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The complete mitochondrial genome from an unidentified *Phalansterium* species.

Jean-François Pombert[1], Alexey Smirnov[3], Erick R. James[2], Jan Janouškovec[2], Michael W. Gray[4], Patrick J. Keeling[2]*

[1]Department of Biological and Chemical Sciences, Illinois Institute of Technology, 3105 South Dearborn, Chicago, Illinois, 60616, USA

[2]Department of Botany, University of British Columbia, 3529-6270 University Boulevard, Vancouver, BC, V6T 1Z4, Canada

[3]Department of Invertebrate Zoology, Faculty of Biology & Soil Sciences, St.Petersburg State University, Universitetskaja nab. 7/9, 199034, St.Petersburg, Russia

[4]Department of Biochemistry and Molecular Biology, Dalhousie University, 5850 College Street, Room 8-F2, PO Box 15000, Halifax, NS, B3H 4R2, Canada

Abstract

We describe the complete sequence and organization of the mitochondrial genome from an unidentified species of *Phalansterium*. This is the first sequenced mitochondrial genome of a member of Variosea clade (Amoebozoa, Conosa). The sequence was assembled from shotgun reads of DNA from a mixed culture containing the euglenid *Monomorphina aenigmatica* and an amoebozoan that we demonstrate here is closely related to *Phalansterium* (in nuclear SSU rRNA phylogenies, it branches between two sequences from described species of *Phalansterium*). Sequence assembly resulted in two distinct mitochondrial genome types, one fragmented and euglenid-like, and the second a single circular-mapping contig of 53,614 bp with an amoebozoan-like set of genes. The *Phalansterium* sp. mitochondrial genome is gene-rich and densely packed, with a large number of tRNAs and an unusually low ratio of identifiable protein-coding genes to unidentified ORFs. These ORFs potentially encode ribosomal proteins exhibiting a divergent character at the sequence level, and whose identification may be hindered by the presence of RNA editing in *Phalansterium* mitochondria, as inferred from numerous acceptor stem mis-matches typical of amoebozoan tRNA 5' editing.

Keywords

Phalansterium • Amoebozoa • Variosea • Mitochondrion • Genome

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Introduction

The genus *Phalansterium* was established by Cienkowsky [1] for two species of colonial uniciliate flagellates: *Monas consociatum* Fresenius 1858, renamed *Phalansterium consociatum*, and newly described species *P. intestinum*, transferred eight years later to the genus *Spongomonas* because it was shown to have two closely apposed flagella instead of one [2]. Cienkowsky observed that, together, *Phalansterium* species fashion their living place together almost like a society in 'heaps of jelly', hence the name *Phalansterium* referring to "le Phalanstère", a palace-like building capable of housing four hundred families imagined in the early 19th century by French utopian socialist Charles Fourier as part of his 'Théorie Sociale' [3]. The genus *Phalansterium* currently unifies four species - *Phalansterium digitatum* [2], *P. solitarium* [4], *P. filosum* (Cavalier-Smith and Chao, in [5]) and the type species *P. consociatum* [1]. Of those, the first three species are relatively well studied (Cavalier-Smith and Chao in [5], [6,7]) whereas *P. consociatum* on the other hand was never reliably re-isolated and Hibberd [6] suggested that it may be co-specific with *P. digitatum*.

Phalansterium cells are uni-flagellate, with the flagellum partiallfy surrounded by a collar-like structure and associated with a single basal body that is situated at the apex of a radiating cone of microtubules [6,7]. This relatively simple structure led to the proposal that *Phalansterium* might represent a primitive, early-branching lineage [8]. However, molecular studies show that *Phalansterium* branches within Amoebozoa, in the clade named Variosea within the subphylum Protamoebae [9]. The phylogenetic position of Variosea was not clear for some time but members of this clade tend to group with archamoebae and mycetozoans rather than with typical lobose amoebae [10–13]. Cavalier-Smith [14] suggests transfer of the class Variosea to the subphylum Conosa Cavalier-Smith 1998, unifying them with two major amoebozoan groups, mycetozoans (dictyostelids, physarids and some of protostelids) and archamoebae (pelobionts and *Entamoeba*). Close relationships of Variosea with other Conosa were confirmed in further studies [15–20] and also are recognised in the systems of amoebae by Smirnov and Cavalier-Smith [5].

The mitochondria of Variosea are of some interest because members of one of the major conosean groups, Mycetozoa, have relatively normal mitochondria, while members of the other group, Archamoebae, are anaerobes possessing highly reduced mitosomes [21]. However few mitochondrial genomes of Amoebozoas are sequenced and most are from mycetozoans while other groups are virtually not covered [22–25]. Here we describe the complete mitochondrial genome from an unidentified

* *E-mail: pkeeling@mail.ubc.ca*

species of *Phalansterium* (the first sequenced mtDNA from a variosean representative) found as a contaminating species in a culture of a photosynthetic euglenid.

Materials and methods

Genome sequencing.

UTEX 1284 culture containing *Monomorphina aenigmatica* (and subsequently also found to contain the *Phalansterium* species) was obtained directly from the University of Texas Culture Collection, cultured, and purified DNA prepared for sequencing as described [26]. 320,000,000 filtered reads corresponding to 16,000,000 pairs of raw sequence were assembled in parallel with Ray [27] version 2.0.0 rc4, as described [26]. From the 1,849,947 contigs generated (546,420,088 bp total), 130,292 contigs larger than 500 bp (241,607,848 bp total) were searched for sequences similar to genes expected to be encoded in a mitochondrial genome. Two general types of contig resulted: small linear mapping contigs with genes sharing high similarity to *Euglena gracilis* mitochondrial genes, and a single, 53,614 bp circular mapping contig (71X coverage) with sequences sharing closest similarity to amoebozoan mitochondria.

Genome annotation.

Open reading frames were located on the circular-mapping *Phalansterium* sp. mitochondrial genome sequence with Artemis 14 [28] and known genes identified by BLAST homology searches [29] against the NCBI non-redundant databases. ORFs showing low or no similarity with known sequences were further queried against the NCBI databases using iterative PSI-BLAST searches [30]. Genes coding for tRNAs were positioned with tRNAcan-SE [31] whereas genes coding for ribosomal rRNAs were located by BLAST homology searches. Artemis annotations were converted to TBL format using the built-in tools, the TBL file further manually curated, and the accession number generated with NCBI's TBL2ASN. The physical map was generated from the accession number using OGDRAW 1.2 [32]. The *Phalansterium* sp. mitochondrial genome has been deposited in GenBank under accession number KC121006.

Phylogenetic analysis.

The number of species present in what was thought to be a uni-eukaryotic culture was inferred by examining the phylogeny of all sampled nuclear small subunit ribosomal RNA (SSU rRNA) genes. From 546,420,088 bp of sequence, three types were found: a 10.8 kb contig (99,073 reads; 910X coverage) corresponding to the euglenid *Monomorphina aenigmatica*, a 5.8 kb contig (3,021 reads, 52X coverage) closely related to *Drosophila melanogaster*, and a 8.5 kb contig (1,573 reads; 19X coverage) with the closest match to *Phalansterium* (deposited under GenBank accession number KF539978).

Phalansterium-like sequences were aligned to an extensive alignment covering all major groups of eukaryotes using SeaView 4 [33]; the alignment was manually polished and Maximum Likelihood phylogenies were inferred with PhyML 3.0 [34] using a nucleotide mask (863 positions). For greater resolution, 89 species (1049 nucleotide positions) representing all branches of Amoebozoa and a limited number of outgroups were selected and the phylogeny reconstructed with PhyML under the GTR model [35] of nucleotide substitution with 8 gamma categories of among-site rate heterogeneity and optimized proportions of invariable sites; a total of 100 bootstrap replicates were performed and mapped on the best likelihood tree. Bayesian analysis was performed using MrBayes 3.2 [36], GTR model with gamma correction for intersite rate variation (8 categories) and the covarion model. The trees were run as two separate chains (default heating parameters) for 4.8 million generations, by which time they had ceased converging (final average standard deviation of the split less than 0.01); the first 25% of the generations were discarded for burn-in.

To confirm that the amoebozoan mitochondrial genome is derived from the same source, we also reconstructed the phylogeny of mitochondrion-encoded cytochrome oxidase b (Cob) from a wide variety of eukaryotes (339 taxa total). Other genes and a concatenation of mitochondrial genes were also examined, but were found to have limited resolution or available species diversity (especially of amoebozoans and euglenids, both of which were important to have represented). Cob protein sequences were retrieved from NCBI's GenBank database, aligned with the L-INS-i algorithm implemented in MAFFT 6.956 [37], and then filtered with trimAl 1.4 [38] with the following parameters: -gt 0.6, -st 0.001, -cons 60. Maximum Likelihood phylogenetic reconstructions were performed on the filtered dataset with PhyML 3.0 under the LG+Γ4+F model of amino acid substitution.

Results and discussion

A complete mitochondrial genome from an amoebozoan related to *Phalansterium*.

We recently reported the complete plastid genome from shotgun sequencing of *Monomorphina aenigmatica* strain UTEX 1284 [26]. This culture is not axenic, so we used the shotgun sequence data to assess its species diversity by characterizing all nuclear SSU rRNA-encoding contigs. From 320,000,000 filtered reads assembled into 130,292 contigs over 500 bp in length (corresponding to 241,607,848 bp of sequence), only three distinct nuclear SSU rRNA types could be found: that of *M. aenigmatica*, a 5.8 kb fragment related to *Drosophila melanogaster*, and a third type corresponding to a novel sequence closely related to *Phalansterium solitarium*. Based on this deep sequencing survey, we conclude the culture was dominated by *M. aenigmatica* and that the other two organisms were low-level contaminants, with the novel strain of *Phalansterium* present at about 2% of the dominant species (the few *Drosophila* sequences are interpreted as a low level sequencing contaminant as is common with such volumes of sequence).

Phylogenetic analyses on a representative alignment containing all major groups of eukaryotes (tree not shown)

indicated that the newly identified *Phalansterium* SSU sequence robustly branches within the Amoebozoa, where it always clusters (with 100% support by all methods used) with two named *Phalansterium* species. This position is genuine, and is confirmed by analyses performed with an expanded number of amoebozoan sequences (Figure 1). In the latter tree, *Phalansterium* species appear as a group inside the Variosea clade, with Filamoeba species and a number of environmental sequences as nearest neighbors. This position is not supported statistically, however, and the *Phalansterium* clade sometimes appears basal to the entire Variosea. Overall in our analysis, Variosea, together with dictyostelids, mixogastrians and archamoebians

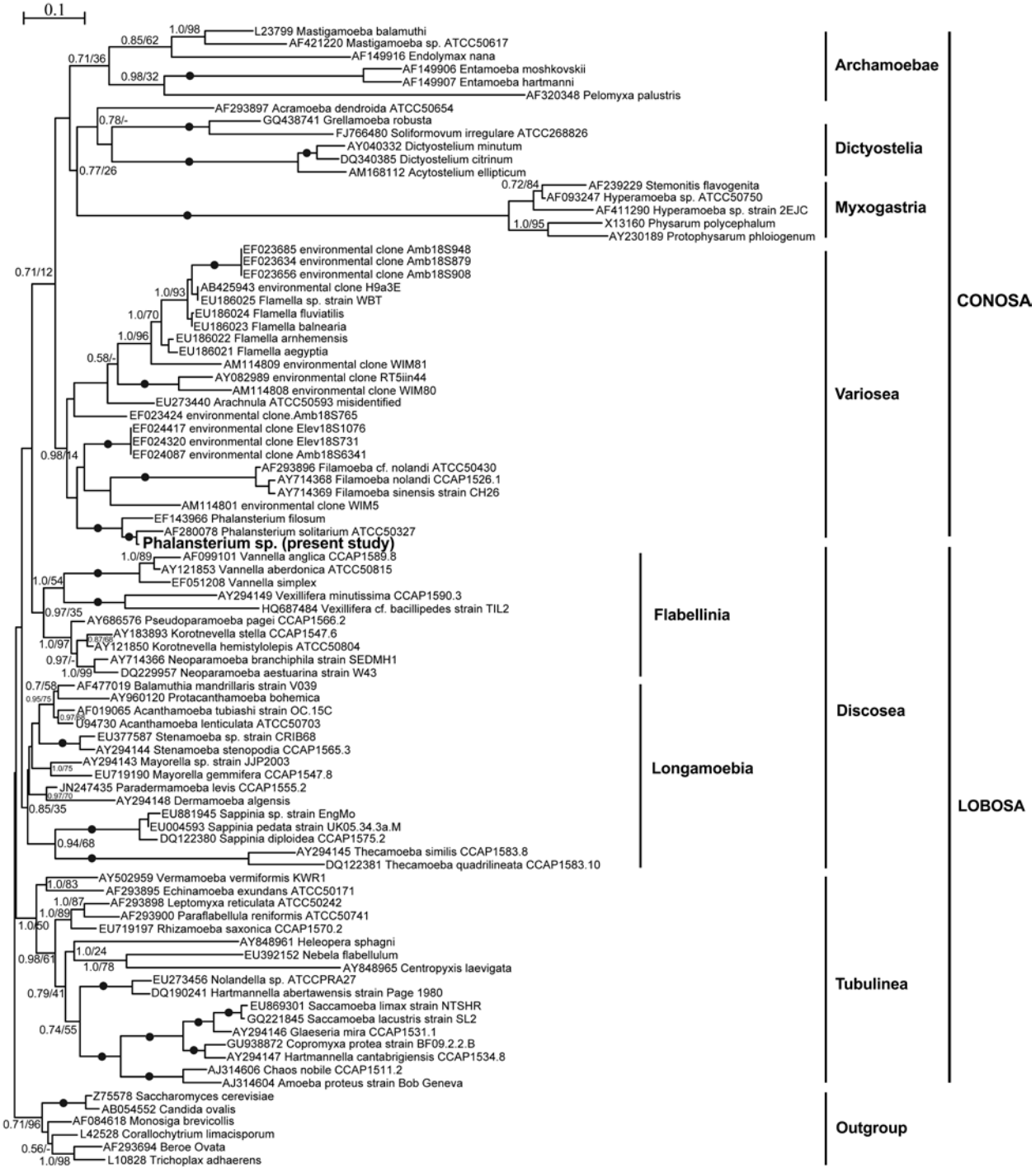

Figure 1. 18S rDNA phylogenetic tree showing the position of Phalansterium sp. (present strain) amongst Amoebozoa. PP/Bootstraps over 0.5/50% are indicated (even if only one of the two values exceeds the thresholds); dashes indicate branches not reproduced in the corresponding Bayesian or ML analyses.

form a monophyletic group corresponding to Conosa while Lobosa appears paraphyletic. All major groups of Amoebozoa are represented in our trees in the same configuration as in Smirnov et al. [5] and the class Discosea remains paraphyletic. The new sequence branched within the *Phalansterium* lineage, specifically related to *Phalansterium solitarium* to the exclusion of *Phalansterium filosum* with complete statistical support, but showing clear differences with both species (the new sequence differs from existing ones at 1.8% and 5.5%, whereas they differ from one another by 6.3%). The phylogeny therefore supports the conclusion that the novel strain represents a new species of *Phalansterium*, and we henceforth refer to it (in the absence of morphological data allowing description and naming) as *Phalansterium* sp.

Searching the assembled sequence (filtered for *Drosophila* contamination) for contigs derived from mitochondrial genomes yielded not one, but two distinct mitochondrial types, as expected. The first is a collection of small linear contigs encoding genes with high similarity to mitochondrial genes from *Euglena gracilis*. We infer that this collection is derived from the *M. aenigmatica* mitochondrial genome (not shown). The second type formed a single, gene-rich 53,614 bp circular-mapping contig (Figure 2). Phylogenetic inferences based on the analysis of mitochondrial cytochrome b (Figure 3) supported a close relationship between this protein and amoebozoan homologues, and we accordingly infer the genome encoding this protein to be that of *Phalansterium* sp.

The *Phalansterium* sp. mitochondrion has an ancestral, gene-rich genome.

The *Phalansterium sp*. mitochondrial genome is in some ways similar to that of other amoebozoans, but in others is unique (Figure 2, Table 1). It falls within the size range of other sequenced amoebozoan mitochondrial genomes, is slightly less AT-rich, and contains no identifiable introns (Table 1). It is gene-rich, with a similar coding density as other amoebozoan mtDNAs, but exhibits an unusually low ratio of identifiable protein-coding genes to ORFs. Only 19 protein-coding genes could be identified as

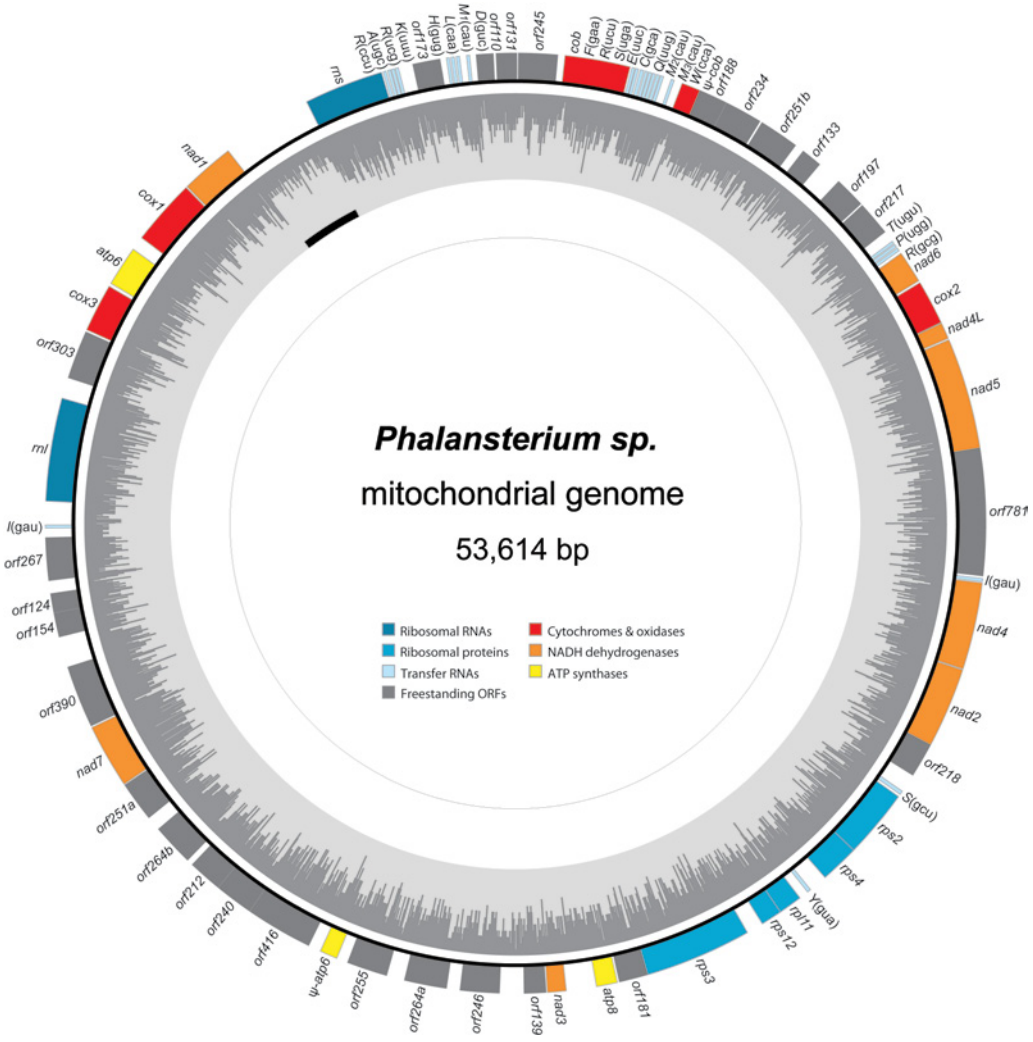

Figure 2. Map of the Phalansterium sp. mitochondrial genome. Genes (filled boxes) are transcribed clockwise. tRNA genes are indicated by the one-letter amino acid code followed by the anticodon in parentheses. All genes were found located on the same strand. ORFs smaller than 100 amino acids are not shown. GC content calculated with OGDRAW is shown underneath the genes in the inner gray circle. The GC-poor region between nad1 and rns is indicated by a black bar.

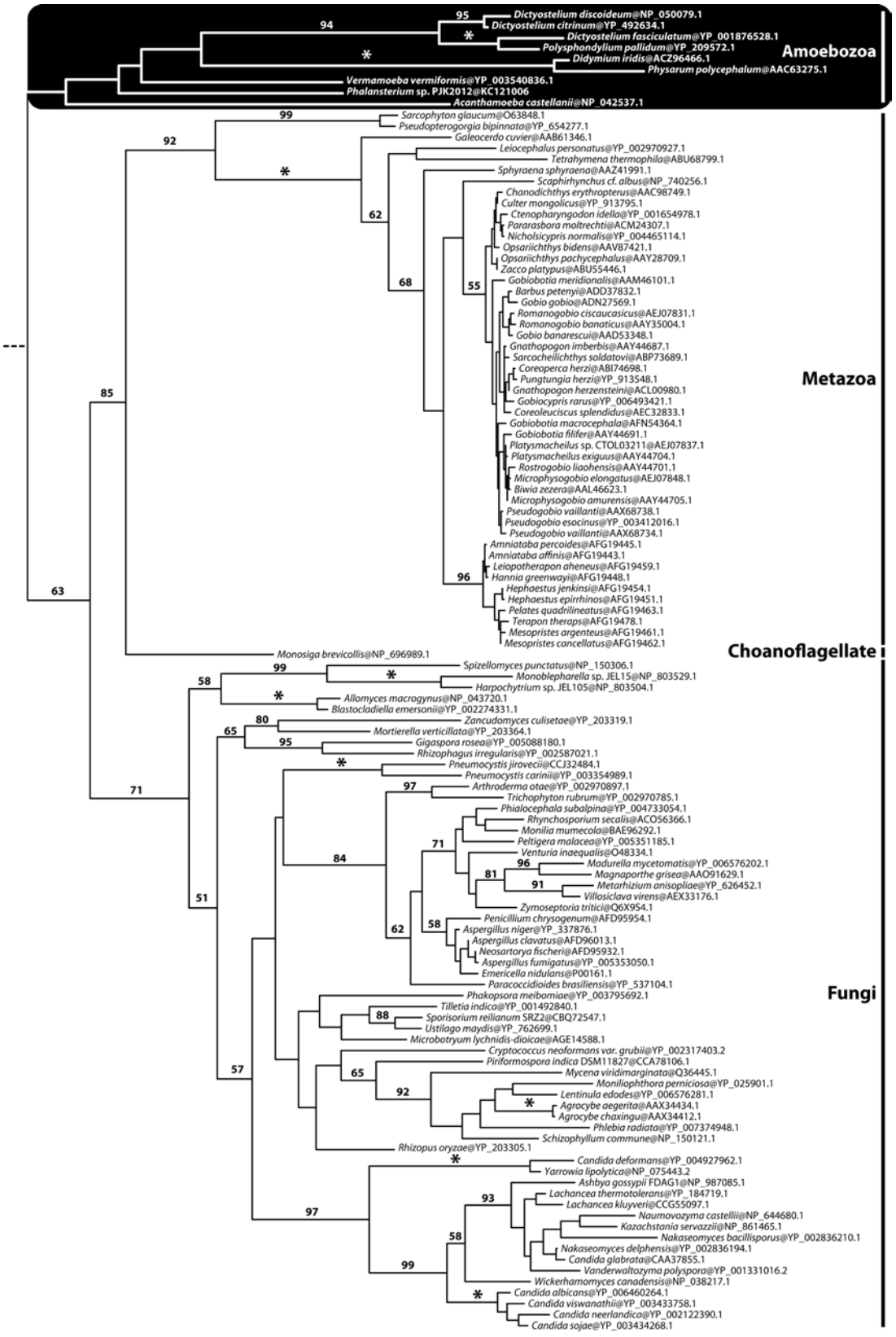

Figure 3. Phylogenetic position of the Phalansterium sp. cytochrome oxidase b mitochondrial protein (Cob). The figure shows a zoom-in of the best ML tree computed with PhyML under the LG+Γ4+F model of amino acid substitution (the full tree is available in Nexus format in Data S1). The dashed line indicates the junction with the rest of the tree (not shown). Bootstrap support over 50% is shown above the major nodes, (with asterisks for 100%).

Table 1. General characteristics of select amoebozoans mtDNAs

Species	size	GC (%)	protein[a]	rRNA	tRNA	ORFs	Density[b] (genes/kbp)	Introns	Intronic ORFs	Accession
Acanthamoeba castellanii	41,591	29.4	36	3	16	3	1.47	3	3	NC_001637
Dictyostelium citrinum	57,820	27.0	35	3	19	11	1.30	5	7	NC_007787
Dictyostelium discoideum	55,564	27.4	35	3	18	3	1.13	5	4	NC_000895
Dictyostelium fasciculatum	54,563	25.5	35	3	17	11	1.25	5	2	NC_010653
Vermamoeba vermiformis	51,645	32.0	37	3	25	9	1.42	0	0	NC_013986
Physarum polycephalum	62,862	25.9	36	3	5	22	1.05	0	0	NC_002508
Polysphondylium pallidum	47,653	24.2	35	3	19	7	1.34	3	0	NC_006862
Phalansterium sp.	53,614	34.6	19[b]	2[d]	24	27	1.34	0	0	KC121006

[a]*The values represent the number of separate coding regions, rather than number of proteins per se. Thus, the fused cox1/2 ORF is counted only once whereas the split rps3 in Dictyostelium species and Polysphondylium is counted twice.*
[b]*The coding density (number of genes per kbp) was calculated from the sum of the genes encoding proteins and RNAs as well as unidentified and intronic ORFs.*
[c]*The two pseudo-genes in the Phalansterium sp. mitochondrial genome (Ψ-cob and Ψ-atp6) were not included in this value.*
[d]*The divergent 5S rRNA encoded in amoebozoan mitochondrial genomes [50] could not be reliably identified in Phalansterium and is therefore not included in this number.*

homologous to other known sequences, in contrast to the 35-37 protein genes of known function found in all other amoebozoan mitochondrial genomes sequenced to date. In contrast, the *Phalansterium* mitochondrial genome contains an abundance of unidentified ORFs—27 longer than 300 bp were identified—whereas typically fewer than 12 are found in other amoebozoans (*P. polycephalum* is an exception with 22 unidentified ORFs). The identifiable genes are also very divergent at the sequence level, which is not surprising for amoebozoans. For examples, amino acid sequence identity between *cox1* homologues (excluding the fused *cox2* segment in *Acanthamoeba*, *Polysphondylium* and the *Dicyostelium* species) ranges between 61% and 87% whereas *cob* homologs share from 51% to 94% identity between species. This variation suggests that some of the unassigned ORFs likely correspond to genes found in other amoebozoan mitochondrial genomes. This inference is consistent with the fact that the 'missing' genes mostly encode small ribosomal proteins, which can be difficult to identify in a divergent genome. On the other hand, in *Physarum* mitochondria, most of the additional unassigned ORFs appear to be transcriptionally inactive, at least under the growth conditions used by investigators to date, suggesting they may not be functional [39].

In *Physarum* mitochondria, transcripts undergo extensive nucleotide insertion editing as well as limited C-to-U substitution editing during maturation [40]. This post-transcriptional editing, superimposed on a relatively high rate of sequence divergence at the genome level, complicates the identification of genes via standard homology searches [41,42]. Extensive RNA editing, if it occurs in *Phalansterium* mitochondria, could be an additional reason for the apparent paucity of identifiable protein-coding genes in this genome.

In *Acanthamoeba castellanii* and other amoebozoans, the Cox1 protein lacks the C-terminal portion of a conventional, mtDNA-encoded Cox1. This C-terminal sequence is instead encoded by a nuclear gene, whose protein product is imported into mitochondria [43]. The *Phalansterium* Cox1 also lacks the C-terminal region in question, indicating that the last common amoebozoan ancestor already had this *cox1* gene fission. On the other hand, as in *Vermamoeba* and *Physarum*, the *cox1* and *cox2* genes are separate in *Phalansterium* mtDNA, whereas they are fused into a single continuous ORF in the mitochondrial genomes of *Acanthamoeba* [44], *Dictyostelium* species, and *Polysphondylium*.

In contrast to its relatively low content of protein-coding genes, *Phalansterium* mtDNA exhibits the second largest repertoire of tRNA genes (24) of completely sequenced amoebozoan mitochondrial genomes. This set includes 3 species with CAU anticodon (putative $tRNA^{Met}$ isoacceptors), 2 of which are virtually identical duplicates. *Phalansterium* uniquely shares with *Vermamoeba* five mitochondrial tRNA genes, encoding three $tRNA^{Arg}$ and two $tRNA^{Ser}$ isoacceptors, that are absent from other amoebozoan mitochondrial genomes. We infer that these tRNA genes were present in the last amoebozoan common ancestor and subsequently lost in later diverging amoebozoans.

In *A. castellanii* [45], *P. polycephalum* [46] and *D. discoideum* [47], mitochondrial tRNAs undergo a form of 5′ editing to correct mis-matches in the first three positions of the acceptor stem. By revealing such acceptor stem mis-matches, secondary

structure modeling of tRNA gene sequences has proven to be a powerful predictor of mitochondrial tRNA 5′ editing [48]. Of the 24 tRNAs encoded by *Phalansterium* mtDNA, we infer on this basis that at least 16 require post-transcriptional 5′ editing to generate the functional species. The almost certain existence of tRNA 5′ editing in *Phalansterium* mitochondria argues that this system was already established in a last common amoebozoan ancestor.

Overall, the *Phalansterium* mitochondrial genome might be summed up as having an 'ancestral' architecture (according to the classification of mitochondrial genome architecture types proposed in [49]), but populated with highly derived genes.

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