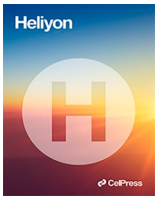
Heliyon 10 (2024) e32807

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

The presence of plasmenyl ether lipids in *Capsaspora owczarzaki* suggests a premetazoan origin of plasmalogen biosynthesis in animals

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ARTICLE INFO

Keywords:
Opisthokonta
Plasmanylethanolamine desaturase
TMEM189
Phylogeny

ABSTRACT

Plasmalogens are glycerophospholipids with a vinyl ether bond, rather than an ester bond, at *sn*-1 position. These lipids were described in anaerobic bacteria, myxobacteria, animals and some protists, but not in plants or fungi. Anaerobic and aerobic organisms synthesize plasmalogens differently. The aerobic pathway requires oxygen in the last step, which is catalyzed by PEDS1. CarF and TMEM189 were recently identified as the PEDS1 from myxobacteria and mammals, which could be of valuable use in exploring the distribution of this pathway in eukaryotes. We show the presence of plasmalogens in *Capsaspora owczarzaki*, one of the closest unicellular relatives of animals. This is the first report of plasmalogens in non-metazoan opisthokontas. Analysis of its genome revealed the presence of enzymes of the aerobic pathway. In a broad BLAST search, we found PEDS1 homologs in Opisthokonta and some genera of Amoebozoa and Excavata, consistent with the restricted distribution of plasmalogens reported in eukaryotes. Within Opisthokonta, PEDS1 is limited to Filasterea (*Capsaspora* and *Pigoraptor*), Metazoa and a small group of fungi comprising three genera of ascomycetes. A phylogenetic analysis of PEDS1 traced the acquisition of plasmalogen synthesis in animals to a filasterean ancestor and suggested independent acquisition events for Amoebozoa, Excavata and Ascomycetes.

1. Introduction

Ether lipids represent a particular subgroup of phospholipids characterized by the substituent at the *sn*-1 position of the glycerol backbone; they have a fatty alcohol attached via an ether bond instead of a fatty acid via an ester bond. Plasmanyl ether lipids have an alkyl group, while plasmenyl lipids, also called plasmalogens, have an alkenyl or vinyl group at *sn*-1 [1].

Plasmalogens have particular physicochemical properties. As a component of a lipid bilayer, they promote an increase in its order. Plasmenyl ethanolamine (1-*O*-alk-1′-enyl, 2-acyl-*sn*-glycero-3-phosphoethanolamine; plasmenyl-PE) favors the formation of non-bilayer phases at lower temperatures [2,3]. Phase transitions are necessary for fusion and fission events; plasmenyl-PE was shown to facilitate rapid membrane fusion [4]. Lipid rafts microdomains are lateral membrane domains, enriched in cholesterol, sphingolipids and proteins, involved in cell signaling, cell-cell interactions, and endocytosis. Lipid rafts isolated from human epidermal

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https://doi.org/10.1016/j.heliyon.2024.e32807
Received 28 November 2023; Received in revised form 7 June 2024; Accepted 10 June 2024
Available online 10 June 2024

carcinoma and CHO cells were also enriched in plasmalogens [5,6], suggesting that they could facilitate signal transduction processes. In addition, plasmalogens are enriched in polyunsaturated fatty acyl residues such as arachidonic and docosahexaenoic acids at the *sn*-2 position, and then function as reservoirs of second messengers that regulate the inflammatory response [7].

Plasmenyl ether lipids make up about 18 % of human phospholipids, mainly as plasmenyl-PE, which it represents up to 70 % of all ethanolamine phospholipids in some tissues such as the nervous system. Plasmenyl choline (plasmenyl-PC) and, to a much lesser extent, serine- and inositol-linked plasmalogens are also present [8]. Reduced plasmalogen levels are associated with rare genetic disorders, such as peroxisome biogenesis disorder (Zellweger spectrum diseases), mutations in peroxisomal enzymes of plasmalogen biosynthesis or in proteins involved in the import of these enzymes into the organelle (rhizomelic chondrodysplasia punctata), metabolic disorders (Barth syndrome), cancer, respiratory diseases (bronchopulmonary dysplasia) and neurodegenerative diseases (including Alzheimer, Parkinson and Niemann-Pick type C diseases) and probably with autism spectrum disorders, just to name some of them [9,10]. Elevated levels of plasmalogens were associated with cancer [11,12].

Plasmalogens are widely distributed in vertebrate and invertebrate animals. They are also present in anaerobic bacteria [13], aerobic mixobacteria such as *Myxococcus xanthus* [14] and some protists, such as *Dictyostelium discoideum* [15], trypanosomatids [16,17] and anaerobic ciliates [18,19]. These lipids do not occur in other aerobic or facultative bacteria [20], plants [21] and fungi [22].

Ether lipids are synthesized differently in anaerobic and aerobic organisms. In anaerobic bacteria such as Clostridium spp., plasmalogens are formed from diacylated phospholipids at a late stage of phospholipid formation [23]. The conversion of the acyl ester to an alk-1′-enyl ether is carried out by the consecutive action of two recently described reductases [24]. In contrast, aerobic organisms use dihydroxyacetone phosphate as primer. A saturated ether bond is formed at an early stage of plasmalogen biosynthesis, which is desaturated in the last oxygen-dependent step, by the enzyme plasmanylethanolamine Δ1′-desaturase (PEDS1) [25]. PEDS1 was an orphan enzyme until very recently. Its genes were independently identified in *M. xanthus* [26] and in mammals [27,28] by three groups, which indicates the interest of the scientific community in the synthesis of this important lipid.

This unusual distribution and the major differences found in their biosynthesis suggest that the ability to synthesize plasmalogens first evolved in anaerobic bacteria but did not persist in the facultative and aerobic bacteria that appeared after the increase in oxygen in the early Earth's atmosphere [13], very probably due to the known lability of the vinyl ether bond to oxidation. The revival of such a capacity could be explained by the ability of some organisms to use these lipids in an advantageous way or having new functions [13], such as in multicellularity or in cell signaling processes, as mentioned above. Interestingly, it was suggested that plasmalogens could prevent the oxidation of polyunsaturated fatty acids (PUFA) and other vulnerable membrane lipids by playing a role as sacrificial oxidants [29]. Two important questions arise from this: i) how many aerobic pathway reacquisition events occurred in nature and ii) what is the evolutionary history of this event in Opisthokonta, the clade to which fungi and metazoans belong.

We detected plasmalogens in the unicellular amoeboid protist *Capsaspora owczarzaki*, one of the closest relatives of animals. *C. owczarzaki* belongs to Filozoa, which includes filastereans, choanoflagellates, and metazoans [30,31]. Its genome appears to have the genes encoding the enzymes required for ether lipid biosynthesis, including PEDS1. Interestingly, in a wide blast search, PEDS1 orthologues in Opisthokonta were found only in Metazoa, Filasterea and a small group of fungi. In this work we also present a phylogenetic analysis using available PEDS1 sequences. This analysis suggests that plasmalogen biosynthesis capacity is restricted to few clades in nature, and probably as a consequence of independent acquisition events. It also shows that the origin of plasmalogen synthesis in animals should be traced back to a unicellular ancestor of Filozoa.

2. Materials and methods

2.1. Capsaspora owczarzaki cultures

C. owczarzaki cells were grown axenically in 25 or 75 cm^2 flasks containing either 7 or 25 ml of ATCC 1034 medium of the following composition: 1 % Bacto peptone, 1 % yeast extract, 0.1 % ribonucleic acid type VI from Torula yeast, 15 mg l^{-1} folic acid, 1 mg l^{-1} haemin, 10 % fetal bovine serum, 2 % phosphate buffer (18.1 g l^{-1} KH_2PO_4, 25 g l^{-1} Na_2HPO_4). Cultivation was carried out in a 23 °C incubator. Adherent filopodiated cells were obtained by starting cultures of approximately 5×10^4 cells ml^{-1}, after stationary incubation during 3–4 days. Aggregates formation was induced by initiating cultures of approximately 1.5×10^4 cells ml^{-1} and incubated with gentle agitation at 70 r.p.m. for 4–5 days. Floating cystic cells were obtained from 14-days-old stationary cultures, started in the same conditions as the adherent cultures [31].

2.2. Lipid extraction and analysis

Cells from each stage were collected by centrifugation at 3000*g* for 5 min at 4 °C, washed twice with 20 ml of phosphate buffer, and the lipids were extracted according to Bligh and Dyer [32]. The organic phase was evaporated to dryness under a N_2 stream, and the lipids were submitted to transmethylation. Acid transmethylation was carried out by addition of 2 % H_2SO_4 in methanol and incubation at 80 °C during 1 h. Basic transmethylation was carried out by addition of 0.5 M sodium methoxyde in methanol and 30 min incubation at room temperature. Fatty acid methyl esters (FAME) were extracted three times with hexane without previous neuralization, the hexane fractions combined, concentrated and analyzed by gas chromatography-mass spectrometry (GC-MS), by running through a ZB-WAX column (30 m × 0.25 mm × 0.25 μm; ZEBRON) in a Shimadzu GC-2010 Plus gas chromatograph. The chromatography was runed at 180 °C during 30 min followed by an increase of 10 °C per min till 240 °C and held 10 min at 240 °C. MS was carried out using a GCMS-QP2010 Plus mass detector, operated at an ionization voltage of 70 eV with a scan range of 20–600 atomic mass units. The retention times and mass spectra of all new peaks obtained were compared with those of standards (Sigma-Aldrich), as

well as those available in the literature and in the National Institute of Standards and Technology mass spectral library. Double bond positions in mono- and di-unsaturated FAMEs were determined by obtaining the dimethyl disulfide adducts as previously described [33]. For PUFA analysis, dimethyloxazoline (DMOX) derivatives were also prepared, by adding 0.25 g of 2-amino-2-methyl-1-propanol to up to 2 mg of lipid samples, as described [34].

High-resolution mass spectra (HRMS) were recorded on a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific). Methanol (Carlo Erba) was used for sample preparation. Samples were directly infused to the mass spectrometer at a 5 μL/min rate via a HESI source. Spray voltage was set at 3500 V, the capillary temperature was 320 °C, sheath gas (nitrogen) 5 L/min. Spectra were analyzed with FreeStyle 1.6 (Thermo Fisher Scientific). Direct infusion MS and MS/MS experiments were carried out using a triple quadrupole (QqQ) mass spectrometer (TSQ Quantum Access Max, Thermo-Scientific), equipped with a heated electrospray ionization (HESI-II) probe to generate the charged ions. The operating conditions were optimized by syringe-pump injection (20 μL/min) of a mixture of standards and are as follows: spray voltage, 4.5 kV; sheath gas (N_2) pressure, 20 psi; auxiliary gas (N_2) pressure, 2 psi; vaporizer temperature, 220 °C; capillary temperature, 250 °C; tube lens offset, 106 AU; collision gas (argon) 1.5 mTorr. Lipid extract was properly diluted to less than 50 pmol of total lipids/μl with chloroform/methanol/isopropanol (1:2:4, v/v/v) prior to infusion to the mass spectrometer. Lithium adducts were obtained by adding 25 μM of LiOH. For each MS/MS mass spectrum, a 2–5-min period of signal averaging was employed. The collision energies used for the different experiments were as follows: neutral loss scanning (NLS) for 183 and 189 fragments and Parent-ion scanning (PIS) of PC P16:0, PC P17:0 and PC P18:0, 35 eV; NLS of 213 fragment and FA substituents ([M + Li-FA-59]+), 50 eV; product-ion scanning of PC P-17:0_16:1 and PC P-17:0_20:3, 40 eV. Data processing and analysis was done using Xcalibur software.

2.3. Phylogenetic and sequence analyses

The data set for the phylogenetic analysis was prepared with 55 sequences obtained from the National Center for Biotechnology

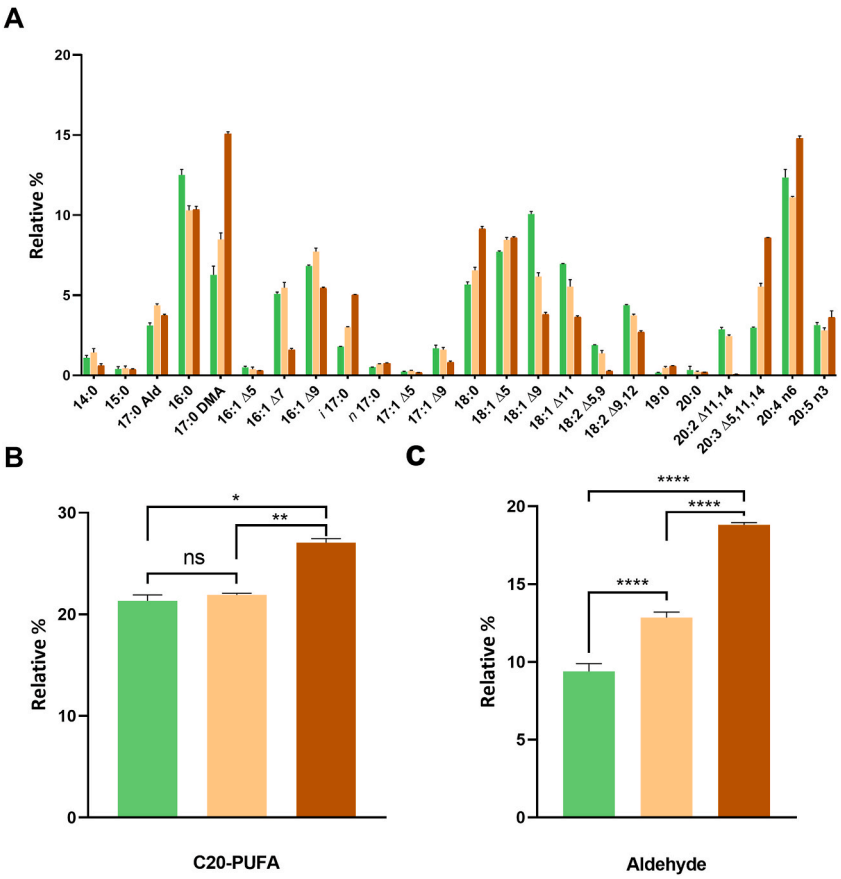


Fig. 1. Fatty acid profile of *Capsaspora owczarzaki* cells along its life cycle. (A) Relative percentages of each fatty acid, as methyl ester derivatives (FAME), fatty aldehyde (17:0 Ald) and its dimethyl acetal (17:0 DMA), obtained after acid transmethylation. (B) Total C20-PUFA and (C) aldehydes (17:0 Ald plus 17:0 DMA) in each stage. Green, orange and red bars: filopodial, aggregative and cystic stages, respectively. i17:0 and n17:0 indicate iso and normal heptadecanoic acid FAME. Data are expressed as mean ± SD (n = 3). *p < 0.05, **p < 0.005, ***p < 0.0005, ns: not significant (one-way ANOVA with Tukey post hoc test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Information (NCBI) by protein BLAST (Basic Local Protein Alignment Search Tool) with the PEDS1 of *C. owczarzaki* (XP_004348333.1) and *H. sapiens* (NP_954580) as queries against the nonredundant protein database. For the species of the Filasterea clade, which genomes were recently published [35], genomes were downloaded (https://www.ebi.ac.uk/ena/browser/view/PRJEB52884) and a local BLAST made with the Blast2GO software [36]. Amino acid sequences in the final data sets were aligned using the MAFFT algorithm [37,38] and the confidence of the alignment was assessed with GUIDANCE2 [39] removing columns with a confidence score below 0.93. The topology and branch lengths were obtained by maximum likelihood method using the RAxML-HPC BlacksBox (8.2.12) algorithm [40] with the PROTCAT model and statistical support obtained by 1000-bootstrap raxml replicates. All tools were implemented in the online resource at CIPRES Science Gateway (https://www.phylo.org/portal2) [41]. The customize process of the tree was carried out online in the Interactive Tree Of Life (iTOL) an internet-based utensil for the display, operation and modification of phylogenetic trees (https://itol.embl.de/) [42].

2.4. Statistical analysis

All data are representative of three independent experiments. Results were expressed as means ± standard errors of the means (SEM). One-way ANOVA with Tukey's post hoc test was used to determine a significant difference between independent groups. The difference between groups was set at *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ns: not significant.

3. Results

3.1. Capsaspora owczarzaki contains plasmalogens

As part of a lipidomic project, we analyzed the fatty acid profile of *C. owczarzaki* throughout its life cycle. *C. owczarzaki* alternates between filopodial (adherent), aggregative and cystic stages [31]. We obtained lipid extracts from each stage, which were subjected to acid transmethylation and GC-MS to identify the FAME. Fig. 1A summarizes the relative percentages of FAME in the three stages.

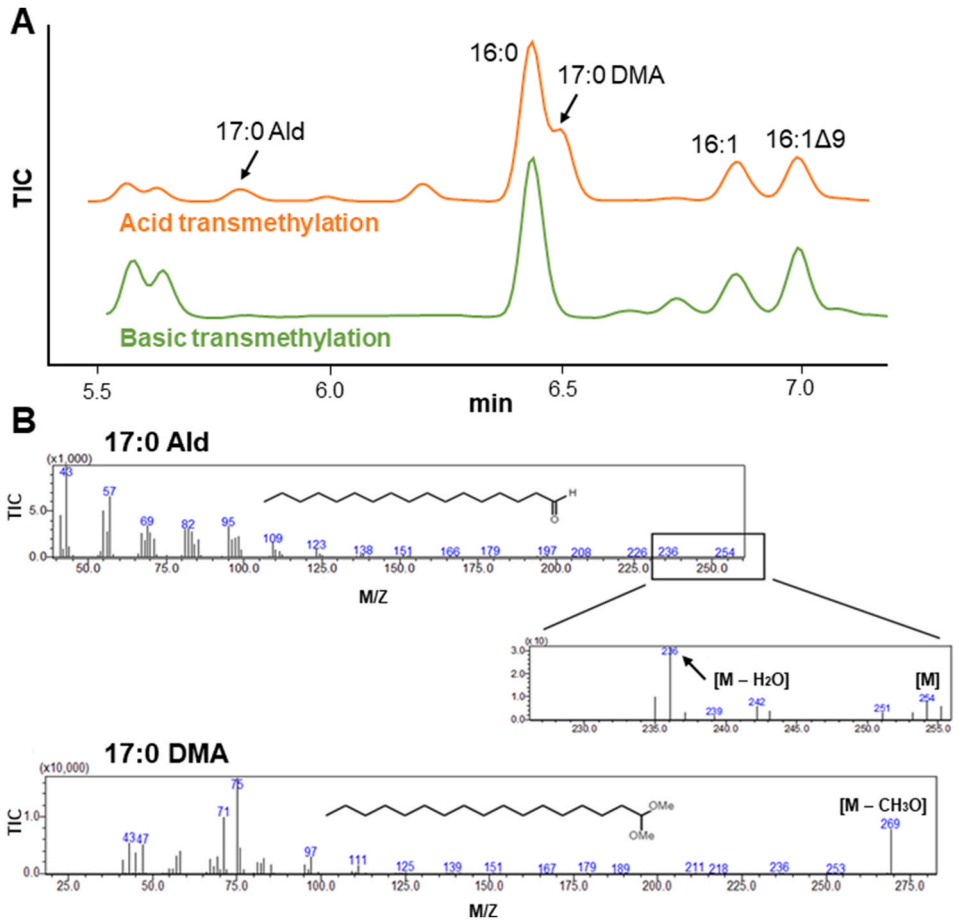
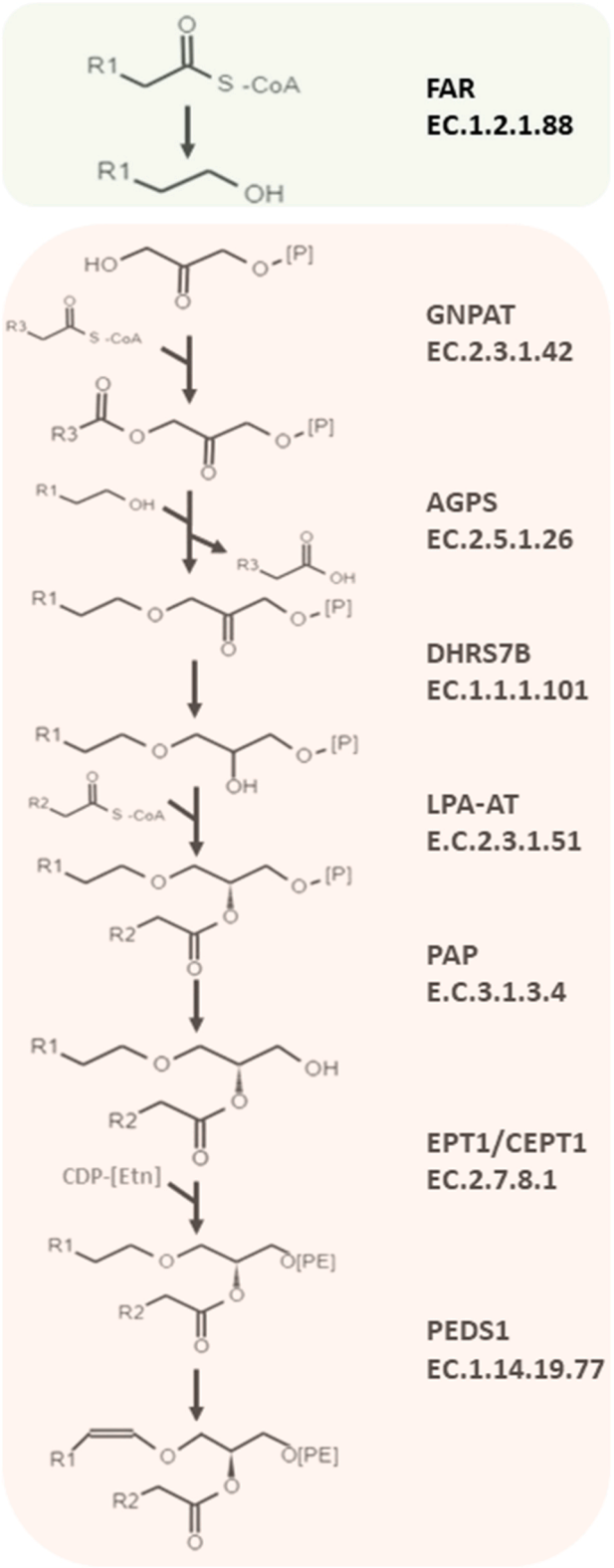


Fig. 2. (A) Partial gas chromatogram obtained after acid or basic transmethylation of filopodiated cells lipid extracts. (B) Mass spectra of 17:0 Ald and 17:0 DMA, detected only under acid transmethylation.

Table 1
High-resolution mass spectrometry (HRMS) of lipid extracts from *Capsaspora owczarzaki* cysts. Fragmentation information was obtained by triple quadrupole mass spectrometry, from parent ions as lithium adducts.

Compound	Formula	[M+H]+ Theoretical	[M+H]+ HRMS	Δ ppm	[M+Li]+ Theoretical	Parent-ion scanning			Neutral loss scanning					
						PC P-16:0	PC P-17:0	PC P-18:0	[M + Li-59-FA]+			183	189	213
									FA16:1	FA18:1	FA20:3			
PC P-17:0/16:1	C41H80NO7P	730.5751	730.5728	3.15	736.58	ND	736.7	ND	736.6	ND	ND	736.1	736	ND
PC P-17:0/18:1	C43H84NO7P	758.6064	758.603	4.48	764.61	ND	763.8	ND	764.4	764	ND	764	763.9	ND
PC P-17:0/20:3	C45H84NO7P	782.6058	782.6064	0.77	788.61	ND	787.1	ND	788	ND	788.4	787.9	788	ND
PC P-17:0/18:2	C43H82NO7P	756.5907	756.5891	2.11	762.6	ND	761.4	ND	762	ND	ND	762	762	ND
PC 16:1/20:3	C44H80NO8P	782.5700	782.5678	2.81	788.57	ND	ND	ND	788	ND	788.4	787.9	788	ND
PC 20:3/20:3	C48H84NO8P	834.6013	834.599	2.76	840.61	ND	ND	ND	ND	ND	840.1	840	840	ND

NA: Not detected.



	H. sapiens	*Capsaspora*
FAR **EC.1.2.1.88**	NP_115604	CAOG_002487 CAOG_001663
GNPAT **EC.2.3.1.42**	NP_055051	CAOG_001663
AGPS **EC.2.5.1.26**	NP_003650	CAOG_009325
DHRS7B **EC.1.1.1.101**	NP_056325	CAOG_004335
LPA-AT **E.C.2.3.1.51**	NP_060309[a] NP_005759	CAOG_004258 CAOG_004992
PAP **E.C.3.1.3.4**	O14494[a]	CAOG_009636
EPT1/CEPT1 **EC.2.7.8.1**	NP_277040/ NP_001007795	CAOG_008392 CAOG_001385
PEDS1 **EC.1.14.19.77**	NP_954580	CAOG_004505

(caption on next page)

Fig. 3. Aerobic pathway for de novo plasmenyl ethanolamine biosynthesis. The IUBMB code number of each enzyme is shown. The protein code of the human enzymes (third column) and the *Capsaspora owczarzaki* code for the corresponding orthologs (fourth column) are also indicated. FAR: fatty acyl-CoA reductase; GNPAT: glycerone phosphate acyltransferase; AGPS: alkyl-glycerone phosphate synthase; DHRS7B: acyl/alkyl-glycerone phosphate reductase; EPT1: ethanolaminephosphotransferase; CEPT1: choline/ethanolaminephosphotransferase. [a]: enzymes not conclusively assigned.

C. owczarzaki showed a high percentage of PUFA of no more than 20 carbons (C18- and C20-PUFA). The relative proportion of C20-PUFA increased significantly in the cystic stage compared to the filopodial and aggregative stages, with arachidonic acid (20:4Δ5,8,11,14) being the main PUFA (Fig. 1B). We found a small but significant amount of saturated and monounsaturated odd fatty acids, 15 and 17 carbons long. Interestingly, a high amount of C17-fatty aldehyde and its dimethyl acetal were also detected (Fig. 1A); they were absent when subjecting the lipid extracts to a basic transmethylation (See Fig. 2A and B). It is indicative of the presence of plasmenyl ether lipids, consistent with the known susceptibility of the vinyl bond only to acid hydrolysis [43]. Acid transmethylation conditions produce the release of aldehydes that are subsequently methylated. In contrast, the alkyl ether linkage of plasmanyl lipids is resistant to hydrolysis [44]. The proportion of plasmalogen-derived aldehydes and acetals increased significantly from one stage to another throughout the life cycle, in the order filopodial < aggregative < cystic stages (Fig. 1C).

To further elucidate the non-hydrolyzed molecules, we employed direct infusion HRMS on an Orbitrap instrument, of cystic cells lipid extract. Ions corresponding to PC O-33:2, PC O-37:4, PC O-35:2, and PC O-35:3 $[M+H]^+$ among other PC molecules were identified. We hypothesized that these molecules could be plasmenyl-PCs containing a C17-vinyl alcohol moiety (Table 1). To confirm the structure of these molecules, we obtained fragmentation information using QqQ mass spectrometry in the presence of lithium hydroxide. Previous studies have shown that plasmenyl-PC can be fragmented as $[M+Li]^+$, providing direct evidence of vinyl alcohol [45]. NLS confirms that they are choline-containing glycerophospholipids, due to characteristic losses at *m/z* 183 and 189. Lack of loss at *m/z* 213 rules out sphingomyelins [46]. In PIS, four of them showed a fragment ion of *m/z* 293, corresponding to $[M + Li–189–FA]^+$, indicating the presence of a C17-vinyl alcohol. No fragment ions of *m/z* 279 and 307, corresponding to C16- and C18-vinyl alcohols, were detected [46]. Neutral losses of *m/z* 313, 341 and 365, indicate loss of trimethylamine plus FA 16:1, FA 18:1, and FA 20:3, respectively (Table 1). It confirms the presence of PC P-17:0/16:1, PC P-17:0/20:3 (see product-ion scanning in Figs. S1 and S2) and PC P-17:0/18:1 in *C. owczarzaki* cystic cells. We found no evidence of plasmenyl-PE in the Orbitrap mass spectral analysis. Although not conclusive, evidence consistent with PE P-17:0/20:3 was found using QqQ spectrometry. The $[M+H]^+$ ion with *m/z* 739.5 was detected in PIS of the fragment with *m/z* 378, compatible with the presence of a C17-vinyl alcohol [47]. Additionally, a $[M - H]^-$ ion was detected, with *m/z* 738.5 in PIS for the fragment of *m/z* 305 (FA 20:3).

3.2. *Capsaspora owczarzaki has a complete set of enzymes for the synthesis of plasmenylethanolamine*

The *C. owczarzaki* genome sequence was mined for genes encoding enzymes of the plasmalogen pathway. We made a BLAST analysis with a set of amino acid sequences of *Homo sapiens* plasmalogen synthesis proteins as queries.

The synthesis of plasmalogens in eukaryotes begins in peroxisomes, by the esterification of the free hydroxyl group of dihydroxyacetone phosphate or glycerone phosphate (GNP) by GNP acyltransferase (GNPAT), using long chain fatty acyl-CoA as substrates (Fig. 3). The resulting fatty acyl moiety in *sn*-1 is later replaced by a long chain fatty alcohol via alkyl-GNP synthase (AGPS). Fatty alcohols are synthesized from fatty acyl-CoA by two reductase isoenzymes in humans, known as FAR1 and FAR2 [9,25]. We identified an ortholog (XP_004349237, *Capsaspora* genome code CAOG_002487) that shares 53 % identity with FAR1. Interestingly, its genome also encodes a 1397 amino acids protein (CAOG_001663, XP_004364531) with a clear PTS-1 (AKL) signal for peroxisome targeting. It appears to be a multidomain enzyme, with its C-terminal half sharing 33 % identity with GNPAT, while the N-terminal half is 26 % identical to FAR1 and 24 % to FAR2. An AGPS ortholog (CAOG_009325, KJE89098) sharing 54 % identity was also retrieved.

The next reaction is carried out by an acyl/alkyl-GNP reductase formerly known as DHRS7B. Reduction of the ketone in *sn*-2 produces 1-alkyl-2-lyso-*sn*-glycero-3-phosphate, the ether linked analog of acyl-lysophosphatidates (LPA). In mammals, the enzyme seems to be involved in the synthesis of ether lipids and diacyl lipids, since it can use alkyl- and acyl-GNP as substrates; the enzyme localizes to both the peroxisome and the endoplasmic reticulum (ER). A clear ortholog of this reductase (CAOG_004335, XP_004348163, 43 % identity) was detected.

The following three reactions are involved in the synthesis of both ether lipids and diacylphospholipids and are found in the ER [10,25]. They are: LPA acyltransferase (LPA-AT) which introduces a fatty acyl residue in *sn*-2; phosphatidate phosphohydrolase (PAP) which removes the phosphate at *sn*-3; and CDP-ethanolamine diacylglycerol ethanolamine phosphotransferase (EPT) which catalyzes the binding of phosphoethanolamine at *sn*-3. There are fourteen LPA-AT isoenzymes in mammals, also known as lysophospholipid acyltransferases (LPLAT) [48]. They have different tissue distribution and substrate specificities, using both alkyl- and acyl-lysophospholipids. Based on their primary structures, LPLATs can be divided into two families, 1-acylglycerol-3-phosphate O-acyltransferases or AGPAT (LPLAT1-10) and membrane bound O-acyltransferases or MBOAT (LPLAT11-14) [48]. *C. owczarzaki* has orthologs for the following AGPAT: LPLAT1 (XP_004349241 or CAOG_002491), LPLAT3 (XP_004364540/CAOG_001672), LPLAT5 (XP_004363330/CAOG_003602), LPLAT6 (XP_004347312/CAOG_004565 and XP_004346081/CAOG_005408), LPLAT8 (XP_004363771/CAOG_002932) and LPLAT9b (XP_004348083/CAOG_004258), sharing 30–45 % identity. We also found orthologs for MBOATs: LPLAT14 (XP_004363955, CAOG_003116), LPLAT13 (XP_004363935, CAOG_003096) and LPLAT12 (XP_004346677, CAOG_004992), which share 33–38 % identity (E values < e^{-75}).

PAP activity was earlier associated to lipins (fat-regulating proteins) in mammals. The phosphohydrolase activity of lipins involved

in de novo lipid biosynthesis resides in ER and is highly specific for PA, while LPA is not a substrate [10]. There are three lipin isoforms in humans, but we were unable to detect orthologs in *C. owczarzaki*. In contrast, lipid phosphate phosphatases (LPPs) are ER integral membrane proteins, having phosphohydrolase activities toward LPA and PA. The putative *C. owczarzaki* orthologs are KJE92150 (CAOG_009636) and XP_004364008 (CAOG_003169), sharing 28–31 % identity with human LPP1, although with low scores (E values $> e^{-20}$).

SELENOI, the EPT1 gene, was identified in humans due to its involvement in a rare inherited disease [49]. EPT1 is highly specific for CDP-ethanolamine and is a relevant enzyme in the synthesis of both alkylacyl-PE and diacyl-PE. Contrary to what was previously

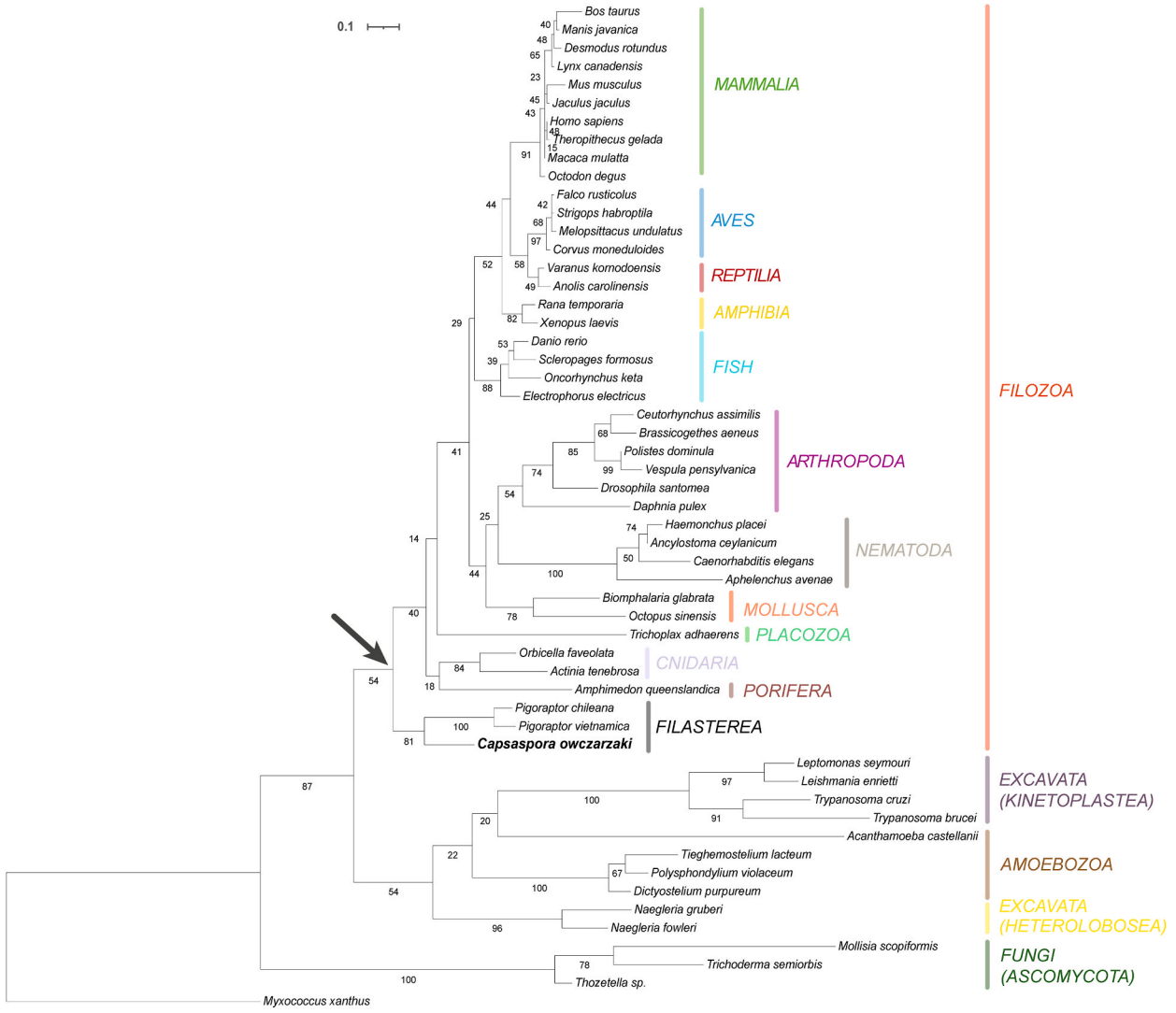


Fig. 4. Phylogenetic analysis of PEDS1. Arrow indicates the proposed acquisition event of PEDS1 in Filozoa. Maximum likelihood phylogenetic reconstruction was performed with amino acid sequences from: *A. castellanii* (XP_004357191), *A. tenebrosa* (XP_031565811), *A. queenslandica* (XP_019858443), *A. ceylanicum* (EYC45081), *A. carolinensis* (XP_008108340), *A. avenae* (KAH7728392), *B. taurus* (NP_001093785), *B. glabrata* (XP_013082447), *B. aeneus* (CAH0557382), *C. elegans* (NP_493036), *C. owczarzaki* (XP_004348333), *C. assimilis* (CAH1130273), *C. moneduloises* (XP_031982549), *D. rerio* (XP_005159144), *D. pulex* (XP_046441868), *D. rotundus* (XP_024415067), *D. purpureum* (XP_003283098), *D. santomea* (XP_039483291), *E. electricus* (XP_026885415), *F. rusticolus* (XP_037259213), *H. placei* (VDO38720), *H. sapiens* (NP_954580), *J. jaculus* (XP_004663924), *L. canadensis* (XP_030165270), *L. enrietii* (KAG5480133), *L. seymouri* (KPI89550), *M. mulatta* (NP_001180988), *M. javanica* (XP_017507389), *M. undulates* (XP_033923137), *M. scopiformis* (XP_018071845), *M. musculus* (NP_663513), *M. xanthus* (WP_140865935), *N. fowleri* (XP_044565043), *N. gruberi* (XP_002676085), *O. degus* (XP_004636098), *O. sinensis* (XP_029652872), *O. faveolated* (XP_020624199.1), *O. keta* (CDQ82662), *P. dominula* (XP_015180136), *P. chileana* (Pchi_g12073), *P. vietnamica* (Pvie_g9176), *P. violaceum* (KAF2069211), *R. temporaria* (XP_040187100.1), *S. formosus* (XP_018582118), *S. habroptila* (XP_030358758), *T. gelada* (XP_025254407), *Thozetella* sp. (KAH8900397), *T. semiorbis* (KAH0527355), *T. adhaerens* (XP_002112059), *T. lacteum* (KYQ89613), *T. brucei* (XP_822479), *T. cruzi* (PWU95043), *V. komodoensis* (XP_044296322), *V. pensylvanica* (XP_043667822), *X. laevis* (AAI29523). Numbers represent bootstrap values. The bar represents the percentage of substitutions.

known, it was recently localized to the Golgi apparatus of HEK293 cells [49,50]. EPT1 has a putative ortholog in *C. owczarzaki* (CAOG_008392, XP_011269962) that shares 39 % identity. A choline/ethanolamine phosphotransferase (CETP1) that is located in the ER is mainly involved in the synthesis of PC and PE [51] and, to a much lesser extent, plasmanyl-PE [50]. XP_004349905 (CAOG_001385) shares 41 % identity with human CEPT1 (Fig. 3).

The final step is carried out by PEDS1, structurally related to fatty acid desaturases, and involves the insertion of a double bond between carbons 1 and 2 of the alkyl chain of plasmanyl-PE, forming the vinyl ether bond. *CarF* and *TMEM189* were identified as the PEDS1 genes in *M. xanthus* [26] and humans [27,28], respectively. XP_004348333 (CAOG_004505) shares 59 % identity with TMEM189 and 47 % with CarF.

3.3. Distribution and phylogenetic analysis of PEDS1

The recent identification of the genes encoding PEDS1 could be of valuable utility to explore the distribution of the aerobic biosynthesis pathway in eukaryotes. We performed a BLAST search on the genome sequences of a large number of eukaryotes using the sequences of the two characterized PEDS1 (CarF and TMEM189) as queries. As expected, we found orthologues in each group containing aerobic organisms known to synthesize plasmalogens. In addition, we found PEDS1 orthologues also in *Acanthamoeba castellanii*, *Naegleria* sp., slime molds, *Pigoraptor* sp., *C. owczarzaki* (as described above) and fungi, only in three genera of ascomycetes (E values $< e^{-40}$), which were not previously reported as having plasmalogen synthesis. It indicates that the aerobic pathway is present in a limited number of organisms belonging to clades too diverse as Amoebozoa (i.e., *Acanthamoeba castellanii* and slime molds), Excavata (Kinetoplastea and *Naegleria* sp.), Fungi (*Trichoderma*, *Thozetella* and *Mollisia*), Filasterea (*Pigoraptor* sp. and *C. owczarzaki*) and Metazoa. Ctenophora is the only metazoan group lacking PEDS1. Within Opisthokonta, we were unable to detect orthologues in any available genome of organisms belonging to Choanoflagellatea, Fonticulida, Nucleariida, Teretosporea (Ichthyosporea and *Corallotrichium*), the filasterean *Ministeria vibrans* and most of fungi, with the exception of the ascomycetes mentioned above.

The search for PEDS1 in plants only retrieved FAD4 like enzymes, although with low score (E values $> e^{-23}$). FAD4 is a novel class of fatty acid desaturase that localizes in chloroplasts and is involved in the formation of a *trans* double bond between carbon 3 and 4 of palmitic acid, which is specifically esterified to the *sn*-2 position of phosphatidylglycerol [52]. It is in agreement to the lack of plasmalogens in plants as previously stated [21]. FAD4-like desaturases were also retrieved from Algae and plastid-containing protists from the SAR supergroup (Stramenopila, Alveolata and Rhizaria).

A maximum likelihood phylogenetic reconstruction was performed with PEDS1 amino acid sequences from representative species of each taxon. The myxobacteria PEDS1 (CarF) was used as outgroup root in the analysis. As shown in the phylogenetic tree (Fig. 4), the metazoan and the filasterean PEDS1 are closely related, suggesting a common ancestor, probably the result of a single event of horizontal gene transfer from an unidentified donor to a primitive filozoan protist. Filozoa, Excavata, Amoebozoa and Fungi PEDS1 are distantly related, suggesting that several independent gene acquisition events occurred during the evolution of eukaryotes.

A multiple alignment of PEDS1 revealed a remarkable conservation in the primary structure of the filozoan enzymes, including the eight histidines proposed to be involved in coordinating the di-iron center of their active sites [28]. A single significant difference is a two amino acids insertion (Met200 and Ala201, in *Capsaspora* numbering) that was unique to filasterean PEDS1 (Fig. 5). The eight histidines appear to be conserved also in the remaining sequences, but lower similarities and several insertions and deletions are evident between amoebozoan, excavates and fungi PEDS1 with respect to the filozoan enzymes.

3.4. Fungi have orthologs of the enzymes for plasmanyl ether lipid synthesis

Next, each Opisthokonta clade was mined with the enzymes involved in plasmanyl ether lipids synthesis. We used the amino acid sequences of FAR, GNPAT, AGPS, DHRS7B, EPT1 and CEPT1 from both *Homo sapiens* and *C. owczarzaki* as queries. Due to the uncertainty in defining candidates for LPA-AT and PAP and that they are involved in the synthesis of both alkyl-acyl- and diacylphospholipids, we excluded them from the analysis. Fig. 6 shows the presence or absence of orthologs of these enzymes plus PEDS1 in each clade. In addition to Filasterea and Metazoa, also Fungi, in most of the genera of each group (Chytridiomycota,

Fig. 5. Alignment of filozoan PEDS1. In red, the essential histidines of the active site. In green, the two-aminoacids insertion of filasterean organisms. Amino acid sequences were aligned using the MAFFT algorithm and visualized by Jalview. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Zoopagomycota, Basidiomycota, Mucoromycota and Ascomycota), seems to have the ability to synthesize plasmanyl lipids. Ctenophora, Nucleariida and Corallotrichium showed the absence of orthologs for any of these enzymes. The BLAST search showed no hits, or retrieved proteins with low score (E values $> e^{-20}$, identity lower than 30 %) in Blastocladiomycota (for FAR, AGPS and DHRS7B), Microsporidia (FAR, GNPAT, AGPS and DHRS7B), *Fonticula* (GNPAT, AGPS and DHRS7B), Ichthyosporea (for FAR, AGPS and DHRS7B) and Choanoflagellatea (for FAR, GNPAT, AGPS, DHRS7B and EPT1), suggesting they are unable to synthesize ether lipids.

4. Discussion

The amoeboid protist *C. owczarzaki* has recently become a model organism for the study of metazoan evolution [30]. This is due to their close phylogenetic relationship to animals, the existence of an aggregative stage in their life cycle, and the presence of multiple metazoan-related genes involved in cell differentiation, adhesion and signaling [31,53]. These characteristics supported the idea that the common single-celled ancestor of metazoans already had a complex repertoire of elements (tools) that were recycled (co-opted) during evolution [30]. One of these tools could be the synthesis of plasmalogens which, as we showed in this report, is present in *C. owczarzaki* and most likely also in Pigoraptor sp. Plasmalogens have been proposed to be involved in multicellularity, cell-cell interactions, signaling and phagocytosis [1,13]. So, these lipids could be behind the ability of *C. owczarzaki* to switch between different forms of its life cycle, including establishing a rudimentary multicellular (aggregative) stage. They could also be responsible for the phagocytic capacity of the filopodial cells. It is interesting to note that the other plasmalogen containing organisms, such as Acanthamoeba, Naegleria and slime molds, are also phagotrophic. Notably, we found the highest plasmenyl lipid content in the cystic stage of Capsaspora (Fig. 1C) suggesting some functions of these lipids in the resistance of this cell type to adverse environmental conditions. Alternatively, it could also indicate the role of cysts as a reservoir of essential lipids that should be used immediately after differentiation into filopodial cells.

Interestingly, we were able to detect plasmenyl ether lipids containing only a C17-vinyl alcohol in *C. owczarzaki*, using different chromatographic and mass spectrometry methods. Even so, the presence of plasmalogens containing C16- and C18- vinyl alcohols cannot be ruled out. We are underway for a more detailed lipidomic analysis, using LC-MS techniques that could also detect other plasmenylphospholipid species. C17-vinyl alcohol is an unusual substituent in mammalian plasmalogens and its presence in Capsaspora must be a consequence of the combined substrate preferences of FAR and AGPS, warranting biochemical characterization of these enzymes.

The ether lipid biosynthesis capacity is not exclusive to filozoan organisms between opisthokonts, as fungi appear to have the enzymes to synthesize plasmanyl lipids (Fig. 6). Actually, in a preliminary search, the genes encoding these enzymes appear to be present in most of the eukaryote phyla (data not shown), indicating that the ether lipids are, most probably, not exceptional but a common component of their membranes. However, plasmalogen biosynthesis is much less common. In Opisthokonta, is unique to Filozoa, as evidenced by the distribution of PEDS1 within the clade, with the exception of the small group of ascomycetes comprising Trichoderma, Thozetella, and Mollisia. Phylogenetic analysis suggests that PEDS1 was acquired by a Filozoa ancestor, retained in

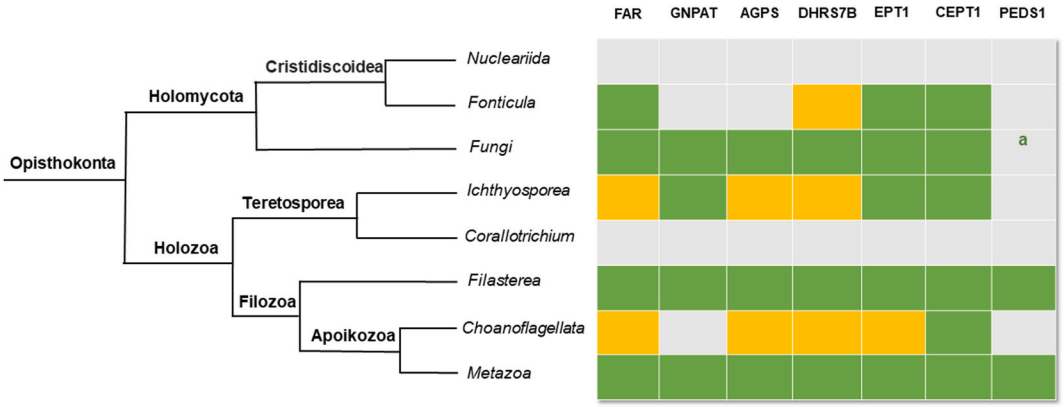


Fig. 6. Putative orthologs of the plasmalogen aerobic pathway in Opisthokonta. Green boxes: highly probable orthologs (BLAST E values $< e^{-39}$); yellow boxes: low probability (E values $> e^{-23}$); grey boxes: no BLAST hits. [a]: no hits, with the exception of Trichoderma, Thozetella, and Mollisia genera. Representative sequences with high score: FAR: *Fonticula alba* XP_009494582, *Synchytrium microbalum*, XP_031026750, *Entomophthora muscae* KAJ9086244, *Linnemannia elongata* KAG0079398, *Capsaspora owczarzaki* XP_004349237, *Homo sapiens* NP_115604; GNPAT: *Trichoderma harzianum* XP_024776632, *Linnemannia schmuckeri* KAF9149574, *Rhodotorula diobovata* TNY20793, *Blastocladiella emersonii* KAI9175858, *Sphaeroforma arctica* XP_014154288, *C. owczarzaki* XP_004364531, *H. sapiens* NP_055051; AGPS: T. harzianum KKP00238, *C. owczarzaki* KJE89098, *H. sapiens* NP_003650; DHRS7B: L. elongata KAF9292753, *C. owczarzaki* XP_004348163, *H. sapiens* NP_056325; EPT1: F. alba XP_9494251, *T. harzianum* XP_024774613, *Linnemannia zychae* KAF9922440, *Blastocladiella britannica* KAI9222539, *S. arctica* XP_014158253, *C. owczarzaki* XP_011269962, *H. sapiens* NP_277040; CEPT1: *F. alba* XP_009494186, *Aspergillus parasiticus* KAB8205464, *Irpex lacteus* KAI0803223, *S. arctica* XP_014158253, *C. owczarzaki* XP_004349905, *Salpingoeca rosetta* XP_004989264, *H. sapiens* NP_001007795; PEDS1: *T. harzianum* PNP52479, *Thozetella* sp. KAH8900397, *Mollisia scopiformis* XP_018071845, *C. owczarzaki* XP_004348333, *H. sapiens* NP_954580. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Filasterea and Apoikozoa (Metazoa plus Choanoflagellatea) but lost during choanoflagellate's evolution. Notably, phylogenetic reconstructions performed for GNPAT and AGPS, the first two enzymes of the pathway, generate trees (Figs. S3 and S4) that have the same topology as that of PEDS1 (Fig. 4). The distant relationship with PEDS1 of ascomycetes suggests an independent acquisition event in the ancestor of this fungal group, which presumably, already had the ability to synthesize plasmanyl lipids. A limited number of similar events occurred during the evolution of other no related clades, allowing the synthesis of plasmalogens in extant slime molds, Acanthamoeba, Naegleria, and trypanosomatids. Whether these events involved the en bloc acquisition of the entire aerobic pathway or were the result of several steps resulting in transitional (ancestral) organisms capable of synthesizing plasmanyl lipids, will require further analysis. This last option seems the most likely for the evolutionary history of Opisthokonta, since the first one would imply the loss of PEDS1 in most of the branches.

CRediT authorship contribution statement

Joaquín Costa: Visualization, Validation, Methodology, Investigation, Formal analysis. **Matías Gabrielli:** Validation, Methodology, Investigation, Formal analysis. **Silvia G. Altabe:** Validation, Methodology, Investigation. **Antonio D. Uttaro:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

A.D.U. and S.G.A. are members of the Carrera del Investigador Científico, CONICET, Argentina. J.C. is a doctoral fellow from CONICET. M.G. was a postdoctoral fellow from CONICET. We would like to thank Dr. Matías Cabruja for his advice on mass spectrometry. This work was supported by CONICET, through Grant PIP 2021 N° 100805CO.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32807.

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