown in Fig. 1B. **B.** Phylogenetic analysis of methyl-end fatty acid desaturases. For phylogenetic analyses, PROMALS3D with default parameters was used to obtain datasets multiple alignments (MSA). Poorly aligned sequences were removed using trimAl (<http://trimal.cgenomics.org/>) with the gappyout-tool. This MSA was used to construct the phylogeny by Maximum Likelihood using PhyML 3.0 optimized by SPR and NNI and a support of 100 bootstraps. The model LG+I+G for the MSA were selected using Prottest 3.0 server [35,36].

Figure 2: **A.** Functional characterization of ciliate $\Delta 12$ FAD expressed in yeast. To test the desaturation activity of the *T. thermophila* $\Delta 12$ FAD, two clones (rDES12.1 and rDES12.2) of yeast transformed with pYES2- $\Delta 12$ FAD were grown in *Saccharomyces cerevisiae* minimal medium lacking uracil (SCMM^{ura}) in the absence of exogenously added fatty acid substrates. Expression of the *T. thermophila* $\Delta 12$ FAD was induced by supplementation of galactose 0.02 % (w/v). A control treatment consisting of yeast transformed with empty pYES2 was also run under the same conditions. The recombinant *T. thermophila* activity $\Delta 12$ FAD was estimated by calculating the $\Delta 12$ desaturation capacity as $[\text{absolute area of } 18:2^{\Delta 9,12} / (\text{absolute area } 18:2^{\Delta 9,12} + 18:1^{\Delta 9}) \times 100]$ in both control yeast and yeast from two yeast clones (rDES12.1 and rDES12.2). ** denote statistical significances ($P \leq 0.05$) in comparison to control yeast. Results are shown as the mean $\pm$ SD (n=3). **B.** Schematic representation of *DES12* gene replacement in the wild-type (WT) locus by homologous recombination, using a cassette in which neo 3 confers paramomycin resistance.

Figure 3: Localization of GFP tagged *DES12* in *T. thermophila*; **A.** Schematic representation of gene replacement in the wild-type (WT) locus of the *DES12* gene by targeting the $\Delta 12$ FAD-GFP construct by homologous recombination, using a cassette in which neo 4 confers paramomycin resistance. Primers used to amplify the fragments necessary to generate the mutant are shown with gray arrows; primers used to check the incorporation of EGFP gene following the *DES12* gene are shown with black arrows. **B.** Immunofluorescence localization of GFP-tagged *DES12*. WT and $\Delta 12$ FAD-GFP strains were grown to Log phase and fixed for indirect immunofluorescence staining. The GFP was localized by anti-GFP primary antibody followed by labeling with anti-mouse-Alexa Fluor 594 secondary antibody. The nucleus of the ciliate was stained with DAPI (4',6-diamidino-2-

phenylindole, diluted in EtOH). The primers used for all amplification reactions are listed in the Supplementary material Table S1.

Figure 4: Phenotype associated with the KO Δ 12 strain. **A.** Growth of WT and KO Δ 12 strains measured at 15°C, 20°C, 30°C and 35°C with a Neubauer chamber a different time points until they reached their maximum cell density. Results are shown as the mean $\pm$ SD (n=3). Different colors indicate different culture temperature: blue for 15°C, pale blue for 20°C, orange for 30°C and red for 35°C. Colored arrows denote statistical significances ($P \leq 0.01$) in comparison to WT strain in the same culture temperature. Arrows followed by a line denote that all the following points of the curves also have statistical significances ($P \leq 0.01$) in comparison to WT strain. **B.** Growth of WT and KO Δ 12 strains at 15°C with the supplementation of oleic acid (18:1 Δ^9), linoleic acid (18:2 $\Delta^{9,12}$) or ethanol (control). Results are shown as the mean $\pm$ SD (n=3). **C.** Cell viability measured as freezing resistance in liquid nitrogen. WT and KO Δ 12 strains were grown at 15°C or 35°C prior to freezing. The percent recovery of viable cells was determined using the Poisson distribution [26]. Results are shown as the mean $\pm$ SD (n=3). **Denote statistical significances ($P < 0.05$) using paired T-Test.

Figure 5: Regulation of membrane fluidity by *DES12*. **A.** WT and KO Δ 12 cells grown at either 35 or 15°C were labeled with the fluorescent probes 6-AS or 12-AS and membrane fluidity was evaluated in the 15-35°C temperature range from the changes in their fluorescence anisotropy values. Results are shown as the mean $\pm$ SEM (n=4). * denotes statistical significance ($P < 0.05$) respect to the value recorded in WT cells at the same temperature. **B.** Lipid headgroup composition of WT and KO Δ 12 strain grown at 15°C and 35°C. The relative content of 2-AEP, phosphatidylethanolamine (PE) and phosphatidylcholine (PC), as well PE/PC ratio are shown. Results are shown as the mean $\pm$ SD (n=3). Statistical analysis was performed by applying one-way ANOVA followed by Brown-Forsythe comparison test. * denotes statistical significance ($P < 0.05$). **C.** Total lipids were extracted from WT and KO Δ 12 cells, and tetrahymanol and phospholipid contents were measured as described under Materials and Methods. Results are shown as the mean $\pm$ SD (n=3). Cell growth and membrane fluidity statistical analysis were performed by applying two-way ANOVA followed by Sidak's multiple comparison test. Tetrahymanol and total phosphorus content analysis was statistically analyzed using *t*-test. *, ** and *** denote statistical significances ($P < 0.05$, $P \leq 0.01$ and $P < 0.001$ respectively) respect to the value recorded in WT cells at the same temperature.

Figure 6: FADs transcription regulation in wild-type (WT) and KO Δ 12 cells at 35°C and 15°C. For q-PCR analyses, cells were first grown at 35°C, gradually chilled to 15°C (during a period of 20 min) and subsequently maintained at this temperature for 2 h. Results are shown as the mean $\pm$ SD (n=3)

of mRNA levels relative to WT values. Statistical analysis was performed using one- way ANOVA. *, ** and *** denote statistical significances ($P < 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively) respect to the value recorded in WT cells at the same temperature.

Table 1: Fatty acid composition in WT and KOΔ12 cells grown at 15 or 35 °C. Wild-type (WT) and KOΔ12 cells were grown either at 15°C or 35°C, and fatty acid composition was evaluated as described under Materials and Methods. Results are shown as the mean $\pm$ SD of three independent cultures. Two-way ANOVA followed by Bonferroni's multiple comparison test was used to assess differences in fatty acid profiles of WT and KO strains. Means in the same row with different superscripts are significantly different ($P < 0.001$) respect to the value recorded in WT cells at the same temperature.

Table 2: Fluorescence anisotropy of 6- and 12-AS measured in WT and KOΔ12 strain cultured either at 35°C or 15°C and measured at their respective growth temperatures. Wild-type (WT) and KOΔ12 cells were grown either at 15°C or 35°C, and membrane fluidity was evaluated as described under Materials and Methods by fluorescence anisotropy. Results are shown as the mean $\pm$ SEM of four independent cultures.

Supplementary information

Figure S1: FADs gene expression at different stages of the organism's life cycle. Results of a genome-wide analysis of gene expression performed during the three major stages of the organism's life cycle (growth, starvation, and conjugation at 30°C), W. Miao et al. 2009 [63]. Results are shown as the mean±SD (n=3).

Figure S2: **A.** Schematic representation of *DES12* gene replacement in the wild-type (WT) locus, primers used to check the incorporation of neo 3 cassette in the *DES12* locus are shown with arrows, grey for WT locus and black for knock-out (KO) locus. **B.** Genomic DNA of the *T. thermophila* WT and KOΔ12 strains analyzed by PCR to check the gene replacement assay. **C.** *DES12* gene transcription in WT and KOΔ12 strains analyzed by RT-PCR. The primers used for all amplification reactions are listed in the Supplementary material Table S1.

Table S1: List of oligonucleotides used for PCR amplifications. ID^a, identity. WT allele and KO allele are the sequences amplified to check the replacement of the endogenous gene in the KO5680 mutant. *DES12* and *α-Tub* are the primers used for the amplification of the first exon of the THERM_00535680 gene and the second exon of the *α*-tubulin gene used in the competitive RT-PCR analysis. *DES12* primers are also used in the Q-PCR analysis. Primers Up and Dw are used to amplify the fragments necessary to generate the Δ12 FAD-GFP mutant; primer Up 8 include SphI restriction site, primer Up 9 BamHI site, primer Dw 10 XhoI site and primer Dw 11 include KpnI site. Primers *DES12*-GFP are used to check the incorporation of the EGFP gene following the *DES12* gene. *OLE1*, *OLE2*, *DES6A*, *DES6B* are the primers used for the amplification of exons of THERM_00463280, THERM_00052620, THERM_00339850, THERM_00196130 genes respectively used in the Q-PCR, respectively, as well as 17S which are primers used for the amplification of 17S rRNA.

Table S2: List of sequences present in the phylogenetic tree. Sequences were retrieved from the National Center of Biotechnology Information (NCBI) and from the ciliates genomes of *E. octocarinatus* (<http://ciliates.ihb.ac.cn/database/species/eo>), *I. multifiliis* (<http://ich.ciliate.org/index.php/home>), *O. trifallax* (<http://oxy.ciliate.org/index.php/home>), *S. lemnae* (<http://stylo.ciliate.org/index.php/home>), *T. thermophila* (<http://ciliate.org/index.php/home>), *P. tetraurelia* (<http://paramecium.cgm.cnrs-gif.fr/>) and *P. persalinus* (<http://ciliates.ihb.ac.cn/database/species/pp>). OrthoMCL database v5 was also used to retrieve the sequences from Δ12 and Δ15 FADs (OG5_129048 and OG5_143101).

Table 1

Table 1. Fatty acid composition in WT and KOΔ12 cells grown at 15 or 35 °C

Species	WT		KOΔ12	
	15°C	35°C	15°C	35°C
14:0	1.45 ± 1.90	2.58 ± 0.04	1.78 ± 0.10	3.48 ± 0.88
15:0	2.01 ± 0.47	2.09 ± 0.80	1.02 ± 0.12	1.71 ± 0.14
16:0	12.16 ± 0.31 ^a	13.75 ± 0.94	12.93 ± 0.57	16.98 ± 1.65 ^a
16:1 (9)	4.55 ± 0.25 ^{ab}	6.64 ± 0.35	8.79 ± 0.55 ^{ac}	8.88 ± 0.75 ^{bc}
16:2 (7,10)	0.00 ± 0.00	0.00 ± 0.00	1.65 ± 0.35	2.16 ± 0.05
17:0	4.03 ± 0.30	6.55 ± 0.70 ^{ab}	1.10 ± 0.12 ^a	1.77 ± 0.82 ^b
18:0	9.29 ± 1.60 ^{abc}	3.87 ± 0.14 ^a	4.22 ± 1.09 ^b	4.31 ± 2.03 ^c
18:1 (9)	15.03 ± 0.97 ^{abc}	8.25 ± 0.25 ^{ade}	34.52 ± 0.59 ^{bdf}	25.27 ± 0.80 ^{cef}
18:1 (11)	0.00 ± 0.00 ^a	6.36 ± 0.59 ^{abe}	0.00 ± 0.00 ^b	0.00 ± 0.00 ^c
18:2 (6,9)	0.00 ± 0.00 ^{ab}	0.00 ± 0.00 ^{cd}	23.42 ± 1.13 ^{ac}	21.94 ± 0.31 ^{bd}
18:2 (9,12)	14.84 ± 0.73 ^{abc}	10.83 ± 0.47 ^{ade}	0.00 ± 0.00 ^{bd}	0.00 ± 0.00 ^{ce}
18:2 (11,13)	0.00 ± 0.00	2.63 ± 3.03	0.00 ± 0.00	0.00 ± 0.00
18:3 (6,9,12)	24.90 ± 0.00 ^{abc}	28.96 ± 0.82 ^{ade}	0.00 ± 0.00 ^{bd}	0.00 ± 0.00 ^{ce}
18:0 (2OH)	1.23 ± 0.16	1.24 ± 0.55	2.67 ± 1.65	1.73 ± 0.44
20:1 (11)	5.29 ± 0.89 ^{ab}	0.00 ± 0.00 ^{acd}	7.19 ± 0.97 ^c	10.06 ± 1.30 ^{bd}
Total SFA	30.2	30.1	23.7	30.0
Total MUFAs	24.9 ^{ab}	21.3 ^{cd}	50.5 ^{ac}	44.2 ^{bd}
Total PUFAs	39.7 ^{ab}	42.4 ^{cd}	25.1 ^{ac}	24.1 ^{bd}
PUFA index	72.2 ^{ab}	82.6 ^{cd}	33.8 ^{ac}	32.5 ^{bd}

WT and KOΔ12 cells were grown either at 15°C or 35°C, and fatty acid composition was evaluated as described under Materials and Methods. Results are shown as the mean ± SD of 3 independent cultures. Two- way ANOVA followed by Bonferroni’s multiple comparison test was used to assess differences in fatty acid profiles of WT and KO strains. Means in the same row with different superscripts are significantly different ($P < 0.001$) respect to the value recorded in WT cells at the same temperature.

The relative content of polyunsaturated fatty acids [PUFA index (%)] was calculated as:

$$\text{Eq. 1. } PUFA \text{ index (\%)} = 100 \times \frac{PUFA}{SFA+MUFA}$$

MUFA: monounsaturated fatty acid, SFA: saturated fatty acid.

Table 2

1 **TABLE 2:** Fluorescence anisotropy of 6- and 12-AS measured in WT and KOΔ12 strain cultured
2 either at 35°C or 15°C and measured at their respective growth temperatures.

3

Growth & measurement temperature	Strain	6-AS anisotropy	12-AS anisotropy
35°C	WT	0.393 ± 0.013	0.377 ± 0.004
	KOΔ12	0.380 ± 0.008	0.367 ± 0.005
15°C	WT	0.380 ± 0.010	0.378 ± 0.010
	KOΔ12	0.398 ± 0.010	0.383 ± 0.014

4

5 WT and KOΔ12 cells were grown either at 15°C or 35°C, and membrane fluidity was evaluated as
6 described under Materials and Methods by fluorescence anisotropy. Results are shown as the mean ±
7 SEM of 4 independent cultures.

8

FIGURE 1

FIGURE 2

A

B

Figure 3

Figure 4

Figure 5

A

Growth temperature: 35°C

6-AS

12-AS

Growth temperature: 15°C

6-AS

12-AS

B

C

Figure 6

Figure S1

Figure S2

Table S1**1 TABLE S1:** List of oligonucleotides used for PCR amplifications.

Description or purpose	Sequence ID ^a	Primers no. and sequence			
		No.	Forward	No.	Reverse
KO5680 mutant	<i>WT allele</i>	1	AGTCTATGCGCTTGTCATGCT	2	GGCTTCCATTTTACTCCTAAGTGG
	<i>KO allele</i>	3	TCGCCTTCTTGACGAGTTC	2	GGCTTCCATTTTACTCCTAAGTGG
RT-PCR analysis	<i>DES12</i>	4	CCAGAGAGGTTAAATAGGCC	5	CCTTTTCACAGCTTTCTTAGC
	<i>α-Tub</i>	6	TGTCGTCCCAAGGAT	7	GCAGTTTCAATACCGACT
Δ 12 FAD-GFP mutant	<i>Up</i>	8	TCCGCATGCTTATATCCTCCATACT GCTTTATTAGTACC	9	CGCGGATCCTTTCCAATAATAGAA ACCTTATTCTCTTTCAG
	<i>Dw</i>	10	TCCGCTCGAGTTCAAAGCTTTAGT GGCAGGA	11	CGGGGTACCCGCATGAATTAGTTG GATTGA
	<i>DES12-GFP</i>	4	CCAGAGAGGTTAAATAGGCC	12	TTGTTGATAATGATCAGCTAAT
	<i>DES12</i>	4	CCAGAGAGGTTAAATAGGCC	5	CCTTTTCACAGCTTTCTTAGC
	<i>OLE1</i>	13	TGCCACATGTTTGGTAGCAG	14	TGGGGTATTCGTGATGCCAG
Q-PCR analysis	<i>OLE2</i>	15	CTAGAGACTGGAGAGCTTGCG	16	ACCAGCCATTAGGATTCCAC
	<i>DES6A</i>	17	TGCACAGCAAGAAGGCTCTC	18	CTCAATTGGCCAGTGAGGTTC
	<i>DES6B</i>	19	TGTAAGAAAGAGCTCCCATGC	20	CACCGAAATTGCGAGTCCG
	<i>17S</i>	21	CCTGGGAAGGTACGGGTAAT	22	AAGGGTCACCAGACCATTTCG

^a ID, identity. WT allele and KO allele are the sequences amplified to check the replacement of the endogenous gene in the KO5680 mutant. *DES12* and α -Tub are the primers used for the amplification of the first exon of the THERM_00535680 gene and the second exon of the α -tubulin gene used in the competitive RT-PCR analysis. *DES12* primers are also used in the Q-PCR analysis. Primers *Up* and *Dw* are used to amplify the fragments necessary to generate the Δ 12 FAD-GFP mutant; primer *Up* 8 include SphI restriction site, primer *Up* 9 BamHI site, primer *Dw* 10 XhoI site and primer *Dw* 11 include KpnI site. Primers Δ 12 FAD-GFP are used to check the incorporation of the eGFP gene following the *DES12* gene. *OLE1*, *OLE2*, *DES6A*, *DES6B* are the primers used for the amplification of exons of THERM_00463280, THERM_00052620, THERM_00339850, THERM_00196130 genes respectively used in the Q-PCR, respectively, as well as 17S which are primers used for the amplification of 17S rRNA.

Table S2

TABLE S2: List of sequences present in the phylogenetic tree.

Sec. ID	Organism	Uniprot Entry	Tree group
A.thaliana_AAM65548	>Eukaryota_Viridiplantae_Streptophyta_Arabidopsis_thaliana	AAM65548	Archaeplastida w6 / Δ12
B.napus_P48627	>Eukaryota_Viridiplantae_Streptophyta_Brassica_napus	P48627	Archaeplastida w6 / Δ12
C.vulgaris_E2CT06	>Eukaryota_Viridiplantae_Chlorophyta_Chlorella_vulgaris	E2CT06	Archaeplastida w6 / Δ12
G.max_P48628	>Eukaryota_Viridiplantae_Streptophyta_Glycine_max	P48628	Archaeplastida w6 / Δ12
G.sulphuraria_M2WYY0	>Eukaryota_Rhodophyta_Bangioophyceae_Galdieria_sulphuraria	M2WYY0	Archaeplastida w6 / Δ12
S.oleracea_P48629	>Eukaryota_Viridiplantae_Streptophyta_Spinacia_oleracea	P48629	Archaeplastida w6 / Δ12
A.protothecoides_A0A087STD1	>Eukaryota_Viridiplantae_Chlorophyta_Auxenochlorella_protothecoides	A0A087STD1	Chlorophyta w3
C.reinhardtii_A8HMC4	>Eukaryota_Viridiplantae_Chlorophyta_Chlamydomonas_reinhardtii	A8HMC4	Chlorophyta w3
L.incisa_U5YCT7	>Eukaryota_Viridiplantae_Chlorophyta_Lobosphaera_incisa	U5YCT7	Chlorophyta w3
A.protothecoides_A0A087SDQ7	>Eukaryota_Viridiplantae_Chlorophyta_Auxenochlorella_protothecoides	A0A087SDQ7	Chlorophyta w6 / Δ12
C.reinhardtii_A8IR24	>Eukaryota_Viridiplantae_Chlorophyta_Chlamydomonas_reinhardtii	A8IR24	Chlorophyta w6 / Δ12
C.subellipsoides_I0YVH9	>Eukaryota_Viridiplantae_Chlorophyta_Coccomyxa_subellipsoides	I0YVH9	Chlorophyta w6 / Δ12
C.vulgaris_Q8W3L4	>Eukaryota_Viridiplantae_Chlorophyta_Chlorella_vulgaris	Q8W3L4	Chlorophyta w6 / Δ12
A.cylindrica_K9ZLJ5	>Bacteria_Cyanobacteria_Nostocales_Anabaena_cylindrica	K9ZLJ5	Cyanobacteria w3 / Δ15 / Δ12
A.marina_B0C5M8	>Bacteria_Cyanobacteria_Synechococcales_Acanyochloris_marina	B0C5M8	Cyanobacteria w3 / Δ15 / Δ12
Calothrix_K9PJQ0	>Bacteria_Cyanobacteria_Nostocales_Calothrix_sp	K9PJQ0	Cyanobacteria w3 / Δ15 / Δ12
M.aeruginosa_I4G9A1	>Bacteria_Cyanobacteria_Oscillatoriothycideae_Microcystis_aeruginosa	I4G9A1	Cyanobacteria w3 / Δ15 / Δ12
N.azollae_D7E3I4	>Bacteria_Cyanobacteria_Nostoc_azollae	D7E3I4	Cyanobacteria w3 / Δ15 / Δ12
Nostoc_A0A0S3PA06	>Bacteria_Cyanobacteria_Nostocales_Nostoc_sp	A0A0S3PA06	Cyanobacteria w3 / Δ15 / Δ12
P.priestleyi_A0A0P8A3U9	>Bacteria_Cyanobacteria_Synechococcales_Phormidesmis_priestleyi	A0A0P8A3U9	Cyanobacteria w3 / Δ15 / Δ12
R.brookii_D4TVI0	>Bacteria_Cyanobacteria_Nostocales_Raphidiopsis_brookii	D4TVI0	Cyanobacteria w3 / Δ15 / Δ12
Synechocystis_Q55240	>Bacteria_Cyanobacteria_Synechococcales_Synechocystis_sp	Q55240	Cyanobacteria w3 / Δ15 / Δ12
A.platensis_Q54794	>Bacteria_Cyanobacteria_Oscillatoriothycideae_Arthrospira_platensis	Q54794	Cyanobacteria w3 / w6 / Δ12
A.variabilis_Q3M5C6	>Bacteria_Cyanobacteria_Nostocales_Anabaena_variabilis	Q3M5C6	Cyanobacteria w3 / w6 / Δ12
D.salina_K9YUA1	>Bacteria_Cyanobacteria_Synechococcales_Dactylococcopsis_salina	K9YUA1	Cyanobacteria w3 / w6 / Δ12

P.priestleyi_A0A0P8BU24	>Bacteria_Cyanobacteria_Synechococcales_Phormidesnis_priestleyi	A0A0P8BU24	Cyanobacteria w3 / w6 / Δ12
Phormidium_A0A0P7Z4L8	>Bacteria_Cyanobacteria_Oscillatoriothricideae_Phormidium_sp	A0A0P7Z4L8	Cyanobacteria w3 / w6 / Δ12
Synechocystis_P20388	>Bacteria_Cyanobacteria_Synechococcales_Synechocystis_sp	P20388	Cyanobacteria w3 / w6 / Δ12
L.major_Q4Q3K9	>Eukaryota_Euglenozoa_Kinetoplastida_Leishmania_major	Q4Q3K9	Euglenozoa Δ12
T.cruzi_Q4DTH7	>Eukaryota_Euglenozoa_Kinetoplastida_Trypanosoma_cruzi	Q4DTH7	Euglenozoa Δ12
T.vivax_G0TRT6	>Eukaryota_Euglenozoa_Kinetoplastida_Trypanosoma_vivax	G0TRT6	Euglenozoa Δ12
M.alpina_Q59J82	>Eukaryota_Fungi_Mucoromycota_Mortierella_alpina	Q59J82	Fungi w3 / Δ12
M.alpina_Q9Y8H5	>Eukaryota_Fungi_Mucoromycota_Mortierella_alpina	Q9Y8H5	Fungi w3 / Δ12
A.nidulans_Q5AWX6	>Eukaryota_Fungi_Dikarya_AspERGillius_nidulans	Q5AWX6	Fungi w3 / w6 / Δ12
C.parapsilosis_C3W956	>Eukaryota_Fungi_Dikarya_Candida_parapsilosis	C3W956	Fungi w3 / w6 / Δ12
C.parapsilosis_C3W957	>Eukaryota_Fungi_Dikarya_Candida_parapsilosis	C3W957	Fungi w3 / w6 / Δ12
C.purpurea_A7KR53	>Eukaryota_Fungi_Dikarya_Claviceps_purpurea	A7KR53	Fungi w3 / w6 / Δ12
C.torrendii_A0A0D7BDW3	>Eukaryota_Fungi_Dikarya_Cylindrobasidium_torrendii	A0A0D7BDW3	Fungi w3 / w6 / Δ12
F.graminearum_I1RP33	>Eukaryota_Fungi_Dikarya_Fusarium_graminearum	I1RP33	Fungi w3 / w6 / Δ12
F.verticillioides_Q27ZJ7	>Eukaryota_Fungi_Dikarya_Fusarium_verticillioides	Q27ZJ7	Fungi w3 / w6 / Δ12
K.pastoris_A1EAA6	>Eukaryota_Fungi_Dikarya_Komagataella_pastoris	A1EAA6	Fungi w3 / w6 / Δ12
K.phaffii_F2R0D5	>Eukaryota_Fungi_Dikarya_Komagataella_phaffii	F2R0D5	Fungi w3 / w6 / Δ12
L.starkeyi_A0A1E3QDX9	>Eukaryota_Fungi_Dikarya_Lipomyces_starkeyi	A0A1E3QDX9	Fungi w3 / w6 / Δ12
N.crassa_Q7S463	>Eukaryota_Fungi_Dikarya_Neurospora_crassa	Q7S463	Fungi w3 / w6 / Δ12
N.irregularis_A0A1U7LKN2	>Eukaryota_Fungi_Dikarya_Neoclecta_irregularis	A0A1U7LKN2	Fungi w3 / w6 / Δ12
P.sporulosa_A0A177CB22	>Eukaryota_Fungi_Dikarya_Paraphaeosphaeria_sporulosa	A0A177CB22	Fungi w3 / w6 / Δ12
W.ciferrii_K0KC75	>Eukaryota_Fungi_Dikarya_Wickerhamomyces_ciferrii	K0KC75	Fungi w3 / w6 / Δ12
A.anophagefferens_F0Y5X8	>Eukaryota_Stramenopiles_Pelagophyceae_Aureococcus_anophagefferens	F0Y5X8	Stramenopiles w6 / Δ12
P.tricornutum_Q84KI9	>Eukaryota_Stramenopiles_Bacillariophyta_Phaeodactylum_tricornutum	Q84KI9	Stramenopiles w6 / Δ12
T.pseudonana_B8C757	>Eukaryota_Stramenopiles_Bacillariophyta_Thalassiosira_pseudonana	B8C757	Stramenopiles w6 / Δ12
A.thaliana_P48623	>Eukaryota_Viridiplantae_Streptophyta_Arabidopsis_thaliana	P48623	Streptophyta w3
B.napus_P48618	>Eukaryota_Viridiplantae_Streptophyta_Brassica_napus	P48618	Streptophyta w3
C.sativa_A0A059V8B3	>Eukaryota_Viridiplantae_Streptophyta_Camellia_sativa	A0A059V8B3	Streptophyta w3
C.sativus_B3VA19	>Eukaryota_Viridiplantae_Streptophyta_Cucumis_sativus	B3VA19	Streptophyta w3
G.max_Q84X84	>Eukaryota_Viridiplantae_Streptophyta_Glycine_max	Q84X84	Streptophyta w3

H.annuus_Q56V/S4	>Eukaryota_Viridiplantae_Streptophyta_Helianthus_annuus	Q56V/S4	Streptophyta_w3
L.usitatissimum_Q3SAG0	>Eukaryota_Viridiplantae_Streptophyta_Linum_usitatissimum	Q3SAG0	Streptophyta_w3
N.tabacum_P48626	>Eukaryota_Viridiplantae_StreptophytaNicotiana_tabacum	P48626	Streptophyta_w3
P.frutescens_O04807	>Eukaryota_Viridiplantae_Streptophyta_Perilla_frutescens	O04807	Streptophyta_w3
P.patens_A9SNL0	>Eukaryota_Viridiplantae_Streptophyta_Physcomitrella_patens	A9SNL0	Streptophyta_w3
V.radiata_P32291	>Eukaryota_Viridiplantae_Streptophyta_Vigna_radiata	P32291	Streptophyta_w3
A.thaliana_Q8LFZ8	>Eukaryota_Viridiplantae_Streptophyta_Arabidopsis_thaliana	Q8LFZ8	Streptophyta_w6 / Δ12
B.juncea_Q39287	>Eukaryota_Viridiplantae_Streptophyta_Brassica_juncea	Q39287	Streptophyta_w6 / Δ12
C.alpina_O81931	>Eukaryota_Viridiplantae_Streptophyta_Crepis_alpina	O81931	Streptophyta_w6 / Δ12
C.officinalis_Q9AT72	>Eukaryota_Viridiplantae_Streptophyta_Calendula_officinalis	Q9AT72	Streptophyta_w6 / Δ12
C.sativus_A0A0A0L CV6	>Eukaryota_Viridiplantae_Streptophyta_Cucumis_sativus	A0A0A0L CV6	Streptophyta_w6 / Δ12
D.carota_A0A164XZA3	>Eukaryota_Viridiplantae_Streptophyta_Daucus_carota	A0A164XZA3	Streptophyta_w6 / Δ12
G.hirsutum_Q2HPG7	>Eukaryota_Viridiplantae_Streptophyta_Gossypium_hirsutum	Q2HPG7	Streptophyta_w6 / Δ12
G.max_P48630	>Eukaryota_Viridiplantae_Streptophyta_Glycine_max	P48630	Streptophyta_w6 / Δ12
I.balsamina_Q9SP62	>Eukaryota_Viridiplantae_Streptophyta_Impatiens_balsamina	Q9SP62	Streptophyta_w6 / Δ12
M.charantia_Q9SP61	>Eukaryota_Viridiplantae_Streptophyta_Momordica_charantia	Q9SP61	Streptophyta_w6 / Δ12
P.granatium_Q84VT2	>Eukaryota_Viridiplantae_Streptophyta_Punica_granatium	Q84VT2	Streptophyta_w6 / Δ12
T.kirilowii_Q84UC0	>Eukaryota_Viridiplantae_Streptophyta_Trichosanthes_kirilowii	Q84UC0	Streptophyta_w6 / Δ12
A.castellani_L8GY15	>Eukaryota_Amoebozoa_Discosea_Acanthamoeba_castellani	L8GY15	No group
A.millepora_A0A2H4NNK7	>Eukaryota_Metazoa_Cnidaria_Acropora_millepora	A0A2H4NNK7	No group
C.elegans_G5EGA5	>Eukaryota_Metazoa_Ecdysozoa_Caenorhabditis_elegans	G5EGA5	No group
C.elegans_Q9NEQ0	>Eukaryota_Metazoa_Ecdysozoa_Caenorhabditis_elegans	Q9NEQ0	No group
C.merolae_M1VHZ7	>Eukaryota_Rhodophyta_Bangioophyceae_Cyanidioschyzon_merolae	M1VHZ7	No group
E.siliculosus_D7FSC6	>Eukaryota_Stramenopiles_PX_clade_Ectocarpus_siliculosus	D7FSC6	No group
E.siliculosus_D7G044	>Eukaryota_Stramenopiles_PX_clade_Ectocarpus_siliculosus	D7G044	No group
E.siliculosus_D7G6D1	>Eukaryota_Stramenopiles_Ectocarpus_siliculosus	D7G6D1	No group
F.alba_A0A058Z2R6	>Eukaryota_Nucleariidae_Forticula_group_Forticula_Forticula_alba	A0A058Z2R6	No group
I.multifiliis_G0QKZ5	>Eukaryota_Alveolata_Ciliophora_Ichthyophthirius_multifiliis	G0QKZ5	No group
I.multifiliis_G0R4K0	>Eukaryota_Alveolata_Ciliophora_Ichthyophthirius_multifiliis	G0R4K0	No group
L.salmonis_A0A0K2TVX7	>Eukaryota_Metazoa_Ecdysozoa_Lepeophtheirus_salmonis	A0A0K2TVX7	No group

M.brevicollis_A9V6W3	>Eukaryota_Choanoflagellida_Craspedida_Monosiga_brevicollis	A9V6W3	No group
M.commoda_C1FD88	>Eukaryota_Viridiplantae_Chlorophyta_Micromonas_commoda	C1FD88	No group
M.pusilla_C1N703	>Eukaryota_Viridiplantae_Chlorophyta_Micromonas_pusilla	C1N703	No group
N.gaditana_12CR09	>Eukaryota_Stramenopiles_Eustigmatophyceae_Nannochloropsis_gaditana	12CR09	No group
N.gruberi_D2VAV7	>Eukaryota_Heterolobosea_Schizopyrenida_Naegleria_gruberi	D2VAV7	No group
P.brassicace_A0A0G4IWE8	>Eukaryota_Rhizaria_Cercozoa_Plasmodiophora_brassicace	A0A0G4IWE8	No group
P.marinus_C5KCW9	>Eukaryota_Alveolata_Perkinsea_Perkinsus_marinus	C5KCW9	No group
P.persalinus_A0A0V0Q827	>Eukaryota_Alveolata_Ciliophora_Pseudocohniembus_persalinus	A0A0V0Q827	No group
P.persalinus_A0A0V0R0V6	>Eukaryota_Alveolata_Ciliophora_Pseudocohniembus_persalinus	A0A0V0R0V6	No group
P.sojae_G4Z5F7	>Eukaryota_Stramenopiles_Oomycetes_Phytophthora_sojae	G4Z5F7	No group
P.tetraurelia_A0DVT3	>Eukaryota_Alveolata_Ciliophora_Paramecium_tetraurelia	A0DVT3	No group
P.tetraurelia_A0DYG0	>Eukaryota_Alveolata_Ciliophora_Paramecium_tetraurelia	A0DYG0	No group
Prochlorococcus_sp._A0A162DSM3	>Bacteria_Cyanobacteria_Synechococcales_Prochlorococcus_sp	A0A162DSM3	No group
R.filosa_X6NBA42	>Eukaryota_Rhizaria_Foraminifera_Reticulomyxa_filosa	X6NBA42	No group
T.pseudonana_B8BWVX9	>Eukaryota_Stramenopiles_Bacillariophyta_Thalassiosira_pseudonana	B8BWVX9	No group
T.thermophila_17MA19	>Eukaryota_Alveolata_Ciliophora_Tetrahymena_thermophila	17MA19	No group
T.trahens_A0A0L0DDP8	>Eukaryota_Apusozoa_Apusomonadidae_Thecamonas_trahens	A0A0L0DDP8	No group